THE EFFECTS OF VARIOUS DIETARY FATS ON FATTY LIVERS AND SELECTED BIOCHEMICAL SYSTEMS IN THE THREONINE DEFICIENT RAT

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ABSTRACT

THE EFFECTS OF VARIOUS DIETARY FATS ON FATTY LIVERS AND SELECTED BIOCHEMICAL SYSTEMS IN THE THREONINE DEFICIENT RAT

by Linda Ann Morris

Feeding weanling rats a 9% casein diet supplemented with methionine and tryptophan, but not threonine, induces fatty livers under specific dietary conditions. The basal diets used in these experiments contained corn oil (5% of the diet) as the fat source and sucrose as the carbohydrate source.

Recently emphasis has been shifted to a study of interrelationships between the threonine deficient state and the kind and proportion of other nutrients in the diet. The experiments reported here were undertaken to determine the effect of the type of dietary fat on the fatty livers associated with rats fed threonine deficient diets.

Male weanling rats of the Sprague-Dawley strain were fed diets containing 9% casein and 30% fat. The effect of the following constituents of the diet on fatty livers was studied: 1) type of fat; 2) choline; 3) threonine. Food intake, weight gains, liver moisture, fat and nitrogen analysis and the electrophoretic determination of serum proteins were done for all animals. In one experiment, liver pyridine nucleotides and the activity of the fatty acid oxidase system and endogenous oxidation were measured in liver tissues. Determinations of total and esterified serum cholesterol were made in the last two experiments.

The results of these experiments were presented and discussed. To explain these data, a theory involving alternate routes of fat transport for different types of dietary fat was discussed.

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By

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REVIEW OF LITERATURE

Nutritional factors which affect the deposition of fat in the liver have been studied for many years. Extensive investigations have been done on the lipotropic action of protein and specific amino acids, choline, vitamins, and other dietary components; and the mechanism of action by which each of these factors affects the fatty liver syndrome. This review is primarily concerned with the role of protein and amino acids in controlling liver fat levels. However, since this area is so closely allied to the related studies with choline and other nutrients, some discussion of these experiments will also be included.

The fact that fatty livers could be produced experimentally in rats by manipulating components of the diet was first shown by Best and Huntsman (1932). They were able to induce fatty livers in rats by increasing the fat content of the diet to 40%. The addition of choline prevented excessive accumulation of fat in these livers. Further experiments by the same researchers (Best and Huntsman, 1935) proved choline to be lipotropic under a variety of dietary conditions.

Tucker and Eckstein (1937) investigated the possibility that compounds acting as choline precursors *in vivo* might prevent liver fat accumulation due to choline deficiency. The addition of 0.5% methionine to a diet containing 5% casein and 40% fat reduced liver fat levels to 11% as compared to the unsupplemented control values of 20%[1]. In 1941,

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[1] Except where indicated to the contrary, liver fat figures are quoted per gram dry weight of liver tissue.

du Vigneaud et al. clarified the role of methionine in preventing fatty livers. Using supplements of deuterium-labelled methionine in low choline diets, they demonstrated that the methyl groups of methionine are used for choline synthesis in the rat. The methionine-sparing action of choline has been confirmed by other investigators (Engel, 1948 and Treadwell, 1948).

At about the same time that the lipotropic action of choline was being verified, data were being collected which suggested a lipotropic action of dietary protein. Best and Huntsman (1935) prefed rats a hypolipotropic diet of mixed grains and containing 40% of fat for a period of three weeks. At the close of this period, the hypolipotropic diet was replaced by sucrose, and the feeding trial continued for 13 days. Replacing the hypolipotropic diet with sucrose resulted in a marked elevation of liver fat levels (14% vs. 23%, respectively). The addition of choline (75 mg/day) to the sucrose caused the reduction of the liver fat to 5%. When sucrose was supplemented with casein (20% of the diet) liver fat levels were also reduced, but not to the same extent as was observed when choline was supplemented to sucrose. The authors postulated that proteins per se are not lipotropic and the apparent lipotropic effect of casein observed in their experiments was due to impurities such as betaines in the protein.

During this period, other workers were supporting a contrary theory to that of Best and Huntsman concerning the lipotropic effect of protein. In 1935, Channon and Wilkinson observed that rats fed a 5% casein diet developed fatty livers to the extent of 13% of the fresh tissue. Increasing the casein level to 20% reduced liver fat to 7%.

A further reduction in liver fat to 6% occurred when casein was increased to 50% of the diet. On the basis of these data, Channon and Wilkinson postulated that proteins did indeed exert a lipotropic effect and that this effect was in proportion to the level of protein in the diet. More recent experiments (Harper *et al.*, 1953a and Harper *et al.*, 1953b) have confirmed the lipotropic action of increasing levels of protein. Beeston *et al.* (1936) observed that feeding rats 5% casein diets containing 0.1 - 0.2% choline resulted in fatty livers about two times the values found when diets containing 30% casein were fed. Thus it became obvious that protein exerted a lipotropic effect separate and apart from either choline or methionine.

These results with protein led to further investigations in which the lipotropic action of various amino acids was studied. Beeston and Channon (1936) fed rats diets containing 5% casein and 40% fat. Under these experimental conditions, animals developed fatty livers, and the amino acids lysine, glutamic acid, aspartic acid, serine, glycine, and phenylalanine were ineffective in preventing this condition. However, cystine exerted an "anti-lipotropic" effect, and promoted the accumulation of fat in the liver. This increase in liver fat could be reversed by feeding additional casein. Tucker and Eckstein (1937) investigated the role of the sulfur amino acids in the production of liver fat. Using dietary conditions similar to those reported by Beeston and Channon (1936), they also observed increased liver fat upon including cystine in the ration.

In the first of a series of experiments done by Singal and his co-workers (1948), it was noted that under certain dietary conditions threonine became the limiting factor for growth. When a diet containing

9% casein supplemented with histidine, valine, threonine, and lysine or with nicotinic acid alone was fed to rats normal growth did not occur. However, the 9% casein diet supplemented with both the four amino acids and either tryptophan or nicotinic acid did promote normal growth. Further experiments by these workers showed that of the four amino acids studied, only threonine produced normal growth when added to a 9% casein diet supplemented with tryptophan or nicotinic acid. The authors noted that the livers from the threonine deficient animals appeared to be fatty although fat analysis was not done.

The concept that one amino acid might be the limiting factor for growth and another the most limiting in liver fat production was further defined by Litwack, Hankes and Elvehjem (1952). They fed rats diets containing 9% casein and supplemented with either tryptophan or threonine. Under these dietary conditions, tryptophan was shown to be the most limiting amino acid for growth and threonine the most limiting amino acid for fatty liver production. Morrison and Harper (1960) recently confirmed these results using diets containing 8% casein and supplemented with L-cystine or DL-methionine. The addition of 0.36% of DL-threonine to the basal diet caused a depression in the growth rate of rats which could be corrected by the addition of either niacin or tryptophan.

On the other hand, under certain dietary conditions, threonine can be shown to be limiting for both growth and liver fat production. Using a 6% casein diet supplemented with 4% of an amino acid mixture which contained tryptophan but no threonine, Harper (1959) showed that between 0.025 and 0.05% of L-threonine was required to overcome the growth depression and the fatty livers caused by the deficient diet. He concluded that the percent threonine originally present in the diet was made unavailable because of the imbalance of amino acids.

A considerable amount of work has centered on studying the relationship between threonine and the appearance of fatty livers. In 1949, Singal _et_ _al_. published a report which clearly showed the effect of threonine deficiency on liver fat. Rats which were fed diets containing 5% fat, 9% casein, and supplemented with choline and either tryptophan or niacin had 14.4% liver fat. When threonine was included in the ration, the liver fat levels dropped to 5.9%. Increasing the level of dietary fat to 40% increased liver fat levels to 21.8% in threonine-deficient animals and 10.1% in those receiving the threonine supplement. Further studies with 9% casein diets (Singal _et_ _al_., 1953b) demonstrated that only the L- isomer of threonine prevents fatty livers and stimulates growth of rats on a deficient diet.

Harper, Benton, Winje and Elvehjem (1954) showed that the lipotropic action of threonine is separate and distinct from that of other known lipotropic factors. The addition of 0.36% threonine to a 9% casein diet supplemented with 1% methionine and 0.15% choline reduced liver fat levels by half (23% to 11%).

When threonine was omitted from an amino acid diet simulating the 9% casein ration (a threonine devoid diet), the rats lost weight and liver lipids were only slightly above normal. Adding increasing amounts of threonine to the amino acid diet simulating the 9% casein ration (Singal _et_ _al_., 1953a), improved growth but also increased liver fat. Increasing the level of threonine to 1.1% of the diet produced good growth and normal levels of liver fat. Thus liver fat levels varied directly with the quantity of threonine in the diet up to a certain level. The authors concluded that a growth rate at least 80% of the optimal is required for maximal fat deposition in threonine- and lysine-induced fatty livers. Harper and his co-workers (Harper et al., 1953a) found that provided a minimal level of growth is maintained, the rate of growth does not affect the degree to which liver fat will accumulate in threonine-deficient rats. Other experiments with amino acid diets containing only essential amino acids and deficient in threonine (Dick et al., 1952) resulted in enlarged and extremely fatty livers. Similar diets deficient in lysine also produced fatty livers although they appeared to be less severe (Dick et al., 1952).

The deposition of fat in the livers of threonine-deficient rats appears to be also a function of age of the animal (Harper, Benton, Winje, Monson and Elvehjem; 1954). The amount of fat in livers of weanling rats fed a 9% casein diet containing methionine and choline decreased gradually as the animals matured and their protein requirement decreased. Fat determinations were made over a ten week period. Deposition of fat reached a peak (30 to 40%) after two weeks on the diet and then tapered off until after ten weeks the liver fat levels were 15%. In order to induce fat to accumulate in livers of mature rats, the protein content of the diet had to be reduced to 5%. The addition of either threonine or glycine to the 5% casein diet reduced the accumulation of fat. Thus Harper, Monson, Benton, Winje and Elvehjem (1954) postulated that three groups of dietary factors must be considered in evaluating the lipotropic activity of proteins: 1. choline which is involved in phospholipid metabolism and can be at least partially replaced by methionine; 2. threonine and/or other essential amino acids which are required for normal liver metabolism; and 3. glycine, serine, and betaine which act in a more non-specific way by sparing essential compounds.

Efforts have been made to identify the mechanism by which threonine regulates fat metabolism. The effect of threonine deficiency on several enzyme and co-enzyme systems has been investigated. Harper _et al_. (1953) reported that the activities of the mitochondrial enzymes, succinic oxidase and choline oxidase, were higher in liver homogenates from threonine deficient as compared with control animals, while endogenous respiration and the activities of the cytoplasmic enzymes, xanthine oxidase and tyrosine oxidase, were lower. The differences in endogenous respiration between the basal and threonine deficient groups were presumed to reflect a metabolic difference in oxidative pathways in the two groups. Arata _et al_. (1954) also noted decreases in endogenous oxidation and the activity of the xanthine oxidase and tyrosine oxidase systems in threonine deficient rats.

The marked reduction in endogenous oxidation seen in threonine-deficient fatty livers, and the known involvement in oxidation-reduction reactions of the pyridine nucleotide co-enzymes, promoted investigations to determine the role of these compounds in threonine deficiency. Arata _et al_. (1956) reported a decrease in the concentration of pyridine nucleotides in livers from threonine deficient rats. Since the pyridine nucleotides are essential co-factors in fat oxidation, (Green, 1954), a deficiency in these co-enzymes could be a major factor in liver fat accumulation in threonine deficiency. Singal and Littlejohn (1963) used an experimental ration containing 7% casein supplemented with tryptophan and cystine. The control diet contained 0.4% threonine. They found a large decrease in all pyridine nucleotide fractions (expressed as μM/gm N) in the livers of deficient animals. However, Falcone et al. (1962) found no decrease in pyridine nucleotides (expressed as μM/gm N) in fatty livers from either choline- or threonine-deficient diet. The conflict between these data and that of Singal and Littlejohn (1963) could be explained by the fact that Singal and Littlejohn included tryptophan in the diets of both the threonine-deficient and control animals, whereas Falcone did not. In a later report, the same group (Methfessel et al. 1964a) repeated these experiments. When the pyridine nucleotides were calculated on a per gram liver basis, both the choline- and threonine-deficient rats had decreased levels of liver pyridine nucleotides, however, the pyridine nucleotides calculated per total liver were not significantly altered in the deficient state. Thus, these data lent little support to the concept that the development of fatty livers in choline- or threonine-deficient rats is related to an altered concentration of pyridine nucleotides.

It has already been noted that the effect of threonine-deficiency on fatty livers varies with the age of the animal (Harper, Benton, Winje, Monson and Elvehjem; 1954). Carroll and her co-workers (1960) confirmed this report and attempted to correlate changes in certain enzyme systems and the accumulation of liver fat with time. Maximum deposition of liver fat (30%) in weanling rats occurred after 24 days on the threonine-deficient diet. After six weeks on the deficient diet liver fat levels had fallen to approximately half of the maximum. The activity of the two enzyme systems, xanthine oxidase and malic dehydrogenase, also varied with time. The activity of these two enzymes decreased in the deficient animals for 19 days followed by a period of increased activity. It appeared that fat could not be mobilized out of the liver until after recovery of the enzyme systems. Arata, Carroll, and Cederquist (1964) reported changes in other enzyme systems as well. Weanling rats were fed a 9% casein diet deficient in threonine and sacrificed after 2, 4, and 6 weeks on the ration. Control animals received the same diet supplemented with threonine. Liver fat levels reached a maximum in the deficient rats after approximately 2 weeks on the ration. During this 2 week period of liver fat deposition, the activity of the fatty acid oxidase system was depressed in the deficient animals as compared with their controls. The activity of this enzyme system returned to normal in the following weeks followed by a return of liver fat levels to nearly normal. The levels of labile phosphorus from adenosine diphosphate and adenosine triphosphate were lower than control values. The activity of the DPN-cytochrome c reductase system, an enzyme of the electron transport chain, was depressed in deficient animals after two weeks. Methfessel _et_ _al_., (1964b) assayed for several enzymes in fatty livers from choline- and threonine-deficient rats. Animals were sacrificed at intervals after 3 to 30 days on the deficient diets. They found no change in the activity of malic dehydrogenase, but the activity of the enzyme system which catalyzes the DPN-mediated oxidation of TPNH by cytochrome c was lower in the two groups which developed fatty livers than in their control groups. Data from these two laboratories (Methfessel and Arata) suggest that a threonine deficiency may alter some phase of the electron transport system.

Other workers have correlated changes in fat metabolism with fatty infiltration of the liver. Yoshida and Harper (1960) found fat synthesis to be stimulated in rats fed a threonine-deficient diet. They injected acetate- and palmitate-1-C^{14} and found the amounts of C^{14} incorporated into body fat and the neutral fat fraction of livers were significantly greater in rats fed the threonine-deficient diet.

Channon and Wilkinson (1936) first investigated the effects of different types of dietary fat on the severity of the fatty liver syndrome. Rats were fed diets containing 5% casein and 40% fat for 2 weeks. Fatty livers developed ranging from 30.7% in the case of butter fat to 7.2% of the fresh liver weight for cod liver oil. In general the amount of fat which would accumulate in the liver was inversely proportional to the iodine value of the fat. They concluded that the amount of C_{14}-C_{18} saturated fatty acids ingested appeared to govern the deposition of fat in the liver. However, the results of Channon and his co-workers are complicated by the very low protein level plus the choline deficiency of the diet.

Harper, Monson, Benton, Winje and Elvehjem (1954) fed a threonine-deficient, 10% casein diet containing either 5% or 20% fat. Liver fat levels were similar regardless of type or level of fat fed (corn oil or butter fat). The addition of threonine caused a reduction of liver fat with each level of both fats.

A subsequent experiment by Benton _et al_. (1956) did show a difference in the effect of dietary fats. A 9% casein diet supplemented with 0.15% choline and deficient in threonine was fed. Fat was included at the 20% level of the diet. The levels of liver fat were high when butter or lard was fed (32.6 and 26.2%, respectively) and lower when corn oil or margarine was used (22.4 and 22.7%, respectively). This measurable difference in biological response to fats of different chemical composition was enhanced when the level of protein in the diet was decreased or when choline was omitted from the diet. When the fatty acids of butter were isolated and fed, the long chain saturated fatty acids produced higher liver fat levels than the unsaturated fatty acids. The authors suggest that the effect of fats containing large amounts of long chain saturated fatty acids on liver fat deposition is important only when large amounts of fat are fed in diets deficient either in choline or protein.

A higher level of choline was required to lower liver fat levels in animals fed 30% butter fat rather than 30% corn oil (Benton et al., 1957). Rats fed butter fat required 0.15% choline chloride to reduce liver fat levels to 16.6%. Rats consuming corn oil required 0.12% choline chloride to reduce liver fat levels to 16.3%. These values were not affected by supplements of cystine, methionine, tryptophan or all three amino acids.

Sidransky and Verney (1964) force fed young rats a threonine-devoid diet containing various levels of corn oil. The highest level of corn oil (25%) produced the highest level of liver fat (338 mg/liver). Feeding the 5% corn oil diet lowered liver fat to 188 mg/liver. A further reduction of dietary corn oil to 0.6% had no further effect.

Carroll (1964) investigated the effects of various carbohydrate-fat combinations on liver fat deposition and serum cholesterol. Diets containing 20% casein, 5% fat, 60.55% carbohydrate and supplements of the known lipotropic factors were fed for 2 or 4 weeks. Various combinations of corn oil, hydrogenated coconut oil, and hydrogenated peanut oil were made with glucose, fructose, sucrose, and rice starch. Each particular combination of fat and carbohydrate appeared to have its own unique effect on the level of liver fat and serum cholesterol.

The apparent toxicity of saturated fats has been studied by Tove (1964). He fed male weanling mice a 20% casein ration. Glycerolmonopalmitate and glycerolmonostearate usually at the 40% level of the diet served as the source of fat. These diets produced poor growth and a high mortality rate. This effect could be prevented by the addition of small amounts of either oleate or linoleate and was therefore not reflective of an essential fatty acid deficiency. The mechanism by which the saturated fat produces the toxicity is unknown. The animals showed signs and symptoms of starvation although they were consuming the diet at the rate of 2.5 to 3.0 g/day. Tove suggests that the high level of saturated fat may promote uncoupling of oxidative phosphorylation as happens in essential fatty acid deficiency (Klein and Johnson; 1954). However, Tove presented no data to support this conclusion.

Viviani *et al*. (1964) have attempted to define the fatty acid composition of fatty livers produced by a deficiency of both lysine and threonine. Low protein diets (rice served as the sole source of protein) containing 3% corn oil and 1.5% cod liver oil were fed to weanling rats for six weeks. The control group received the same diet supplemented with lysine and threonine. The deficient diet caused a fatty liver in the rats in addition to a lower growth rate. No differences in liver phospholipid levels were observed between the two groups but the deficient animals had less of the 20:5, 22:5 and 22:6 fatty acids in the phospholipid fraction. The livers of the lysine- and threonine-deficient rats contained more neutral fat with marked increases in myristic, palmitic, oleic and linoleic acids. The total percentage of polyunsaturated fatty acids of the C_{20} to C_{22} series was higher in the livers of rats receiving the supplemented diet.

Fatty livers produced by various dietary means appear to have the same fatty acid composition. Tesluk and Stewart (1964) produced fatty livers by two different means. They fed diets which were choline-deficient or contained corn meal as the source of protein. Analyses of fatty acid composition was done on liver, depot, and dietary fat. Fatty acid composition of depot fats were unchanged in deficient animals as compared to the control animals. Fatty acid patterns in fatty livers produced by either the choline-deficient or corn meal diets were similar. Fatty livers produced by either diet showed decreases in palmitic and stearic, and increases in oleic and linoleic acids as compared to the controls. The authors conclude that the above data support the concept that the increase in hepatic fat is due to interference with transport of fat through the liver rather than to partial interference with fat oxidation.

PART I

EFFECT OF VARIOUS DIET FATS ON THREONINE IMBALANCE[1,2]

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[2] This investigation was supported in part by Public Health Research Grant A-6093 from the National Institute of Arthritis and Metabolic Diseases.

INTRODUCTION

Feeding weanling rats a 9% casein diet supplemented with methionine and tryptophan, but not threonine, induces fatty livers under specific dietary conditions (1, 2, 3). The basal diets used in these experiments contained corn oil (5% of the diet) as the fat source and sucrose as the carbohydrate source. Under these dietary conditions, the deposition of fat in liver tissues reaches a maximum after 2 to 3 weeks of feeding and then slowly decreases (4,5).

Recently emphasis has been shifted to a study of interrelationships between the threonine deficient state and the kind and proportion of other nutrients in the diet. In 1961 Sidransky and Clark (6) suggested the pathological changes observed in young rats fed a threonine devoid diet were due to an imbalance between the amino acid and the calorie intake. Later Sidransky and Verney (7) observed an accentuation of said pathological changes by increasing the corn oil content of the diet from 5% to 25%.

In addition to the quantity, the type of fat present in the diet has also been shown to influence the serverity of lesions induced by nutritionally inadequate diets. Benton _et_ _al_. (8) reported higher liver fat concentrations when butterfat or lard provided the diet fat source than when corn oil or margarine was used as the dietary fat in threonine deficient diets. In all instances, the addition of threonine significantly lowered liver fat levels. Benton _et_ _al_. postulated the long chain fatty acid composition of the fat was the determining factor in regulating liver fat deposition. Carroll (9,10), using different dietary conditions also observed a marked effect of different diet fats on glycolysis and lipogenesis.

The experiments reported in this paper were undertaken to determine the effects of various types of fats and oils on growth and liver composition of rats fed threonine deficient diets.

METHODS

Weanling male rats of the Sprague-Dawley strain were used as the experimental animals. They were divided by weight into groups of ten and housed in individual screen-bottomed cages in a temperature-controlled room. Data from replicate groups were combined at the close of the study. Food and water were supplied *ad libitum* except in one experiment when the paired feeding technique was used.

The basal diet was of the following percent composition: sucrose, 25; casein, 9; salts W[3], 4; fat[4], 30; vitamin mix, 0.25; choline, 0.15; DL-methionine, 0.30; DL-tryptophan, 0.10; alphacel[5], 31.20. The composition of the vitamin mix has been described previously (11). When threonine was added to the diet, 0.36% of the DL isomer of the amino acid replaced an equal amount of sucrose. These diets, containing 30% fat were isocaloric with the 5% fat diets used in previous experiments (4,5); alphacel was used to make up the weight differences. The animals were divided into the following experimental groups:

Group 1 - basal diet (corn oil)
Group 2 - corn oil + 0.36% DL-threonine
Group 3 - olive oil
Group 4 - olive oil + 0.36% DL-threonine
Group 5 - cottonseed oil
Group 6 - cottonseed oil + 0.36% DL-threonine

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[3] Wesson modification of Osborne and Mendel salt mixture. Wesson, L. G. Sci. 75, 339 (1932).

[4] Containing 75 mg α-tocoperol acetate per kilogram of diet.

[5] Purchased from Nutritional Biochemicals, Cleveland, Ohio.

Group 7 - hydrogenated vegetable oil[6]

Group 8 - hydrogenated vegetable oil[6] + 0.36% DL-threonine

Group 9 - hydrogenated corn oil[7].

The rats were weighed at weekly intervals during the study and food consumption records were kept. At the end of the experimental period the animals were lightly anesthetized with ether, approximately 2/3 of the tail severed with a sharp razor blade, and blood samples collected. By this method 1 ml of blood could be collected from very young (2 weeks post weaning) rats. The blood samples were allowed to stand overnight and sera harvested the next morning. The sera were stored frozen until analysed for proteins by paper electrophoresis in a Spinco apparatus. The strips were scanned in a model RB Spinco analytrol and results expressed as percent of total protein separated.

After collecting the blood, the rats were killed by decapitation. Livers were excised, weighed, homogenized with distilled water, and dried at 90°C to constant weight. The dried livers were weighed to determine moisture content, and ground in a Wiley mill. Fat content was determined on one gram samples by continuous ether extraction for 3 hours on a Goldfisch apparatus. The concentration of fat in the tissues was expressed as percent dry weight. The percent nitrogen was determined on the residue by the macro kjeldahl method and calculated as fresh weight of tissue.

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[6] Obtained from Hunt Foods and Industries, Fullerton, California. Iodine value = 74. It is composed of approximately 90% hydrogenated soybean oil and 10% hydrogenated cottonseed oil.

[7] Corn oil hydrogenated by Proctor and Gamble to iodine value of 74. The authors are indebted to Proctor and Gamble for their fine cooperation and assistance.

Standard errors of the means were calculated for all data. Student's t test was used as a measure of significance. Only those differences with a probability of less than 0.01 were considered significant.

RESULTS

Food intake and weight data for rats fed different types of fat in 9% casein diets with and without threonine supplements are summarized in table 1. No marked changes were observed in these parameters when the corn oil in the basal diet was replaced by olive oil, cottonseed oil, or hydrogenated vegetable oil. Likewise, there were no significant changes in food intake or weight gain when threonine was added to a diet containing any one of these fats. However, by changing the type of fat in the diet, or by the addition of threonine significant changes in liver composition were induced (table 2).

The most marked changes in liver composition centered in the lipid component (table 2). Replacing the corn oil in the basal diet (group 1) with either cottonseed oil (group 5) or hydrogenated vegetable oil (group 7) caused a significant reduction in liver fat concentration. Substituting olive oil (group 3) for corn oil (group 1) slightly increased the quantity of fat in livers of rats fed these diets (25.6% _vs_ 22.5%, respectively). In every instance, the addition of threonine significantly lowered liver fat levels (groups 1 _vs_ 2, 3 _vs_ 4, 5 _vs_ 6, 7 _vs_ 8).

In an effort to draw a more controlled comparison between the effects of corn oil and hydrogenated vegetable oils on threonine deficient animals, corn oil was hydrogenated to the same iodine value determined for the hydrogenated fat used in groups 7 and 8. When hydrogenated corn oil was used as the diet fat in a threonine deficient diet (group 9), liver fat levels were again significantly decreased below those in the control group (1). Since a slight difference in food consumption was observed between these 2 groups (table 1), the experiment was repeated using the paired feeding technique. Liver fat levels in rats pair fed a diet containing hydrogenated corn oil (9P) were still significantly lower than those in control animals (corn oil).

A more detailed study comparing groups 1 and 9 was initiated. Ten animals from each group were sacrificed at 2 weeks and again at 4 weeks, because liver fat levels in threonine deficient rats have been shown to vary with time (4). Results are presented in table 3. Replacing corn oil (group 1) with hydrogenated corn oil (group 9) in threonine deficient diets significantly reduced liver fat levels after 2 weeks; this effect persisted for 4 weeks. The decreased fat concentrations in livers from rats in group 9 were accompanied by an increased concentration of nitrogen and moisture after 4 weeks as compared with rats in group 1.

Significant changes with time were also seen in the serum protein patterns; the most striking change was observed in the gamma globulin fraction. This fraction increased approximately 3 fold between the 2nd and the 4th week of the experiment. The magnitude of the increase was of the same order in both groups of rats. In group 9, the increase in concentration of gamma globulin after 4 weeks was approximately counterbalanced by a decrease in the concentration of serum albumin between weeks 2 and 4. Since the changes observed in γ globulin concentrations were not correlated with diet changes, the observed increase in this fraction with time must have reflected a normal pattern of growth or an artifact. Subsequent experiments suggeste the later.

DISCUSSION

The data reported here support the contention that the severity of fatty livers associated with a threonine deficiency varies to a considerable extent with the chemical composition of the fat source in isocaloric diets. Of the various fats incorporated in threonine deficient diets, oilive oil produced the most severe fatty livers (25.6%) followed closely by corn oil (22.5%). Fat accumulation in livers from rats fed deficient diets containing cotton seed oil or hydrogenated vegetable oil was comparable (17.9% and 17.6% respectively), while rats fed hydrogenated corn oil had the least severe fatty livers (13.7%).

Since all diets were isocaloric and since no significant differences in food intake or weight gain were observed among any of these threonine deficient groups except group 9, the "protective" action of some diet fats is apparently not mediated <u>via</u> a more equitable balance between the amino acid and calorie ratio. Food intake of rats in group 9 (hydrogenated corn oil) was significantly greater than the control group.[1] However, when rats in this group were pair-fed with the control animals, liver fat levels were still significantly depressed below those in rats fed the basal diet. This observation substantiates the suggestion that the calorie intake is not of major importance in regulating the degree of fat accumulation in threonine deficient animals.

Of the fats tested, hydrogenated fats tended to be more protective against fatty livers associated with threonine deficiency. Feeding threonine deficient rats corn oil hydrogenated to the same iodine number as the commercial hydrogenated fat resulted in a significantly lower

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[1] The small standard error in group 9 probably accounts for the significance of the difference between these groups. In subsequent experiments no differences in food intake were observed.

concentration of liver lipids as compared with those in rats fed the unhydrogenated corn oil. However, the oils did not offer equal protection to threonine deficient animals; cottonseed oil was far more effective than was olive oil despite the fact that the iodine value of olive oil is lower than that of cottonseed oil. Thus the degree of hydrogenation did not solely determine the effectiveness of the fat in limiting the development of fatty livers in threonine deficient animals.

The data reported here do not support the suggestion made by Benton _et al_. (8) or Channon and Wilkinson (12), that the chain length of the constituent fatty acids determines the serverity of fatty livers associated with threonine deficiency, since the hydrogenation of corn oil would not alter the length of the fatty acid chains. However, the hydrogenation process may have resulted in the appearance of isomeric forms which actively reduce liver fats in threonine deficient animals.

These data suggest both the fatty acid composition and the degree of hydrogenation of the dietary fat serve to influence the degree of fat accumulation in threonine deficient rats. The combination of these two factors apparently determines the metabolic deposition of a given fat in the liver. The presence of various isomers in the triglyceride may further delineate the metabolic deposition. Thus, an intricate interrelationship must exist between the amino acid threonine and the metabolic path taken by dietary fat. Studies on this problem are in progress.

Table 1. Food intake and weight gain of rats fed 9% casein diets with and without threonine and containing different sources of dietary fat.

Group #	Diet[1]	N	Food Intake g/wk	Wt. Gain g/wk
1	corn oil	50	70 ± 2[2]	23 ± 2[2]
2	corn oil + threo.	40	70 ± 2	25 ± 1
3	olive oil	10	68 ± 2	22 ± 1
4	olive oil + threo.	10	70 ± 4	25 ± 2
5	cottonseed oil	10	68 ± 2	20 ± 1
6	cottonseed oil + threo.	10	64 ± 2	22 ± 1
7	hydrog. veg. oil[3]	30	77 ± 4	27 ± 1
8	hydrog. veg. oil[3] + threo.	30	81 ± 2	30 ± 1
9	hydrog. corn oil[4]	10	75 ± 1	22 ± 1

[1] Fat content of all diets = 30% w/w. Length of experimental period = 2 weeks.

[2] Standard error of the means.

[3] Maxim brand. Iodine value = 74.

[4] Corn oil was hydrogenated to an iodine value of 74 by Proctor and Gamble.

Table 2. Liver composition of rats fed 9% casein diets with and without threonine and containing different sources of dietary fat.

Group #	Diet[1]	Moisture %	Nitrogen % Wet Wt.	Fat % Dry Wt.
1	corn oil	68.4 ± 0.5[2]	2.53 ± 0.04[2]	22.5 ± 1.0[2]
2	corn oil + threo.	70.6 ± 0.3	2.69 ± 0.04	14.8 ± 0.5
3	olive oil	68.1 ± 0.2	2.45 ± 0.02	25.6 ± 0.9
4	olive oil + threo.	71.0 ± 0.4	2.62 ± 0.03	17.6 ± 1.2
5	cottonseed oil	69.9 ± 0.2	2.60 ± 0.03	17.9 ± 0.9
6	cottonseed oil + threo.	70.9 ± 0.3	2.76 ± 0.03	11.4 ± 0.5
7	hydrog. veg. oil[3]	71.0 ± 0.2	2.47 ± 0.05	17.6 ± 0.8
8	hydrog. veg. oil[3] + threo.	70.6 ± 0.7	2.62 ± 0.03	15.4 ± 0.5
9	hydrog. corn oil[4]	72.2 ± 0.2	2.53 ± 0.03	13.7 ± 0.4
9P	hydrog. corn oil[4] (pair fed with group 1)	71.1 ± 0.3	2.74 ± 0.03	13.2 ± 0.3

[1] Fat content of all diets = 30% w/w. Length of experimental period = 2 weeks.

[2] Standard error of the means.

[3] Maxim brand. Iodine value = 74.

[4] Corn oil was hydrogenated to an iodine value of 74 by Proctor and Gamble.

Table 3. Liver composition and serum proteins of rats fed threonine deficient diets containing either corn oil or hydrogenated corn oil[1] for two or four weeks.

Diet[2]	Group 1 (Corn Oil)		Group 9 (Hydrogenated Corn Oil[1])	
	2 weeks	4 weeks	2 weeks	4 weeks
Liver Composition (%)				
Moisture	69.9 ± 0.4[3]	69.2 ± 0.4[3]	72.2 ± 0.2[3]	71.8 ± 0.3[3]
Nitrogen	2.51 ± 0.02	2.63 ± 0.04	2.53 ± 0.03	2.80 ± 0.03
Fat	22.8 ± 0.8	20.2 ± 1.0	13.7 ± 0.4	12.3 ± 0.6
Serum Proteins (%)				
α_1 globulin	13.2 ± 0.5	12.0 ± 0.8	9.9 ± 0.3	11.2 ± 0.8
α_2 globulin	10.8 ± 0.7	8.5 ± 0.5	8.8 ± 0.6	8.3 ± 0.4
β globulin	15.0 ± 0.5	13.8 ± 0.4	12.7 ± 0.5	13.1 ± 0.5
γ globulin	4.3 ± 0.9	13.7 ± 1.9	3.7 ± 0.9	12.4 ± 1.6
[albumin	56.8 ± 2.0	52.0 ± 1.9	65.0 ± 1.7	55.2 ± 2.3

[1] Corn oil was hydrogenated to an iodine value of 74 by Proctor and Gamble.

[2] Fat content of both diets = 30% w/w.

[3] Standard error of the means.

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PART II

EFFECT OF TWO DIET FATS ON SELECTED ENZYME AND COENZYME SYSTEMS IN THREONINE DEFICIENT RATS

INTRODUCTION

Changes in certain enzyme and coenzyme systems have been demonstrated in fatty livers induced in rats by a threonine deficient diet containing 5% of corn oil. Endogenous oxidation is reduced in livers from threonine-deficient rats (Harper et al., 1953; and Arata et al., 1954), as is the activity of the fatty acid oxidase system (Arata et al., 1964). Arata and her co-workers (1964) also reported that liver pyridine nucleotides were lower in threonine-deficient animals. Other reports confirm this observation (Arata et al., 1956; and Singal and Littlejohn, 1963). However, Falcone et al. (1962) reported no decrease in pyridine nucleotides (expressed as µM/mg N) in either choline- or threonine-deficient rats. In a later experiment the same group (Methfessel et al., 1964a) did observe decreased levels of pyridine nucleotides in fatty livers from choline- and threonine-deficient rats when the pyridine nucleotides were calculated on a per gram liver basis.

It was established in the preceding section that animals fed a threonine deficient diet containing 30% hydrogenated corn oil do not develop fatty livers whereas animals fed the identical diet containing 30% corn oil do develop fatty livers. Thus, by judicious choice of diet fat one can control the appearance of fatty livers in threonine-deficient rats. The purpose of this experiment was to determine the extent to which the nature of the diet fat altered the enzymatic changes associated with threonine deficiency. By comparing activities of selected enzyme systems in threonine-deficient rats fed corn oil with those in deficient animals fed hydrogenated corn oil, it should be possible to differentiate those enzyme changes which are more closely associated with the amino acid deficiency *per se* and which are more closely related to the disruption of fat metabolism.

In the experiment reported here, rats were fed a 9% casein diet containing either corn oil or hydrogenated corn oil and deficient in threonine. Control groups received the same diets supplemented with 0.36% DL-threonine. The effect of both type of dietary fat and the threonine deficiency on growth, liver fat, endogenous oxidation, fatty acid oxidase activity and liver pyridine nucleotides was studied at intervals over a period of 6 weeks. Since serum cholesterol levels are known to vary with the type of dietary fat, determinations of both total and esterified cholesterol were also made.

METHODS

The composition of the basal diet used in this experiment is identical with that described in the preceding section.

Diet 1. corn oil (basal)

Diet 2. corn oil + 0.36% DL-threonine

Diet 9. hydrogenated corn oil

Diet 10. hydrogenated corn oil + 0.36% DL-threonine.

The animals were divided into groups of ten. Immediately upon arrival 15 rats were sacrificed. After 2, 4, and 6 weeks on the experimental ration, 5 animals from each group were sacrificed and blood samples taken as previously described for electrophoretic determination of serum proteins. Sera were also analysed for total and esterified cholesterol according to the method of Haung _et al._, (1963) with the following modifications: 7.5 ml of the fat solvent [a mixture of 95% ethyl alcohol-acetone-ether (6:3:1) by volume] was added to 0.5 ml serum. A 5 ml aliquot was taken for the ester determination and 0.5 ml tomatine reagent was added to this aliquot. Livers were excised and analysed for moisture, fat and nitrogen as described in Part I.

At the same time, the remaining animals in each group were sacrificed and livers removed for determination of endogenous oxidation, fatty acid oxidase activity and liver pyridine nucleotides. The livers were rapidly removed and chilled in ice. A portion of the liver (500 - 800 mg) was placed in a tared weighing bottle containing 20 mg $Ce(SO_4)_2$ in 10 ml of 2% nicotinamide solution. The exact weight of the sample was obtained by difference and the liver pyridine nucleotides determined according to the method of Robinson _et al._ (1947).

A second sample of the liver was used to determine the activity of fatty acid oxidase system and endogenous oxidation. These systems were measured manometrically using the Warburg apparatus. The method of Lehninger (1955) was modified so that one ml of whole homogenate (33.3%) replaced 0.5 ml of mitochondrial suspension.

Standard errors of the mean were calculated for all data. Significant differences were calculated by student's "t" test. Except where noted those differences cited in the text as being significant were significant at the 1% level ($P < 0.01$).

RESULTS

Threonine-deficient animals fed either type of fat (groups 1 and 9) showed no differences in food intake over the experimental period (table 1). Rats receiving the threonine supplement likewise showed no significant differences in the amount of food eaten regardless of type of fat fed (groups 2 and 10). Adding threonine to these diets significantly increased food consumption after 4 weeks in those animals fed hydrogenated corn oil and after 6 weeks in those rats fed the diets containing corn oil.

The differences in food intake were reflected in the growth patterns of the animals (table 2). The addition of threonine produced significant increases in growth after 4 weeks with the hydrogenated corn oil diet (groups 9 _vs_ 10) and after 6 weeks with the corn oil diet (groups 1 _vs_ 2). Substituting hydrogenated corn oil for corn oil in threonine-deficient diets had no effect on growth (groups 1 _vs_ 9).

Livers from animals fed the hydrogenated corn oil diets in general had a significantly greater moisture content than livers from rats fed the corn oil rations (groups 1 _vs_ 9, 2 _vs_ 10; table 3). Rats receiving the unsupplemented hydrogenated corn oil diets (group 9) had a significantly higher percent of liver moisture than did the threonine supplemented control animals (group 10) after 2, 4, and 6 weeks of the experiment.

Supplementing either the corn oil or hydrogenated corn oil diets with threonine significantly increased the percent nitrogen in the liver (groups 1 _vs_ 2, 9 _vs_ 10) at each time period studied (table 3). Feeding either of the hydrogenated corn oil diets for 6 weeks resulted in higher liver nitrogen values than feeding the respective corn oil control diets (groups 1 vs 9, and 2 vs 10) for the same period.

Livers from rats fed the hydrogenated corn oil diets had significantly less fat than livers from rats on the corn oil rations regardless of whether or not they were threonine deficient (groups 1 vs 9, 2 vs 10; table 3). Adding threonine to the hydrogenated corn oil diet did not significantly lower liver fat levels (group 9 vs 10) although a trend towards lower liver fats in the supplemented rats was observed in every period. Animals fed the threonine-deficient corn oil diet for 4 weeks had a significantly higher level of liver fat than the group fed the supplemented corn oil diet (group 1 vs 2).

Pyridine nucleotide data are reported in table 4. Liver pyridine nucleotides were significantly reduced in threonine-deficient rats fed diets containing either type of fat for 2 weeks as compared with their respective controls (groups 1 vs 2, 9 vs 10). This difference between the supplemented and unsupplemented groups persisted for 4 weeks in animals fed the unsupplemented corn oil diet (group 1 vs 2). After 6 weeks, pyridine nucleotide concentrations in all animals had returned to normal values. The type of fat included in the diet produced no significant difference in pyridine nucleotide levels in rats fed either the supplemented or unsupplemented diets (groups 1 vs 9, 2 vs 10).

The activities of the fatty acid oxidase system and endogenous oxidation are reported in table 5. The addition of threonine to either the corn oil or hydrogenated corn oil diet caused no significant change in endogenous oxidation (groups 1 vs 2, 9 vs 10). Livers from rats fed diets supplemented with threonine had similar values for endogenous oxidation regardless of type of fat used (group 2 vs 10). In animals fed the threonine-deficient, corn oil diet (group 1), endogenous oxidation increased more slowly than in the threonine deficient hydrogenated corn oil group (group 9) for the first 4 weeks of the experiment. However, after 6 weeks on the experimental ration, endogenous oxidation was higher in group 1 than group 9.

The differences observed in fatty acid oxidase activity between groups were most marked at 4 weeks. The activity of this system, which was decreasing slowly from zero time in all four groups, decreased precipitously in groups 9 and 10 between the second and fourth weeks of the experiment. A similar precipitous decline in fatty acid oxidase activity was not observed in the corn oil fed groups (1 and 2). The addition of threonine to rats fed either corn oil (groups 1 vs 2) or hydrogenated corn oil (groups 9 vs 10) had no significant effect on the activity of the fatty acid oxidase system in liver tissues.

Serum protein patterns (table 6) showed little variation between groups although serum albumin levels tended to be higher in animals receiving the hydrogenated corn oil diets regardless of whether threonine was supplemented or not (groups 1 vs 9, 2 vs 10).

The animals receiving either of the threonine-deficient diets had significantly lower total and esterified serum cholesterol after 2 weeks on the experiment than did the threonine supplemented controls (groups 1 vs 2, 9 vs 10; table 7). This difference between control and threonine deficient animals had disappeared by the fourth week. The nature of the diet fat appeared to exert a more lasting effect on serum cholesterol levels. For the duration of the experiment, animals fed the hydrogenated corn oil diets (groups 9 and 10) had lower cholesterol values than did the rats fed the corn oil diets (groups 1 and 2) regardless of whether threonine was or was not supplemented.

DISCUSSION

The results of this experiment indicate threonine deficiency and the type of dietary fat have somewhat different effects on the parameters measured.

The type of dietary fat did not influence either the amount of food eaten or the weight gained by the animals fed either a threonine-deficient or threonine-supplemented diet. Therefore, the different biochemical responses to the nature of the diet fat observed in this experiment were not simple reflections of a different food consumption and/or growth rate between groups. However, the increased food consumption and growth in threonine supplemented rats, as compared with the unsupplemented controls, is supportive of the existence of a threonine deficiency in groups 1 and 9. While rats in both groups were threonine deficient, fatty livers appeared only in group 1, whereas fat concentrations in livers from rats in group 9 were not elevated throughout the 6 week study (figure 1). Therefore, an accumulation of fat in the liver is not necessarily a reflection of a threonine deficiency. These data also refute the hypotheses (Sidransky and Clark; 1961) that fatty livers appear in threonine deficient animals because of the decreased food intake and concomitant disruption of the calorie-amino acid ratio. Since the food intake of rats in group 1 was not significantly different than that of rats in group 9, the appearance of fatty livers in group 1 and not in group 9 cannot be explained on the basis of food intake.

The observation that the addition of 0.36% DL-threonine to corn oil fed rats (group 1) did not completely reduce liver fat levels to normal was unexpected. The high quantity of corn oil in this diet may have increased either the threonine or the choline requirement of the animal (see Part III).

The liver pyridine nucleotide data show a marked correlation with the state of threonine nutrition of the animal. The pyridine nucleotide levels were depressed in both groups of rats fed threonine-deficient diets. This depression was most severe after two weeks of the experiment. In the subsequent weeks, the pyridine nucleotide concentrations in the deficient rats returned to levels comparable with the controls (figure 2). These data are in agreement with Arata _et al_. (1956), Arata _et al_. (1964), Singal and Littlejohn (1963) and Methfessel _et al_. (1964a), who also found decreased pyridine nucleotide levels in threonine deficient rats after 2 weeks.

There was no marked response in endogenous oxidation to either threonine deficiency or type of dietary fat. Nor were significant differences due to either threonine deficiency or type of dietary fat observed in fatty acid oxidase activity. The activity of this enzyme was depressed in all groups from zero time values (figure 3). The marked reduction in fatty acid oxidase activity in animals fed either of the hydrogenated corn oil diets for 4 weeks is as yet unexplained. The data on fatty acid oxidase activity and endogenous oxidation do not agree with other reports in the literature. Harper _et al_. (1953), Arata _et al_. (1954), and Arata _et al_. (1964), all reported decreases in these systems in threonine-deficient rats. However, the higher fat levels used in this experiment (30% _vs_ 5%) may be altering the animals response to the threonine deficient diet.

The six-fold increase in fat content of these diets as compared to those used by Arata et al. (1964) might cause a substrate inhibition-like phenomenon on the activity of the fatty acid oxidase system. However, since inhibiting the activity of the fatty acid oxidase system does not result in an increased liver fat concentration in all groups, these data lend support to the suggestion by Tesluk and Stewart (1964) that elevated liver fat concentrations induced by low protein diets do not result from interference with the fat oxidative pathway but rather from interference with fat transport out of the liver.

The changes in serum protein fractions with time which were noted in the preceding section were not observed in this experiment. Neither threonine deficiency nor the source of dietary fat appeared to appreciably change the proportions of the various fractions.

The levels of serum cholesterol appear to be influenced both by the type of fat in the diet, and by threonine deficiency. The former appeared to be a more important factor in the control of serum cholesterol while threonine exerted a more transient effect. The animals fed either of the hydrogenated corn oil diets had consistently lower levels of both total and esterified cholesterol than did the animals fed corn oil. The addition of threonine to diets containing either type of fat increased cholesterol levels after 2 weeks on the diets, but this effect was not observed after 4 weeks. The cholesterol data are complicated by the high levels of serum cholesterol in the zero time animals and the large proportion of esterified cholesterol in all groups after 4 weeks on the diets. In these animals, virtually all the cholesterol is in the ester form. The data are in conflict with a series of published reports wherein unsaturated fats tend to lower serum cholesterol levels below those established with more saturated fats. The combination of low protein, high fat content of the diets used in these experiments must somehow modify the action of unsaturated fatty acids in lowering serum cholesterol levels.

Summary:

Thus threonine deficiency has a definite effect on growth, liver composition, liver pyridine nucleotide levels and, to a lesser extent, on serum cholesterol in rats. The type of fat in the diet has a marked effect on liver fat and serum cholesterol concentrations. The slow decline in fatty acid oxidase activity in all groups with time is suggestive of a substrate inhibition. However, the more marked decline after 4 weeks in hydrogenated fat-fed rats suggests an influence on this system by diet fat which is additive to that exerted by the total fat content of the diet.

Table 1. Food intake in gms/wk of rats fed diets containing either corn oil or hydrogenated corn oil with and without threonine supplements for 2 to 6 weeks.

	Diet	2 weeks	4 weeks	6 weeks
1	CO[1]	70 ± 1[2]	82 ± 3[2]	87 ± 2[2]
2	CO + threonine	80 ± 4	89 ± 2	97 ± 2
9	HCO	68 ± 3	81 ± 2	89 ± 2
10	HCO + threonine	69 ± 3	90 ± 1	98 ± 2

[1] CO = corn oil.
HCO = hydrogenated corn oil.

[2] Standard error of the mean.

Table 2. Weight gain in gms/wk of rats consuming diets containing either corn oil or hydrogenated corn oil with and without threonine supplements.

	Diet	2 weeks	4 weeks	6 weeks
1	CO[1]	25 ± 1[2]	25 ± 1[2]	24 ± 1[2]
2	CO + threonine	26 ± 2	28 ± 1	29 ± 1
9	HCO	25 ± 2	24 ± 1	25 ± 1
10	HCO + threonine	24 ± 2	29 ± 1	29 ± 1

[1] CO = corn oil.
HCO = hydrogenated corn oil.

[2] Standard error of the mean.

Table 3. Liver composition of rats fed diets containing either corn oil or hydrogenated corn oil with and without threonine supplements.

Weeks on Diet	Diet 1[1]			Diet 2[1]		
	% H_2O	% N[2]	% Fat[3]	% H_2O	% N[2]	% Fat[3]
0	75.7±1.1[4]	2.45±0.05[4]	11.0±0.6[4]	75.7±1.1[4]	2.45±0.05[4]	11.0±0.6[4]
2	71.8±0.7	2.15±0.06	20.6±1.6	69.4±0.3	2.52±0.04	18.5±0.9
4	69.3±0.7	2.15±0.03	27.8±3.0	68.3±0.3	2.77±0.03	15.8±0.6
6	71.1±0.5	2.24±0.02	21.9±1.8	68.3±0.5	2.76±0.04	19.3±1.5
	Diet 9			Diet 10		
0	75.7±1.1	2.45±0.05	11.0±0.6	75.7±1.1	2.45±0.05	11.0±0.6
2	75.0±0.3	2.31±0.02	12.4±0.6	71.1±0.9	2.55±0.05	9.7±0.7
4	74.6±0.5	2.30±0.04	11.8±0.3	70.5±0.2	2.79±0.03	9.7±0.7
6	74.0±0.5	2.48±0.04	12.5±0.7	70.7±0.1	3.02±0.02	10.0±0.7

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[1] Diet 1 = corn oil.
Diet 2 = corn oil + threonine.
Diet 9 = hydrogenated corn oil.
Diet 10 = hydrogenated corn oil + threonine.

[2] % nitrogen calculated on wet weight of liver.

[3] % fat calculated on dry weight basis.

[4] Standard error of the mean.

Table 4. Liver pyridine nucleotides in µg per gm liver in rats fed diets containing either corn oil or hydrogenated corn oil with and without threonine supplements.

	Diet	weeks 0	weeks 2	weeks 4	weeks 6
1	CO[1]	1121 ± 73[2]	738 ± 32[2]	736 ± 29[2]	1147 ± 72[2]
2	CO + threonine	1121 ± 73	1205 ± 93	1013 ± 67	930 ± 77
9	HCO	1121 ± 73	639 ± 69	767 ± 83	866 ± 72
10	HCO + threonine	1121 ± 73	1148 ± 70	815 ± 60	907 ± 67

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[1] CO = corn oil.
HCO = hydrogenated corn oil.

[2] Standard error of the mean.

Table 5. Endogenous oxidation and fatty acid oxidase activity in rats fed corn oil or hydrogenated corn oil diets with and without threonine supplements.

Weeks on Diet	Diet 1[1]		Diet 2		Diet 9		Diet 10	
	Fatty Acid[2] Oxidase	Endo. Oxidation[3]	Fatty Acid Oxidase	Endo. Oxidation	Fatty Acid Oxidase	Endo. Oxidation	Fatty Acid Oxidase	Endo. Oxidation
0	614±50[4]	1164±72[4]	614±50[4]	1164±72[4]	614±50[4]	1164±72[4]	614±50[4]	1164±72[4]
2	448±66	1289±212	494±55	1674±73	318±85	1649±106	524±55	1701± 7
4	449±69	1505±64	359±50	1728±61	197±66	1872±134	299±40	1800±41
6	317±81	1958±72	430±50	1642±115	396±111	1634±37	419±75	1701±98

[1] Diet 1 = corn oil.
Diet 2 = corn oil + threonine.
Diet 9 = hydrogenated corn oil.
Diet 10 = hydrogenated corn oil + threonine.

[2] Fatty acid oxidase activity calculated on µl O_2/hr/gm tissue.

[3] Endogenous oxidation calculated on µl O_2/hr/gm tissue.

[4] Standard error of the mean.

Table 6. % Serum electrophoresis data for rats fed corn oil or hydrogenated corn oil diets with and without threonine supplements.

	Zero Time					2 Weeks				
Diets	γ	β	α_2	α_1	Alb	γ	β	α_2	α_1	Alb.
1[1]	4.5±.2[2]	14.3±.3[2]	8.3±.3[2]	9.5±.3[2]	63.9±.8[2]	4.6±.3[2]	16.1±1.5[2]	13.0±.5[2]	16.2±.9[2]	50.2±2.4[2]
2	4.5±.2	14.3±.3	8.3±.3	9.5±.3	63.9±.8	4.8±.4	15.4±.6	12.5±.7	13.4±.5	54.0±1.1
9	4.5±.2	14.3±.3	8.3±.3	9.5±.3	63.9±.8	3.8±.5	13.0±2.0	12.8±1.4	12.3±1.1	58.3±4.7
10	4.5±.2	14.3±.3	8.3±.3	9.5±.3	63.9±.8	3.5±.4	13.0±.9	11.7±.5	12.1±1.0	59.9±1.8
	4 Weeks					6 Weeks				
1	5.8±.04	13.0±.6	12.6±.6	15.1±.5	53.6±.9	5.9±.6	13.0±.6	10.6±.6	16.6±.9	54.0±2.0
2	4.2±.5	14.1±.3	10.3±.5	13.4±.4	58.1±.8	4.5±.7	12.1±.4	10.2±.3	13.3±.2	60.0±1.2
9	4.7±.6	10.0±.5	10.7±.8	12.3±.5	62.5±1.7	5.0±.4	11.9±.2	10.1±.3	12.7±.5	60.5±.7
10	3.7±.3	11.6±.9	10.4±.6	13.4±.4	61.1±.9	4.2±.3	10.9±.5	10.9±.3	11.9±.6	62.3±1.3

[1] Diet 1 = corn oil. Diet 2 = corn oil + threonine. Diet 9 = hydrogenated corn oil. Diet 10 = hydrogenated corn oil + threonine.

[2] Standard error of the mean.

Table 7. Serum cholesterol in rats fed corn oil or hydrogenated corn oil diets with and without threonine supplements.

	Zero Time			2 Weeks		
Diet	Total[1]	Ester[2]	%[3]	Total[1]	Ester[2]	%[3]
1. corn oil	158±8[4]	131±4[4]	83	150±8[4]	132±4[4]	88
2. corn oil + threonine	158±8	131±4	83	185±4	166±7	90
9. hydro. corn oil	158±8	131±4	83	103±7	65±4	63
10. hydro. corn oil + threonine	158±8	131±4	83	152±11	133±8	88
	4 Weeks			6 Weeks		
1. corn oil	138±5	134±5	97	139±6	119±4	86
2. corn oil + threonine	147±8	148±7	101	152±10	141±7	93
9. hydro. corn oil	124±6	111±2	90	121±4	99±5	82
10. hydro. corn oil + threonine	120±6	125±7	104	112±6	102±6	91

[1] Total cholesterol in mg/100 mls serum.

[2] Cholesterol ester in mg/100 mls serum.

[3] Percent ester of total cholesterol.

[4] Standard error of the mean.

Figure 1. Changes in liver fat with time in rats fed corn oil or hydrogenated corn oil with and without threonine supplements.

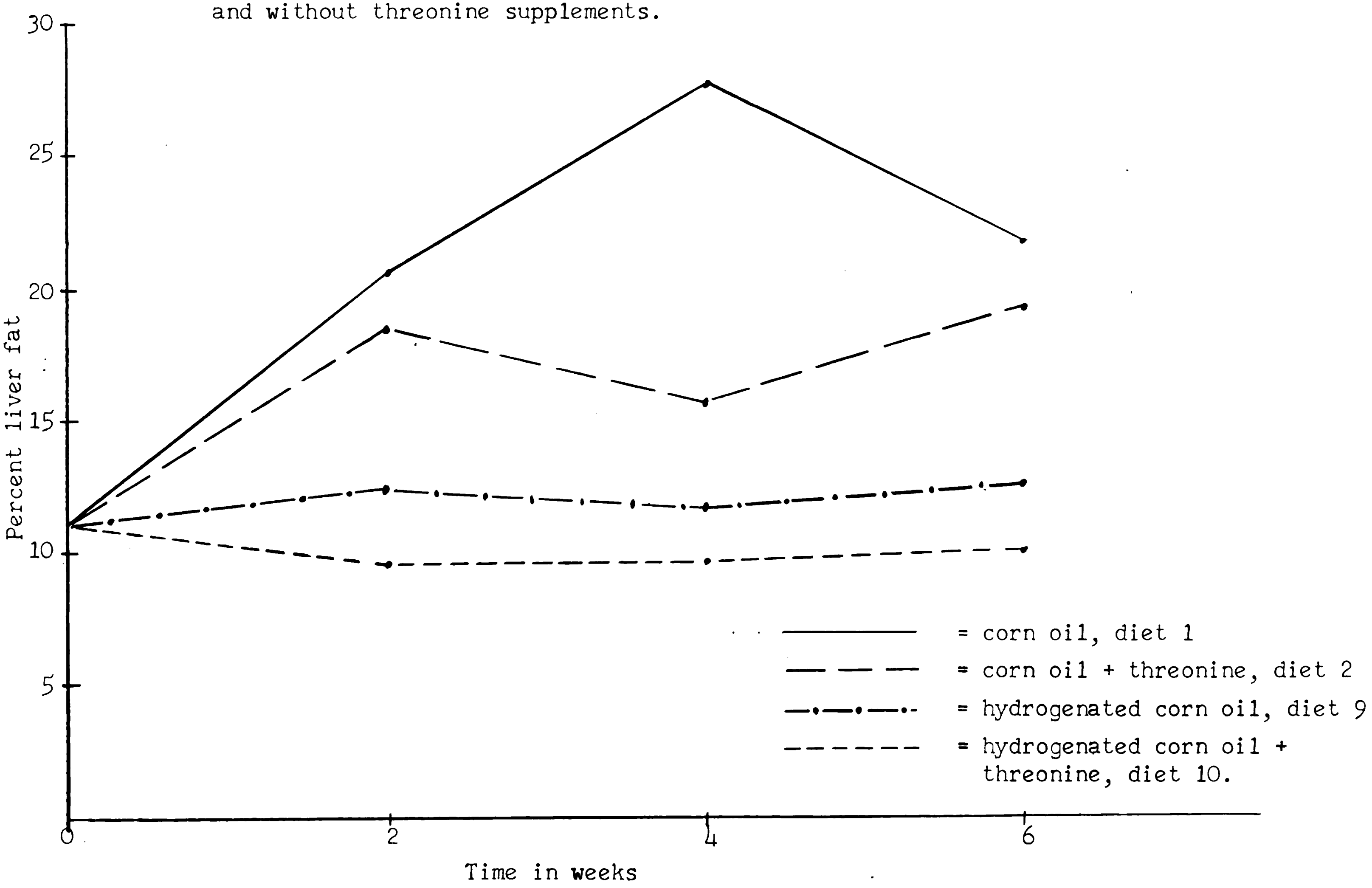


Figure 2. Changes in liver pyridine nucleotides with time in rats fed corn oil or hydrogenated corn oil diets with and without threonine supplements.

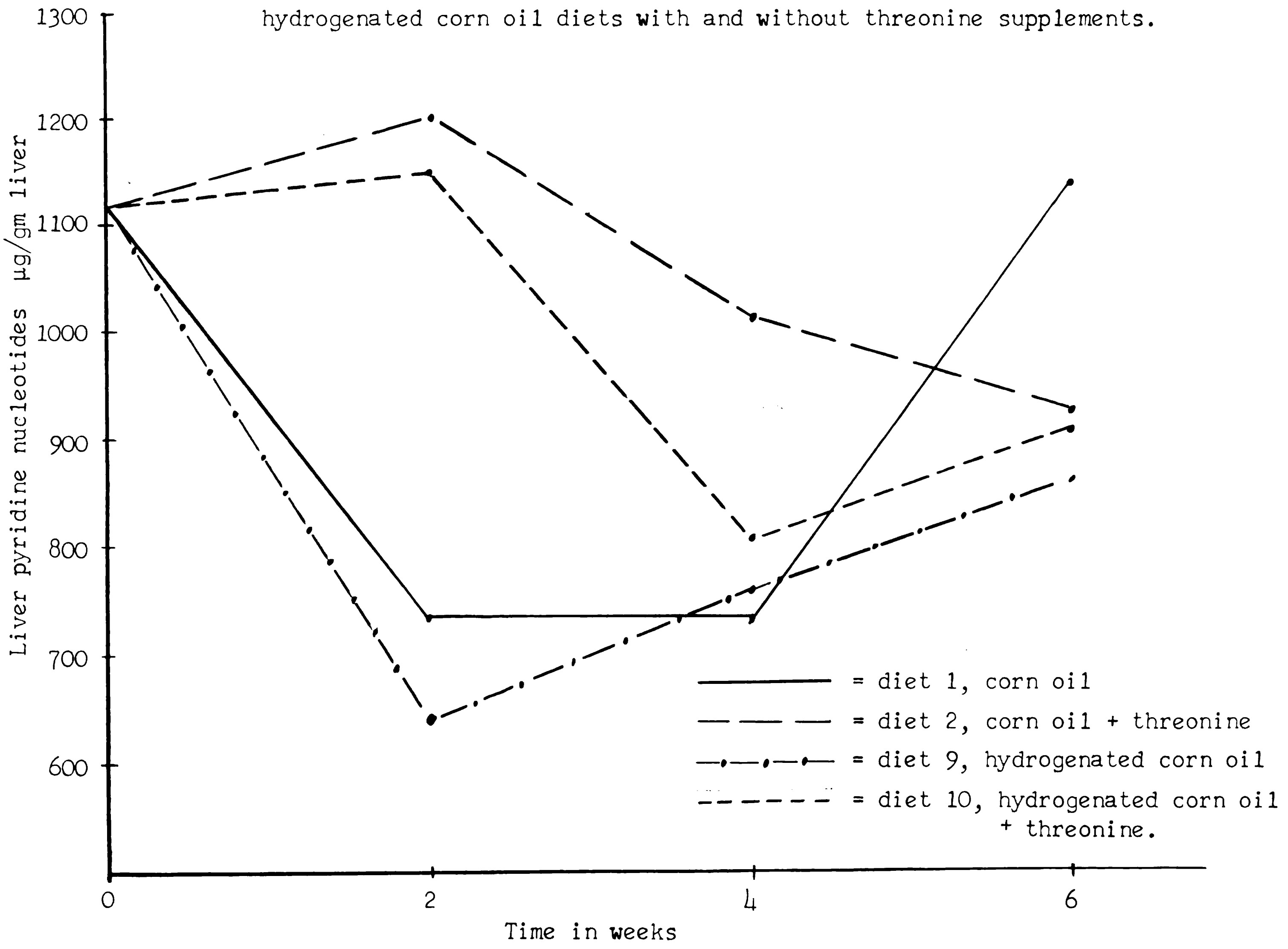
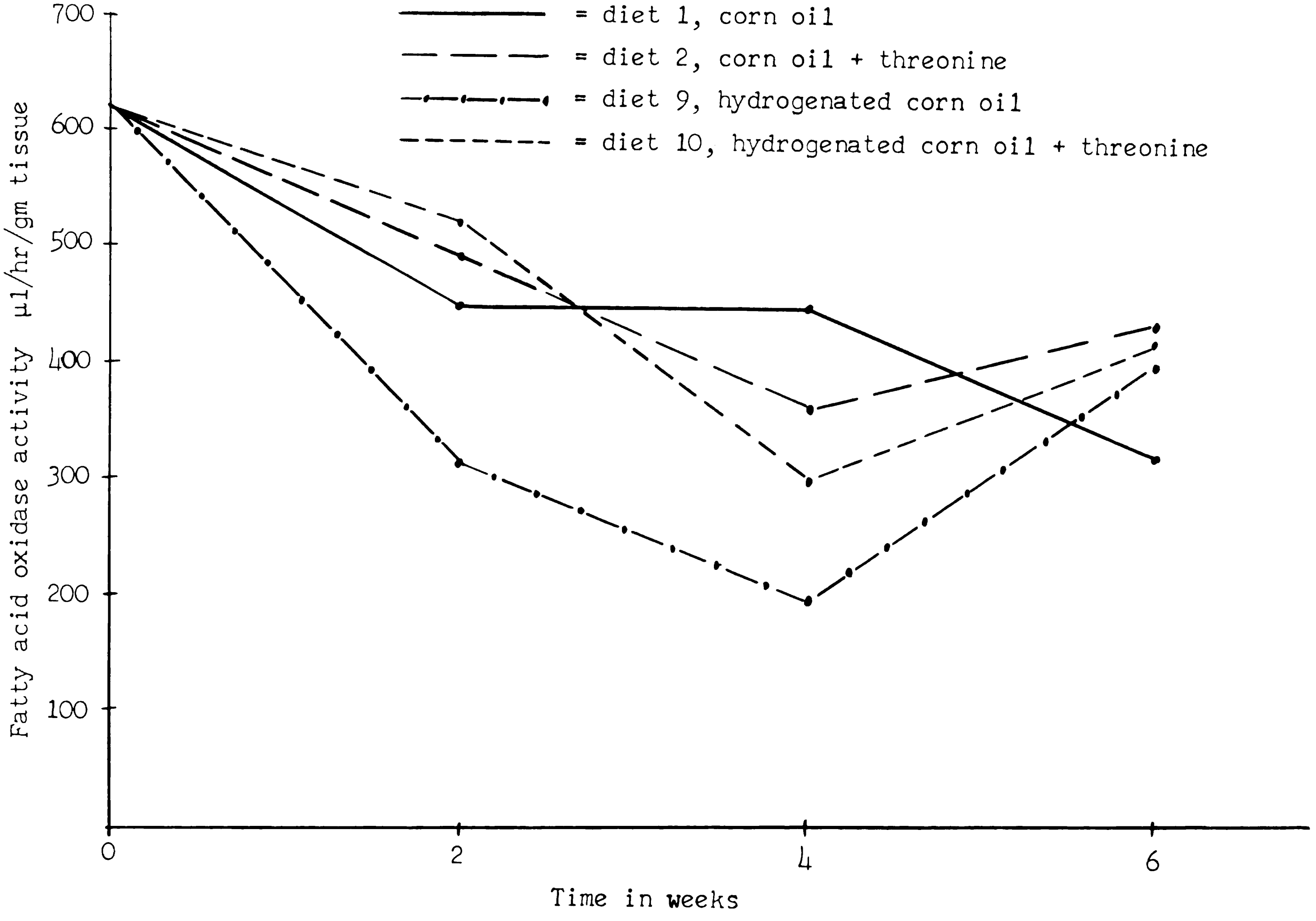


Figure 3. Changes in fatty acid oxidase activity with time in rats fed corn oil or hydrogenated corn oil diets with and without threonine supplements.



PART III

EFFECT OF CHOLINE ON FATTY LIVERS INDUCED IN THREONINE DEFICIENT RATS FED CORN OIL

INTRODUCTION

During the course of the preceding study, the possibility that a choline deficiency might be complicating the experiment was proposed. When dietary fat is at the 5% level, 0.15% choline is adequate for normal growth and liver fat. However, under the dietary conditions reported here (30% fat), 0.15% choline may not meet the rat's requirement since the requirement for choline does increase with increased diet fat (Griffith, 1948). Benton et al. (1957) have also shown that the choline requirement varies with the type of fat fed. In addition to these considerations, the data presented in Part II combined with those of Tesluk and Stewart (1964) suggest a disruption in fat transport out of the liver as being a controlling factor in the appearance of fatty livers in low-protein or choline-induced fatty livers. Since choline is intimately tied to transport of fat, the effect of this compound in corn oil containing diets must be measured.

The experiment reported here was a preliminary one to determine if an increased choline level could reduce liver fat levels in rats fed threonine-deficient, corn oil containing diets.

METHODS

The diets used in this experiment were identical to those of the previous studies except for the following modifications:

Diet 1. corn oil (basal)

Diet 2. corn oil + 0.36% DL-threonine

Diet 11. corn oil + 0.50% choline

Diet 12. corn oil + 0.50% choline + 0.36% DL-threonine.

The basal diet contained 0.15% choline.

Ten animals were sacrificed at zero time and then 10 animals killed from each group after 2, 4, and 6 weeks of the experiment. However, since critical changes were observed at 4 weeks, only those data are presented here except in one instance as noted in the text. Methods of sacrificing and of collecting liver and blood samples have been described.

RESULTS

Increasing the choline in the diet from 0.15 to 0.50% had no effect on the amount of food consumed by the rats or on weight gain (group 1 *vs* 11; table 1). Adding threonine to diets containing either level of choline significantly increased weight gain (groups 1 *vs* 2, 11 *vs* 12).

The results of liver analysis are given in table 2. Animals receiving 0.50% choline had higher levels of liver moisture than did control animals (groups 1 *vs* 11, 2 *vs* 12). Neither the increase in choline nor the addition of threonine increased the percent of liver nitrogen (groups 1 *vs* 2, 11 *vs* 12, 1 *vs* 11, 2 *vs* 12). Increasing the choline level to 0.50% significantly lowered liver fat in threonine deficient rats (groups 1 *vs* 11). However, when additional threonine was present in the diet, (groups 2 and 12) increasing the choline had no significant effect on liver fat concentration.

When 0.36% DL-threonine was added to the diet containing 0.15% choline (groups 1 and 2), liver fat levels were significantly reduced as expected. When the same quantity of threonine was added to the diet containing 0.50% choline (groups 11 and 12), liver fat levels were not reduced at the end of 4 weeks. Under these conditions, the reversal of fatty livers by threonine was not observed until the sixth week when threonine decreased percent liver fat from 16.2±0.8 to 12.8±0.5. Thus after 6 weeks, liver fat levels in the threonine-supplemented, high choline group (12) had returned to approximately normal while the threonine-deficient rats still had fatty livers.

The serum protein patterns were similar in all groups for the entire experiment (table 3). No effect of either increased choline or threonine supplementation could be demonstrated.

Significant differences in serum cholesterol were not observed in this experiment (table 4). Although the animals receiving more choline (groups 11 and 12) tended to have higher cholesterol both total and ester than their low choline controls (groups 1 and 2), these differences were not statistically significant.

DISCUSSION

Increasing the choline content of the diet from 0.15% to 0.50% of the diet resulted in a lower concentration of fat in liver tissues in threonine deficient animals. The further addition of 0.36% DL-threonine to the diet containing 0.50% choline caused no further reduction in liver fat. Therefore under the conditions of this experiment liver fat levels may be reduced by the addition of either threonine or choline. Since the effects of threonine and choline supplements are not additive, the threonine deficiency may adversely affect the synthesis of choline either directly or indirectly. These data do not refute the theory of Tesluk and Stewart (1964) who suggested that it is an interference in fat transport rather than a change in fat oxidation which produces fatty livers.

The data presented here could be explained by proposing different routes of metabolism for different dietary fats. Some diet fats may, upon absorption, go directly to the fat depots which they more closely resemble in composition; while other fats may be transported to the liver for chemical alteration before they can be transported to the depots. Under these conditions, some fats would put a greater stress on the fat metabolizing, particularly the transporting, systems of the liver than others. The observation by Benton *et al*. (1957) that choline requirements vary with the type of dietary fat would support this theory. The theory is also compatible with the differences observed in response of rats fed various types of dietary fat to threonine deficiency (Parts I and II).

Table 1. Food intake and weight gains of rats fed 30% corn oil diets containing two levels of choline chloride with and without threonine supplements for four weeks.

	Diet	Food Intake grams/week	Weight Gain grams/week
1	0.15% choline	73 ± 2[1]	20 ± 1[1]
2	0.15% choline + threonine	82 ± 2	27 ± 1
11	0.50% choline	69 ± 2	20 ± 1
12	0.50% choline + threonine	76 ± 2	25 ± 1

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[1] Standard error of the mean.

Table 2. Liver composition of rats fed 30% corn oil diets containing two different levels of choline chloride with and without threonine supplements for four weeks.

	Diet	% Moisture	% Nitrogen[1]	% Fat[2]
1	0.15% choline	67.0 ± 0.6[3]	2.94 ± 0.01[3]	23.2 ± 0.4[3]
2	0.15% choline + threonine	68.2 ± 0.3	3.11 ± 0.07	17.2 ± 1.3
11	0.50% choline	69.1 ± 0.2	3.04 ± 0.04	14.6 ± 0.7
12	0.50% choline + threonine	69.8 ± 0.3	3.02 ± 0.03	14.2 ± 0.5

[1] Percent nitrogen was calculated on a wet weight basis.

[2] Percent fat was calculated on a dry weight basis.

[3] Standard error of the mean.

Table 3. Serum electrophoresis data for rats fed 30% corn oil diets containing two different levels of choline chloride with and without threonine supplements for four weeks.

Diet		Globulins %				Albumin %
		γ	β	α_2	α_1	
1	0.15% choline	3.7± 0.2[1]	13.9± 0.6[1]	11.8± 0.3[1]	16.5± 0.7[1]	54.2± 1.2[1]
2	0.15% choline + threonine	3.2± 0.2	13.4± 0.5	12.3± 0.7	13.0± 0.9	58.2± 1.5
11	0.50% choline	3.8± 0.5	13.2± 0.3	12.1± 0.5	15.0± 0.4	56.1± 1.0
12	0.50% choline + threonine	3.0± 0.2	13.8± 0.3	9.7± 0.2	17.0± 0.5	56.6± 0.5

[1] Standard error of the mean

Table 4. Serum cholesterol values for rats fed 30% corn oil diets containing two different levels of choline chloride with and without threonine supplements for four weeks.

Diet		Cholesterol Total mg/100 ml	Cholesterol Ester mg/100 ml	%[1]
1	0.15% choline	150 ± 3[2]	133 ± 4[2]	89
2	0.15% choline + threonine	154 ± 7	130 ± 6	84
11	0.50% choline	163 ± 7	141 ± 4	87
12	0.50% choline + threonine	181 ± 4	154 ± 3	85

[1] Percent of total represented by esterified cholesterol.

[2] Standard error of the mean.

GENERAL SUMMARY AND CONCLUSIONS

Male weanling albino rats of the Sprague-Dawley strain were fed diets containing 9% casein and 30% fat. The effect of the following constituents of the diet on fatty livers was studied: 1) type of fat; 2) choline; 3) threonine. Food intake, weight gains, liver moisture, fat and nitrogen analysis and the electrophoretic determination of serum proteins were done for all animals. In one experiment, liver pyridine nucleotides and the activity of the fatty acid oxidase system and endogenous oxidation were measured in liver tissues. Determinations of total and esterified serum cholesterol were made in the last two experiments.

The following observations were made:

1. Liver fat levels do not appear to be a function of food intake or weight gain.

2. By the judicious choice of the dietary fat, one can control liver fat levels in either threonine-deficient or threonine-supplemented rats.

3. Of the fats tested, hydrogenated fats tended to be more protective against the fatty livers associated with threonine deficiency and against elevated serum cholesterol levels.

4. Threonine deficiency had a definite effect on growth, liver composition, liver pyridine nucleotides and to a lesser extent serum cholesterol.

5. There was no marked response in endogenous oxidation or fatty acid oxidase activity to either a threonine deficiency or the type of dietary fat.

6. In animals fed 30% corn oil diets, increasing the choline content of the diet from 0.15% to 0.50% or supplementing the diet with threonine lowers liver fat levels. However, the effects of the threonine and choline supplements are not additive.

These observations do not refute the theory of Tesluk and Stewart (1964) who suggested that it is an interference in fat transport rather than a change in fat oxidation which produces fatty livers.

These observations could be explained by proposing different routes of metabolism for different dietary fats. Some diet fats may, upon absorption, go directly to the fat depots which they more closely resemble in composition, while other fats may be transported to the liver for chemical alteration before they can be transported to the depots. Under these conditions, some fats would put a greater stress on the fat metabolizing, particularly the transporting, systems of the liver than others. The observation by Benton _et al_. (1957) that choline requirements vary with the type of dietary fat would support this theory. The theory is also compatible with the differences observed in response of rats fed various types of dietary fat to threonine deficiency.

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