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Jayne Tigert Morris
University of Tennessee, Knoxville

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To the Graduate Council:

I am submitting herewith a dissertation written by Jayne Tigert Morris entitled "An Explanation for the Apparent Effect of Cod Liver Oil on Sulfur Metabolism in the Albino Rat." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.

John T. Smith, Major Professor

We have read this dissertation and recommend its acceptance:

Mary Rose Gram, Kenneth J. Monty, Frances A. Schofield

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

150

July 27, 1971

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Accepted for the Council:

Vice Chancellor for
Graduate Studies and Research

AN EXPLANATION FOR THE APPARENT EFFECT OF COD LIVER OIL ON SULFUR METABOLISM IN THE ALBINO RAT

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Jayne Tigert Morris

August 1971

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ABSTRACT

An interaction between inorganic sulfur and cod liver oil (CLO) has been proposed in the normal metabolism of the albino rat after a gross malformation of the hind limbs was observed in rats fed a diet low in sulfate and without CLO. The rats were fed these diets from the time they were two weeks of age through the seventh week of life. Toward the end of this period, the fourth week after weaning, the lesion was observed. The study included four basic variations of a diet low in sulfate: with and without CLO and with and without vitamin E. It was concluded that there was present in CLO a factor which enhanced normal production of the sulfated mucopolysaccharides. The present study was an attempt to identify the "active factor" in CLO.

Methods for extracting vitamin D sulfate, sulfolipids or modifications of either procedure were followed in an attempt to fractionate CLO and isolate the "active factor." The extensive fractionation of CLO failed to concentrate the desired factor in any one fraction of CLO but rather indicated the factor to be associated with any lipid fraction. Rather than continue with an apparently fruitless fractionation of CLO, model systems were sought that would mimic the effect of CLO.

Thus, two sources other than CLO of long-chain polyunsaturated fatty acids were fed (safflower oil or 55 percent pure linolenic acid). The results of the first system confirmed the effect of long-chain polyunsaturated fatty acids to be similar to that of CLO when fed to rats. Since the synthesis of lecithin, especially in female rats, involves

the utilization of S-adenosylmethionine, a lecithin deficiency could develop in those rats fed diets low in sulfate and without CLO. Thus, diets with added lecithin, choline and 0.1 percent of sulfate were fed to rats. Results of that study supported the theory that dietary long-chain polyunsaturated fatty acids such as those supplied by CLO accelerated the metabolism of lecithin.

The relationship between increased lecithin metabolism and increased endogenous sulfur was then investigated. When methionine-l-^{14}C was given to rats fed 0.5 percent methionine and 5 percent CLO or no CLO, there was a statistically significant increase in the metabolism of the carbon skeleton of methionine by rats fed diets low in sulfate but supplemented with CLO. Since this result indicated that more methionine was metabolized to provide more S-adenosylmethionine for the methylation of phosphatidylethanolamine, then presumably more homocysteine escaped from the remethylation cycle and was converted to cysteine and eventually to sulfate. Consequently, the oxidation of methionine-^{35}S to $^{35}SO_4^=$ was measured using liver preparations taken from rats fed the above diets. The results of this experiment showed a statistically significant increase in oxidation of methionine-^{35}S to $^{35}SO_4^=$ by the liver homogenates from rats fed diets low in sulfate and supplemented with CLO. Therefore, it is concluded that rather than containing an "active factor" it is the long-chain polyunsaturated fatty acids in CLO which are responsible for its apparent effect on sulfur metabolism.

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CHAPTER I

INTRODUCTION

Several studies in the laboratory of the Nutrition Department of The University of Tennessee, Knoxville, have contributed a number of interesting facts concerning sulfur metabolism in the rat. This work has shown that restriction of the inorganic sulfur intake of rats causes a potentiating effect on avitaminosis E symptoms, a decrease in the hexosamine content of cellular lipoproteins, an alteration in collagen metabolism and a decrease in $^{35}SO_4^{=}$-fixation by rib cartilage mucopolysaccharides (1-4). During the course of investigating these effects, a diet was introduced replacing cod liver oil (CLO) with stripped lard in an attempt to increase the severity of vitamin E deficiency. Albino rats fed this diet suddenly developed a gross and dramatic lesion of the hind legs which appeared to be due to a malformation of the joints. Button (1) proposed an interaction between inorganic sulfur and CLO in the normal metabolism of the albino rat. She designed a study with a basic diet containing 0.0002 percent of inorganic sulfur and adequate levels of organic sulfur as sulfur amino acids, and four variations, with and without CLO and with and without vitamin E. Animals of both sexes and of two strains of white laboratory rats were fed the diet from weaning over a period of from three to twenty-one weeks. After injection of ^{35}S-sulfate, the specific activity of sulfomucopolysaccharide from costal cartilage was determined, and

autoradiograms of sections from the proximal tibial epiphysis were prepared.

The animals fed diets with CLO especially exhibited an increased uptake of ^{35}S-sulfate into the costal cartilage mucopolysaccharide. The cartilage of the epiphyseal plate had a more uniform distribution of radiation 24 hours after ^{35}S injection. The animals fed diets without CLO had a greater accumulation of radioactivity in their chondrocytes. The cartilage plates of animals which were fed the diet without CLO for three or four weeks were decreased in width; this was not seen in older animals. Observation of the intact animals showed generally that CLO in the diet improved the growth and the efficiency of feed utilization and decreased the incidence of abnormal symptoms.

Button (1) concluded that a factor was present in CLO which enhanced normal production of sulfated mucopolysaccharides and that the absence of this factor coupled with the appropriate stress conditions caused the gross lesion initially observed.

As a result of this study, it was proposed that fractionation of CLO and incorporation of the fractions into diets be carried out in an attempt to identify the factor(s) responsible for the above results. Since inclusion of low levels of inorganic sulfate in the diet of the rat has been shown to improve tissue sulfation, it was postulated that cod liver oil contained either some form of sulfur or some factor which would stimulate sulfur metabolism that could be isolated.

CHAPTER II

REVIEW OF THE LITERATURE

The increase in the number of investigations of phospholipid synthesis and functions may, in part, be attributed to increased interest in membrane function, in which phospholipids seemingly play a central role. Day and Levy (6) include the following in a list of roles of phospholipids: myelin, plasma mitochondrial, chloroplast, and endoplasmic reticular membranes; membrane-bound enzymes; mitochondrial electron transport chain; lipoproteins; and blood coagulation. The versatility of the phospholipids is a result of their ionic and hydrophobic nature which permits both lipid-lipid and lipid-protein associations. Day and Levy (6) propose the primary function of phosphatides in living organisms is to act as a molecular "glue." They envision the DNA coding system as directing the synthesis of cellular organelles in such a manner so as to code phospholipid binding sites into the structure of the protein. For example, Day and Levy (7) suggest lipoprotein synthesis and release may be limited by a change in the species of lecithin, thereby limiting the ability of the lecithin to conjugate with the apolipoprotein as it would no longer have the proper code for the species of lecithin being synthesized. Spritz and Mishkel (8) theorize that the decrease in plasma lipids caused by dietary unsaturated fatty acids is a result of changing the spatial configuration of the lipids into which they are incorporated. Since

the more highly unsaturated lipids would occupy more space, fewer unsaturated lipids would be attached to the apoprotein of the low-density lipoproteins, thus causing the observed effect. Whatever the mechanism by which the phospholipids act, they are essential to the living organism and,for that reason, their biosynthesis is also important.

Following the discovery by Kennedy and Weiss (9) that cytidine diphosphate was an essential factor for the synthesis of lecithin, the major pathways of phospholipid synthesis in animal and bacterial cells have been elucidated (10,11). Although the pathways for phospholipid synthesis in plants have not yet been clearly defined, Kates has recently summarized the known information (12).

The key intermediate in all systems is phosphatidic acid which may be formed in a number of ways. Probably the least important is that catalyzed by diglyceride kinase. Here a triglyceride is converted to a 1,2-diglyceride which reacts with ATP in the presence of the kinase to give L-α-phosphatidic acid. This reaction may be involved in membrane phenomena (13).

A somewhat similar path converts the diglyceride to a monoglyceride which, in brain tissue, reacts with ATP in the presence of monoglyceride kinase to form L-α-lysophosphatidic acid. This compound is then converted to L-α-phosphatidic acid by acylation with a fatty acyl-CoA ester (13,14). This reaction possibly occurs in the intestinal mucosa during fat absorption.

A monoglyceride pathway yielding diglyceride has been shown to exist in a microsomal fraction of the intestine of rat, rabbit (14) and hamster (15) and involves a reaction between monoglyceride plus a fatty acyl-CoA to yield diglyceride plus CoA. Johnston et al. (15) established via double labeling experiments that the 1,2-diglycerides (synthesized by the glycero-3-phosphate pathway) and the 1,2-diglycerides (synthesized via the monoglyceride pathway in intestine), do not equilibrate, and thus both are not involved in the synthesis of phospholipid even though these compounds are of the same stereochemical configuration. The authors suggest two plausible explanations: the enzyme systems for the two pathways are located at different sites in the microsomes or their microsomal fraction actually consists of several different subcellular fractions with different activities. Which of these postulates, if either, is correct remains to be seen.

Yet another rather unusual pathway for the synthesis of phosphatidic acid has recently been published. Dihydroxyacetone phosphate was converted to acyl dihydroxyacetone phosphate in the presence of fatty acyl-CoA esters (preferentially saturated) and guinea pig liver enzymes. After reduction of this intermediate, catalyzed by NADPH + H^+ and a mitochondial enzyme, the 1-acylglycerol-3-phosphate formed undergoes a second acylation that requires a microsomal enzyme which shows a preference for unsaturated fatty acyl-CoA esters. Thus, even this unique pathway, of which the quantitative significance has not

been determined, supports the non-random distribution of the fatty acids (16,17).

Quantitatively the most important pathway for the synthesis of phosphatidate is via the acylation of α-glycerophosphate. The initial reaction, catalyzed by glycerophosphate acyltransferase (EC 2.3.1.15), was first reported by Kornberg and Pricer (18,19) and shows an absolute specificity for α-glycerophosphate. In addition, palmityl-CoA and stearyl-CoA are the preferred fatty acids for the acylation. It seems probable that there are two acyltransferases involved (20) but this has not yet been proven (21). Hübscher (13) has summarized the evidence for the occurrence of phosphatidate in animal tissues and cites evidence for its presence in ox, pig, guinea pig and rat liver; rat brain and intestinal mucosa; ox and guinea pig pancreas; and human blood cells.

Even though recent investigations have permitted a greater understanding of the de novo synthesis of phosphatidic acid, the question of the origin of the asymmetric distribution of fatty acids found in naturally occurring lipids has yet to be unequivocally answered. It has been well established that the major rat liver phospholipids possess distinct fatty acid patterns with a non-random distribution of fatty acids: the saturated fatty acids occurring primarily in the 1-position, the unsaturated at the 2-position (21,22,23). Further, in a structural analysis of the neutral lipids and phospholipids of rat liver plasma membranes, Wood (24) verified that the 1-position of the phosphoglycerides, with the exception of diphosphatidyl glycerol, were predominately esterified with saturated acids, and the 2-positions with

unsaturated acids. This is in agreement with the data of Yurkowski and Walker (15) on the composition of phosphatidylcholines from rat intestinal mucosa.

The primary question has been whether phosphatidic acid was randomly acylated and incorporated into phospholipid where the fatty acids were redistributed by deacylation-reacylation or if the d-glycerophosphate was non-randomly acylated with the resultant phosphatidic acid being used to synthesize phospholipid with a non-random distribution of fatty acids. Lands and Merkl (26) first presented evidence that different acyl esters of CoA would acylate 2-acylglycero-3-phosphorylcholine in a liver microsomal system and suggested that the metabolism of each fatty acid was different and selective. Lands and Hart (27) later modified that theory to suggest that the initial phospholipid was formed by de novo synthesis with a random distribution of fatty acids which were subsequently non-randomized by the action of phospholipases and specific acylating enzymes. The random acylation of glycero-3-phosphate observed by these investigators in guinea pig liver microsomes gave strong support to the random distribution theory. Later data from the same laboratory indicated that the enzyme(s) did not respond to unsaturation per se but rather to configurational differences (28). Although these workers recognized from later studies with rat liver slices that non-random acylation of phosphatidic acid existed in some animals, they still supported the non-specific acylation of the 1-position of glycerophosphate (29).

Acyl transfer by specific acylating enzymes was first shown to occur with the acylation of 1-acyl-glycerophosphatidylcholine (lipolecithin) by oleyl-CoA using rat liver microsomal preparations (30). Subsequently, it was shown that the reaction also took place in rat brain, liver, kidney, heart, lung, testis, spinal cord, brown fat, and erythrocytes as well as in human brain (31,32), and that the reacylation was not limited to lysolecithin (33). Although this mechanism could account for the pattern of label incorporated into lecithin, it is not absolutely clear if this mechanism can direct the original fatty acid distribution or if it only maintains the distribution (34). The reaction is quite active in the intestinal mucosa and is of physiological significance in the absorption of dietary phospholipids (35,36).

Recently, considerable evidence has been accumulated that reacylation of the individual molecular species of phosphatidylethanolamine and phosphatidylcholine is the preferred pathway for the incorporation of the more highly unsaturated fatty acids (21,28,37-39).

In contrast to the evidence for random distribution of fatty acids, Possmeyer et al. (21) performed an experiment in which the simultaneous esterification of saturated and unsaturated fatty acids to glycero-3-phosphate was considered. They found added glycero-3-phosphoric acid was esterified at the 1-position primarily by palmitic or stearic acid, whereas at the 2-position esterification was mainly by linoleic and linolenic acid, with the asymmetry of the phosphatidic acid molecule almost as pronounced as that observed in lecithin. Phosphatidic acid isolated from rat liver showed the same asymmetry as that

from the incorporation studies. It would seem from this study that the observed fatty acid asymmetry of phospholipids is introduced at the time of synthesis of phosphatidic acid.

Van Golde, Scherphof and van Deenen (39) investigated this problem using rat liver microsomes and slices which were incubated in the presence of (2-^{3}H) glycerophosphate or (2-^{3}H) glycerol and (1-^{14}C) fatty acids. They reported that esterification of glycerophosphate proceeds in a non-random fashion and that in rat liver slices de novo synthesis of phosphatidylcholine proceeds primarily via phosphatidic acid containing mono- and di-enoic fatty acyl groups. These data were in agreement with those of Hill et al. (29), also using rat liver slices, who found preferential incorporation of unsaturated fatty acids in the 2-position and saturated fatty acids in the 1-position of the glycerol.

As a result of the above experiments, the workers in van Deenen's laboratory (21,38) suggest that "the fatty acid composition and distribution of lecithin in rat liver is determined during de novo synthesis, but that final adjustments and maintenance are taken care of by the deacylation-acylation cycle" (38).

Montfoort et al. (22) using argentation TLC, evaluated the molecular species of phosphatidylcholine from lung, brain, liver and kidney taken from rat, rabbit, pig and cow in order to assess a possible tissue specificity with regard to the composition of phosphatidylcholine. Despite the fact that considerable variability in the molecular composition of lecithins from the analyzed tissues was found, the

authors were tempted to speculate that there is a certain degree of tissue specificity, citing the trend in patterns of lung and brain lecithins from the various animals investigated as evidence supporting this specificity. (Dipalmitoyl)-lecithin was the predominant lecithin found in lung, but was found only in trace amounts in the liver and kidney of all animals examined. Quantitatively, (1-palmitoyl-2-oleoyl)-lecithin was the most important lecithin species of the brain; this compound was also found in substantial levels in the remaining organs examined of all the animal species. The authors confirmed the arrangement of one saturated and one unsaturated fatty acid in the 1- and 2-position, respectively, of the glycerol moiety of lung, brain, and liver lecithins, but found this positional distribution was reversed in the kidney lecithins. This observation had previously been reported (40). In contrast, Kuksis et al. (41) reported that the (1-palmitoyl-2-arachidonoyl)-lecithin species predominated in rat kidney lecithins.

It is clear from the preceding discussion that much work remains to be done before the question of positional specificity of fatty acids will be correctly solved and/or the mechanism for the natural asymmetry of fatty acids in lipids clearly defined. Whatever the answer, before phosphatidic acid may be further involved in phospholipid synthesis, it must be converted to its active form, cytidine diphosphate diglyceride (CDP-diglyceride). The active compound is derived from a reaction of the phosphatidic acid with cytidine triphosphate (CTP) to yield CDP-diglyceride and pyrophosphate. The central role assigned CDP-diglyceride

in the synthesis of the neutral glycerides and phospholipids in higher and lower forms of life has been reinforced by recent detection of the enzyme catalyzing its synthesis, CTP: phosphatidic acid cytidyltransferase, in bacteria and yeast (11) and plants including Chorella (12). The relationship of CDP-diglyceride to synthesis of the phospholipids may be briefly summarized as follows: CDP-diglyceride reacts with L-serine yielding phosphatidylserine plus CMP. Decarboxylation of the serine group will give phosphatidylethanolamine which can be methylated by S-adenosylmethionine to yield phosphatidylcholine. Investigations of the phospholipid composition of tissue indicate that phosphatidylcholine is the most abundant phospholipid in nature. For this reason most of the definitive work of phospholipid synthesis has been via phosphatidylcholine. Consequently the purposes of this review can best be served by describing further the pathways of biosynthesis of phosphatidylcholine and some factors affecting those pathways.

Based on the previous lipid research of others, as well as their own, Kennedy and Weiss in 1956 (9) suggested a pathway for the synthesis of lecithin from phosphorylcholine involving cytidine triphosphate as a cofactor (4). They had initially observed that impure but not purified ATP stimulated the incorporation by rat liver of ^{32}P-phosphorylcholine into lecithin. Observations with CTP followed and were supported by further studies showing that chemically synthesized CDP-^{14}C-choline was more rapidly incorporated into lipid than ^{14}C-phosphorylcholine plus CTP. Further, additional proof was supplied when CDP-choline was isolated from rat liver, synthesis was supported by a supernatant

fraction of rat liver, and a net synthesis of lecithin was found upon addition of 1,2-diacylglycerols to CDP-choline.

The following year, the same laboratory reported the isolation from guinea pig liver of a partially purified preparation of the enzyme that catalyzes the formation of CDP-choline, CTP choline phosphate cytidyltransferase (EC 2.7.7.15) (37). The maximal conditions for the enzyme are pH 7.2 and the presence of divalent cations (Mg^{++} or Mn^{++}), CTP, and phosphorylcholine. Wilgram and Kennedy (43) established the presence of the enzyme in the soluble fraction of liver although the presence of CTP choline phosphate cytidyltransferase has been questioned in studies with skeletal muscle, where synthesis was reported to occur largely in the microsomal fraction (44). Investigations with other nucleotides indicated that only deoxy-CTP (dCTP) was active with the enzyme. dCTP formed dCDP-choline with the enzyme (45).

The reaction between CDP-choline and the 1,2-diacyl glycerol is catalyzed by cholinephosphotransferase (CDP-choline: 1,2-diglyceride choline phosphotransferase EC 2.7.8.2). The pH optimum was found to be 8.6 in the presence of Tween 20-solubilized 1,2-diacylglycerol (46). CDP-choline and, to a much lesser extent, dCDP-choline were active with this enzyme (47). The enzyme is most active in the microsomal fraction of the liver (43) followed by kidney, adrenal, lung, brain, muscle, and intestine (48).

Mudd et al. (38) studied the substrate specificity of choline phosphotransferase as a means of determining if fatty acid distribution is brought about during de novo synthesis of lecithin. They measured

the amount of lecithin synthesized in the presence of CDP-^{14}C-choline and three molecular species of diglycerides isolated from rat liver lecithin containing four, two, and one double bond(s) or with different diglycerides derived from egg lecithin. In both instances the rate of synthesis was the same regardless of the fatty acid composition of the diglyceride. In addition, when ^{3}H- or ^{14}C-labeled mono- and di-ene diglycerides were incubated in different ratios with unlabeled CDP-choline there were no changes in the label ratios of the lecithins produced. The authors concluded that "there was no specificity of choline phosphotransferase depending on fatty acid composition of the diglyceride acceptor at least for the molecular species of diglycerides investigated so far."

Since the discovery of the lipotropic function of choline, many researchers have devoted their time to elucidating the mechanism by which this action is exerted. Although the mechanism is still speculative, one attractive theory has been that choline exerts a lipotropic effect by regulating phospholipid metabolism. However, results reported so far give little support to this theory (49).

Numerous reports have been made on the effect of dietary changes on the synthesis of lecithin, especially regarding choline deficiency. Lombardi et al. (50) studied the effect of an injection of ^{14}C-labeled 2-dimethylaminoethanol or methionine on choline-deficient and supplemented rats. They observed that when the choline-deficient rats were given a choline supplement, there was an increased concentration of liver and plasma lecithins and a decreased labeling of liver and plasma

lecithins to levels found in control rats. The authors interpreted these results to indicate that while the lack of the substrate, choline, caused a decrease in overall synthesis of lecithin by the major pathway (CDP-choline), there was an increase in lecithin production via the secondary pathway (methylation of phosphatidylethanolamine).

This interpretation is consistent with the data of Thompson et al. (51) who showed increased incorporation of ^{32}Pi and decreased synthesis of phosphorylcholine in livers of choline-deficient rats. However, these workers suggested that since the decreased amount of phosphorylcholine was not limiting and levels of CDP-choline are not reduced in livers of choline-deficient rats, animals may possess an increased capacity to utilize methyl groups from methionine for lecithin synthesis. Support is given this idea by the work of Fallon et al. (52) who found no change in the CDP-choline pathway of either homogenates or microsomal fractions of choline-deficient rat livers, but observed an increase in activity of the sequential methylation pathway. Chronic ethanol ingestion produced similar results, which were reversed by the addition of 2 percent choline to the diet. These workers propose that the increased production of lecithin via methylation is a "response to an increased requirement for endogenous choline and lecithin synthesis in dietary choline deficiency."

A decrease in the degradative enzyme, glycerylphosphorylcholine diesterase, has been reported in choline deficiency and the suggestion made that this may be a mechanism by which the cell is able to conserve phospholipid choline for such functions as it may be needed (53).

More recently, choline deficiency has again been reported to have no effect on the incorporation of choline into phospholipids by a rat liver microsomal system (54). The authors interpreted this to mean that the relative pool size of phospholipids synthesized by the cytidine pathway determines the magnitude of choline incorporation into phospholipids.

Rats fed corn oil after being fed for three months an essential fatty acid-deficient diet showed increased unsaturation at the 2-position of the isolated molecular species, with arachidonic acid replacing eicosatrienoate almost completely during a nine day period (37). Besides this circumstantial evidence that a monoacyl phosphoglyceride intermediate had been reacylated, a concomitant increase over a three day period in a lecithin species containing linoleic acid suggested that several metabolic pathways may influence the change in molecular species of phospholipids induced by a change of diet.

Of interest is the recent verification by Yurkowski and Walker (25) that a deficiency of essential fatty acids results in higher levels of oleic and eicosatrienoic acids and lower levels of linoleic and arachidonic acids in mucosal lecithins. Overall, the tetraenoic acids were partially replaced by trienoic, and the dienoic acids were replaced by monoenoic acids. In addition, the ratios of the saturated acids, palmitic and stearic, were altered so that palmitic acid was significantly lower in the deficient rats. Yurkowski and Walker pointed out that this situation also exists in erythrocyte lipids from fatty acid-deficient rats but not in rat liver lipids where palmitate is not reduced.

Apparently, the major pathway for lecithin synthesis in lung and sphingomyelin synthesis in lung and liver is via CDP-choline (55). From their data comparing (^{3}H-methyl) choline and (1,2-$^{14}C_2$) choline incorporation in vivo into liver lecithins, they suggest there is choline incorporation into linoleoyl molecular species not involving CDP-choline. The implication is that this in vivo exchange of free choline has a substrate specificity for linoleoyllecithin. This is in contrast to other reports that CDP-choline is the preferred pathway for the synthesis of this species (39,56).

Regenerating as well as fetal and newborn rat liver has also been used as a means of determining the biosynthesis of the choline glycerophosphatides. Weinhold (57) noted that there could be two separate pools of phosphorylcholine in the cell. In prenatal rats (57) and rabbits (58), low liver enzyme activity has been observed of CDP-choline transferase. Although both activities increased after birth, the increase was greater for the CDP-choline transferase. Noble et al. (56) reported data for newborn lamb consistent with the above data. In regenerating rat liver, Fex (59) described changes in diglycerides which were reflected in the fatty acid compositions of phosphatidylcholine. He also observed that the methylation of phosphatidylethanolamine was directed toward the polyunsaturated species in both regenerating and normal rats, although in the regenerating liver there was a significantly higher amount of the monoenoic and dienoic species. He concluded that the changes were probably due to altered composition of the acyl-CoA pool in the regenerating liver.

Several years after the first reports on the CDP-choline pathway for lecithin synthesis, two separate groups suggested from in vivo tracer studies that phosphatidylethanolamine was converted to phosphatidylcholine by a stepwise methylation. S-adenosylmethionine was proposed as the methyl donor (60,61). Many reports have appeared in the literature supporting this pathway (62). Although there is clear evidence in rat liver preparation for methylation of phosphatidylmonomethylethanolamine to phosphatidyldimethylethanolamine to phosphatidylcholine, the methylation of phosphatidylethanolamine has not yet been elucidated (10,62). The methylation pathway is primarily found in hepatic tissues and seems to be of little importance elsewhere (44,55,63). However Morgan (64) reported the isolation of an N-methyltransferase from dog lung which was fairly specific for disaturated phosphatidylethanolamine. This finding would offer an explanation for the data of Spitzer et al. (65) showing that methionine methyl groups are heterogeneously incorporated into lung lecithin even though the methylation pathway is not considered important in this tissue.

Lyman et al. (66) noted sex differences in the fatty acid composition of phospholipids from rat liver, plasma and erythrocytes. After considering that, according to the literature, methyl label was incorporated more rapidly into the liver phospholipids of female rats than into phospholipids of male rats, these investigators designed a study to determine if the composition differences were due to a difference in the synthetic pathway of lecithin.

They found from the analysis of molecular species that female rats had higher proportions of stearic and arachadonic acids than did males. In addition, the incorporation of the label from (^{14}C-methyl) methionine was also greater in the females and was correlated with the phospholipids containing stearic and arachadonic acids. Thus, if phosphatidylethanolamine contains more stearic-arachadonic acids (67, 68) and females methylate phosphatidylethanolamine more rapidly than males (63), this would account for the differences in phosphatidylcholine composition between sexes.

More recently Glenn and Austin (49) have also noted a sex difference in the levels of liver microsomal phosphatidylethanolamine: S-adenosylmethionine methyltransferase activity in choline-deficient rats. They found a significant increase in methylation in both males and females, but the increased activity of the above enzyme occurred considerably sooner in the males. The authors also reported a change in molecular species of phosphatidylethanolamine and rates of methylation under the conditions of this experiment and suggest that the final composition of phosphatidylcholine may be more dependent on the concentration of the various species of phosphatidylethanolamine than on the acyl pattern. This concept is in agreement with the data and interpretation of Lyman et al. (69) from studies with fatty acid-deficient rats and is in general agreement with Lombardi et al. (50,51). On the other hand, Haggard and Parks (54) found no effect of choline deficiency on the ability of a microsomal fraction to support the incorporation of the methyl group of S-adenosylmethionine into

phospholipid. However, when cell supernatant fluid was added, there was decreased incorporation. To the authors this indicated that dietary choline acts to spare the utilization of S-adenosylmethionine via the methylation pathway. Haines (70) also reported a decreased incorporation of (1,2-$^{14}C_2$) ethanolamine into lecithin of choline-deficient rats.

The third manner by which phosphatidylcholine may be formed is that of base exchange, i.e. free choline may be exchanged with the choline moiety of an existing phosphatidylcholine with no net synthesis. This is a calcium-stimulated pathway and was first reported in preparations of rat liver mitochondria and microsomes (71). The pathway is separate from those involving either CDP derivatives or methylation of phosphatidylethanolamine and is active for the exchange of serine and ethanolamine as well as choline (72). The reaction is inhibited by Mg^{++} (62).

Arienti et al. (73) have reported a Ca^{++}-stimulated enzymic system in purified microsomal membranes from chick brain. The system exchanged both serine and ethanolamine, but not choline. The presence of Mg^{++} did not inhibit the reaction but EDTA did; Mn^{++}, Hg^{++}, Co^{++}, and Cu^{++} depressed the reaction.

Several investigators have found the administration of (^{3}H-methyl) choline, ^{32}Pi and (^{14}C-methyl) methionine resulted in formation of lecithins with a $^3H/^{32}P$ ratio much higher than that of phosphorylcholine, implying base exchange. Treble et al. (72) used this technique to establish that choline exchange takes place in lecithin production in vivo and seems to have a substrate specificity for linoleoyllecithin.

The authors warn that estimates of the rate of phospholipid turnover based on studies of phospholipid bases are probably not reliable as they could include base exchange values. Spitzer et al. (55) studied the in vivo incorporation of labelled choline into the lecithins of rat liver and lung. Their data could best be explained by incorporation of free choline into liver linoleoyllecithin. However neither lung lecithin nor liver or lung sphingomyelin incorporated choline via the free choline pathway. Beeler et al. (74) report the occurrence of base exchange in hamster liver and suggest essential fatty acid deficiency may cause an increase in lecithin formation by this pathway. Similar results have been reported in choline-deficient animals. Thompson et al. (51) found the specific activity of phosphorylcholine almost equal, rather than much higher, to that of lecithin at the first time interval after injection of (1,2-^{14}C)-choline, indicating the CDP-choline pathway was not functioning.

Although early workers on the biosynthesis of phospholipids concluded that synthesis took place in the microsomes and the phospholipids then transferred to the mitochondria, there is still considerable uncertainty as to the origin of mitochondrial phospholipids. Lately, some evidence has been presented that would seem to validate the occurrence of phospholipid synthesis in the outer membrane of mitochondria. In a study utilizing marker enzymes to distinguish between the inner and outer mitochondrial membranes, Shephard and Hübscher (75) concluded that the outer membrane contained enzymes catalyzing the synthesis of phosphatidate. Under the conditions of their experiment, microsomal

contamination accounted for not more than 20 percent of the total phosphatidic acid formation observed.

Bygrave (76) injected ^{14}C-choline or ^{14}C-acetate and observed maximum incorporation of the label from choline into the microsomes, mitochondria, and outer membrane six hours after injection, while maximum incorporation into the inner membrane required an additional six hours. A similar pattern was observed with acetate. In view of these results, as well as similar results of earlier experiments in his laboratory, Bygrave concluded that mitochondria are able to synthesize a part of their own phospholipid and that since the enzymes are located in the outer membrane, synthesis occurs there.

In a more recent study, however, Williams and Bygrave (77) considered the possibility that the results reported above were due to exchange reactions rather than de novo synthesis of phospholipid. In order to investigate this possibility, the in vitro incorporation of ^{32}P-phosphate into phospholipid was measured. Using optimal conditions for incorporation, it was found that maximal incorporation of the label occurred only when all three fractions, mitochondria, microsomes, and supernatant, of the rat liver homogenate were present. Re-evaluation of the literature and their data led Bygrave to agree that most of the phospholipid in the mitochondrial membranes are indeed synthesized in the endoplastic reticulum, as originally reported by Wilgram and Kennedy (43), and subsequently transferred to the mitochondria.

The rate at which the exchange between microsomes and mitochondria occurs is dependent on a protein factor contained in the 105,000

x g supernatant fraction (78,79). Since it had previously been shown that arachidonic acid is introduced to phosphatidylcholine and phosphatidylethanolamine primarily through a deacylation-reacylation reaction involving 1-acyl lysoderivatives (21,37), and since it had been proposed that the arachidonyl species is firmly bound to structural protein, Wirtz et al. (78) chose to investigate the exchange of molecular species. They concluded that there is no difference in the rate of exchange between arachidonate- and eicosatrienoate-containing lecithins and other molecular species, at least in the presence of 105,000 x g supernatant.

Currently, Bloc et al. (80) have been unable to show a mitochondrial factor comparable to the above supernatant factor, although incorporation of ^{32}P-phosphate was found to be highest in the microsomes, intermediate in the outer membrane and lowest in the inner membrane. This result, along with kinetic studies carried out by the authors, show that in addition to exchange between microsomes and mitochondria, there is intramitochondrial exchange, suggesting the possibility that the inner and outer membranes are contiguous structures.

S-adenosylmethionine (SAM), the methyl donor for the methylation of phosphatidylethanolamine, is formed by a reaction between L-methionine and ATP in the presence of the enzyme ATP: L-methionine-S-adenosyltransferase (EC 2.4.2.13). In addition to SAM, pyrophosphate (PPi) and inorganic phosphate (Pi) are formed. The reaction takes place with the products bound to the enzyme so that tripolyphosphate is initially formed and subsequently hydrozyed to form PPi and Pi.

The PPi is derived from the inner two phosphate molecules of ATP; Pi from the outer phosphate molecule (81). The enzyme is specific for methionine, probably in a trans configuration (82). The requirement for ATP and Mg^{++} is also specific (83).

The mechanism of the reaction as reviewed by Mudd (82) has been modified somewhat by recent kinetic studies of the yeast enzyme (84). Optimal conditions for synthesis of SAM were reported to be pH 9, 0.1 M K^{+}, and an excess of 0.005 M Mg^{++} over that bound to ATP. Hydrolysis of tripolyphosphate is about twice as fast as SAM synthesis (84). Inhibitors of the enzyme have been found to be of three types: (1) straight carbon chain amino acids; (2) cyclic amino acids; and (3) O-acetyl-L-serine, O-carbamyl-L-serine, and S-carbamyl-L-cysteine (85).

The further reaction of SAM is catalyzed by S-adenosylmethionine: homocysteine S-methyltransferase (EC 2.1.1.10) and transfers the methyl group to an acceptor such as homocysteine or another sulfonium compound. The reaction mechanism has been studied extensively by Shapiro and associates (86) who also purified the enzyme from yeast, but the role of the enzyme has not yet been determined. Shapiro demonstrated that there are no cofactor requirements and equimolar amounts of the products of the reaction, methionine and S-adenosylhomocysteine (SAHC), were consistently produced. The methylating reactions of SAM have been reviewed extensively (87,88).

Cantoni demonstrated the activity of S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) from cell-free extracts of rat liver for the synthesis of SAHC from adenosine and homocysteine (89). Although the

in vitro reaction is reversible, the products of the reaction must be removed for hydrolysis to take place, suggesting an explanation for not finding homocysteine in mammalian tissues. A similar mechanism has been described in yeast (90). Duerre et al. (90) reported that the label from an intravenous injection of SAHC-^{3}H (homocysteine labeled) into rats appeared mostly in the urine, suggesting the mammalian system did not efficiently utilize the homocysteine from SAHC. Furthermore, the excreted label was not associated with α-ketobutyrate as was expected, but rather with a keto acid tentatively identified as S-adenosyl-α-thio-γ-ketobutyrate. The authors suggest the compound is probably formed by direct deamination of SAHC. However, because the compound has not been found in normal urine, Finkelstein (91) suggests that Duerre et al. (90) observed only the metabolism of extracellular, not endogenously synthesized, SAHC. Further studies must be completed before the mechanism of this enzyme can be defined.

The remethylation of L-homocysteine to methionine primarily occurs by two pathways. The first path, present in mammalian liver systems, causes the remethylation of homocysteine by betaine in the presence of betaine-homocysteine methyltransferase (EC 2.1.1.5). Choline has been shown to be the precursor of betaine for this reaction (91). Rats fed diets adequate in choline, folic acid, vitamin B_{12}, serine, and glycine utilize newly formed methyl groups less than preformed methyl groups (92). Thus, the betaine pathway probably is the major contributor to the overall rate of homocysteine methylation under normal circumstances (93).

The second pathway is known to exist as both a cobalamin-dependent and -independent transfer reaction. The research leading to elucidation of this pathway has been reviewed (94). The mammalian reaction is cobalamin-dependent and utilizes N^5-methyltetrahydrofolate (N^5-methyl-FH_4) as the methyl donor. Although not directly involved in the path, SAM is required for the activity of the enzyme, N^5-methyl-tetrahydrofolate-homocysteine methyltransferase. Taylor and Weissbach (95,96) have presented evidence that the bacterial enzyme contains a site that accepts a methyl group from both SAM and N^5-methyl-FH_4, generating enzyme-bound methyl-B_{12}. These authors propose that in a flavin-reducing system SAM is needed initially to donate a methyl group to the cobalamin enzyme and is not needed again until the system (probably a nucleophilic Co^{++}) becomes oxidized. Under these conditions the methylated enzyme can alternate between methylated and demethylated forms and thus transfer the methyl from N^5-methyl-FH_4 to homocysteine.

Recently Burke et al. (97) have presented strong evidence that the above bacterial system is almost identical with that isolated from hog kidney. Kinetic studies with the purified hog kidney transmethylase have shown that increased levels of methionine inhibited the transfer so that the methyl-B_{12} intermediate could be isolated.

If homocysteine is not remethylated to methionine, it is non-reversibly reacted with serine to form cystathionine. The enzyme which catalyzes the reaction, cystathionine synthetase (EC 4.2.1.21) has heretofore been regarded in mammals as a multifunctional enzyme identical to both serine dehydratase (EC 4.2.1.13) and threonine

dehydratase (EC 4.2.1.16). However, data now exists to conclusively prove that cystathionine synthetase is a separate enzyme (98). These authors also present some properties of the enzyme, which probably has a cofactor requirement for pyridoxal phosphate.

Cystathionine is cleaved by cystathionase (homoserine dehydratase, L-cysteine desulfurase; EC 4.2.1.15) to cysteine and α-ketobutyrate. The sulfur of cysteine is oxidized via several pathways to sulfate (99,100), which has been shown to be a dietary requirement of the rat for optimal growth and sulfation of mucopolysaccharides (1-5).

Although methionine metabolism takes place in all tissues, different pathways and different rates may occur in each different tissue. Mudd et al. (101) have summarized the occurrence and activity of the enzymes of methionine metabolism in various tissues. Mudd has also reported that the five enzymes are decidedly altered by dietary changes, but, again, not to the same extent at the same time.

If one considers the steps of methionine metabolism discussed above as a cycle in which the 4-carbon chain is converted to homocysteine and remethylated back to methionine, then one can consider various factors affecting the tissue concentration of methionine.

Finkelstein and Mudd (93) fed rats diets low in methionine and supplemented with cystine or adequate in methionine, with or without cystine. They observed that the levels in hepatic tissue of cystathionine synthase and methionine activating enzyme were significantly lower in the first case than in the second. Cystathionase levels were slightly lowered under the same conditions, but betaine-homocysteine

methyltransferase was not affected. These studies supported the concept of methionine-sparing effects of cystine although the mechanism is not yet known. Finkelstein and Mudd (93) suggest that the mechanism is such that the amount of methionine converted irreversibly to cystathionine is reduced when adequate cystine is available.

Since homocysteine interrupts the cycle by conversion to cystathionine, a decrease in the capability to methylate homocysteine would probably cause an increase in both homocysteine and cystathionine concentrations and a decrease in methionine concentration. The activity of the B_{12}-dependent enzyme, N^5-methyltetrahydrofolate methyltransferase, is decreased when chickens and rats are fed excess methionine (91,102). It would seem likely that a deficiency of methionine would increase the activity of the methyltransferase. In addition, some metabolic disorders of sulfur metabolism are caused by the lack of this enzyme (102). Betaine-homocysteine methyltransferase activity is affected by hormonal change and decreased by low protein diets (91,102). Also, it should be recalled that the utilization of SAM is dependent on sex (49) and dietary changes, such as choline deficiency (51).

The literature concerning CLO has been thoroughly reviewed by Button (1). In general, since then the antagonistic relationship between relatively high levels of CLO and vitamin E (103,104) or the change in fatty acid structure and thus tissue structure (105,106,107) have been reported. The latter changes would result from the inclusion of long-chain, polyunsaturated fatty acids in the diet of animals

as discussed earlier in this review. Century and Horwitt (107) have studied the effect of structural fatty acid changes in relation to enzyme activities. They found in liver of rats fed diets containing 7 percent levels of CLO an increased activity of some enzymes concerned with biogenic amine metabolism. Recently, Iverson (108) has reported an extensive analysis of the fatty acids of CLO determined by means of urea fractionation and modified programmed temperature gas chromatography.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

It has previously been established that the lack of inorganic sulfur in the diet of rats resulted in a decrease in the uptake of a test dose of ^{35}S-sulfate by rib cartilage mucopolysaccharides (2) and that the effect was intensified by the removal of all tocopherol sources (109). A requirement for cod liver oil (CLO) became apparent when the animals were fed a diet containing low levels of inorganic sulfate (0.0002 percent) with the fatty acids supplied by stripped lard. Also, under these dietary conditions, a lack of CLO caused a deliterious effect on the growth and feed efficiencies of rats. CLO was fractionated by several methods in an attempt to identify the factor previously reported in this laboratory to be responsible for the increased sulfation of rat tissues (1).

Thus, diets were prepared containing the various fractions of CLO and evaluated for the "active fraction" on the basis of the incorporation of a test dose of $^{35}SO_4^=$ into rib cartilage mucopolysaccharides, efficiency of feed utilization, and weight gain or all three. Additional evaluations are described in the section which delineates each individual experiment.

Rats for the first three experiments were furnished from the stock colony of albino Wistar rats of the Nutrition Department, The

University of Tennessee, Knoxville. During this period, a new strain of rats was being developed by crossing male rats of the Long Evans-Sprague Dawley strain with the female Wistar rats of our colony. The rats from the resulting cross-bred strain were tested for response to the diets with the methods of evaluation being used for this study. As the results were comparable, the cross-bred strain of rats was used for experiments 4 through 7.

The animals were housed individually in galvanized, wire mesh cages and separated according to diet. A sheet of paper was placed between cages down the center of the rack to prevent cross-contamination of diets. The weights of the animals were recorded weekly. All animals were fed the experimental diets when weaned at 21 days of age. The mothers and young were fed the basal diet for a period of one week prior to weaning in order to prevent the pups from ingesting any of the stock colony diet. This process was referred to as "prepping"; therefore, the basal diet is referred to as the "prep" diet.

Sacrifice was by decapitation after a stunning blow to the head. For ^{35}S tracer studies, 10 μc radioactive sulfur as sodium ^{35}S-sulfate[1] in 0.5 ml isotonic saline were administered by subcutaneous injection 24 hours prior to sacrifice since this is the maximum labeling time for cartilage mucopolysaccharides (110). Radioactivity of the samples was determined using a Picker Nuclear Liquimat 220 Liquid Scintillation Counter.

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[1]Union Carbide Nuclear Company, Oak Ridge, Tennessee.

Diets

The composition of the basal (prep) diet and that of the diet with added CLO is given in Table 1. They are the basic diets used by Button (1) as modified from the diet initially reported by Caputto et al. (111). The possibility of contamination by tocopherol was further reduced by the use of stripped lard and vitamin-free casein. The salt mixtures used are presented in Table 2. In all experiments except one the salt mix yielding 0.0002 percent of inorganic sulfate was used and is hereafter referred to as the low sulfate diet. The salt mixtures were those modified by Pendergrass (109) from Hubbell et al. (112). Deviations from the above composition will be given in the description of each experiment.

Experimental diets were compared to several standard diets which were prepared by additions to the prep diet, itself a negative control diet containing no tocopherol, no CLO, and low inorganic sulfate. For experiments 1 through 4 the standard diets were: prep; prep + CLO, $\bar{s}$ E; prep + CLO, $\bar{c}$ E; and prep + E, $\bar{s}$ CLO. These will be referred to hereafter as prep diet or by designating only the addition, i.e., + CLO. Since the addition of tocopherol consistently produced a slight improvement but did not affect the overall comparison of experimental diets, the diets for experiments 5, 6, and 7 were prepared without tocopherol.

Diets for experiments 1 through 3 were mixed in 1 kg lots; for experiments 4 through 7 in 2 kg lots, usually once every two weeks. The feed was stored in plastic or amber-colored glass jars at 0 to

COMPOSITION OF EXPERIMENTAL DIETS

Component	$\bar{s}$ CLO	+ CLO
	g/100 g	g/100 g
Casein (vitamin free)[a]	15.00	15.00
DL-Methionine[a]	0.35	0.35
Sucrose	30.83	30.83
Cornstarch	31.82	31.82
Stripped Lard[b]	8.00	6.00
"Alphacel," non-nutritive bulk[a]	10.00	10.00
Cod-Liver Oil	0.00	2.00
Vitamin Mixture[c]	1.00	1.00
Salt Mixture[d]	3.00	3.00

[a]Nutritional Biochemicals Company, Cleveland, Ohio.

[b]Distillation Products Industries, Inc., Rochester, New York.

[c]Synthetic vitamins as supplement to each 100 g of diet: (in mg) niacin, 20.0; pyridoxine-HCl, 0.5; thiamine-HCl, 0.5; riboflavin, 0.5; calcium pantothenate, 1.0; folic acid, 0.5; biotin, 0.005; 2-methylnaphthoquinone, 0.025; vitamin B_{12} (0.1 percent in mannitol), 4.5; choline chloride, 100.0; i-inositol, 100.0; para-aminobenzoic acid, 7.5. These vitamins were triturated in sucrose, q.s. to make 1.0 g. DL-alpha-tocopherol acetate was added to the sufficient diets at the level of 28 mg/100 g of the diet. Vitamin A acetate and Viosterol were added to the diets $\bar{s}$ CLO at the level of 3400 I. U. of vitamin A per 100 g of diet and 340 I. U. of vitamin D per 100 g of diet.

[d]See Table 2.

TABLE 2

COMPOSITION OF SALT MIXTURES[a]

Component	0.0002 Percent of Dietary Sulfate	0.10 Percent of Dietary Sulfate
	g	g
$CaCO_3$	44.75	41.25
$CaSO_4 \cdot 2H_2O$	0.00	6.00
$MgCO_3$	3.06	3.06
NaCl	6.90	6.90
KCl	11.20	11.20
KH_2PO_4	21.20	21.20
$FePO_4 \cdot 2H_2O$	2.05	2.05
KI	0.008	0.008
NaF	0.010	0.010
$AlK(SO_4)_2$	0.017	0.017
$Cu(C_2H_3O_2)_2 \cdot H_2O$	0.072	0.072
$MnCl_2 \cdot 4H_2O$	0.040	0.040
Cornstarch	10.693	8.193

[a] Hubbell *et al.* (112) as modified by Pendergrass (109).

5°C until used. Distilled water and weighed quantities of feed were given ad libitum. The area of the collection tray beneath each cage was lined with waxed paper from which feed was recovered every third day, sifted, weighed, and returned to the cups. Feed efficiency was calculated by dividing the total grams of weight gained times 100 by the total feed eaten in grams.

In those diets $\bar{s}$ CLO, vitamin A acetate and Viosterol were added at a level equivalent to that contributed by 2 percent CLO in the diet. DL-α-tocopherol acetate was added to the tocopherol sufficient diets at a level of 28 mg/100 g of the diet.

A summary of the experimental groups and treatments other than the general procedure described above follows.

Experiment 1

Since the absence of CLO in the diet affected the growth, feed efficiency and sulfation of cartilage mucopolysaccharides in both male and female (1), littermates of both sexes of the Nutrition stock colony rats were used as available to evaluate the diets fed in this experiment for the "active factor." At weaning two prepped animals were fed each of three experimental diets and three control diets. The experimental diets were those labeled "ether," "Saponified," and "MeOH" prepared as described in the methods. The control diets were the prep; the $\bar{s}$ E + CLO; and the + E, $\bar{s}$ CLO. After the animals had been fed the test diets for four weeks they were injected with approximately 10 µc $^{35}SO_4^{=}$ in 0.5 ml isotonic saline, sacrificed as previously described

and their ribs removed and frozen at -20°C until analysis could be carried out. The three fractions incorporated into diets were also analyzed for sulfate content.

Experiment 2

A sulfolipid and two residual fractions were prepared from CLO fractionation and incorporated into diets in place of CLO. The procedure followed was the same as that described above except that only male rats were used as test animals and the control diet + E, $\bar{s}$ CLO was omitted.

Experiment 3

a. Saponification in the cold of CLO resulted in six fractions which were substituted for CLO in six diets. The procedure was the same as that followed in experiment 1.

b. The procedure above was repeated using cold separation of the saponified CLO.

c. The results of the fractionation procedure followed in 3a., and b. above indicated that it would be of value to prepare another lot of CLO by the same procedure and attempt further purification of the fractions. By the use of both column and thin layer chromatography, six fractions were obtained and incorporated into diets. The procedure outlined in experiment 1 was again followed with a total of eight diets including the control diets.

Experiment 4

Evaluation of the results from the first three experimental procedures strongly indicated that the beneficial effects of CLO were associated with any fraction that had lipid properties. With this in mind, experiment 4 was designed to determine the effects of the addition to separate diets of the phospholipid lecithin and two polyunsaturated oils, safflower oil and 55 percent pure linolenic acid, in place of CLO. Lecithin was incorporated into the diet at a level of 1 percent with the percentage of stripped lard incorporated being increased to 7 percent. Two lecithin diets were prepared, one with tocopherol and one without. To insure homogeneous mixing of the lecithin, it was added with the lard and a minimal amount of chloroform to a beaker and warmed to dissolve the lecithin. The mixture was then placed into an open container and the chloroform evaporated off in a convection oven heated to about 55°C. When the odor of chloroform could no longer be detected, the lecithin-lard mixture was incorporated into the diet as usual.

The strain of rats used for this and the following experiments was a hybrid cross between male Long Evans/Sprague Dawley and female albino Wistar rats from our stock colony. Five weanling, prepped, female rats were fed each of the four experimental diets as well as four control diets, the three listed in experiment a. and a + E, + CLO diet. Since sufficient numbers of littermates could not be obtained for distribution among eight diets, an animal from each litter was always fed the $\bar{s}$ E, + CLO control diet so that a comparison could

be made between all eight diets.

Ribs, livers and lungs were excised and frozen at -20°C until analyses could be carried out. The incorporation of ^{35}S into rib cartilage mucopolysaccharides was routinely analyzed; in addition, the sulfate content of the mucopolysaccharides was determined. Since data have been obtained by another investigator in this laboratory showing that a relationship exists between inorganic sulfate in the diet of the rat and total sulfur as sulfate in rat lung, total sulfur as sulfate was determined on the lyophilized lungs from these rats.[2]

Total lipids were extracted from two livers of each group of rats. Aliquots were removed for determination of total lipid weight and total phosphorus. The remainder of the sample was concentrated and the major phospholipid classes separated by TLC. The distribution of phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin was determined as a percent of the total.

Experiment 5

Since the polyunsaturated fatty acids of CLO at this point appeared to be somehow related to lecithin, experiment 5 was designed to investigate the incorporation of a test dose of ^{14}C-methyl-methionine into liver lipids of prepped rats fed three experimental diets composed of 1 percent lecithin, 0.3 percent additional choline, or 0.1 percent SO_4 and the control (the prep) plus one standard diet (the $\bar{s}$ E, + CLO) for a period of four weeks. A dose of 0.5 ml ^{14}C-

[2] J. T. Smith, unpublished observations.

methyl-methionine (35 mg ^{14}C-methyl-methionine/ml of 0.01 N HCl; 6.6 x 10^4 CPM/mg) from Schwartz Bioresearch, Inc., was administered by stomach tube to rats one hour before sacrifice. The livers were removed and treated as in experiment 4 except that total lipid weights were not determined but the total incorporation of ^{14}C into the liver lipid was determined.

Experiment 6

In view of the results of experiment 5 and those of another investigator in this laboratory regarding adaptation to low sulfate diets (100), adult female rats were fed a modification of the low sulfate diets used previously in this investigation for a period of two weeks. Untreated casein, Crisco, and 2 percent commercial vitamin mix[3] were substituted in the diet shown in Table 1, page 32. Two diets were prepared, both with 0.5 percent methionine: one $\bar{s}$ CLO and one $\bar{c}$ 5 percent CLO. Carbon dioxide collection assembly limitations made it necessary to begin the experiment with two rats, and to add two rats daily until the desired number had been placed under test. At the end of the two weeks the first two rats were weighed, given a 0.5 ml dose of methionine 1-^{14}C (25 mg methionine-1-^{14}C/ml 0.01 N HCl; 4.5 x 10^4 CPM/mg) by stomach tube and each placed in a jar fitted with a two-hole stopper so that air could be drawn into the jar through a 0.1 N NaOH carbon dioxide trap and out through three tubes containing a solution of ethyl cellosolve-50 percent KOH (1:1) by the vacuum of a water pump.

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[3]Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

At the end of three hours, the animals were removed and the solutions of ethyl cellosolve:KOH from each section of the trap were combined, diluted to 120 ml and 2 ml aliquots immediately removed for the determination of ^{14}C activity. The cocktail used was 6 ml ethyl cellosolve and 10 ml dioxane containing 8 g PPO (2,5-diphenyloxazole), 0.6 g M_2POPOP (1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene) and 150 g napthaline per liter.

The livers from each of the animals were removed to a weighed beaker of cold phosphate buffer, the weight recorded, and the liver homogenized for use as an enzyme preparation to incubate with ^{35}S-methionine for the purpose of measuring the oxidation of methionine-sulfur to sulfate.

Experiment 7

Since the livers removed from the rats used for experiment 6 were needed to perfect the technique for determining the amount of methionine-^{35}S oxidized to sulfate under the conditions of the experiment, experiment 6 was repeated in order to obtain the desired data. CO_2 expiration was not measured. The incubation mixture was purified by ion exchange and the radioactivity of the sulfate determined.

II. METHODS

Fractionation of CLO by a Method for Extracting Vitamin D

The method of Chen et al. (113) was followed with slight modifications in an attempt to isolate the vitamin D fraction of CLO. It was presumed that vitamin D-sulfate would also be in this fraction.

Five hundred ml of 5 percent KOH in methanol were added to 50 g (55 ml) of Squibb CLO in a flask fitted with a reflux condenser. The mixture was refluxed for two hours. After cooling, 200 ml of water was added for each 10 ml of oil. The saponified mixture was extracted by the dropwise addition of dichloromethane (DCM) (200 ml/10 ml oil) followed by mixing with a magnetic stirrer for two hours. The extraction was repeated with DCM followed by two ethyl ether extracts. The ether was added to the bottom of the saponification mixture through a small bore glass tube attached by Teflon tubing to a glass funnel. During the addition of the ether the container was covered. The DCM phase was then washed by dropping through 250 ml of water. When the two phases were completely separated, the DCM phase was drawn off and concentrated to near dryness under vacuum in a rotary evaporator. The saponified fraction was dried in the convection oven and stored until it was incorporated into a diet at a level equal to 2 percent CLO.

The concentrated DCM phase was partitioned by first dissolving in 50 ml methanol then adding 75 ml of petroleum ether (B. P. 30°-60°C) and 25 ml water. After complete equilibration, the petroleum ether phase was removed, partially concentrated, and quantitated to 50 ml. Three ml were removed for sulfate determination by the method of Häkkinen and Häkkinen (114); the remainder was evaporated to dryness and added to a diet in place of CLO. The methanol-water phase was also quantitated (200 ml), a portion (25 ml) removed for sulfate determination and the remainder evaporated to dryness and incorporated into a diet in place of CLO.

Colorimetric Determination of Sulfate

Aliquots of the three fractions produced by the technique described in experiment 1 were combusted in a Parr sulfur bomb apparatus, quantitated and analyzed for their sulfate content.

Aliquots of the sample solution, 3 ml of 95 percent ethanol, 1 ml of 1 M, pH 4.0 acetate buffer, and sufficient water to make 6 ml were mixed in test tubes. An excess of barium chloranilate, 30 mg ± 5 mg, was added to each tube after which it was shaken for 15 minutes and centrifuged to remove the precipitated barium sulfate and excess barium chloranilate. The absorption of the liberated chloranilic acid was measured in the Beckman B spectrophotometer at a setting of 530 mμ. The 1 M acetate buffer, pH 4.0, was prepared by mixing 180 ml of 1 M sodium acetate and 820 ml of 1 N acetic acid.

Standard samples including a blank were prepared in the same manner with each set of samples. The blank was used to adjust the instrument to zero absorbance. The amount of sulfate in the samples was calculated from the standards.

Fractionation of CLO by a Method for Isolating Sulfolipid

A modification of the technique reported by Malone and Stoffyn (115) was developed as a method for fractionating CLO. The three resultant fractions were substituted for CLO in the prep diet.

One hundred g of Squibb CLO were mixed with 100 ml of chloroform: methanol (2:1). Twenty ml of this mixture were pipetted onto a column (8 x 2 cm) of Norit A. The Norit A previously had been washed with 6 N HCl, 95 percent ethanol, and chloroform:methanol (2:1). The CLO mixture

was washed with 200 ml chloroform:methanol (2:1) followed by 200 ml of water-saturated chloroform:methanol (2:1). The sulfatides were eluted with chloroform:methanol (2:1) saturated with 20 percent ammonium hydroxide.

After the above procedure was repeated five times, the combined sulfatide fractions (from the equivalent of 50 g CLO) were combined and evaporated to near dryness. It was found that this material was not totally soluble in chloroform. It was extracted with a small portion of water, completely equilibrated, and the chloroform portion removed, concentrated, and placed on a 1 x 4 cm Florisil (magnesium silicate) column. The water phase was evaporated to near dryness and incorporated into a diet.

The sulfatide fraction that had been placed on the Florisil column was first eluted with 200 ml of dry chloroform then the sulfatides brought down with 100 ml chloroform:methanol (2:1). The solvent was removed from the eluate by evaporation; the lipid remaining was incorporated into the prep diet and subsequently labeled as a sulfolipid (SL) diet.

The eluates collected during the process of separating other material from the sulfatide were combined, the solvents removed, and the residue incorporated into a diet. This diet was labeled, for want of a more exacting term, phospholipid (PL).

Fractionation of CLO by Sodium Hydroxide Extraction at 4°C

a. One hundred g Squibb CLO was mixed with 150 ml of 0.5 N sodium hydroxide and shaken for 16 hours at 4°C. The resultant mixture after being centrifuged at 1000 x g for a total of about four hours separated into three layers. The bottom aqueous layer was withdrawn. The top two layers, which had not clearly separated, were re-extracted with 50 ml sodium hydroxide. The three layers again formed, the small lower phase was drawn off and combined with the above aqueous layer which was then acidified to a pH of 4-4.5 with glacial acetic acid and allowed to equilibrate. A clear brown layer rose to the top. It was removed and labeled free fatty acids (FFA). The remaining aqueous phase was concentrated in a rotary evaporator, labeled aqueous and refrigerated. Of the two top phases, the middle appeared as a white foamy layer; the upper phase was a straw colored oil. The two top phases were washed with water which formed an emulsion with the "white" layer. The top oily phase was removed and treated separately. The "white" layer would not go into ether until it was acidified. This fraction gave off a faint smell of sulfur; it was labeled "white fraction" and stored until diets were mixed. The oily phase was further treated with acetone in which it was completely miscible. To the acetone mixture were added 100 ml of sodium hydroxide causing four layers to separate. The bottom phase was small; it was removed but not incorporated into a diet. The next to the lowest phase was an amber color. It was drawn off, and when neutralized with glacial acetic acid turned to a light yellow. This fraction was evaporated

to dryness and labeled oil-2. The third layer from the bottom was a light yellow oil which was withdrawn and labeled oil-3. The top phase, a dark amber color, was removed, concentrated and labeled oil-4.

One kg of diet was made with the addition of approximately half of each fraction except for the oil-3 which was liquid. It was weighed and half of it added to the prep diet. Thus, from this procedure of fractionating CLO, six diets were fed: "white," aqueous, FFA, oil-2, oil-3, and oil-4.

b. The same method was repeated with several modifications. All centrifugation was carried out in a Lourdes refrigerated centrifuge at 10,000 x g at 4°C. The initial mixture was centrifuged for about one hour. The procedure described above was followed with the same results until the straw-colored oily layer was treated with acetone followed by sodium hydroxide. Instead of the four layers that had previously separated out, only two phases formed. This mixture was centrifuged at 10,000 x g and decanted into a separatory funnel. After equilibration there were still only two phases--the lower a dark brown, the upper phase a light yellow oil. The lower phase was drawn off and labeled acetone-1. The oily phase was then called acetone-2. The four fractions now separated (aqueous, FFA, acetone-1 and -2) were placed in a convection oven to evaporate. The remaining "white" fraction, after washing with water was centrifuged at 10,000 x g. It was then repeatedly extracted with ether until only a colorless, watery phase was left. The extract was concentrated and labeled "white-ether."

Five diets were prepared from half of each fraction except that all of FFA was incorporated.

c. Four of the five fractions obtained as described in part b. above were further treated in an attempt to identify the composition of the fractions. The FFA fraction was not treated. Samples were separated on Silica Gel G using a number of solvent systems and visualizing compounds as suggested by Stahl (116). A partially purified sulfolipid standard, prepared from rat brains, was used as a reference material. Although varying degrees of separation were achieved, it was decided to attempt partial purification of the fractions by column chromatography and then to enlist TLC. Subsequently, CLO was treated as described in parts a. and b. but the fractions were determined on the basis of polarity with the end result being two main fractions.

The non-polar fraction was concentrated, dried over disodium sulfate, and placed on an activated silicilate column. The material was separated by gradient elution and collected in 10 ml fractions by an automatic fraction collector. The gradient was: 300 ml chloroform, 250 ml chloroform:methanol (4:1), 200 ml chloroform:methanol (1:1), and 200 ml chloroform:methanol (1:4). The contents of the collection tubes were combined by visually determining bands according to color. Each combined fraction, ten in all, was chromatographed on Silica Gel G with the solvent system n-propanol: 12.5 percent ammonium hydroxide (80:20). This solvent system, and the visualizing agent, Rhodamine B, with which the plate was sprayed, had been found in the earlier experiments to separate and visualize the largest number of

fractions. From the results of this plate, fractions collected in tubes 44-87 (beginning with the fourth tube after the chloroform:methanol (4:1) had been added) were combined, concentrated, and reapplied to a silicic acid column. Gradient elution proceeded as before. TLC of the fractions obtained in this manner (eleven in all) was carried out finally in a solvent of petroleum ether:ether:glacial acetic acid (90:10:1). Visualization was first by Rhodamine B, followed by charring after spraying with a solution of chromic sulfuric acid (5 g potassium dichromate in 100 ml 40 percent sulfuric acid). From this plate, three fractions were selected as sufficiently pure for dietary tests. The three samples corresponded to single collections of which two were distinct bands eluted by the chloroform:methanol (4:1). The latter two fractions were labeled lipid-2 and lipid-3; the former sample, lipid-1.

The polar fraction, dark brown in color, was concentrated and placed on a 3.5 cm x 71.5 cm column of Johns-Manville Hyflo Super-cel that had been washed with the solvent for elution. This solvent was composed of 233 ml isopropanol, 13 ml formic acid, 116.5 ml ethanol, 136 ml water plus 100 ml pyridine. No distinct bands were separated. The eluate was again grouped by the color of the liquid collected and resulted in four fractions (clear, yellow, clear, pale yellow). Every fourth tube from these four was subjected to the Molish test. The tubes making up the second fraction (which ranged in color from light yellow to dark amber to light yellow) reacted positively. The other three fractions reacted negatively. All fractions were chromatographed according to standard techniques for carbohydrate-containing compounds

(117,118). Since no further purification appeared likely, the reaction to the Molish test was used to distinguish which of the fractions from the original aqueous fraction would be incorporated into diets. The two diets were labeled aqueous-1 and aqueous-2.

Determination of the Specific Activity of Costal Cartilage Sulfomucopolysaccharides (MPS)

A sulfomucopolysaccharide fraction was prepared from the costal cartilage according to the modified method of Bostrom (119). The excised ribs were boiled in distilled water for approximately five minutes. The cartilage was completely freed of muscle and weighed. The cartilage was then sonified with approximately 5 ml acetone by using a Bronwell, Biosonik probe. The acetone was discarded, and the cartilage sonified with 4.0 ml of 0.5 N NaOH for four minutes. The liquid portion was decanted into a 15 ml centrifuge tube. The cartilage was then sonified a third time using 2.0 ml of 0.5 N NaOH for two minutes. The liquid was decanted, combining it with the product of the first extraction. The pH of the decanted liquid was adjusted to 6 with 10 percent acetic acid, and the solution was centrifuged for ten minutes at 1000 x g in an International Model SBV centrifuge. The supernatant fluid was poured into a 50 ml centrifuge tube, three or four drops of 20 percent sodium acetate were added, and the solution was precipitated overnight at -20°C with three volumes of 95 percent ethanol. The next day, the mixture was centrifuged as above for ten minutes, and the supernatant fluid was discarded. The residue was dissolved with

stirring in 3.0 ml of 0.5 N NaOH and again centrifuged. The supernatant fluid was poured into a 15 ml centrifuge tube, adjusted to pH 6, 20 percent sodium acetate added, and precipitated overnight as described above. The following day, the ethanol solution was centrifuged, the supernatant fluid discarded, the precipitate washed with 95 percent ethanol and then dried with ethyl ether. Duplicate samples of the mucopolysaccharide were weighed into 25 ml Erlenmeyer flasks, combusted with 1 ml of a combustion mixture (600 ml HNO_3, 300 ml $HClO_3$ and 9 g $Cu(NO_3)_2$) and evaporated to dryness.

The samples were quantitatively transferred with distilled water to 15 ml conical glass centrifuge tubes. Two ml of a 1 percent benzidine solution were added and the samples placed in the freezer for at least two hours. The resulting precipitate was separated from the solution by centrifugation, the supernatant discarded, and 1.0 ml Soluene added. The dissolved samples were quantitatively transferred to counting vials with 15 ml of scintillator solution of PPO in toluene.

Determination of Total Sulfur as Sulfate

Lungs from the animals fed the diets described in experiment 4 were lyophilized, weighed, and combusted in a Parr sulfur bomb apparatus. The residue from each set of lungs was diluted to 25 ml with distilled water, and portions of the dilutions were analyzed by the method of Roe et al. (120) as described by Whittle (100).

One ml of 5 percent lanthanum chloride and 2 ml of 15 percent barium chloride were added to 2 ml of the lung dilution in a 50-ml

centrifuge tube. The tube was shaken well and centrifuged at 2000 x g for ten minutes. The supernatant was discarded and the precipitate was washed with 5 ml of distilled water to remove excess barium from the tube. The sample was centrifuged again, the supernatant discarded, and the washing repeated. After the supernatant from the third centrifugation had been removed, the precipitate was dissolved in 10 ml of an alkaline disodium ethylenediamine tetracetate (EDTA) solution which was prepared by dissolving 10 g EDTA in 500 ml of distilled water, adding 20 g of sodium hydroxide, and diluting to 2000 ml.

A stock sulfate solution was made by dissolving 1.479 g of sodium sulfate in 500 ml of distilled water to yield a concentration of 2000 ppm of sulfate. Duplicate working standards, which contained 0.00, 0.10, 0.25, 0.40 and 0.50 ml, respectively, of the stock sulfate solution plus enough distilled water to bring the volume of each one up to 5 ml were treated in the manner described for the sample. A set of standards, containing 0, 20, 50, 80, and 100 ppm of sulfate, were run with each group of samples.

Samples and standards were aspirated into the flame of a Perkin Elmer Model 303 Atomic Absorption Spectrophotometer and the percent absorption of each was recorded. Percent absorption, an exponential function, was converted to absorbance, a linear function, so that the following equation could be used for determining the absolute values of sulfate:

$$\frac{\text{Absorbance of Unknown}}{\text{Absorbance of Standard}} \times \frac{\text{Concentration of Standard (Mg)}}{1} \times \frac{\text{Dilution}}{\text{Dry Weight of Tissue (Mg)}} = \text{Mg S as } SO_4/\text{Mg of Tissue} .$$

Estimation of Phosphorus

An aliquot of the sample was pipetted into an acid-washed micro-Kjeldahl flask and evaporated to dryness. To the flask were added 2.2 ml perchloric acid and a glass bead. Digestion was carried out on a medium setting of the hot plate until the solution was colorless. When the completely digested sample had cooled 20 ml of distilled water, followed by 2 ml of a hydroquinone solution and 1 ml molybdate solution were added. The extinction coefficient at 650 mμ was determined between five and thirty minutes after all of the reagents had been added with the Beckman B spectrophotometer. If the phosphorus was to be determined from samples which had been separated on Silica Gel G, the portion of the adsorbant containing the sample was quantitatively scraped into the micro-Kjeldahl flask and the same procedure followed. However, during the period of color development the samples were centrifuged and the supernatant decanted into the spectrophotometric tubes. A blank containing Silica Gel G was also scraped from the plate and processed.

Standards containing 0.1, 0.2, and 0.5 mg phosphorus were routinely included with the set of samples being processed. The standards were prepared from a dilution of a standard phosphate solution containing 1 mg phosphorus per ml. The stock solution was prepared by dissolving 1.0967 g of oven-dried potassium phosphate in distilled water and diluting to 250 ml. The hydroquinone solution was prepared by dissolving 2 g of hydroquinone and 50 g sodium bisulfite in distilled water and diluting to 200 ml. It was stored in an amber bottle in the refrigerator and prepared fresh every few days. The molybdate

solution was an 8.3 percent solution of ammonium molybdate in distilled water (121).

Oxidation of Methionine ^{35}S-Sulfur to Sulfate

Each weighed liver was homogenized in a Thomas glass homogenizer with a Teflon pestle with sufficient 0.067 M phosphate buffer, pH 7.4, to make a 10 percent homogenate. The homogenate was centrifuged at 4°C at 750 x g for ten minutes and the denucleated supernatant fluid decanted into cold vials which were packed in ice until used (within ten minutes). Each preparation was analyzed for percent nitrogen by the micro-Kjeldahl procedure.

Each reaction vessel (a 50 ml Erlenmeyer flask) contained 200 µM of ^{35}S-methionine, 10 µM ATP, 600 µM $MgCl_2$, and 1 ml of the enzyme preparation and sufficient 0.067 M phosphate buffer to make a final volume of 4 ml. A control sample contained the same reactants except that the enzyme preparation had been boiled. The samples were incubated in a shaking water bath for 30 minutes at 37°C. The reaction was stopped by the addition of 1 ml of 10 percent trichloroacetic acid. The precipitate was separated by centrifugation and the cake was washed with 5 ml distilled water and recentrifuged. The supernatants were combined and applied to a column packed with Dowex 50W-X8 (200-400 mesh) resin in the hydrogen form. The column was 1 cm in diameter and contained 4 cm of resin. When the sample had passed into the resin, the column was washed with a volume of distilled water equal to the hold-up volume of the column.

The sample was precipitated, collected, and counted by a modification of the method of Katz and Golden (122). One ml of carrier sulfate solution was added to the column eluate followed by 1 ml of 10 percent barium chloride. The carrier sulfate solution, calculated to yield 12 mg of barium sulfate, was prepared by diluting 2.85 ml of concentrated sulfuric acid to 1 liter with distilled water. The resulting precipitate was collected as $BaSO_4$ on a weighed glass-fiber filter paper disc. During the collection process the 24 mm filter paper disc was supported by a small piece of Whatman No. 1 filter paper and secured between a perforated rubber disc and a glass funnel cut from glass tubing. The side arms of the glass funnel were attached to a vacuum flask with springs. The precipitate was washed onto the paper with 5 to 10 ml portions of distilled water, 0.5 N HCl, and 95 percent ethanol followed by acetone. The filter paper was again weighed and then placed in a glass counting vial in 15 ml of a scintillator of PPO in toluene. Previous testing of this procedure, in which the counting efficiency of a solubilized sample was compared with that of a precipitate on a glass fiber filter paper, showed a 50 percent reduction in counting efficiency. Thus, corrections in total radiation reported were made where appropriate. The column procedure was checked by passing a facsimile of the incubation mixture containing either ^{35}S-methionine or ^{35}S-sulfate through the column and determining the counts recovered in the eluate.

Extraction of Lipid from Rat Livers

The livers removed from the rats fed diets as described in experiment 4 were frozen until extracted. For extraction, a liver was partially thawed, weighed, and placed in a Teflon-coated micro-blender with a few crystals of hydroquinone and about 45 ml of methanol. The liver was blended for two minutes, then approximately 90 ml of chloroform were added and the mixture blended an additional minute. Following the addition of 16 ml of distilled water containing 0.4 g zinc acetate, the mixture was homogenized for ten seconds. The extraction mixture was transferred to a Buchner funnel and filtered with suction through Whatman No. 1 filter paper. The residue of liver and paper was re-extracted with chloroform, the filtrates quantitatively transferred to a separatory funnel and placed in the freezer at 4°C to equilibrate. The chloroform layer was removed and quantitated to 200 ml for further testing.

Separation of Lipids by Thin Layer Chromatography

The lipids from the livers of the rats fed the diets described in experiments 4 and 5 and standard solutions of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin were fractionated by thin layer chromatography. Silica Gel G, 5 mm in thickness, was used as the adsorbant and the lipid samples were applied as small dots in the center of vertical channels ruled into the adsorbant. Development of the chromatogram was achieved by using chloroform:methanol:water (80:35:5) as an ascending solvent. Visualization of the developed chromatograms was achieved by spraying them with Rhodamine B. The appropriate

spots were scraped off and quantitatively transferred to micro-Kjeldahl flasks for phosphorus determinations.

III. STATISTICAL METHODS

The data were analyzed by the method of paired or unpaired comparisons as described by Steel and Torrie (123). All computations were performed using the Olivetti-Underwood Programma 101.

CHAPTER IV

RESULTS

Experiment 1

Observations of weight gain and efficiency of feed utilization have long been used to evaluate dietary regimes. In addition, determination of the specific activity of mucopolysaccharides isolated from rib cartilage of rats injected with ^{35}S-sulfate has been used in this laboratory as a measure of sulfation. These three methods of evaluation were used to evaluate the diets investigated in the preliminary experiments.

The fractions partitioned from CLO as described for experiment 1 and fed to male rats resulted in total weight gains and feed efficiencies comparable to the control diet with added CLO, with the possible exception of weight gains when fed the MeOH diet; however, the female rats did not gain weight as well when fed the saponified or MeOH diets as when fed the ether or the + CLO diet. The feed efficiencies of the female rats followed the same trend: the ether diet utilization was comparable to the + CLO, and the saponified and MeOH diets were comparable to the prep diet. The data from males fed these diets was not sufficient to say that the uptake of labeled sulfur was affected by diet. Although the results were not definitive, the uptake of labeled sulfur was decidedly higher in the female rats fed the ether diet than in those fed the other two diets and appeared comparable to the + CLO diet. Overall, the parameters evaluated

were more affected by the diet of female than of male rats.

An aliquot of each fraction investigated was analyzed for sulfate content by the barium chloranilate method. The results indicated that the saponified fraction contained about three times as much sulfate as did the ether fraction (788 μg vs 233 μg), and that the MeOH fraction did not contain sulfate. Thus, it was concluded that this method was not a satisfactory procedure for isolating a fraction with unique properties that could account for the effect of CLO in a diet.

Experiment 2

Since CLO had previously been shown in this laboratory to contain 0.16 percent sulfate (124), and not yet relinquishing the theory of a sulfur compound in cod liver oil being responsible for growth improvement, the second experiment was designed to fractionate cod liver oil by a method known to isolate sulfolipids. The fraction labeled PL had an offensive odor and was poorly accepted by the rats; therefore, even though the average feed efficiencies were the same as that for rats fed the prep diet, the weight gains were less than half as much as for rats fed either the prep or SL diets. In contrast, uptake of ^{35}S-sulfate by the rib cartilage MPS of rats fed the PL diet was slightly better than the uptake by rats fed the SL diet and more than five times the uptake by rats fed the prep diet. The utilization of feed by the rats fed the SL diet was equal to that of rats fed the + CLO diet, but was a third more than the feed efficiency of those animals that consumed the control (prep) diet; however, the

total weight gain of those rats fed the SL diet was only slightly better than that of the rats fed the prep diet. The feed efficiencies and weight gains of the rats fed the H_2O fraction were similar to those of the rats fed the + CLO diet; however, the uptake of the label by rib cartilage MPS from these animals was less than the uptake by animals fed either of the other two experimental diets and was somewhat less than half that of the animals fed the + CLO diet.

Experiment 3

The diets prepared from the saponification in the cold of CLO and fed to male and female rats as outlined in experiment 3 (a and b) were all found to generally improve the growth, feed efficiency and sulfation of rib cartilage mucopolysacchardies. The most interesting change was that observed in rats fed the acetone-2 fraction of part b. Uptake of radioactive sulfur in rib cartilage MPS was approximately three times more than the uptake of radiation by rats fed the remaining experimental diets. In agreement with the data reported from experiment 1, male rats were noted to achieve higher feed efficiencies than female rats. Although the fractions obtained by saponification were further purified by the procedures described in 3 c., little evidence was obtained to indicate that this fractionation procedure contributed to the further identification of the "active factor" in CLO. Neither the weight gains nor the feed efficiencies demonstrated much difference between diets as compared to the + CLO diet. All of the feed efficiency values were high (medians of 36 and 43 percent for

females and males, respectively) with the highest being found in rats fed the aqueous-2 (females) or lipid-1 (males) of diets of part c. The weight gains, however, were highest for male rats fed the aqueous-1 or lipid-3 diets and for the female rats fed the FFA diets. Although the sulfur uptake data are erratic, the data were interpreted as an indication that all of the diets increased the uptake of $^{35}SO_4$ into rib cartilage mucopolysaccharides, but that the aqueous-1 diet had the most activity. Thus, the first three experiments of this project, while not completely disproving the theory of an "active" fraction, strongly suggested that the effect of CLO was dispersed through several fractions and was generally associated with lipid fractions.

Experiment 4

A comparison of the percent utilization of feed by the rats fed the diets described in experiment 4 is summarized in Table 3. Statistical analysis of these data show that the only significant differences are found when the feed efficiencies of rats fed the $\bar{s}$ E, + CLO standard diet are compared with those of rats fed the diets with added lecithin. Although there is not a statistically significant difference among the other diets, the rats fed the prep diet achieved the lowest average feed efficiency followed by that of the rats fed diets without CLO or without vitamin E. The data presented in Table 3 may be interpreted as showing that feeding the standard $\bar{s}$ E, + CLO diet to rats resulted in an average weight gain which was statistically significantly higher than that of rats fed any other diet. In addition, the weight gains of rats

TABLE 3

PERCENT FEED EFFICIENCY AND FINAL WEIGHT GAIN OF RATS FED DIETS OF EXPERIMENT 4 FOR FOUR WEEKS

Diet	Feed Efficiency[1] %	Weight Gain[1] g
Prep	25 ± 1[2ab]	90 ± 4[2a]
$\bar{s}$ E, + CLO	28 ± 0[a]	119 ± 2
+ E, $\bar{s}$ CLO	26 ± 1[ab]	97 ± 6[ab]
+ E, + CLO	28 ± 1[ab]	96 ± 7[ac]
$\bar{s}$ E, $\bar{s}$ CLO + lec[3]	26 ± 1[b]	95 ± 6[ad]
+ E, $\bar{s}$ CLO + lec[3]	27 ± 0[b]	104 ± 4[bcd]
Safflower	27 ± 1[ab]	106 ± 3[bc]
Linolenic	27 ± 1[ab]	84 ± 3[a]

[1]Results are the averages of five animals ± the standard error of the mean.

[2]Mean values in a column followed by the same superscript letter do not differ significantly ($P > 0.05$) by the method of paired comparisons (123).

[3]lec = lecithin.

fed the + E, $\bar{s}$ CLO, + lecithin diet and the safflower diet were significantly higher than weight gains of rats fed either the prep or the linolenic supplemented diets. Further, the safflower diet supported growth significantly better than the $\bar{s}$ E, $\bar{s}$ CLO, + lecithin diet.

The data which represent the uptake of a test dose of $^{35}SO_4$ by the rib cartilage mucopolysaccharides are presented in Table 4. Rats fed the safflower oil diet incorporated a significantly higher level of radioactivity into MPS only when compared with rats fed the prep or $\bar{s}$ E + CLO diets. As was observed when feed efficiencies were compared (Table 3), a statistically significant difference exists in the uptake of label into MPS when rats are fed the $\bar{s}$ E, + CLO diet as compared with those fed either diet containing lecithin. Furthermore the uptake of ^{35}S-sulfate into rib cartilage MPS of rats fed the standard diets (+ E, $\bar{s}$ CLO and + E, + CLO) was significantly higher than rats fed the + E, $\bar{s}$ CLO, + lecithin diet. Possibly a statistically significant difference would be found between the other diets as is suggested by direct comparison of the values presented in Table 4 except that the variance of these data are high and several samples were lost during isolation of the MPS.

When the sulfur as sulfate was determined (see Table 4), the lungs of rats fed the prep diet were found to contain a significantly lower level of sulfur as sulfate than did the lungs of rats fed any other diet. The statistically significant increase in lung sulfate caused by the addition of vitamin E to the + CLO diet is not unexpected;

TABLE 4

UPTAKE OF ^{35}S-SULFATE INTO RIB CARTILAGE MUCOPOLYSACCHARIDE AND TOTAL SULFUR AS SULFATE IN LUNG TISSUE OF FEMALE RATS FED THE DIETS OF EXPERIMENT 4 FOR FOUR WEEKS[1]

Diet	Rib Cartilage Mucopolysaccharide CPM/mM SO_4	Total Sulfur as Sulfate in Dry Lung μg SO_4/mg
Prep	0.85 ± 0.15^{ab}[2]	7.79 ± 1.08^{a}[2]
$\bar{s}$ E, + CLO	1.04 ± 0.04^{a}	11.48 ± 0.75^{b}
+ E, $\bar{s}$ CLO	1.00 ± 0.32^{ad}	13.12 ± 1.03^{bc}
+ E, + CLO	0.99 ± 0.03^{ad}	14.74 ± 4.17^{c}
$\bar{s}$ E, $\bar{s}$ CLO + lec[3]	0.62 ± 0.19^{bcd}	13.48 ± 1.64^{bcd}
+ E, $\bar{s}$ CLO + lec[3]	0.76 ± 0.09^{bc}	14.32 ± 1.24^{cd}
Safflower	1.48 ± 0.44^{cd}	12.67 ± 0.96^{bc}
Linolenic	0.97 ± 0.42^{ac}	12.16 ± 1.03^{bd}

[1]Values are means ± the standard error.

[2]Mean values in a column followed by the same superscript letter do not differ significantly ($P > 0.05$) by the method of unpaired comparisons (123).

[3]lec = lecithin.

however, the statistically significant increase in sulfur as sulfate in lungs from rats fed the + E, $\bar{s}$ CLO, + lecithin diet as compared with that of lungs from rats fed the $\bar{s}$ E, + CLO diet was surprising. A significant increase in lung sulfur as sulfate was also found when rats were fed the + E, + CLO diet as compared with rats fed the linolenic acid supplement diet. In addition, a statistically significant difference was found when the correlation coefficients comparing feed efficiencies with lung sulfur as sulfate were determined.

In addition to the above analyses, lipids of the livers from two rats of each dietary group were extracted and separated by TLC. Although the results of the TLC can only be regarded as intriguing, it appeared that all of the diets with a source or potential source of unsaturated fatty acids retained an average of approximately 46 percent lecithin in the liver whereas the diets with no added unsaturated fatty acids (prep and + E, $\bar{s}$ CLO) retained an average of approximately 65 percent.

Experiment 5

The decision was made to repeat the feeding of the " lecithin diets and to feed diets with additional choline to be sure there was no choline deficiency. A new standard diet, one with a "normal" level of inorganic sulfate (0.1 percent) was included in the test group. The uptake of the ^{14}C-methyl radical from ^{14}C-methyl methionine was added as another evaluation parameter for this experiment.

The data presented in Table 5 may be used to demonstrate that there were only small differences in feed efficiency or weight gain between the rats fed any of the diets of experiment 5. The only statistically significant difference in the average weights of the livers was between the weights of livers taken from rats fed either the + choline or the + lecithin diets (Table 6). The data which are presented in Table 6 may also be used to show that there were several significant changes in the uptake of ^{14}C into the liver lipid of these rats. A statistically significantly lower uptake of ^{14}C was observed only when the prep diet was compared with the $\bar{s}$ E, + CLO diet. On the other hand, ^{14}C-methyl uptake by rats fed the + choline diet was not statistically significantly different except when compared to the ^{14}C-methyl uptake by rats fed the + lecithin diet, where the ^{14}C-methyl uptake was significantly increased. The $\bar{s}$ E, + CLO and + 0.1 percent SO_4 diets fed to the rats also resulted in a significantly higher uptake of label than did the + lecithin diet when fed to rats.

When a comparison was made of the lipid phosphorus per mg liver (Table 7) there were no changes due to the diet. The change in uptake of radiation reported in Table 6 was reflected in the data of Table 7 where the only significant difference in counts/min/mg phosphorus was between animals fed the prep diet and those fed the + choline diet. The effect on rats of choline in the diet was also seen in the total lipid phosphorus where there was a significant increase in total lipid phosphorus over that found in the livers of rats fed the $\bar{s}$ E, + CLO diet.

TABLE 5

PERCENT FEED EFFICIENCY AND FINAL WEIGHT GAIN OF RATS FED DIETS OF EXPERIMENT 5 FOR FOUR WEEKS

Diet	Feed Efficiency[1] %	Weight Gain[1] g
Prep	29 ± 1[a2]	115 ± 5[a]
+ CLO	29 ± 1[a]	100 ± 8[a]
+ 0.1% SO_4	30 ± 2[a3]	114 ± 6[a3]
+ Lecithin	29 ± 3[a]	106 ± 13[a]
+ Choline	30 ± 1[a]	109 ± 4[a]

[1]Results are the averages of five animals ± the standard error of the mean.

[2]Mean values in a column followed by the same superscript do not differ significantly ($P > 0.05$) by the method of paired comparisons (123).

[3]Results are the averages of three animals ± the standard error of the mean. Statistical treatment was by the method of unpaired comparisons (123).

TABLE 6

EFFECT OF DIETS OF EXPERIMENT 5 ON THE UPTAKE OF ^{14}C FROM ^{14}C-METHYL-METHIONINE BY RAT LIVER LIPIDS[1]

Diet	No. Per Group	Average Liver Weight	Average Counts/Min/Mg Liver x 10^5
		g	
Prep	5	6.31 ± 0.38^{ab}	15.7 ± 1.5^{ab}[2]
+ CLO, $\bar{s}$ E	3	5.79 ± 0.65^{ab}	22.6 ± 2.9^{c}
+ Choline	4	6.30 ± 0.25^{a}	18.8 ± 1.7^{ac}
+ Lecithin	3	5.42 ± 0.39^{b}	12.4 ± 1.3^{b}
+ 0.1% SO_4	2	6.01 ± 0.06^{ab}	18.4 ± 2.0^{ac}

[1]Values are means $\pm$ the standard error.

[2]Mean values in a column followed by the same superscript letter do not differ significantly ($P > 0.05$) by the method of unpaired comparisons (123).

TABLE 7

EFFECT OF DIETS ON THE UPTAKE OF ^{14}C FROM ^{14}C-METHYL-METHIONINE AND PHOSPHOLIPID CONTENT OF RAT LIVERS[1]

Diet	No. Per Group	Total Lipid P, mg	µg Lipid P/ mg Liver	Counts/Min/ mg P
Prep	5	6.93 ± 0.96[ab2]	0.11 ± 0.01[a]	1.28 ± 0.25[a]
+ CLO, $\bar{s}$ E	3	6.06 ± 0.81[a]	0.12 ± 0.00[a]	1.56 ± 0.60[ab]
+ Choline	4	7.53 ± 2.01[b]	0.12 ± 0.03[a]	1.86 ± 0.70[b]
+ Lecithin	2	6.50 ± 0.25[ab]	0.12 ± 0.00[a]	0.76 ± 0.02[ab]
+ 0.1% SO_4	1	7.31	0.12	1.07

[1]Values are means ± the standard error.

[2]Mean values in a column followed by the same superscript letter do not differ significantly ($P > 0.05$) by the method of unpaired comparisons (123).

Experiment 6

When rats were fed diets moderately high in CLO and methionine, the effect on the expiration of $^{14}CO_2$ is illustrated by the data presented in Table 8. Those animals fed a diet with a 5 percent level of CLO expired a statistically significantly higher amount of $^{14}CO_2$ as a percent of dose than the rats not given CLO in their diets. Since these data could be interpreted as demonstrating that more methionine was being metabolized by those rats fed diets supplemented with CLO, it was assumed that more sulfate was also being formed.

Experiment 7

Since it was assumed that more sulfate was being formed by those rats fed diets supplemented with CLO this assumption was tested by determining the amount of ^{35}S-methionine sulfur that was oxidized to ^{35}S-sulfate by liver preparations from animals fed the above diets. The data which are presented in Table 9 may be used to document this assumption. Expressed either as the total micromoles converted to sulfate or as micromoles sulfate per mg nitrogen, the oxidation of ^{35}S-sulfate was statistically significantly higher by the liver homogenates from those rats fed CLO.

TABLE 8

THE EFFECT OF COD LIVER OIL ON THE EXPIRATION OF $^{14}CO_2$ FROM METHIONINE-1-^{14}C

	$^{14}CO_2$ Expiration as Percent of Dose	
Rat	$\bar{s}$ CLO	$\bar{c}$ CLO
1	0.029	0.045
2	0.060	0.069
3	0.022	0.040
4	0.022	0.024
5	0.031	0.038
6	0.030	0.042
Mean[1] $\pm$ SE	0.032 $\pm$ 0.01	0.043 $\pm$ 0.01

[1]Means are significantly different ($P < 0.01$).

TABLE 9

EFFECT OF COD LIVER OIL ON THE OXIDATION OF METHIONINE SULFUR TO SULFATE[1]

Rat	Total μmole Converted[2] x 10^{-2}		μmole x 10^{-2}/mg N^2	
	$\bar{s}$ CLO	$\bar{c}$ CLO	$\bar{s}$ CLO	$\bar{c}$ CLO
1	71.5	96.5	42.0	53.0
2	46.4	66.1	30.6	38.4
3	64.3	82.2	39.4	44.0
4	53.9	59.5	28.1	32.2
5	74.4	111.6	45.6	64.9
Mean ± SE	64.1[3] ± 5.3	83.2[3] ± 9.6	37.2[4] ± 3.4	46.5[4] ± 5.7

[1]Incubation mixture contained 200 μM methionine-^{35}S, 10 μM ATP, 600 μM $MgCl_2$, and 1 ml of the enzyme preparation with the total volume adjusted to 5 ml with 0.067 M phosphate buffer. After incubation for 30 minutes at 37°C, the reaction was stopped by the addition of 1 ml of 10 percent trichloroacetic acid.

[2]Calculated on the basis of the specific activity of the methionine.

[3]Means are significantly different ($0.02 > P > 0.01$).

[4]Means are significantly different ($0.05 > P > 0.02$).

CHAPTER V

DISCUSSION

Button (1) proposed an interaction between inorganic sulfur and CLO in the normal metabolism of the albino rat after she observed a gross malformation of the hind limbs of a rat fed a diet low in sulfate and without CLO. The rats were fed these diets from the time they were two weeks of age through the seventh week of life. Toward the end of this period, the fourth week after weaning, the lesion was observed. Her study included four basic variations of a diet low in sulfate: with and without CLO and with and without vitamin E. She concluded, after eliminating many possible causes of abnormal sulfation, that there was present in CLO a factor which enhanced normal production of the sulfated mucopolysaccharides. She further postulated, on the basis of autoradiograms of rat epiphyses, that the site of the lesion was the cell membrane "where an increased permeability, resulting from the incorporation of long-chain, highly unsaturated fatty acids of the oil into the structural lipid of the chondrocyte membrane, facilitated the passage of the sulfomucopolysaccharide from the cell" (1). The diet low in sulfate would create the stress necessary to make the lesion apparent. The present study was undertaken in an attempt to identify the "active factor" in CLO that was proposed by Button (1).

Since Button's studies (1) had indicated that the ossification of cartilage was impaired and other investigators (125) had suggested

that vitamin D-sulfate might be the active form of vitamin D, it was assumed that cod liver oil might be supplying preformed vitamin D-sulfate. If indeed vitamin D-sulfate were the active form of vitamin D, it could exert a profound influence on mineral metabolism and alleviate the symptoms observed by Button (1) by supplying a preformed coenzyme without having the vitamin D compete with other systems for the limited amount of inorganic sulfur available. Thus it seemed logical that the first experiment would be to attempt the isolation of vitamin D-sulfate from cod liver oil. Since no clear cut directions for the isolation of vitamin D-sulfate were found in the literature a method for the isolation of vitamin D was followed (113).

The diets prepared from the fractions of CLO which were obtained in the attempt to isolate vitamin D-sulfate were fed to both male and female rats. Based on weight gains and feed efficiencies the male rats thrived better when fed any of these diets than did the females, although both male and female rats had higher weight gains and feed efficiencies when fed the diet labeled "ether." All of the fractions supported growth sufficiently well that the possibility of their containing the "active factor" was not ruled out. Therefore, since the active factor could not be concentrated in a fraction which should represent vitamin D-sulfate the possibility that vitamin D-sulfate might be the "active factor" was abandoned and a search for some other source of sulfate was begun.

A previous report (124) had shown that although sulfolipids, when fed as an exogenous source of sulfur, were poorly absorbed the

efficiency of utilization of the sulfate that was absorbed to sulfate mucopolysaccharides was higher than that of any source investigated. Therefore, since the lesion observed previously in rats fed diets low in sulfate and without CLO appeared to be associated with mucopolysaccharide metabolism (1), cod liver oil was next fractionated in an attempt to isolate sulfolipids. When the CLO fractions which resulted from an attempt to isolate sulfolipids were fed to rats, once again the "active fraction" was not associated only with the sulfolipid fraction although this fraction was effective. But the active fraction was associated with the phospholipid fraction as well. Based on the incorporation of a test dose of $^{35}SO_4^{=}$ into rib cartilage mucopolysaccharides, the aqueous fraction from the Norit column was inferior to all of the other fractions. It may be recalled that the method for isolating vitamin D-sulfate from CLO required a saponification step and that the "active factor" appeared to be saponifiable even when a similar operation was repeated in the cold. The fact that vitamin D should not be saponifiable and the failure to concentrate the active factor in the sulfolipid fraction, plus the inferior dietary supplement properties of the aqueous fraction all indicated that the "active factor" was associated with any lipid fraction from CLO. Therefore, rather than continue with apparently fruitless fractionation of cod liver oil, model systems were sought to be used to mimic the effect of cod liver oil.

In general, two different types of model systems were chosen. The first was one which would merely supply other sources of long-chain

polyunsaturated fatty acids, i.e., safflower oil and a semi-purified source, linolenic acid. The rationale for the other model system is somewhat more difficult to explain. Previous investigations (1) have shown that the lesions which developed in rats fed diets low in sulfate without cod liver oil were accentuated in female rats. Literature reports (66) have shown that the pathway to lecithin synthesis in the female rat is predominately by the ethanolamine pathway rather than the CDP-choline pathway. Since the ethanolamine pathway requires three molecules of S-adenosylmethionine it seemed reasonable to assume that a lecithin deficiency might be developing in those rats fed diets low in sulfate without cod liver oil. Therefore, both preformed lecithin and additional choline were included as model systems. The data which are presented in Tables 3 and 4, pages 59 and 61, respectively, may be used as an additional argument that the "active factor" in cod liver oil is really due to the effect of long-chain polyunsaturated fatty acids. For example, the effect on weight gain of including safflower oil in the diet is nearly as good as that of including cod liver oil. The beneficial effect of vitamin E cannot be ignored. However feeding large levels (7 percent) of CLO without additional vitamin E is one way to produce vitamin E deficiency symptoms quickly in a rat (109). Therefore, vitamin E cannot be the "active factor" in CLO. Admittedly, although the beneficial effect of safflower oil may be clearly documented by these data, the effect of added lecithin remained somewhat obscure. Therefore an additional experiment was conducted in which choline, lecithin, and sulfate addition were compared

with CLO (Table 5, page 64). Although no statistically significant differences were obtained, the highest average values for feed efficiency were observed in rats fed the diets containing added choline and sulfate.

These data and the foregoing discussion have indicated that the beneficial effects of incorporating CLO into a diet low in inorganic sulfur is somehow related to the metabolism of lecithins. Therefore it seemed reasonable to study the metabolism of lecithin by rats fed diets supplemented not only with CLO but also with the model system. These experiments have shown that the addition of CLO or the model systems results in a decrease in liver lecithin as a percent of total liver phosphorus when compared to diets without CLO or the model systems. Two possible interpretations of these data have been considered. One interpretation is that less lecithin is synthesized in the liver when CLO or the model systems are added to the diet. Another interpretation would be that increases in dietary long-chain polyunsaturated fatty acids resulted in an accelerated movement of lipid from the liver to the depot lipids with a resultant decrease in liver lecithin since it is serving as a vehicle to carry the lipid to the lipid depots. Although perhaps not unequivocal, the data which are presented in Tables 6 and 7, pages 65 and 66, may be used to support the latter interpretation. These data included the incorporation of ^{14}C from ^{14}C-methyl-methionine into liver lipid as well as the total lipid phosphorus in the liver of rats fed diets with added CLO, choline, lecithin, and sulfate. The most striking feature of these data is that

without any statistically significant difference in total liver phosphorus, there was a statistically significant increase in ^{14}C from ^{14}C-methyl-methionine in the lipid fraction from those rats fed diets which contained added CLO. If there is no significant change in the total lipid phosphorus between dietary treatments but an increased incorporation of ^{14}C from ^{14}C-methyl-methionine into the lipid fraction, then it would seem that there was an increased turnover of lecithin in the livers of those rats fed CLO. Therefore, it seems that the second interpretation presented above must be the correct one.

Although these data and the above discussion seem to interrelate CLO and lecithin metabolism the original objective of this investigation must not be forgotten. That is, that the "active factor" was demonstrable when the rats were fed a diet low in inorganic sulfur and without CLO. Therefore, the problem seemed to be one of interrelating increased lecithin metabolism with increased endogenous sulfur. The data which were presented in Table 6, page 65, demonstrated an increased incorporation of ^{14}C from ^{14}C-methyl-methionine into liver lipid. If this increased methyl metabolism also means increased methionine metabolism and cystathionine synthesis, then this could conceivably mean an increase in endogenous sulfate. The diets which were fed were 15 percent casein diets supplemented with 0.35 percent methionine. These diets contain 0.42 percent sulfur-containing amino acids from the casein which with the added methionine gives 0.77 percent sulfur-containing amino acids. According to Rose (126) 0.6 percent sulfur-containing amino acids are required for optimal growth,

which leaves a theoretical excess of 0.17 percent sulfur-containing amino acids. Admittedly the oxidation of cysteine sulfur to sulfate has been shown to be a wasteful process (100). But assuming 50 percent oxidation to sulfate and 50 percent to taurine there would still be 0.09 percent sulfur-containing amino acids to yield sulfate approximately equivalent to 0.04 percent. Recent results have shown 0.02 percent inorganic sulfate to be an optimum level for methionine sparing.[1] Therefore, if CLO would cause increased methionine metabolism it could conceivably supply sufficient endogenous sulfur for the rat. The nature of the dietary fat has been shown to alter the pathway to lecithin to one which uses phosphatidylethanolamine (8,50). Therefore, it seemed reasonable since other experiments in this study have shown that CLO increased the methyl metabolism of methionine to determine if CLO could increase the metabolism of the carbon skeleton of methionine. The data which are shown in Table 8, page 68, are representative of the metabolism of the carbon skeleton of methionine since they represent the percent expiration of a test dose of methionine. It may be seen that there is a statistically significant increase in the metabolism of the carbon skeleton of methionine by rats fed diets low in sulfate but supplemented with cod liver oil. Presumably this increase is a result of cod liver oil triggering the formation of not only increased amounts of lecithin but also more of the lecithin

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[1] J. T. Smith, unpublished observations.

formed is synthesized by the phosphatidylethanolamine pathway with the associated extra demands for S-adenosylmethionine resulting in sufficient S-adenosylhomocysteine to overload the recycling system (93). Therefore, the extra homocysteine provides additional substrate for cystathionine biosynthesis thereby increasing cysteine biosynthesis and increased metabolism of the carbon skeleton of methionine. The increased cysteine would result in increased sulfate.

For this explanation to be acceptable it would seem that methionine-^{35}S incubated with liver homogenates from rats fed diets low in sulfate and supplemented with CLO should give more $^{35}SO_4^=$ than methionine-^{35}S incubated with liver homogenates from rats fed diets low in sulfate but not supplemented with CLO. The data which are presented in Table 9, page 69, may be used to confirm increased oxidation of methionine-^{35}S to $^{35}SO_4^=$ by the liver homogenates from rats fed diets low in sulfate and supplemented with CLO. Therefore, it is concluded that the "active factor" in CLO is the long-chain polyunsaturated fatty acids.

CHAPTER VI

SUMMARY

Previous investigations in the laboratory of the Nutrition Department, The University of Tennessee, Knoxville, have shown that rats fed from weaning diets low in sulfate and without CLO grew less well, had lower feed efficiencies, and incorporated lower levels of $^{35}SO_4^=$ into rib cartilage mucopolysaccharides than did rats fed from weaning diets low in sulfate but supplemented with CLO (1). This investigator (1) proposed that there was an "active factor" in CLO which enhanced normal production of the sulfated mucopolysaccharides. The present study was undertaken in an attempt to identify the "active factor" in CLO.

Extensive fractionation of CLO failed to concentrate the "active factor" in any one fraction of CLO. Instead, any fraction which contained lipid appeared to be active. These observations were extended to show that CLO and other systems containing long-chain polyunsaturated fatty acids would influence the metabolism of lecithin. The increased metabolism of lecithin appeared to be associated with an increased metabolism of methionine.

It was concluded that the "active factor" in CLO is the long-chain polyunsaturated fatty acids. These long-chain fatty acids increase the endogenous sulfate by catalyzing increased lecithin synthesis

by the phosphatidyl ethanolamine pathway, placing extra demands on S-adenosylmethionine. The increased S-adenosylmethionine results in increased S-adenosylhomocysteine, which provides increased substrate for cystathionine biosynthesis and therefore increased cysteine. The increased cysteine consequently supplies the necessary endogenous sulfate.

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VITA

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