A STUDY OF FATTY LIVERS INDUCED IN RATS BY A THREONINE IMBALANCE WITH EMPHASIS ON ENZYME, COENZYME, AND LIVER FAT INTERRELATIONSHIPS

by

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AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Foods and Nutrition
College of Home Economics

1960

Approved Dorothy Arata

ABSTRACT

Fatty livers were produced in male, weanling rats by feeding a low protein diet deficient in threonine. Animals were sacrificed as specified intervals during experimental periods of 4 to 6 weeks. Livers were removed and assayed for fat, several apoenzymes, and labile and inorganic phosphorus.

When rats were fed a 9 per cent casein ration with no threonine supplement, fat accumulated in the liver to the extent of 20 to 30 per cent of the dry weight of tissue. Liver fat increased rapidly during the first two or three weeks, then either decreased or leveled off for the remainder of the experimental periods. Increasing the fat content of the threonine deficient diet from 5 to 20 per cent did not increase the amount of fat deposited in the liver, indicating that the accumulated liver fat was not of dietary origin.

Feeding weanling rats a 9 per cent casein ration deficient in threonine depressed the activity of several liver enzyme systems below that of control rats fed 9 per cent casein supplemented with 0.36 per cent DL-threonine. The activities of the xanthine oxidase and malic dehydrogenase systems in the threonine deficient rats were decreased maximally in 19 days, after which they began to recover. The rate of fatty acid oxidation in livers from threonine deficient rats declined precipitously for three weeks, then leveled off during the 4th week. The

activities of DPN-cytochrome c reductase and cytochrome oxidase did not appear to be a function of time, but were consistently depressed in the threonine deficient rats throughout the experimental periods.

A deficiency of threonine induced a significant depression in the ADP and ATP content of the liver. The decreased concentration of these cofactors was most marked at three weeks, then leveled off. Changes in labile phosphorus levels roughly paralleled changes in fatty acid oxidase activity determined in livers from the same rats.

In view of the marked fluctuations with time of the enzyme and coenzyme systems studied in these experiments, a time study was suggested to insure collection of data at periods of maximum difference between control and deficient groups of rats.

The changes in enzyme activity and in coenzyme concentration varied inversely with the fat content of the liver. As liver fat increased, the concentration of the enzyme and coenzyme systems decreased. In every system studied, the maximum depression in the activity of apoenzymes and in coenzyme levels occurred before the peak in liver fat deposition was attained. In systems where subsequent recovery was observed, the enzyme or coenzyme recovery took place before fat was mobilized out of the liver. These results suggest the biochemical lesions could be the causative factors in the development of fatty livers in rats fed a diet low in protein and threonine.

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A THESIS

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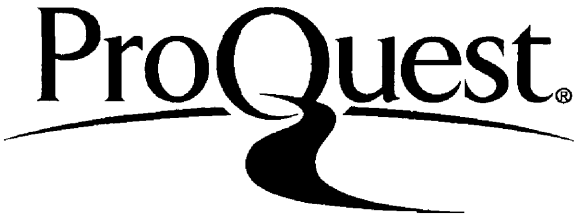

ACKNOWLEDGEMENTS

The author would like to express her deepest gratitude to Dr. Dorothy Arata, her major professor, for her never failing interest and encouragement, for the generosity with which she gave of her time and effort, for many thought-provoking discussions, and for her very stimulating guidance. She is especially indebted to Dr. Arata for a deeper insight into the meaning of research.

Her sincere thanks also go to Dr. Thelma Porter for first awakening a desire to do graduate work, and for her continued interest; and to Dr. Dena Cederquist for her always wise and friendly advice.

She would also like to thank Mrs. Marilyn Pearson for her ever cheerful cooperation in whatever task was to be done.

Finally, the author gratefully acknowledges receipt of the Marie Dye and the General Foods Fund Fellowships, which enabled her to complete the course requirements much more rapidly than would otherwise have been possible.

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Fatty livers were produced in weanling rats by feeding a low protein diet deficient in threonine. The supplementation of a 9 per cent casein diet with methionine and tryptophan made threonine the most limited amino acid (Harper, '59). Harper ('58) has demonstrated that such an "amino acid imbalance" causes not only a decreased efficiency of protein utilization, but also an increased requirement for the amino acid which has been made most limiting.

A threonine deficiency produces widespread metabolic disturbances in weanling rats: fat accumulates in the livers (Singal *et al.*, '53), the activities of several enzyme systems are markedly altered (Harper *et al.*, '53), and the metabolism of at least one coenzyme is disrupted (Arata *et al.*, '56). The mechanism by which a threonine deficiency effects these various metabolic lesions is not understood.

An investigation into the relationship between enzyme changes and accumulation of fat produced in livers of rats by an amino acid imbalance has yielded further information about the complicated interrelationships, *viz.* when a double amino acid deficiency was produced in rats by restricting tryptophan as well as threonine, the enzyme changes observed on threonine deficient diets did not occur, but fat

accumulated in the livers to the same extent as with the single deficiency (Arata et al., '54).

Fatty livers resulting from an amino acid imbalance are apparently produced by a different type of metabolic derangement than those resulting from choline deficiency or carbon tetrachloride poisoning. In the case of amino acid imbalance or protein deficiency, fat accumulates in the peripheral cells of the lobules, while choline deficiency or carbon tetrachloride poisoning causes centrolobular fat deposition (Glynn et al., '48; Hartroft, '50; Nino-Herrara et al., '54).

This series of experiments was undertaken in an attempt to correlate liver fat accumulation in threonine deficient rats with alterations in the activities of several enzyme systems and with changes in the concentration of several coenzymes. The variations in these cellular constituents were studied as a function of time.

Part I is a report of two experiments in which the activity of xanthine oxidase, malic dehydrogenase, and succinic oxidase enzyme systems were observed over a period of six weeks. In Part II, a study of the DPN-cytochrome c reductase and fatty acid oxidase enzyme systems, and of adenosine phosphate coenzymes is described. Part III includes data on the activity of the cytochrome oxidase enzyme system, and on the effects of altering the fat level of the threonine deficient ration. Liver fat data are included for all experiments.

REVIEW OF LITERATURE

"FATTY LIVERS"

In the livers of normal animals the fat present in the cells is not visible, but dispersed in a "concealed" form by emulsifying and stabilizing substances. Dixon ('58) uses the term "deposition" to describe the appearance of visible droplets of liquid fat and cholesterol esters in so-called "fatty livers." The concentration of fat in such livers is usually increased, although stainable fat droplets may be produced by a disruption of the dispersion mechanism without a concomitant rise in total lipid (Best et al. '55). The term "lipotropic" was first used by Best et al. ('35) to describe substances which "decrease the rate of deposition or accelerate the rate of removal of liver fat." For nearly thirty years a great deal of research has been directed towards elucidating the etiology of fatty livers. Many factors, both dietary and non-dietary, have been implicated in this syndrome.

LIPOTROPIC FACTORS

Choline

Best and Huntsman ('32) were the first to demonstrate the lipotropic effect of lecithin was due to its choline content. They showed the fatty infiltration produced in the livers of rats fed diets high in saturated fats could be prevented by choline supplementation. In order to determine

whether the intact choline molecule or only the labile methyl groups of choline were responsible for the lipotropic effect, Welch ('37) and Welch and Landau ('42) fed rats a high fat, low choline ration supplemented with arseno-choline. The lipotropic effect of the arsenic analogue was approximately equivalent to that of choline chloride, and lecithins isolated from the livers after 3 weeks' treatment contained significant amounts of arsenic. Arseno-choline contains no labile methyl groups, therefore the lipotropic activity of choline is not a function of the methyl portion of this molecule.

Methionine

A few years after their demonstration of the lipotropic activity of choline, Best and Huntsman ('35) found the fat content of livers was not regulated solely by the choline content of the diet. Casein, added to diets low in choline, produced a "choline-like action." They suggested that some of the amino acids of casein might be choline precursors. The lipotropic effect of protein was confirmed by Channon and Wilkinson ('35).

Tucker and Eckstein ('37) also investigated the possibility that substances providing precursors for choline synthesis *in vivo* might prevent liver fat accumulation caused by a deficiency of dietary choline. They established the fact that methionine acts as a lipotropic agent in rats fed choline-deficient diets. By supplementing low choline diets with deuterium-labeled methionine, du Vigneaud ('41) demonstrated that the methyl groups of methionine are

used for choline synthesis in the rat. A methionine-sparing action of choline was proposed by Engel ('48) and Alexander and Engel ('52). They observed both preventative and curative effects of choline or methionine in the nutritional edema accompanied by fatty livers produced in rats by low protein, low choline diets. After choline supplementation, hemoglobin level and serum protein returned to normal.

Protein

However, when adequate amounts of choline were present in the diet, protein still exerted a lipotropic action (Beeston et al., '36). When rats were fed a 5 per cent casein diet supplemented with choline, liver fat was double that in rats fed a 30 per cent casein diet. This finding indicated the lipotropic action of protein could not be due to precursors for choline synthesis present in the protein molecule.

A similar observation was made by Best and Ridout ('40), who showed that although the addition of methionine to a 5 per cent meat powder diet caused some decrease in liver fat accumulation, a further decrease was achieved by the addition of 30 per cent casein. The inability to establish a quantitative relationship between the amount of dietary methionine and the rate of deposition of liver fat led other workers to suggest some factor or factors other than methionine must be involved in the lipotropic action of casein (Tucker and Eckstein, '38; '40; Channon et al., '40). The issue was further complicated by the unexpected

"antilipotropic" effect obtained when low protein diets containing choline were supplemented with another sulphur-containing amino acid, cystine (Griffith and Wade, '40; Griffith and Mulford, '41; Mulford and Griffith, '42; Treadwell, '48). In a series of studies in which the effects of varying the levels of casein, methionine, cystine, and choline were observed, Griffith and his associates showed the stimulation of growth, produced by raising the casein level of the diet or by providing cystine supplements, increased liver fat deposition on low choline diets. However, restricting the food intake of the cystine supplemented group to that of the unsupplemented group did not cause an increase in liver fat. From these and similar results, the authors concluded both the amount and composition of the dietary protein affected the lipotropic effectiveness of choline.

Beveridge et al. ('44; '45) did much to clarify the seemingly conflicting results by controlling the levels of all the essential amino acids in the diets. They demonstrated the relative effectiveness of methionine and casein supplements depended on both the total amount and the proportions of amino acids provided by the diet. They proposed "the amount of dietary methionine available for lipotropic action is limited to that portion not utilized by metabolic processes of apparently higher priority, such as growth and maintenance. The amount required for these non-lipotropic activities is dependent upon the total protein intake, and

is further modified by the adequacy of the essential amino acids supplied in the diet." They attributed previous discrepancies to different dietary levels of casein.

Further support for the hypothesis proposed by Beveridge et al. ('45) was recently provided by Snyder and Cornatzer ('58), who measured the incorporation of labeled methionine into liver proteins of rats on 5 per cent casein diets with and without choline supplements, and on a 25 per cent casein diet. The addition of choline to the low protein, low choline diet did not result in any increase in the amount of labelled methionine used in protein synthesis, indicating when the diet was deficient in both protein and methyl groups very little methionine was utilized as a methyl donor. On the 25 per cent protein diet, however, more methionine was apparently used to supply methyl groups, as a smaller percentage of the injected radioactive methionine was found in liver proteins. These authors, too, concluded protein synthesis takes precedence over lipotropic action when animals are subjected to the double deficiency of protein and methyl groups.

Other Amino Acids

The indications that the methionine content of protein was not the only factor contributing to its lipotropic action, led research workers to investigate the lipotropic activity of various amino acids. In 1949 Singal et al. reported the addition of threonine to a 9 per cent casein

diet supplemented with cystine, choline, and tryptophan resulted in a decrease of liver lipids in rats from 16.0 per cent to 6.6 per cent. The lipotropic action of threonine was not due to an increased food intake, since liver fat levels with and without threonine supplements were 5.9 per cent and 14.4 per cent respectively when animals were pair-fed. In a subsequanet investigation using both 9 per cent casein rations and purified diets providing essential and non-essential amino acids in amounts simulating the 9 per cent casein ration, Singal et al. ('53a) produced fatty livers on both threonine- and lysine-devoid diets. A similar observation was made by Harper et al. ('53c), who showed the level of liver fat in rats fed 9 per cent casein diets could be reduced by supplements of threonine, gelatin, or casein. The relative effectiveness of methionine, choline, protein, and threonine in preventing liver fat accumulation was investigated by Harper et al. ('54a; '54b) and by Lucas and Ridout ('55). They demonstrated the lipotropic action of methionine was mediated through the synthesis of choline. However, when adequate choline and methionine were included in a low protein diet, threonine exerted an additional lipotropic effect, indicating its action was distinct from that of choline. Singal et al. ('53a) also noted the lipotropic effects of threonine and lysine were evident only in the presence of adequate dietary choline.

Factors other than the absolute amounts of amino acids present in the diet have been shown to affect the

lipotropic activity of these compounds. Rats fed a 9 per cent casein ration supplemented with choline and tryptophan had normal liver fat levels, but the addition of 0.1 per cent DL-methionine resulted in fatty livers, which were prevented by threonine supplementation or by pair-feeding the methionine-supplemented animals with the controls (Harper, et al., '54c). The growth stimulation caused by methionine supplementation apparently precipitated the threonine deficiency. The authors concluded the proportions as well as the absolute amounts of amino acids provided by a low protein diet influenced the deposition of liver fat.

Fatty livers had been produced by supplementing the diet with the most limiting amino acid (or acids), thereby causing a deficiency of the second (or third) most limiting, usually threonine (Harper et al., '54c; Winje et al., '54). In 1958 another method of creating an imbalance was developed, viz. by supplementing the protein with a mixture of all the amino acids except the most limiting amino acid (or acids) (Deshpande et al., '58a). A severe imbalance was produced by adding methionine and phenylalanine to a 6 per cent fibrin diet, causing a deficiency of the 4 almost equally limiting amino acids: leucine, isoleucine, valine and histidine. Subsequently, addition of these 4 amino acids stimulated growth to the extent that supplements of threonine and lysine were necessitated to prevent accumulation of liver fat. This complicated picture emphasized the interrelationship between protein synthesis and liver fat deposition.

Because of the intimate relationship between growth and deposition of fat in the liver, experiments were initiated to determine the effect of food intake on the severity of the lesions produced. Two different methods of studying this problem were utilized; force-feeding (Deshpande et al., '58b), and pair-feeding (Kumpta et al., '58) imbalanced fibrin diets. In the former experiment, most of the deficient animals died within two or three days, suggesting they were unable to metabolize the diet efficiently. In the pair-feeding experiment, nitrogen balance was used as a criterion of protein utilization, and the animals pair-fed the 6 per cent fibrin diet retained as little or less of the ingested nitrogen as did those fed the imbalanced diet (6 per cent fibrin supplemented with methionine and phenylalanine). In a previous experiment (Harper et al., '53a) in which dietary carbohydrates were varied on a threonine-deficient 9 per cent casein diet, no differences in growth rates were observed when control rats were pair-fed with deficient rats, but liver fat levels remained elevated in the deficient group. Thus nitrogen retention, reflecting mainly changes in growth rate, is probably not as sensitive an index of amino acid imbalance as is liver fat level and would not give a complete picture of the metabolic states of rats fed nutritionally inadequate diets.

It was noted that the food intake of rats on the imbalanced fibrin diets did not decrease until the second day, suggesting impalatability of the ration was not a

factor (Deshpande et al., '58b). Observations in this series of experiments led Kumpta et al. ('58) to propose that the lowered food intake of the imbalanced diet by rats fed ad libitum reflected a "physiologic adjustment of the rat" to a diet he was unable to metabolize efficiently, while a subsequent increase in consumption was an indication of "an ability of the animal to adapt to this diet."

Sauberlich ('53) also stressed the importance of the amino acid balance of dietary protein, by using a variety of protein sources (corn, sesame meal, wheat gluten, and casein) and supplementing them with various combinations of amino acids. He also found liver fat was deposited only when the diet was adequate to support growth. These data were corroborated by Vennart et al. ('58), who reversed the fat infiltration in livers of rats fed 78 per cent corn diets with supplements of tryptophan and lysine.

Spector and Adamstone ('50) produced fatty livers in both male and female rats by feeding a 20 per cent acid-hydrolysed casein diet supplemented with methionine. When tryptophan was supplemented to this diet, fat did not accumulate in the liver. Samuels et al. ('51) likewise produced a tryptophan deficiency in rats, as well as isoleucine and phenylalanine deficiencies, by force-feeding purified diets devoid of the respective amino acids. They reported an increase in both liver and carcass fat levels, and a decrease in amino nitrogen excretion in all three deficiencies.

Sidransky and Farber ('58a; '58b) showed force-feeding rats purified diets devoid of threonine, methionine, or histidine caused more severe deficiency symptoms than feeding the same diets ad libitum. No gross or microscopic changes were observed in the livers of rats fed the deficient diets ad libitum, while in the force-fed groups, omission of threonine or histidine from the rations resulted in infiltration of considerable lipid and glycogen, and omission of methionine, in increased lipid alone. They pointed out that the amount of diet consumed is an important factor in determining the responses of the rat to dietary variations. In the above experiments the animals were sacrificed after 3, 6, or 7 days. Force-feeding the deficient diet may have accelerated liver changes which would have appeared in the ad libitum fed animals later. Singal's pair-feeding experiments mentioned earlier (Singal et al., '53) illustrated that threonine exerted a marked lipotropic effect independent of food intake.

Other Lipotropic Factors

Substances other than those already discussed (choline, methionine, protein, and amino acids) have been implicated as lipotropic agents intermittently since investigations into the etiology of fatty livers began. The role of essential fatty acids was recently reinvestigated, and results indicated liver fat levels were increased in rats fed a diet deficient in essential fatty acids. The ratio of saturated to unsaturated fats also seemed to

exert an effect distinct from the essential fatty acid deficiency. Barnes et al. ('59) noted that the accumulation of fat in livers of rats fed hydrogenated cocoanut oil was not prevented by the presence of essential fatty acids in the diet. Also, a high ratio of saturated to unsaturated fatty acids promoted the onset of essential fatty acid deficiency symptoms in rats (Peifer and Helman, '59). Vitamin B_6 was also implicated in this type of fatty liver, probably because of the role this vitamin plays in the utilization of linoleate (Tupule and Williams, '55). Inositol was shown to have lipotropic activity on fat free diets (Best et al., '51).

Vitamin B_{12} and folacin have been associated with choline and methionine synthesis (Schaeffer et al., '51), and hence indirectly involved in fat metabolism. Alexander and Sauberlich ('57) and Sauberlich ('59) found vitamin B_{12} and folacin were as effective as choline and methionine in reversing the blood changes which accompanied nutritional edema induced by low protein diets deficient in choline, but were not as effective as choline and methionine in reducing the liver fat accumulation associated with this syndrome.

HISTOLOGICAL CHANGES IN FATTY LIVERS

Histological studies of fatty livers were first carried out on hepatic tissues into which fat had infiltrated as a result of a deficiency of choline or of carbon

tetrachloride poisoning (Glynn et al., '48; Hartroft, '50). Hartroft noted small droplets of fat in the centrolobular areas of livers from rats which had been on a choline deficient ration for only 24 hours. After 7 to 10 days the small droplets had fused into large spherules, and were present in every parenchymal cell. This same centrolobular distribution was noted by Glynn et al. in both choline deficiency and carbon tetrachloride poisoning, and they attributed the subsequent fibrosis to poor intralobular circulation.

Fatty livers resulting from a deficiency of protein or of specific amino acids presented a different histological picture (Nino-Herrara et al., '54; Best et al., '55). Under these dietary conditions, there was a peripheral fat distribution. Shils and Stewart ('54) demonstrated the difference between the two types of fat deposition very clearly in a series of experiments in which they fed rats varying amounts of corn and casein with and without choline supplements. Feeding imbalanced corn proteins resulted in a periportal distribution of fat in the liver lobule, while feeding a diet deficient in choline caused the characteristic centrolobular distribution of fat. Cole and Scott ('54), using a tryptophan deficiency to induce fatty infiltration in livers of rats, also observed cells around the portal tracts laden with neutral fat. Force-feeding of a tryptophan devoid diet resulted in a more complete infiltration of the lobule, involving the central zone after two

weeks, although the peripheral cells were affected first (Adamstone and Spector, '50). These workers also noted reversible changes in size, shape, and number of mitochondria in liver cells which had been infiltrated with fat (Spector and Adamstone, '50).

A short term study of the relative effects of ethionine and carbon tetrachloride on fatty infiltration and necrosis was conducted by Koch-Weser et al. ('51). Lesions were observed at intervals over a period of 120 hours. Carbon tetrachloride resulted in necrosis, while the administration of ethionine produced no necrosis but caused a greater accumulation of fat in the liver. In contrast to reports by other workers (Glynn et al., '48; Dianzani, '55), Koch-Weser found that no fat was deposited in the centrolobular area after CCl_4 injection, although that area eventually became necrotic. Fat droplets appeared first in the intermediate zone, and later spread to periportal cells of the lobule. After ethionine administration, on the other hand, fat appeared first in the periportal zone, spread to the intermediate zone, and eventually distended the entire lobule. The largest droplets were still in the peripheral area at the peak of deposition, 48 hours. At 120 hours, practically all the liver cells of both groups were again normal.

The localization of fat in the liver lobule depends on the factors causing its deposition. Popper and Schaffner ('57) have classified fatty metamorphosis regionally as follows:

(a) Scattered fat - isolated fat rich cells throughout the lobule; may appear normally after a high carbohydrate diet.

(b) Centrolobular fat, eventually involving the greater part of the lobule - appears first and disappears last from the center; typical in anemic or hypoxemic conditions, or in nutritional conditions such as choline deficiency or alcoholism.

(c) Intermediary fat - mainly if central zone is destroyed or damaged, and the intermediary zone is then the most central intact area; found in CCl_4 poisoning.

(d) Peripheral fat - predominantly found in toxemias caused by many diseases in which the liver is not the primary target, and in some forms of malnutrition such as protein deficiency.

The source of the accumulating fat probably influences its site of deposition, i.e. it would be expected fat mobilized from the depots would be deposited in different areas from that synthesized within the liver cells, while newly absorbed fat might be deposited differently, depending on hepatic blood circulation. Barrett et al. ('38) labeled depot fat of mice with deuterium, and observed a rise in the concentration of deuterium in liver fat following injection of anterior pituitary extract and carbon tetrachloride. Under the conditions of this experiment, the fat deposited in the liver as a result of carbon tetrachloride poisoning had been mobilized from the depots. On the other hand, the

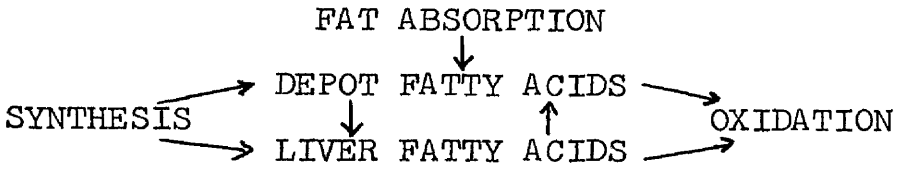
fat present in livers of mice fed high carbohydrate diets, or diets low in choline and protein, showed no increase in deuterium content. The authors assumed that in this case liver fat was derived from the diet. In a similar study, Stetton and Salcedo ('44) detected impaired transport of fatty acids from the liver to the depots in rats subjected to choline deficiency; in cystine deficiency or after thiamine injection there was an increase in fatty acid synthesis; while after anterior pituitary injection there was excessive mobilization of fat from the depots to the liver.

Jaffe et al. ('49) used a more gross approach to determine the source of liver fat in protein-depleted rats. They observed the effects of protein depletion on liver and carcass fat levels at intervals over an 111-day experimental period. Liver fat accumulated most rapidly between 11 and 28 days, after which there was a decrease in fat as well as in water, protein, and glycogen. After prolonged depletion, however, there was a secondary rise in liver fat level. This secondary rise was accompanied by marked atrophy of the cytoplasm and irregularity of nuclear arrangement. The ratio of fat to protein in the liver rose steadily throughout the 111-day period. Concurrent determinations of carcass fat indicated its mobilization could have accounted for the sudden liver fat accumulation between 11 and 28 days.

METABOLIC PROCESSES POSSIBLY INVOLVED IN LIVER FAT DEPOSITION

A diagram of liver and depot fat metabolism presented by Artom ('58) gives a graphic description of the basic processes which affect the level of liver fat:

Sources and Fates of Liver and Depot Fatty Acids



Each of these basic processes is in turn influenced by many metabolic factors, directly and indirectly, therefore it is understandable that no single, clearcut mechanism has been found that will satisfactorily explain the occurrence of all types of fatty livers.

Phospholipid Metabolism

As lecithin and its component choline were the first lipotropic substances to be discovered, disturbed phospholipid metabolism was believed to be the causative factor of fatty infiltration. As early as 1939, Perlman and Chaikoff began a series of studies on phospholipid metabolism in the liver, using radioactive phosphorus (Perlman and Chaikoff, '39a; '39b; '39c; Perlman *et al.*, '40). The administration of choline to rats which had been maintained on high fat, low protein, low choline diets for three days to three weeks increased both the rate of phospholipid synthesis and the rate of removal of phospholipids from the liver. Cholesterol

administration depressed the stimulation of phospholipid turnover caused by choline. Betaine, methionine, cystine, and cysteine all had effects similar to that of choline, in stimulating the rate of phospholipid turnover. Entenman et al. ('46a) separated the choline-containing and non-choline-containing phospholipids, and demonstrated the administration of choline increased the turnover of lecithins but depressed the rate of turnover of other phospholipids.

Artom and Fishman also conducted an extensive series of studies of the effects of various levels of protein, fat, and lipotropic factors on liver phospholipid metabolism in intact rats (Artom and Fishman, '43a; '43b; '43c; Fishman and Artom, '44a; '44b; '46). On low protein, low choline diets, while total liver lipid was increased, phospholipid was decreased, chiefly in the lecithin fraction. Although choline supplementation always reduced total liver lipid, its effect on raising the choline-containing phospholipid levels to normal varied with other experimental conditions. For example, the addition of choline to a 10 per cent casein, 10 per cent fat diet increased the lecithin content of the liver to normal levels in weanling rats, but not in adult rats on the same diet. When the fat content of the 10 per cent casein diet was raised to 20 per cent or more, choline supplementation resulted in an increase of liver lecithins to normal levels in adult rats. The effect of fat on phospholipid synthesis was further investigated by Artom and Cornatzer ('47): rats maintained on 5 per cent casein,

5 per cent fat diets were tube fed a single dose of water, choline, partially hydrogenated cottonseed oil and choline, or cottonseed oil and water, and the subsequent incorporation of injected P^{32} into liver lipids was measured. Choline alone stimulated lipid phosphorylation, and the effect was enhanced by the cottonseed oil. The oil alone had no effect on phospholipid synthesis.

Further studies by this group (Artom and Swanson, '50) and by others (Bollman and Flock, '46a; Horning and Eckstein, '46) indicated the role of choline in phospholipid synthesis was more complicated than was first apparent. Although single doses of choline stimulated the incorporation of injected P^{32} into liver phospholipids, daily supplements of choline did not have a comparable effect. Choline stimulated the formation of phospholipids in vitro only when the diet was inadequate in protein. The addition of choline to the medium enhanced the incorporation of P^{32} into liver slices from rats fed 5 per cent casein diets, but inhibited P^{32} incorporation into slices from rats fed 25 per cent casein diets.

A lack of correlation between the action of choline in phospholipid turnover and in removal of fat from the liver was noted. Horning and Eckstein ('46) observed that in rats fed 5 per cent casein, 40 per cent lard rations, administration of choline or methionine by stomach tube 8 hours before sacrificing was usually followed by increased incorporation of injected P^{32} into liver lipids, but the

increase was not always accompanied by a fall in total liver lipid level. After CCl_4 poisoning or 70 per cent hepatectomy, Bollman and Flock ('46b) detected an increased rate of phospholipid turnover per unit of intact liver tissue, with the result that total turnover rate in the livers was essentially unchanged.

Phospholipid synthesis has also been shown to be impaired in fatty livers resulting from feeding threonine-deficient diets. Singal et al. ('53b) suggested that the depressed rate of phosphorus incorporation into liver phospholipids and nucleoproteins observed in threonine deficiency might be due to impairment of the activity of the phospholipid synthesizing enzymes.

When it was first shown that choline stimulated phospholipid synthesis, fatty acids were believed to be transported in the form of phospholipids. Therefore, choline was assumed to exert its lipotropic effect by stimulating the removal of fatty acids as phospholipids (Best and Lucas, '43). More recent studies with labeled fatty acids have indicated this is not the major pathway of fatty acid transport in either plasma (Entenman et al., '46 b; Goldman et al., '50) or lymph (Bloom et al., '51). However, phospholipids are still considered to be involved in the lipotropic action of choline.

Since phospholipids are integral constituents of lipoprotein (Macheboeuf, '53), and since choline is an

integral constituent of a phospholipid, the importance of choline in lipoprotein metabolism is established. Beveridge ('56) and Harper ('58) have suggested that choline appears to be involved in fat transport *via* the synthesis of phospholipid in liver (Fishler *et al.*, '43; Goldman *et al.*, '50), and the corresponding synthesis of serum lipoprotein. In support of this thesis, several groups have noted decreases in various serum lipid or lipoprotein fractions in animals deficient in choline (Ridout *et al.*, '54a; Wilgram *et al.*, '55; '57; Passananti *et al.*, '58; Ohlson *et al.*, '58; Best, '56).

Another serum lipid fraction which has received much attention, and which is also associated with fatty livers, is cholesterol. In rats fed a high cholesterol, low choline diet, a close correlation was observed between liver and serum cholesterol levels (Nath *et al.*, '59). In choline deficient fatty livers, a direct relationship was observed between glyceride and cholesterol deposition (Best *et al.*, '46). When cholesterol was added to the choline deficient ration, there was a marked increase in deposition of both neutral fat and cholesterol esters in the liver. At moderate intakes of cholesterol (0.2 per cent), sufficient dietary choline (0.64 per cent) prevented excessive accumulation of both glycerides and cholesterol, while at high intakes of cholesterol (0.85 and 1.6 per cent), choline prevented the accumulation of excess glycerides but not of excess cholesterol esters.

Fatty Acid Metabolism

Another path by which fat can be removed from the liver is by oxidation. The rate of oxidation of C^{14}-stearate or -palmitate by liver slices from choline deficient rats was increased by the addition of choline *in vivo* (Artom, '53). Addition of choline to the liver slice preparation *in vitro*, however, did not result in a similar increased rate of oxidation. Artom proposed that some substance was formed from choline *in vivo* which enhanced fatty acid oxidation, and suggested the possibility of choline-containing phospholipids. In substantiation of this theory, the injection of choline *in vivo* produced increases in lecithin levels roughly parallel to increases in fatty acid oxidation rates in livers from rats subjected to a choline deficient diet (Artom, '56). Artom suggested various ways in which choline-containing phospholipids could mediate fatty acid oxidation viz. as essential constituents of enzymes such as ATP-ase and DPN-cytochrome c reductase, as physical agents to maintain the spatial configuration of enzyme systems in the cell, or as some essential minor component of lecithins with a specific role in fatty acid oxidation (Artom, '58).

The direct relationship demonstrated between the rates of fatty acid oxidation and phospholipid synthesis in choline deficient fatty livers was not observed in fatty livers induced by ethionine injections (Artom, '56; '59a; '59b). Although the rate of fatty acid oxidation was

decreased in fatty livers induced by ethionine, the concentration of lecithin in these livers was slightly increased, and the rate of incorporation of P^{32} into liver phospholipids was unchanged. Moreover, the accumulation of fat and the depression of fatty acid oxidation in the livers were prevented by administration of methionine but not of choline. Artom therefore concluded the effects of injected ethionine on fat metabolism were not due to the rapid development of choline deficiency. These results suggest that a different mechanism is involved in the depression of fatty acid oxidation in fatty livers produced by a methionine deficiency per se from those produced by a choline deficiency. Artom ('59b) suggested that ethionine may have interfered with the synthesis of some enzymes or coenzymes required for fatty acid oxidation.

Enzymes and Coenzymes of Intermediary Metabolism in Fatty Livers

Observations of the movement of fat into and out of the liver, and changes in liver lipid constituents under various conditions give some indication of the gross changes which have taken place in the process of fat accumulation. To suggest how and why these alterations have come about, an understanding of the changes occurring in the basic cellular mechanisms of intermediary metabolism is necessary.

Many studies have indicated that feeding low protein diets to albino rats results in an alteration of the activity of liver enzyme systems. Xanthine oxidase activity is depressed by both low protein diets and diets containing poor quality proteins (Litwack et al., '50; '52; '53; Westerfield and Richert, '49; McQuarrie and Venosa, '45; Williams and Elvehjem, '49). In a pair-feeding experiment with adult male rats, Seifter et al. ('48) observed that a protein-free diet resulted in a decrease in the activity of arginase and D-amino acid oxidase. Benditt et al. ('49) noted that the activities of the cytochrome oxidase and succinoxidase enzyme systems of protein deficient rats decreased progressively with time, and faster than total liver protein. Protein depletion in adult rats produced a reversible depression in the activity of phosphatase, xanthine dehydrogenase, cathepsin, and arginase (Miller, '50). Contrary to some of these findings, Millman ('51) found no change in liver cytochrome oxidase or arginase activities (expressed per unit dry weight of tissue) in rats on 2 per cent protein or on protein-free diets, although succinoxidase activity was depressed to approximately half of that in livers from rats on 18 per cent casein diets. Comparing low protein and choline deficient fatty liver, Koch-Weser et al. ('53) found that although large doses of choline removed a considerable part of the fat accumulated on low protein diets, no corresponding effect on the depression in the activities of liver and

serum alkaline phosphatase and esterases was observed.

Wainio et al. carried out a series of experiments concerned with the effect of protein depletion on the activities of several enzyme systems in various tissues of the body (Wainio et al., '53; '54; '59). They found the activity of cytochrome oxidase per total liver of rats fed protein-free rations equivalent to that of pair-fed controls (18 per cent casein), but when the data were expressed on a per gram of nitrogen basis there was an increase in the activity of this enzyme in the depleted animals. Both unit and total activities of the other enzyme systems studied (succinic dehydrogenase, D-amino acid oxidase, DPN-cytochrome c reductase, uricase, and xanthine oxidase) were depressed in livers of the protein depleted rats. After comparison of these enzyme systems in the liver, heart ventricle, brain, kidney, skeletal muscle, and spleen, Wainio concluded liver enzymes are by far the most labile in protein depletion.

Omission of a single amino acid from the diet has also been reported to result in changes in a variety of enzyme systems in rats (Williams et al., '49; Williams and Elvehjem, '50; Bothwell and Williams, '51; '54). Specific amino acid analogues have been used to depress the activity of the xanthine oxidase enzyme system (Younathan et al.,'56).

Attempts have been made to correlate fluctuations in liver fat levels with changes in the activities of various enzyme systems in the livers of rats subjected to an

amino acid imbalance. Harper et al. ('53b) observed a decrease in the rates of endogenous oxidation and in the activities of the xanthine and tyrosine oxidase enzyme systems in rats fed a 9 per cent casein ration supplemented with choline, tryptophan, and methionine. When a double amino acid deficiency was produced in rats by restricting tryptophan as well as threonine on a 9 per cent casein diet, the enzyme changes observed on the threonine-deficient diet did not occur, but fat accumulated in the livers to the same extent as with the single deficiency (Arata et al., '54). In subsequent studies undertaken to discover the underlying metabolic lesions associated with the threonine-deficiency syndrome, Arata et al. ('56; '60) observed a significant decrease in the level of total pyridine nucleotides in livers of threonine-deficient rats. The ratio of oxidized to reduced pyridine nucleotides was decreased to about one-sixth of the ratio found in control rats (Arata et al. '60).

Dianzani ('55) also found a decrease in the total pyridine nucleotides and in the ratio of oxidized to reduced pyridine nucleotides in the fatty livers of rats subjected to a choline deficiency, carbon tetrachloride poisoning, or white phosphorus poisoning. In subsequent studies of fatty livers produced by the above methods, decreases were also noted in levels of cytochrome c and of adenosine polyphosphates (Dianzani and Viti, '55; Dianzani, '57). Decreases in coenzyme A content of fatty

livers induced by CCl_4 were reported by Severi and Fonnesu ('56). CCl_4 poisoning also caused increases in succinoxidase and choline oxidase activities (Richter '51).

Carbohydrate Metabolism

Evidence has recently accumulated to support the thesis that the level and/or type of carbohydrate in the diet have an effect on fatty acid synthesis in the liver. In the low protein, low to moderate fat diets used to promote fatty livers in rats, the necessarily large proportion of carbohydrate present in these diets might exert a considerable influence. In rats fed low casein diets, the activity of xanthine oxidase was increased, liver fat deposition was decreased, and growth was improved by substituting dextrin for sucrose (Harper et al., '53a). The action of glucose was similar to that of dextrin, and of fructose similar to sucrose. The authors suggested that dextrin increased the efficiency of protein utilization. Similar results were observed by Womack and Marshall ('55). Yoshida et al. ('58) also noted a beneficial effect of substituting dextrin, cerulose, or fat for part of the sucrose in low protein diets.

Other workers have correlated the glycogen content of the liver with the ability of the rat to synthesize fatty acids from C^{14}-glucose (Masoro et al., '50) or C^{14}-acetate (Haugaard and Stadie, '52). Results from both of these studies indicated fatty acid synthesis was markedly enhanced as the glycogen level in the liver was increased.

In the last few years, still another approach has been taken to clarify the relationship between carbohydrate metabolism and fatty acid synthesis, viz. the relative participation of the glycolytic pathway and of the hexose-monophosphate shunt (HMS) under different conditions. In 1953, Bloom et al. reported that the HMS accounted for at least 75 per cent of the CO_2 formed from glucose in liver slices. TPNH[1] is required as a specific electron donor in the reduction of b-unsaturated acyl CoA[2] derivatives (Langdon, '55). Several research groups have suggested that the level of TPNH available (derived chiefly from the HMS) is an important factor in the control of fatty acid synthesis (Kaplan et al., '56; Langdon, '57; Brady et al., '56; Sipperstein and Fagan, '57; Cahill et al., '58; Abraham and Chaikoff, '59). Kaplan and coworkers ('56) suggested the chief function of TPNH is probably to mediate fat synthesis, while that of DPNH[3] is in the generation of ATP[4] via the electron transport system. There may be a direct relationship between the relative activities of the HMS and glycolytic pathways, and the disturbed pyridine nucleotide metabolism in fatty livers.

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[1]Reduced triphosphopyridine nucleotide.

[2]Coenzyme A.

[3]Reduced diphosphopyridine nucleotide.

[4]Adenosine triphosphate.

The composition of the diet with respect to the type of carbohydrate and relative proportions of protein, fat and carbohydrate affects the level of glucose-6-phosphatase in the liver of rats (Freedland and Harper, '57; '58a; '58b; '59). Fitch et al. ('59) also observed increases in the levels of phosphoglucomutase and glucose-6-phosphatase activities in fructose-fed rats. Hepatic glucokinase activity was increased on a high glucose diet and decreased on a high galactose diet (Landau et al., '58). As the inclusion of 1 per cent ethionine in 8 per cent casein diets high in sucrose completely inhibited the glucose-6-phosphatase adaptation, Freedland and Harper ('58c) concluded that this enzyme adaptation involved a true protein synthesis rather than activation of the existing enzyme or an inactive precursor. Since the intermediary metabolism of proteins, fats, and carbohydrates are so closely interrelated, the products of one process acting as rate limiting factors on other processes, it seems quite possible that in fatty livers produced by a protein deficiency, the metabolic path taken by carbohydrate would influence the rate of fatty acid synthesis and/or oxidation. These studies indicate that the type and amount of dietary carbohydrate determine the relative participation of different metabolic paths in carbohydrate metabolism, and hence possibly the availability and state of oxidation of some of the coenzymes.

Hormone Influences on Liver Fat Accumulation

The extensive literature on the various metabolic effects of hormones which could directly or indirectly influence liver fat accumulation cannot be reviewed here. However, recent investigations have yielded some interesting results which seem especially applicable to the relationship between amino acids, particularly methionine, and fatty livers.

Farber, with various associates, has done extensive research on the effects of ethionine on liver lipid and protein metabolism in male and female rats, and the influence of various hormones on these effects. Ethionine, injected intraperitoneally into female fasted rats resulted in fatty livers within 12 hours. Simultaneous administration of methionine prevented the lesion. Equimolar amounts of carbohydrate administered with the ethionine had no effect, but large doses of carbohydrate prevented the occurrence of fatty livers (Farber et al., '50). When ethionine was administered to male rats under the same experimental conditions, liver fat did not accumulate. Castrated male rats, however, reacted in the same way as did females. Administration of testosterone protected both female and castrated male rats from liver fat deposition when ethionine was injected (Farber et al., '51). The apparent ability of male sex hormones to protect rats from the ill effects of ethionine administration was also

demonstrated with other androgens (Farber and Segaloff, '55). Swendseid et al. ('52) found ethionine decreased choline oxidase activity in male rats, but did not produce fatty livers. Farber and Segaloff ('55) showed that pre-treating female rats with growth hormone decreased the liver lipid level produced by ethionine injection. Cortisone and ACTH[5], on the other hand, increased the severity of the fatty livers induced by ethionine. The authors noted only those hormones believed to be anabolic or nitrogen-sparing were effective in the prevention of ethionine fatty livers, and therefore proposed the hypothesis that ethionine fatty liver is causally related to interference with protein metabolism (Farber and Segaloff, '55). Further support for this theory was provided by their studies with S^{35}-methionine (Simpson et al., '50) and C^{14}-glycine, -valine, and -leucine (Farber, '55), in which it was shown that ethionine inhibited amino acid incorporation into liver and plasma proteins of female rats by 25 to 40 per cent, while no such inhibition was observed in males. The interference of ethionine in other functions of methionine, however, such as transmethylation, maintenance of choline oxidase activity, pancreatic protein synthesis, etc., was not observed to be a function of the sex of the animal (Farber and Corban, '58).

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[5]Adrenocorticotrophic hormone.

Sidransky and Farber ('58c) compared the effects of force-feeding purified rations devoid of methionine, with the effects of ethionine injection. Both of these treatments resulted in periportal accumulation of fat in livers of female rats but not of male rats. When a threonine deficient diet was force-fed, the livers of both male and female rats exhibited the typical periportal fat deposition of protein or amino acid deficiency. The authors suggested methionine may have a metabolic role associated with the action of androgens, in addition to its role as a methyl donor.

The suggestion that methionine and/or proteins are closely associated with hormone activity is supported by the observation that methionine is required for the inactivation of estradiol (Unna et al., '44). Feeding low protein diets resulted in a marked depletion of the liver estrogen inactivating system (Vanderlinde and Westerfield, '50).

Amino acids other than methionine have been associated with the activity of various hormones. Bromley and Soderberg ('56) noted changes in plasma amino acid levels in adrenalectomized rats force-fed an isoleucine deficient diet. From the same laboratory, Van Pilsum et al. ('57) fed intact rats diets deficient in tryptophan, isoleucine, or phenylalanine, and observed a decrease in the activity of liver xanthine oxidase and catalase, but not of arginase and aconitase. Correlating the two studies, Van Pilsum et al. ('57) suggested the increased production of steroid

hormones, induced by the dietary stress, mobilized amino acids from peripheral tissue, and that these were used for synthesizing only those enzymes with highest priority; catalase and xanthine oxidase presumably having low priority.

Various coenzymes involved in intermediary metabolism are also interrelated with hormone activity. Talalay et al. ('58) and Talalay and Williams-Ashman ('58) observed an increased rate of reduction of DPN[6] promoted by steroid hormones. They later isolated a hormone-dependent transhydrogenase from human placenta which catalyzed the reaction:

$$TPNH + DPN \rightleftharpoons TPN^{7} + DPNH$$

by reversibly converting estrone to estradiol.

Bosch and Harper ('59) noted a decreased pyridine nucleotide level in hyperthyroid rats, and tentatively associated the effect with interference with oxidative phosphorylation and ATP production.

PHYSICAL FACTORS ASSOCIATED WITH FATTY LIVERS

Physical factors may also play a prominent role in the fatty liver syndrome. Dianzani and Scuro ('56) related the biochemical changes in the fatty livers produced by choline deficiency, CCl_4 poisoning, and white phosphorus poisoning to an increased permeability of mitochondria. Recknagel et al. ('57); '58; '59) on the other hand,

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[6]Diphosphopyridine nucleotide.

[7]Triphosphopyridine nucleotide.

maintain that in CCl_4 poisoning infiltration of liver fat and mitochondrial degeneration are not causally related, at least in their initial steps. Calvert and Brady ('58) attributed increased ATP-ase and choline oxidase activity and decreased DPN level in CCl_4 fatty livers to increased permeability of mitochondria.

Phospholipid metabolism was discussed at some length earlier in this review, but the phospholipids are also important in fat infiltration on a purely physical basis. According to Dixon ('58), neutral fat is normally held in a finely dispersed state in the cells of the liver, in laminar micelles of phospholipids. As long as the ratio of neutral fat to phospholipid remains sufficiently low, no fat droplets will be visible. An increase of this ratio, produced by either a decrease in phospholipids or an increase in neutral fat, will result in the appearance of globules of stainable fat. This globular fat is not as readily metabolized or transported from the cell as is the finely dispersed fat, and hence tends to accumulate.

PART I

THE EFFECTS OF A THREONINE DEFICIENCY ON THE ACTIVITIES OF SEVERAL ENZYME SYSTEMS AND ON LIVER FAT LEVELS IN WEANLING RATS

INTRODUCTION

INTRODUCTION

The enzyme systems chosen for investigation in this first section include xanthine oxidase, which has been shown to be depressed by low protein diets (Litwack *et al.*, '50) and by a threonine deficiency (Harper *et al.*, '53); succinic oxidase, a citric acid cycle enzyme whose response to a threonine deficiency has been variable (Arata *et al.*, '54); and malic dehydrogenase, an oxidative enzyme functioning in the citric acid cycle beyond succinic oxidase.

The study was divided into two parts: Experiment 1 was designed to follow the development of biochemical lesions as a function of time. This approach was undertakento determine which enzyme systems would be affected, at what point in time maximum changes would occur, and to correlate these changes with accumulation of liver fat. Experiment 2 was designed to collect data pertaining to enzyme activities and liver fat levels at the time of maximum changes in Experiment 1, and also 4 weeks later, in order to observe the course of the changes with time.

EXPERIMENTAL PROCEDURE

EXPERIMENTAL PROCEDURE

Male weanling rats of the Sprague-Dawley strain were used in the two experiments. In experiment 1, three groups of 14 to 20 animals each were fed the experimental diets ad libitum for two to 36 days. Group I received a 25 per cent casein ration, and served as the primary control group. This ration was composed of casein, 25; choline, 0.15; salts W[1], 4.0; vitamin mix[2], 0.25; corn oil[3], 4.0; cod liver oil, 1.0 ; and sucrose, to make 100 grams. Rats in group II were fed a 9 per cent casein ration supplemented with 0.36 per cent DL-threonine, 0.30 per cent DL-methionine, and 0.10 per cent DL-tryptophan, and served as the secondary control group. Other constituents of this diet were identical with those in the diet for group I; the weight difference was made up with sucrose. The diet for group III was identical with that for group II except that no threonine was added to this ration.

After two, 7, 12, 19, 24, 31, and 36 days on the diets, representative rats from each group were stunned

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[1]Obtained from Nutritional Biochemicals Corporation.

[2]The vitamin mix provided 0.5 mg. thiamin, 0.5 mg. riboflavin, 1.0 mg. niacin, 0.25 mg. pyridoxine, 2.0 mg. calcium pantothenate, 10.0 mg. inositol, 0.02 mg. folic acid, 0.002 mg. vitamin B_{12}, and 0.01 mg. biotin per 100 grams diet.

[3]Containing 7.5 mg. a tocopherol acetate and 0.375 mg. menadione.

by a sharp blow on the head and decapitated. The livers were removed as rapidly as possible, chilled for a few seconds in crushed ice, blotted free of excess moisture, weighed, and homogenized in cold sodium potassium phosphate buffer, pH 7.3. A portion of the homogenate was used for enzyme determinations. The remainder of the homogenates were stored in the cold and later analysed for fat.

Endogenous oxidation and xanthine oxidase (Axelrod and Elvehjem, '41), succinic dehydrogenase (Umbreit et al., '51), and malic dehydrogenase (Potter, '46), were measured by manomentric procedures using the Warburg apparatus. The xanthine oxidase method was modified by using only 1 ml of 16.7 per cent liver homogenate in the reaction mixture. The method for malic dehydrogenase was modified to the extent that livers were homogenized in 0.039M sodium potassium phosphate buffer rather than in water.

All flasks were incubated at 37° C. and allowed to equilibrate for 10 minutes. Substrates were tippped in from the side arms, and readings were taken at 10-minute intervals for the first three systems, and at 5-minute intervals for malic dehydrogenase. Cytochrome c preparations were isolated from beef hearts according to the method of Keilin and Hartree ('45).

In addition to the enzyme systems mentioned, the rat livers were analysed for fat. The homogenates remaining after the aliquots were taken for enzyme determinations were transferred to evaporating dishes, and dried at 90° C.

for 12 hours. The dried livers were ground, and the per cent fat determined by ether extraction on one gram samples.

In experiment 2, similar groups and diets were used, except that Vitamin A and D concentrates[4] were used instead of cod liver oil in the rations. Eleven of the 22 rats in each group were fed ad libitum for 15 days, and the remainder for 43 days, at the end of which time they were sacrificed as above.

The rate of endogenous oxidation and the activities of the xanthine oxidase and malic dehydrogenase enzyme systems were measured in this experiment. At 15 days, endogenous oxidation and xanthine oxidase activity were determined with and without the addition of DPN (0.2 ml 0.5 per cent DPN plus 0.3 ml 0.1M nicotinamide substituted for 0.5 ml water in reaction mixtures).

Livers were analysed for fat as previously described. Nitrogen was determined by the macro Kjeldahl method on 300 mg samples of the ground dried liver from which the fat had been extracted.

"Student's" t test was used to evaluate the statistical significance of the data.

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[4]Providing 200 I.U. vitamin A and 150 units vitamin D per 100 gms. diet.

RESULTS

RESULTS

Growth data are presented in table 1, and liver fat data in table 2, for groups I, II, and III for each time period. The average per cent liver fat in rats fed the 25 per cent casein ration (group I) remained fairly constant over the 36 day period, ranging from 5.1 to 7.9 per cent in experiment 1. There was a slightly higher fat content in livers from rats fed the 9 per cent casein ration supplemented with threonine (group II), a range of 7.3 to 13.8 per cent. In group III (9 per cent casein ration with no added threonine) there was a progressive increase in liver fat to 30.4 per cent on the 24th day, after which time the level decreased steadily to 17.4 per cent by the 36th day, and finally to 14.7 per cent on the 43rd day.

Livers taken from rats in group I (25 per cent casein) at 15 days contained 2.6 $\pm$ 0.1 gm. nitrogen per 100 gm. liver tissue, and at 43 days, 2.9 $\pm$ 0.1 gm. nitrogen. Per cent nitrogen in livers from rats in groups II and III was significantly lower at the 1 per cent level for each time period, viz. 2.1 $\pm$ 0.1 for group II, and 2.0 $\pm$ 0.1 for group III at 15 days; and 2.5 $\pm$ 0.1 for group II, and 2.4 $\pm$ 0.1 for group III at 43 days.

Enzyme activities for group I (25 per cent casein) and group II (9 per cent casein plus threonine supplement) approached similar values when calculated on a per gm. nitrogen basis, e.g. at 15 days the endogenous oxidation

in group II was 110 per cent of that in Group I. The corresponding value for the activity of xanthine oxidase was 79 per cent and of malic dehydrogenase 91 per cent. No significant differences in activity could be observed between groups I and II in any enzyme system studied except xanthine oxidase, therefore group II was chosen as the primary control in interpretation of these data. Since no differences were observed in nitrogen content of the rat livers in group II when compared with those from group III, enzyme activities were reported in terms of grams of liver tissue rather than grams of nitrogen (table 3).

When the enzyme activities per gm. liver of groups II and III are plotted against time, a relationship can be observed between time and the activities of two of the enzyme systems, xanthine oxidase and malic dehydrogenase (fig. 1). In each of these systems, the greatest differences between groups II and III occurred at about the 19th day of the experiment. With the use of a larger number of rats in each group, statistically significant differences at the 1 per cent level were observed between group II and III for both systems on the 15th day ($P < 0.01$). On the 43rd day, these differences had decreased in the case of the xanthine oxidase ($P < 0.05$) system, and disappeared entirely in the malic dehydrogenase system (table 3).

The data collected for succinic oxidase were inconclusive in character. For this system there was sufficient variation within a group to negate any difference

that might have existed between groups, therefore no statistically significant comparisons can be made. Endogenous oxidation also proved to be variable over the time interval studied.

DISCUSSION

DISCUSSION

The activity curves of the xanthine oxidase and malic dehydrogenase enzyme systems plotted against time (fig. 1) show that in both systems maximum depression in the threonine deficient group (group III) occurred on the 19th day, and was followed by a period of recovery. A significant difference ($P=<0.05$) in the activity of xanthine oxidase between the threonine-deficient and control groups was still present on the 43rd day, but the absolute difference between these groups was not as great as it was on the 15th day (table 2). A more complete recovery of the malic dehydrogenase system was observed in the threonine deficient group. The activity of this enzyme system in the threonine deficient animals was not significantly different from that in the control animals on the 43rd day of the experiment.

There was a marked similarity in the general shape of the xanthine oxidase activity curves for groups II and III (fig 1a). The enzyme activity curve for group III (threonine deficient) described a more precipitous decline and a more pronounced lag before recovery than did the control group (group II). The rate of recovery, after its onset, was identical in the two groups.

When xanthine oxidase and malic dehydrogenase activities of group III (threonine deficient) are expressed as

per cent of control (group II), and superimposed on a bar graph showing per cent fat in livers from the threonine deficient rats, the temporal relationships between enzyme activity and liver fat deposition become more evident (fig. 2). The fat was not mobilized out of the livers of the threonine deficient rats until after the activities of the enzyme systems had started to return to control levels. A similar observation was made by Arata et al. ('56), when they observed maximum stimulation of endogenous oxidation by added DPN in livers of threonine deficient rats before the peak in liver fat deposition. These data suggest the importance of choosing the proper point in time to observe maximum differences between groups when enzyme systems are to be used as assay tools. In view of the marked changes in enzyme activity which occurred at various points in this experiment, a time study is strongly indicated as a preliminary approach to this type of investigation.

The metabolic relationships between threonine and the various enzyme systems studied is not clear. A threonine deficiency could affect the apoenzymes indirectly. The primary target of this dietary stress may be a more elemental constituent of the cell than the enzymes themselves. Evidence for this possibility was provided by Arata et al, who showed that a threonine deficiency resulted in a marked decrease in total pyridine nucleotide levels (Arata et al., '56), and a greatly reduced DPN/DPNH ratio (Arata et al. '60).

SUMMARY

An observation made during the course of measuring xanthine oxidase in this study led to a consideration of the possible involvement of DPN as a cofactor in this enzyme system. The time required for liver homogenates to begin oxidizing xanthine was considerably longer in the threonine deficient group, viz. an average of 133 minutes for group III as against 77 minutes for group II. The difference between the two groups was more marked at 15 days than at 43 days, when some degree of recovery had taken place. It is apparent some factor or factors other than the concentration of the apoenzyme was responsible for the changes in activity observed in this system. It has been shown in studies with deuterium labeled substrates (Vennesland, '56) that xanthine oxidase can catalyse the reduction of DPN. This observation could implicate DPN as a coenzyme for xanthine oxidase, despite the unfavorable oxidation-reduction potentials. The deficiency of this coenzyme observed in threonine-deficient rats (Arata et al., '56) could be limiting the rate at which xanthine oxidase oxidized the substrate in the experiments reported here.

This possibility was investigated by the addition of DPN in vitro to xanthine oxidase flasks. The addition of DPN stimulated xanthine oxidase activity in liver homogenates from threonine deficient rats, but the results were variable and not proportional to the decreased activity of this system observed without added DPN. Thus, the metabolic defect observed in this system is more complex than a simple deficiency of DPN.

SUMMARY

Albino rats were fed a low protein diet (9 per cent casein) deficient in threonine. The activities of several enzyme systems were measured at intervals over a period of 43 days. Control rats were fed the same diet supplemented with 0.36 per cent threonine.

The deposition of liver fat in the threonine deficient rats reached a peak in 24 days. After 6 weeks the level of fat in the livers of these rats had fallen to approximately half of this maximum.

The activity of two enzyme systems, xanthine oxidase and malic dehydrogenase, varied with time. The maximum decrease in activity of these systems in the threonine deficient group, as compared with the control, occurred on the 19th day of the experiment. This phase of decreasing activity was followed by a period of recovery.

The fat was not mobilized out of the livers of the threonine deficient rats until after the enzymes had begun to recover.

Because enzyme activities and liver fat levels changed markedly with time over the 43 days of this experiment, a time study was strongly suggested as a preliminary approach to this type of research.

TABLE 1

Weight records of rats fed casein diets with and without threonine supplements.

DAYS ON DIET	NO. RATS	GROUP I[1]	GROUP II[2]	GROUP III[3]
		g	g	g
0	66	50 ± 1[4]	50 ± 1[4]	49 ± 1[4]
7	66	79 ± 1	66 ± 1	59 ± 1
14	66	126 ± 1	95 ± 1	81 ± 1
21	33	173 ± 2	125 ± 1	105 ± 2
28	33	217 ± 2	160 ± 5	130 ± 4
35	33	261 ± 2	192 ± 7	159 ± 4
42	33	307 ± 5	231 ± 9	191 ± 5

[1]25 per cent casein.

[2]9 per cent casein plus 0.36 per cent DL-threonine.

[3]9 per cent casein with no threonine supplement.

[4]Standard error of the mean.

TABLE 2

Per cent liver fat in rats fed casein diets with and without threonine supplements

DAYS ON DIET	NO. RATS	GROUP I[1]	GROUP II[2]	GROUP III[3]
		%	%	%
2	8	5.1	7.3	7.0
7	8	6.6	13.6	21.7
12	8	5.3	12.9	20.8
19	6	6.9	11.5	22.6
24	6	6.7	10.2	30.4
31	6	5.5	13.8	18.6
36	6	7.9	8.3	17.4
12-17	33	9.5±1.3[4]	11.4±1.3[4]	23.0±1.1[4]
40-45	33	7.6±0.5	8.6±0.3	14.7±0.8

[1]25 per cent casein.

[2]9 per cent casein plus 0.36 per cent DL-threonine.

[3]9 per cent casein with no threonine supplement.

[4]Standard error of the mean.

TABLE 3

Enzyme activities in rats fed casein diets with and without threonine supplements (experiment 2)

DAYS ON DIET	NO. RATS	GROUP II[1]	GROUP III[2]
		XANTHINE OXIDASE	
		ul O_2/hr/g liver	
12-17	22	136+ 9[3]	76+ 9[3]
40-45	22	197±16	145±13
		MALIC DEHYDROGENASE	
		ul O_2/hr/10 mg liver	
12-17	22	151+ 6	115+10
40-45	22	133±14	111±34

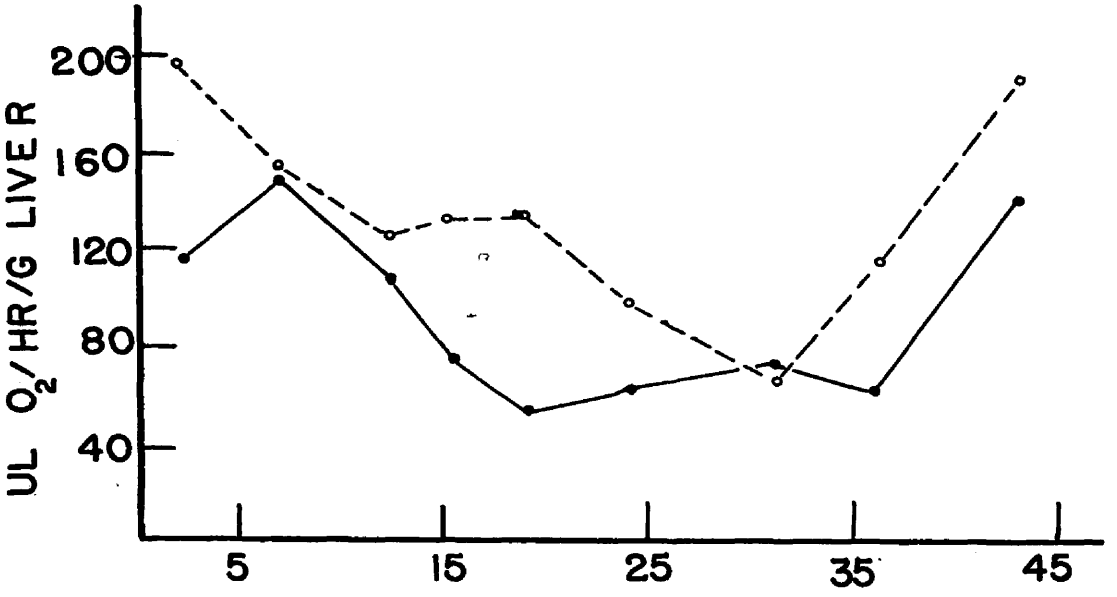
[1] 9 per cent casein plus 0.36 per cent DL-threonine.

[2] 9 per cent casein with no threonine supplement.

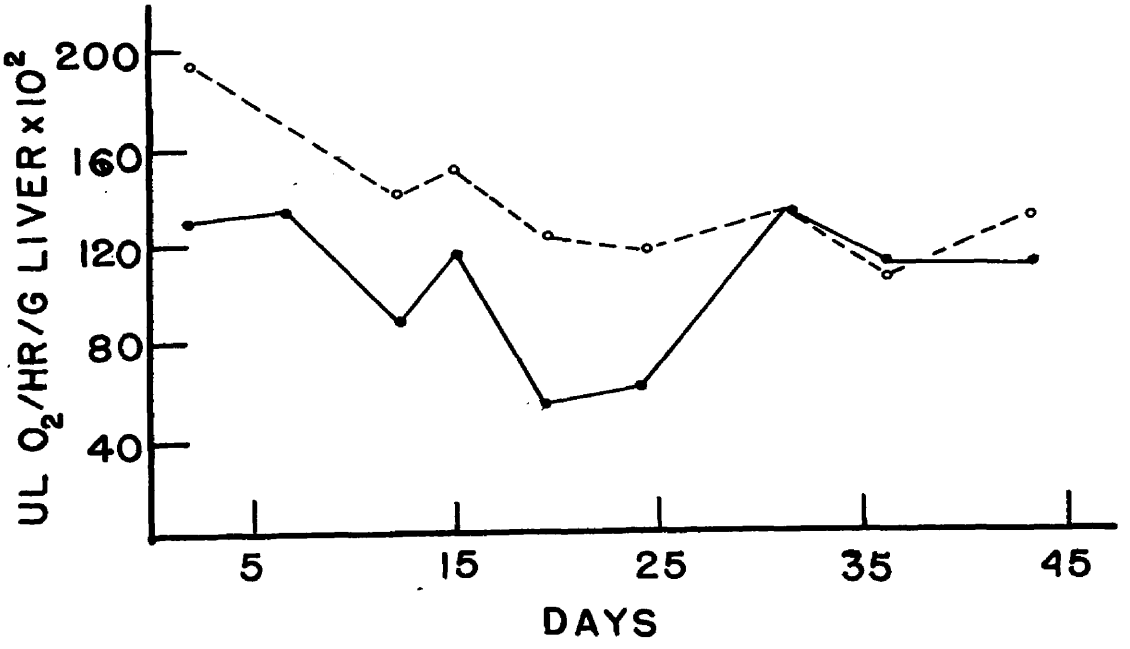
[3] Standard error of the mean.

FIGURE 1

A XANTHINE OXIDASE ACTIVITY



B MALIC DEHYDROGENASE ACTIVITY

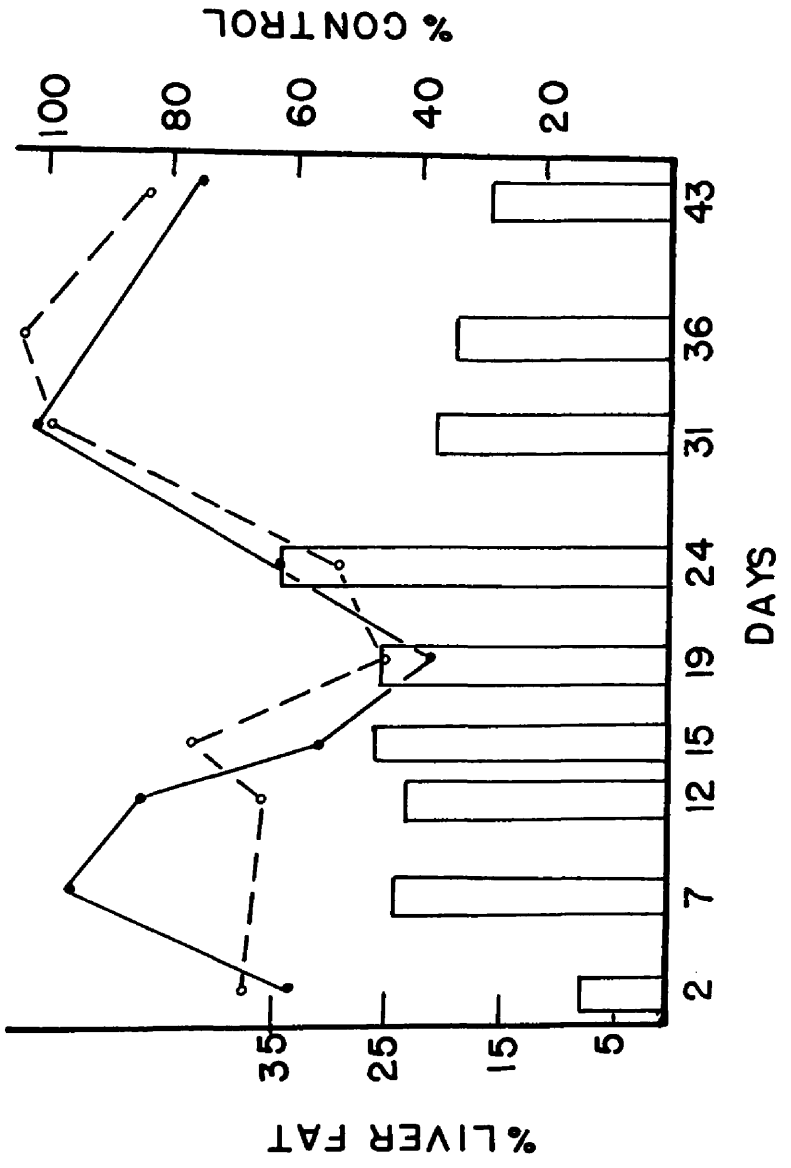


GROUP II (+THREONINE) -------

GROUP III (-THREONINE) ————

FIGURE 2

ENZYME ACTIVITIES IN THREONINE DEFICIENT RATS (%CONTROL)



PART II

LEVELS OF LABILE PHOSPHORUS AND ACTIVITY OF THE FATTY ACID OXIDASE AND DPN-CYTOCHROME c REDUCTASE ENZYME SYSTEMS IN FATTY LIVERS INDUCED BY A THREO-NINE IMBALANCE IN RATS

(Supported by a grant from the United States Air Force Contract No. AF 49(657)276)

INTRODUCTION

INTRODUCTION

In a continuing effort to isolate biochemical lesions associated with fatty livers induced by a threonine deficiency, this study centered around the assay of fatty acid oxidation. There are four basic mechanisms which control liver fat (Artom, '58) viz. synthesis, transport to and from the depots, and oxidation (see diagram p. 18). Failure of the oxidative system to function normally would upset the balance among these four mechanisms and lead to accumulation of liver fat. In this experiment, the activity of the fatty acid oxidase system was measured at specified time intervals in control and threonine deficient rats.

In addition to fatty acid oxidation rates, activity of the DPN-cytochrome c reductase enzyme system and levels of adenosine polyphosphate coenzymes were determined in both groups of rats. These were chosen for study because of their involvement in the series of reactions comprising fatty acid oxidation. As DPN is an obligatory cofactor for this system (Van Baalen, '53), DPN-cytochrome c reductase is associated with the oxidation of fatty acids inasmuch as it functions to maintain an adequate supply of DPN in the oxidized state. ATP is required for the synthesis of DPN (Preiss, '58), in addition to being an essential cofactor for fatty acid oxidation (Van Baalen, '53; Lehninger, '45).

EXPERIMENTAL PROCEDURE

EXPERIMENTAL PROCEDURE

Male, weanling rats of the Sprague-Dawley strain were used in this experiment. Ten rats were sacrificed the first day, to obtain zero time values. The remaining animals were divided into two groups. The rats in group I were fed a 9 per cent casein ration consisting of casein, 9.0; choline, 0.15; salts W, 4.0; vitamin mix, 0.25; corn oil, 5.0; DL-methionine, 0.3; DL-tryptophan, 0.10; DL-threonine, 0.36; and sucrose to make 100 grams. The diet fed to the rats in group II was identical, except that threonine was omitted.

Labile phosphorus, inorganic phosphorus, liver fat, DPN-cytochrome c reductase, and fatty acid oxidase activities were determined at intervals throughout the experimental period.

The method used for the determination of labile phosphorus was a slight modification of the method of Seits ('56), which was derived from Crane's method for separating various organic and inorganic phosphates from liver tissue by charcoal adsorption (Crane, '53). Rats were stunned by a sharp blow on the head, and decapitated. The livers were removed as rapidly as possible, chilled for a few seconds in crushed ice, blotted free of excess moisture, weighed, and homogenized in ice cold 0.25M sucrose. A two ml. aliquot of the homogenate was pipetted into each of two centrifuge tubes containing two ml. cold

10 per cent trichloracetic acid (TCA), and the volume made to 10 ml. with 5 per cent TCA. The duplicate tubes were placed in an ice bath for approximately 30 minutes while fatty acid oxidase activity was determined. The tubes were centrifuged for 10 minutes, after which 5 ml. aliquots of the supernatants were pipetted into centrifuge tubes containing 200 mg. activated charcoal. A blank was prepared with 5 ml. 5 per cent TCA. The tubes were shaken for 10 minutes, centrifuged for 5 minutes, and the resulting supernatants decanted through filter paper (to collect any floating charcoal) into test tubes calibrated to 20 ml. The charcoal was washed twice with distilled water, the washings decanted as above, and made to volume with water. The combined supernatants from each centrifuge tube were frozen and saved for inorganic phosphorus determinations.

Centrifuge tubes containing the washed charcoal were drained on filter paper. The charcoal was suspended in two ml. of 2N HCl (any charcoal that had been collected on the filter papers during decanting was washed into the respective tubes with the HCl), and placed in a boiling water bath for 15 minutes to hydrolyze off the labile phosphorus. The charcoal was filtered off and washed with distilled water to make the total volume of the filtrate not more than 6 ml. Filtrates were stored frozen overnight. The following day, the filtrates were brought to room temperature and neutralized with two ml. freshly

prepared 2N NaOH. Inorganic phosphorus was determined on all solutions by the method of Fiske and Subarrow ('25).

Fatty acid oxidation rates of the liver homogenates were measured manometrically in the Warburg apparatus, using octanoate as substrate. The method used was essentially that of Lehninger for mitochondrial preparations ('55), except that one ml. of whole homogenate (33.3 per cent) was used in place of 0.5 ml. of mitochondrial suspension equivalent to 0.25 gm liver. Quantities of the other reaction mixture components were adjusted in proportion to make the total flask volume 3.2 ml. Flasks were equilibrated at 25° C. for 5 minutes, and readings taken at 5-minute intervals.

DPN-cytochrome c reductase activity was determined by the method of Potter ('51). Crude malic dehydrogenase preparations necessary for this system were isolated from fresh beef hearts as described by Potter ('46).

Fat was determined in dried, ground liver homogenates by ether extraction of one gram samples.

"Student's" t test was used to evaluate the statistical significance of the data.

RESULTS

RESULTS

The activity of the DPN-cytochrome c reductase system was consistently depressed in the threonine deficient group (group II) as compared with the control group (group I). Changes in the activity of this enzyme system did not appear to be a function of time, therefore data collected over the entire experimental period of 5 weeks were averaged:

Group I (9% casein plus 0.36% DL-threonine).....182±13

Group II (9% casein with no threonine supplement).139±11

Each figure represents an average of 14 to 15 rats. The difference between groups was significant at the 5 per cent level ($P < 0.05$).

Growth data from rats fed the 9 per cent casein ration supplemented with 0.36 per cent threonine (group I) and with no added threonine (group II) are presented in table 1. The rats in both groups gained weight more slowly than did those used in part I, but the differences between groups were still significant at the 1 per cent level ($P < 0.01$).

Results of the liver analyses for labile and inorganic phosphorus, for fatty acid oxidase activity, and for fat are summarized in table 2. A progressive accumulation of fat in livers of threonine deficient rats was again observed. This fatty deposition in group II, like

the growth rate, proceeded at a slower rate than in the experiments reported in part I. The differences between groups I and II were significant at the 1 per cent level at 14 and 21 days. Liver fat levels in the control group (group I) did not rise significantly above the level established at zero time.

The fatty acid oxidase activity of the rats in the threonine deficient group was significantly lower than that of the control group after two weeks ($P < 0.01$), and continued to decline throughout the experimental period (table 2).

The values for labile phosphorus in the deficient group are somewhat variable, but hover in the vicinity established at zero time. In the control group, on the other hand, the quantity of labile phosphorus in the livers increased sharply in the first two weeks, continued to rise during the third week, then plateaued between the third and fourth weeks (fig. 1a). Levels of labile phosphorus in group II (threonine deficient) were significantly lower ($p < 0.01$) than those in group I (control) throughout the experimental period of four weeks. The rate of increase of inorganic phosphorus in the liver was similar in the two groups for three weeks, then rose much more rapidly in the control group than in the deficient group (fig. 1b).

Since the control group showed certain fluctuations over the 4 week period, labile phosphorus and fatty acid

oxidase data for group II (threonine deficient) were calculated as per cent of the control group (group I), and plotted on a bar graph representing per cent liver fat in group II (fig. 2). This graph shows the time relationship of changes in labile phosphorus level and fatty acid oxidase activity with that of liver fat accumulation.

DISCUSSION

DISCUSSION

One cause for the abnormally high quantity of fat accumulating in the livers of rats fed a diet deficient in threonine is a loss in the ability of that organ to oxidize fatty acids. The activity of the fatty acid oxidase system was shown to fall rapidly as fat accumulated in the liver tissues (fig. 2).

The decreased supply of labile phosphorus in threonine deficient rats mirrors a restricted quantity of adenosine di- and tri-phosphates (ADP and ATP), while the depressed activity of the DPN-cytochrome c reductase system would probably contribute to an inadequate supply of oxidized DPN (Arata, et al., '56) and the pileup of reduced DPN (Arata et al., '60). The failure of the fatty acid oxidase system to function normally in the deficient group is thus probably reflective of the disturbed metabolism of ATP and DPN, both obligatory cofactors in fatty acid oxidation (Van Baalen, '53; Lehninger, '45).

An examination of fig. 2 suggests that decreased labile phosphorus and depressed fatty acid oxidase activities are closely related. The total labile phosphorus in livers from deficient rats, expressed as per cent of that of control rats, was depressed to approximately the same extent as was the fatty acid oxidase activity at the end of three weeks, after which both leveled off. No concomitant decrease in liver fat level was observed during the

4th week. This phenomenon was observed in part I in the assay of xanthine oxidase and malic dehydrogenase activities in relation to liver fat, namely, that the enzyme systems started to recover before the accumulated fat was mobilized out of the liver.

The repeated pattern in the temporal relationship between biochemical changes and fatty infiltration in livers of threonine deficient rats suggests the possibility of a causal relationship. Certainly the decreased rate of oxidation of fatty acids in the liver would favor the accumulation of fat in that organ. Faulty metabolism of both ATP and DPN would contribute to malfunctioning of the fatty acid oxidase system. DPN supply would be affected by both the amount of ATP available for its synthesis (Preiss, '58), and by the rate at which DPN-cytochrome c reductase reoxidized DPNH. DPN-cytochrome c reductase is a link in the electron transport chain, by means of which the major part of ATP is generated (Lehninger, '58). Therefore, although the evidence is by no means conclusive, a threonine deficiency could interfere with electron transport, and so precipitate a chain of reactions leading to an increase in the fat content of the liver.

Dianzani ('57) observed a decrease in ATP preceding accumulation of fat in livers of rats subjected to CCl_4 poisoning, and suggested a causal relationship. Recknagel ('59), on the other hand, has suggested that biochemical

lesions and liver fat accumulation are not interdependent, at least in the case of CCl_4 poisoning. However, the data presented here and in part I strongly suggest a definite temporal relationship between some enzyme systems, coenzymes, and liver fat accumulation.

SUMMARY

Labile phosphorus levels in the livers from threonine deficient rats were significantly lower than those from control rats.

The activity of the DPN-cytochrome c reductase system was consistently depressed in the threonine deficient group as compared with the control group throughout the experimental period.

The fatty acid oxidase activity of the threonine deficient group decreased rapidly for three weeks then leveled off, as compared to that of the control group.

Liver fat accumulated progressively in the deficient livers for four weeks.

Possible interrelationships between enzyme and coenzyme lesions and liver fat deposition are discussed.

TABLE I

Weight records of rats fed 9 per cent casein supplemented with 0.36 per cent DL-threonine, and 9 per cent casein with no threonine supplement

Days on Diet	NO. RATS	GROUP I[1]	GROUP II[2]
0	32	47±1[3]	47±1[3]
7	32	60±2	50±1
14	32	80±7	59±2
21	16	111±2	75±4
28	8	142±4	85±8

[1]9 per cent casein plus 0.36 per cent Dl-threonine.

[2]9 per cent casein with no threonine supplement.

[3]Standard error of the mean.

TABLE 2

Labile and inorganic phosphorus levels, fatty acid oxidase activity, and per cent liver fat in rats fed casein diets with and without supplements of threonine

DAYS ON DIET	NO. RATS	GROUP I[1]	GROUP II[2]	GROUP I[1]	GROUP II[2]
		LABILE PHOSPHORUS		INORGANIC PHOSPHORUS	
		ug/total liver		ug/total liver	
0	2	307[3]	307[3]	735[4]	735[4]
14	16	560±53[5]	343±33[5]	880±69[5]	723±44[5]
21	6	627±15	289±48	1008±91	865±62
28	8	639±66	359±47	1829±183	1079±145
		FATTY ACID OXIDASE		LIVER FAT	
		ul O_2/hr./g/liver		% dry wt.	
0	8	583±56	583±56	10.2±0.8	10.2±0.8
14	16	532±39	449±40	7.0±0.8	15.4±1.5
21	6	782±83	318±24	8.4±1.2	20.2±2.1
28	8	621±60	267±76	10.3±1.3	23.2±6.6

[1]9 per cent casein plus 0.36 per cent DL-threonine.

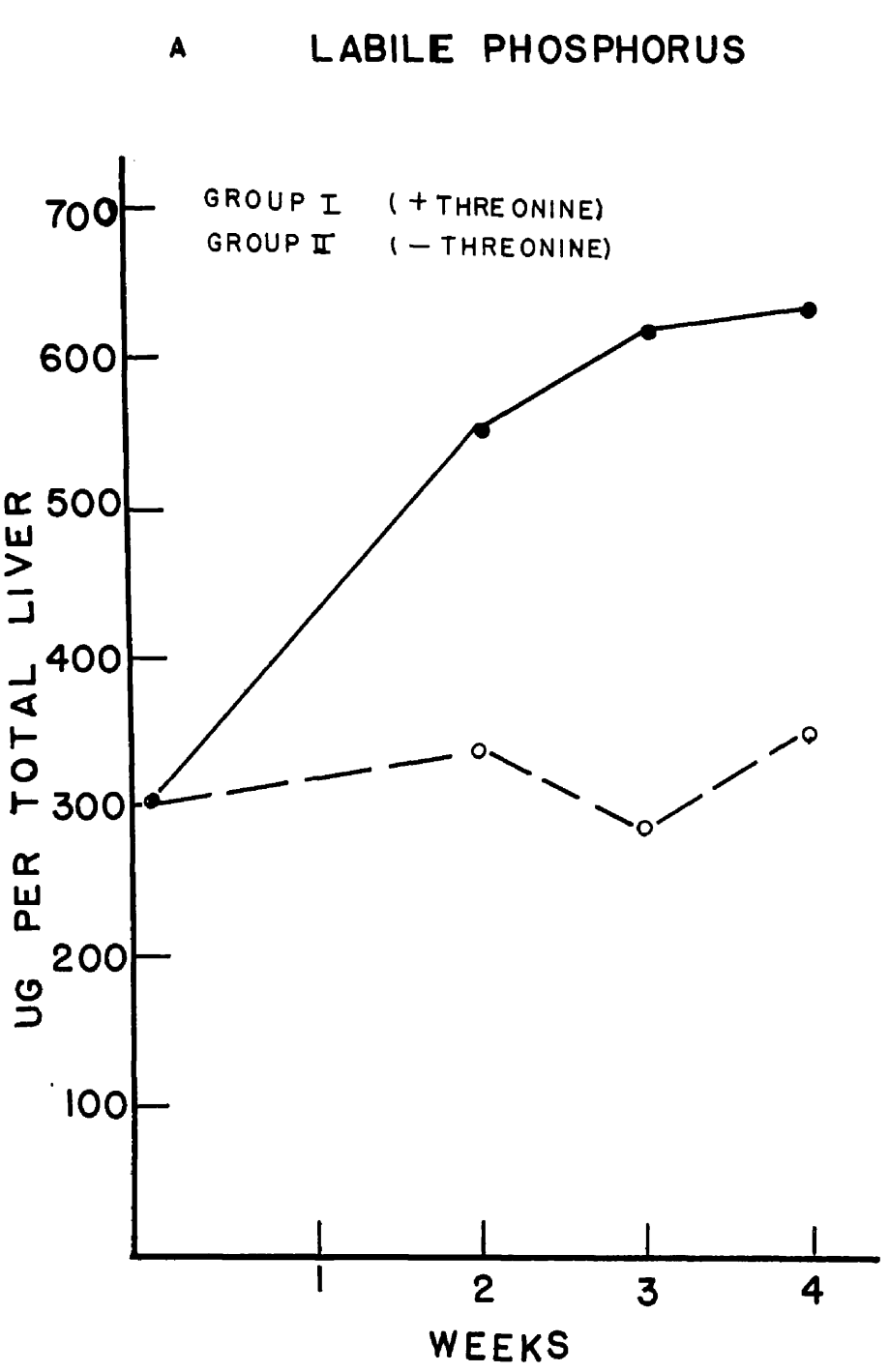
[2]9 per cent casein with no threonine.

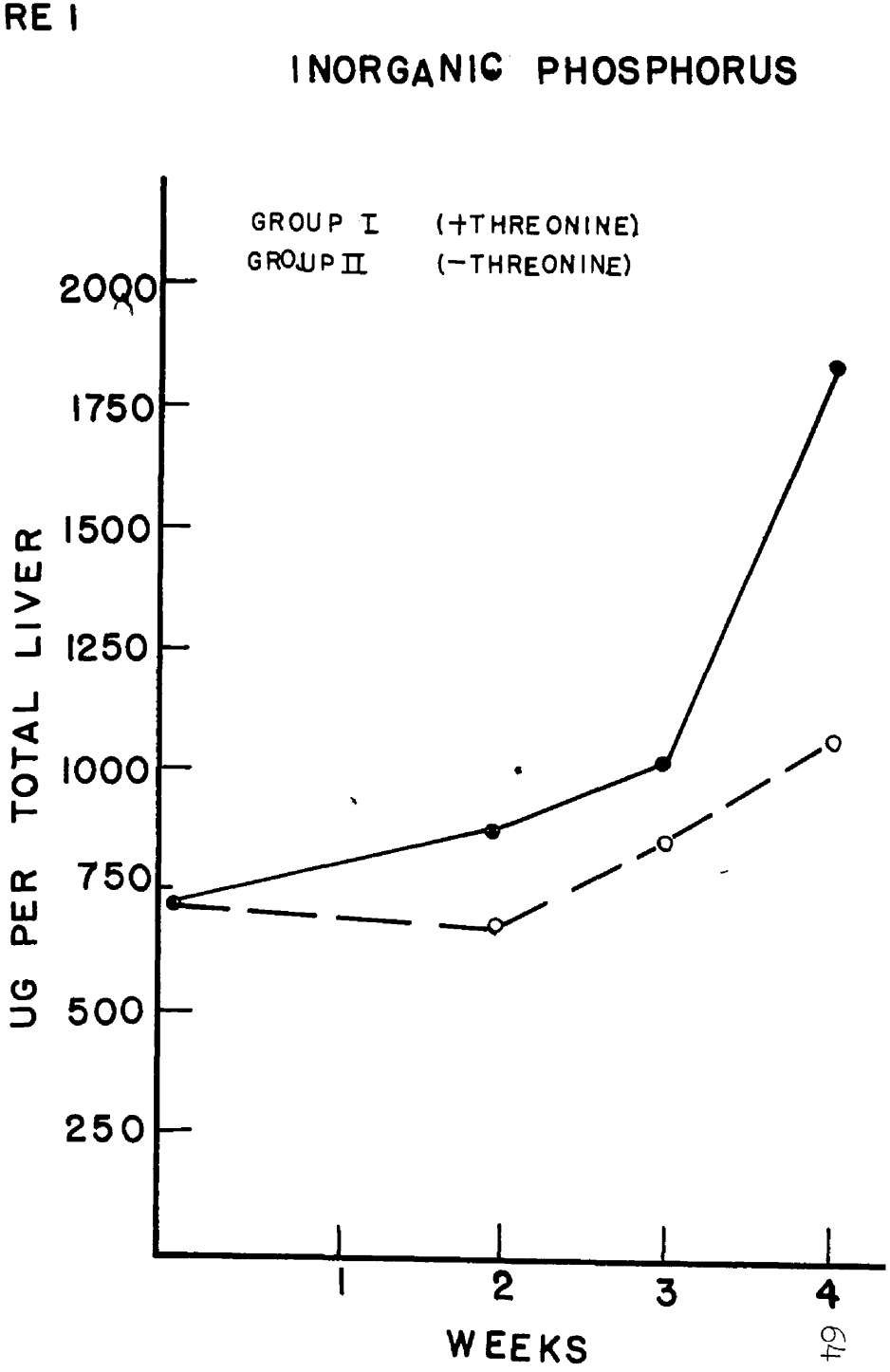
[3]Range 223 - 391 ug.

[4]Range 732 - 738 ug.

[5]Standard error of the mean.

FIGURE I


A
LABILE PHOSPHORUS
GROUP I (+THREONINE)
GROUP II (−THREONINE)
700
600
500
400
300
200
100
UG PER TOTAL LIVER
1
2
3
4
WEEKS


INORGANIC PHOSPHORUS
GROUP I (+THREONINE)
GROUP II (−THREONINE)
2000
1750
1500
1250
1000
750
500
250
UG PER TOTAL LIVER
1
2
3
4
WEEKS

PART III

ACTIVITY OF THE CYTOCHROME OXIDASE ENZYME SYSTEM AND LIVER FAT LEVELS IN RATS FED LOW AND HIGH FAT DIETS DEFICIENT IN THREONINE

(Supported by a grant from the United States Air Force
Contract No. AF 49(657)276)

INTRODUCTION

INTRODUCTION

Feeding rats a low protein diet deficient in threonine resulted in the disruption of the intermediary metabolism of the liver at several different sites (parts I and II). It was suggested that some basic factor or factors in the oxidative processes of cellular metabolism were affected by a lack of threonine in the diet.

The decrease observed in the level of ATP present in livers of threonine deficient rats suggested that a basic lesion might involve electron transport, which provides a large part of the energy for ATP synthesis (Lehninger, '58). Other observations implicating a derangement in electron transport were the persistent depression in the activity of the DPN-cytochrome c reductase system observed in part II, and the abnormal pyridine nucleotide metabolism demonstrated by Arata et al. ('56; '60).

Since cytochrome oxidase is required for the next step in the transport of electrons from DPNH to oxygen, the activity of this enzyme system was chosen for study. An additional variable included in this experiment was the level of fat in the diets. In part II, a precipitous decrease in fatty acid oxidase activity was found to accompany the accumulation of excess fat in the livers of rats fed a diet deficient in threonine. These data infer that threonine deficient rats cannot oxidize fatty acids at a

normal rate. If this is true, increasing the fat content of the diet might exert an additional stress on the animal. Therefore, the effect of increasing the fat content of the diet from 5 per cent to 20 per cent was studied.

EXPERIMENTAL PROCEDURE

EXPERIMENTAL PROCEDURE

Eighty-five male weanling rats of the Sprague-Dawley strain were used in this experiment. Zero time determinations were run on 10 animals, and the remainder were divided into three groups. The rats in group I were fed the 9 per cent casein ration supplemented with 0.36 per cent DL-threonine, as described in part II. The diet for group II consisted of the 9 per cent casein ration with no added threonine. Rats in group III received the same ration as those in group II, except that the corn oil was increased from 5 per cent to 20 per cent at the expense of sucrose.

Cytochrome oxidase activity was measured at zero time, and after two, three, four, five and six weeks in livers from rats fed each of the experimental diets. Livers of all rats used for the cytochrome oxidase determinations were analysed for fat, except at zero time when, due to the small size of the livers, 5 extra rats had to be used for liver fat analyses.

The method of Schneider and Potter ('43) was used for determination of cytochrome oxidase activity. Livers were homogenized in distilled water, and volume of homogenate equivalent to one, two, three, and four mg. liver were pipetted into reaction flasks.

Livers were analysed for fat by ether extraction using the Goldfisch apparatus.

RESULTS

RESULTS

Weight records of rats fed 9 per cent casein, 5 per cent corn oil rations with and without added threonine; and 9 per cent casein, 20 per cent fat with no added threonine are included in table 1. A significant weight depression ($P < 0.01$) was observed in group II (5 per cent fat, no threonine). Increasing the fat content of the threonine deficient diet to 20 per cent imposed no additional stress on the animal as measured by growth. The weights of rats in group III (20 per cent fat, no threonine) was not significantly different than those of rats in group II.

Liver fat data for each of the three groups are presented in table 2. After a comparatively small rise from zero time to two weeks, the level of fat in livers of the control group of rats (group I) remained constant throughout the experimental period. In group II (5 per cent fat, no threonine), liver fat level reached a peak of 23.0 per cent in two weeks, remained elevated through the 5th week, and decreased to 13.3 per cent by the end of 6 weeks. In group III (20 per cent fat, no threonine), liver fat accumulated more slowly than in group II (5 per cent fat, no threonine). The deposition of fat in livers from rats in group III did not reach a peak until the 4th week. By the end of 6 weeks, liver fat was still elevated, and no significant decrease from the peak level was observed.

Cytochrome oxidase activity of all groups showed a substantial increase from zero time to two weeks (table 3). As there were no marked fluctuations within groups for the remainder of the experimental period, activities for the six time intervals for each group were averaged. The average cytochrome oxidase activities in livers from both threonine deficient groups (II and III) were depressed significantly below that of the control group ($P < 0.01$). The activity of this enzyme in the low fat, threonine deficient group (II) was depressed to a greater extent than that in the high fat, threonine deficient group (III). This difference between groups II and III was significant at the 1 per cent level ($P \quad 0.01$).

DISCUSSION

DISCUSSION

Both the liver fat and cytochrome oxidase data indicate that additional fat in the diet tends to delay the appearance of lesions induced by a threonine imbalance (fig. 1). The peak in liver fat accumulation occurred at two weeks in the threonine deficient group receiving 5 per cent fat, but not until four weeks in the group receiving 20 per cent fat. Cytochrome oxidase activity in the low fat group was depressed below that in the control group at two weeks, but this depression was not observed until three weeks in the high fat group. Also, the severity of the enzyme lesion was lessened by the additional 15 per cent dietary fat. The average cytochrome oxidase activity was significantly lower in the low fat group as compared to that in the high fat group ($P < .01$).

Since the total amount of fat deposited in livers from rats in group III (20 per cent fat, no threonine) was no higher than that in group II (5 per cent fat, no threonine), dietary fat apparently does not contribute to the rapid accumulation of liver fat induced by a threonine deficiency.

Although accumulation of fat and depression of cytochrome oxidase activity in the liver both result from a threonine deficiency, data from this experiment suggest that fatty infiltration is not the cause of the depression

in the activity of the cytochrome oxidase enzyme system. The decrease in per cent liver fat observed in group II (low fat, threonine deficient) from 23.0 per cent at two weeks to 13.3 per cent at six weeks was not accompanied by a significant increase in cytochrome oxidase activity. The per cent liver fat in group III (high fat, threonine deficient) was as high or higher than that in group II after three weeks, yet the cytochrome oxidase activity in group III was not depressed to the same extent as was observed in the low fat group.

The suggestion was made in part II that a deficiency of threonine might have an adverse effect on electron transport. The decrease observed in activity of the cytochrome oxidase system, an integral part of the electron transport chain, supports this possibility.

Unlike the majority of oxidative enzyme systems measured in these experiments (parts I and II), the activity of cytochrome oxidase in livers from threonine deficient rats did not vary appreciably with time. Of all the enzyme and coenzyme systems studied, only DPN-cytochrome c reductase behaved in a similar manner. The activities of both cytochrome oxidase and DPN-cytochrome c reductase were persistently depressed throughout the experimental period in threonine deficient rats as compared with control rats.

SUMMARY

SUMMARY

The effect of a threonine deficiency on liver fat level and cytochrome oxidase activity was observed over a period of six weeks in rats fed 9 per cent casein diets containing either 5 per cent or 20 per cent corn oil.

Liver fat in the low fat, threonine deficient group rose to a peak of 23.0 per cent dry weight at two weeks, and had decreased to 13.3 per cent at six weeks. In the high fat, threonine deficient group, liver fat accumulation was greatest at four weeks, and was not significantly decreased by six weeks.

The activity of cytochrome oxidase was significantly depressed in both threonine deficient groups, but to a greater extent in rats fed the lower fat ration. No marked fluctuations with time were observed in this system in any of the three dietary groups.

TABLE I

Weight records of rats fed casein diets with and without threonine supplements

WEEKS ON DIET	NO. RATS	GROUP I[1]	GROUP II[2]	GROUP III[3]
		g	g	g
0	85	46±1[4]	46±1[4]	46±1[4]
1	75	56±2	52±1	49±2
2	75	79±3	66±3	62±3
3	60	107±4	87±2	74±6
4	39	126±5	101±5	97±6
5	24	147±8	114±5	100±5
6	14	191±11	133±10	121±6

[1]9 per cent casein, 5 per cent corn oil, plus 0.36 per cent DL-threonine.

[2]9 per cent casein, 5 per cent corn oil, with no threonine supplement.

[3]9 per cent casein, 20 per cent corn oil, with no threonine supplement.

[4]Standard error of the mean.

TABLE 2

Per cent liver fat in rats fed casein diets with and without threonine supplements

WEEKS ON DIET	NO. RATS	LIVER FAT GROUP I[1]	GROUP II[2]	GROUP III[3]
		% dry weight		
0	5	6.2±1.1[4]	6.2±1.1[4]	6.2±1.1[4]
2	15	11.3±3.1	23.0±2.4	16.0±2.6
3	20	8.6±1.0	19.5±1.6	17.6±1.1
4	14	10.6±1.1	17.1±2.5	20.3±0.9
5	12	10.8±0.6	18.5±1.7	19.8±0.7
6	14	10.1±1.3	13.3±1.7	18.2±2.0

[1]9 per cent casein, 5 per cent corn oil, plus 0.36 per cent DL-threonine.

[2]9 per cent casein, 5 per cent corn oil, with no threonine supplement.

[3]9 per cent casein, 20 per cent corn oil, with no threonine supplement

[4]Standard error of the mean.

TABLE 3

Cytochrome oxidase activity in rats fed casein diets with and without threonine supplements

WEEKS ON DIET	NO. RATS	CYTOCHROME OXIDASE ACTIVITY		
		GROUP I[1]	GROUP II[2]	GROUP III[3]
		ul O_2/hr/g liver		
0	5	570±21[4]	570±21[4]	570±21[4]
2	15	937±33	793±44	903±46
3	20	1010±21	856±11	941±31
4	14	1034±61	851±36	895±71
5	12	1100±69	834±84	934±84
6	14	1090±40	882±72	1013±75
2-6	80	1033±22	845±21	936±24

[1]9 per cent casein, 5 per cent corn oil, plus 0.36 per cent DL-threonine.

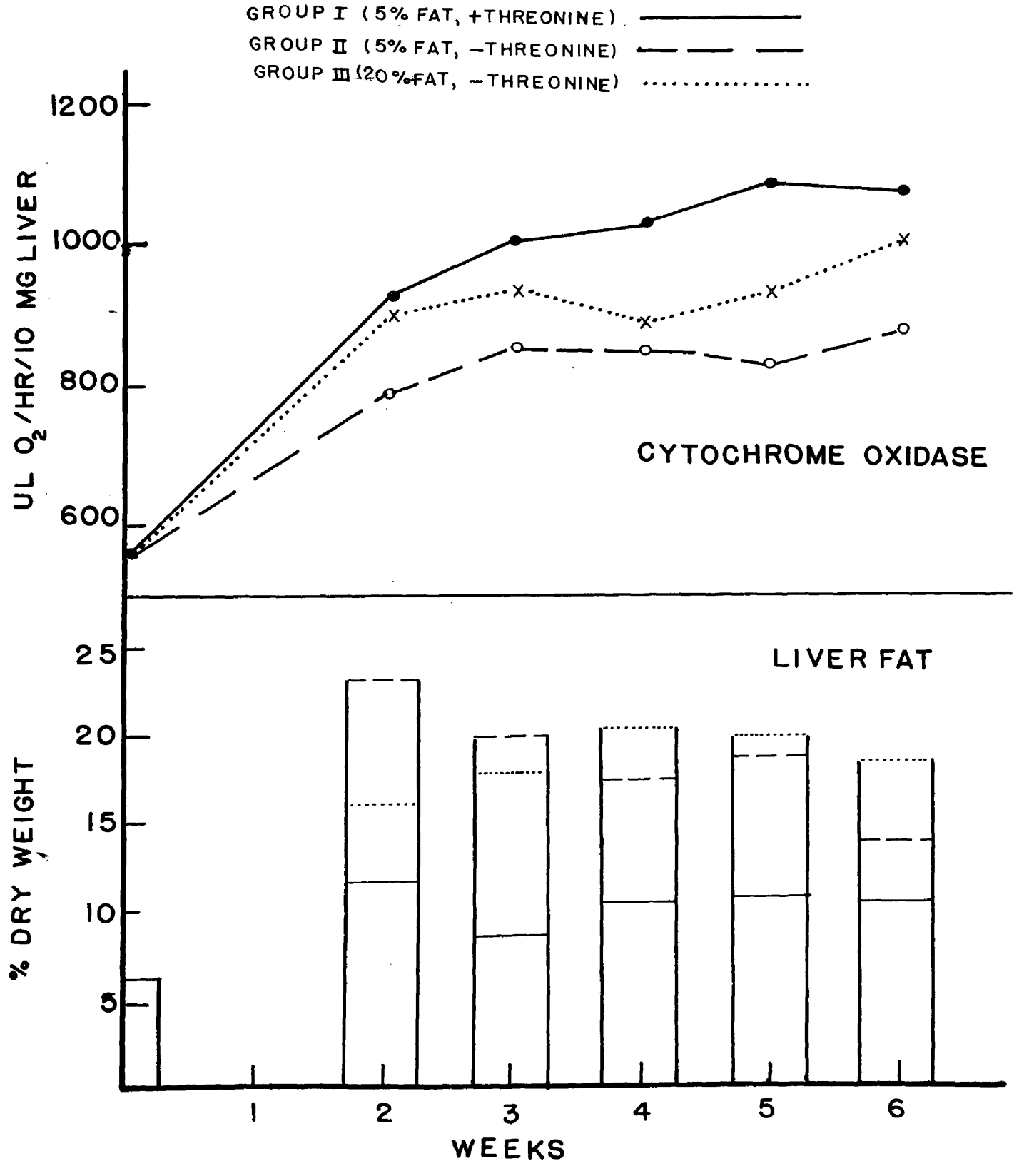
[2]9 per cent casein, 5 per cent corn oil, with no threonine supplement.

[3]9 per cent casein, 20 per cent corn oil, with no threonine supplement.

[4]Standard error of the mean.

FIGURE 1

CYTOCHROME OXIDASE ACTIVITY AND PER CENT LIVER FAT



GENERAL SUMMARY

GENERAL SUMMARY

Male, weanling rats were fed 9 per cent casein, 5 per cent corn oil diets with and without supplement of 0.36 per cent DL-threonine. Additional diets used in individual experiments included a 25 per cent casein ration (part I) and a 9 per cent casein, 20 per cent corn oil ration with no threonine supplement (part III).

Rats were sacrificed at specified intervals during experimental periods of 4 to 6 weeks. Livers were removed and assayed for fat, succinic dehydrogenase, xanthine oxidase, malic dehydrogenase, DPN-cytochrome c reductase, fatty acid oxidase, cytochrome oxidase, and labile and inorganic phosphorus.

Most of the metabolic changes observed in this study were found to be a function of time. A time study was suggested as a tool to insure collection of data at periods of maximum difference between control and deficient groups of rats.

When rats were fed 9 per cent casein rations with no threonine supplement, abnormally large amounts of fat deposited in the livers. The quantity of fat accumulating as a result of this nutritionally inadequate diet ranged from 20 to 30 per cent of the dry weight of tissue. Liver fat increased rapidly during the first two or three weeks, then either decreased or leveled off for the remainder of

the experimental periods. Increasing the fat content of the threonine deficient diet from 5 per cent to 20 per cent did not increase the amount of fat deposited in the liver. Therefore the fat accumulating in the livers of threonine deficient rats did not originate from the fat present in the diet.

Feeding weanling rats 9 per cent casein rations deficient in threonine depressed the activity of several liver enzyme systems below that of control rats fed 9 per cent casein supplemented with 0.36 per cent DL-threonine. The activities of the xanthine oxidase and malic dehydrogenase systems in the threonine deficient rats were decreased maximally in 19 days after which they began to recover. The rates of fatty acid oxidation in livers from threonine deficient rats declined precipitously for three weeks, then leveled off during the fourth week. The activities of the other two enzyme systems studied, DPN-cytochrome c reductase and cytochrome oxidase, did not appear to be a function of time, but were consistently depressed in the threonine deficient rats throughout the experimental periods.

Livers from rats fed 9 per cent casein diets with and without threonine supplements were analysed for labile phosphorus in order to determine the levels of the adenosine di- and tri-phosphate coenzymes. A deficiency of threonine induced a significant depression in the ADP and ATP content of the liver. The decreased concentration of both these cofactors was most marked at three weeks and then leveled

off. Changes in labile phosphorus levels closely paralleled changes in fatty acid oxidase activity determined in livers from the same rats.

The changes in enzyme activity and in coenzyme concentration induced by a threonine deficiency vary inversely with the fat content of the liver. As liver fat increases, the concentration of the enzyme and coenzyme systems decreases. In every system studied, the maximum depression in the activities of apoenzymes and in coenzyme levels occurred before the peak in liver fat deposition was attained. In systems where subsequent recovery was observed, the enzyme or coenzyme recovery took place before fat was mobilized out of the liver. These results suggest that if there is a causal relationship between infiltration of liver fat and enzymatic lesions, the enzymatic lesions are the causative factors.

The common biochemical defect observed in fatty livers from threonine deficient rats in this series of experiments was an abnormality in oxidative processes. All the systems studied require an intact electron transport mechanism to maintain their normal level of activity. Moreover, the activities of two enzyme systems directly involved in electron transport, DPN-cytochrome c reductase and cytochrome oxidase, were found to remain depressed throughout the experimental periods, while the xanthine oxidase and malic dehydrogenase systems tended to recover, and the activity of the fatty acid

oxidase system leveled off. The decreased level of ATP, generated chiefly by energy derived from electron transport (Lehninger, '58) yields further support to the thesis that the electron transport system is the primary target of a threonine deficiency. It is possible that defective functioning of this system could precipitate a chain of lesions resulting in infiltration of fat into the liver.

It is not clear what causes the recovery of the enzyme systems not directly associated with electron transport. Evidence about the sequence of participating units and the number of pathways involved in the electron transport chain is conflicting. However, alternate pathways have been proposed for the oxidation of both DPNH (Estabrook, '57; Cooperstein, '59) and cytochrome c (Devlin and Lehninger, '56) and for the reduction of DPN (Jacobson and Kaplan, '57). It is also believed that there are links between the DPNH and succinate chains (Crane et al., '59; Chance, '59). The persistent decrease observed in this study in the activity of the enzyme systems responsible for reducing and reoxidizing cytochrome c, may indicate that the activity of an alternate pathway is increased, thus enabling other oxidative enzymes to resume normal activity.

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