RELATIONSHIP BETWEEN PLASMA LIPIDS AND LIVER LIPIDS IN THE LAYING HEN

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CHAPTER I

INTRODUCTION

Abnormal liver lipid accumulations in laying hens have received considerable attention since Couch (1956) described a condition known as the "Fatty Liver Syndrome" (FLS). This condition occurs in commercial production systems and is characterized by (1) increased body weight, (2) decreased egg production by up to 50% (Abbott and Couch, 1971), (3) an excessive amount of abdominal fat, (4) fatty livers, and (5) liver capillary hemorrhages and hematomas. Involvement of kidney tissues also has been reported (Abbott and Couch, 1971; Weiss and Fisher, 1957). Wolford and Polin (1972a) have suggested the more inclusive term "Fatty Liver--Hemorrhagic Syndrome" (FLHS) to describe the condition. Associated with the economic loss of decreased egg production, an increase in mortality has been reported (Couch; 1956; Nesheim et al., 1969; Ringer and Sheppard, 1963). These two factors constitute an appreciable economic loss to the poultry producer.

Excessive energy consumption has been implicated as an important predisposing factor in the occurrence of FLHS (Nesheim and Ivy, 1970; Wolford and Polin, 1972). Under certain conditions, restriction in caloric intake by approximately 10% has been successful in preventing the syndrome (Sallmann and Schole, 1973; Wolford and Murphy, 1972). As a prophylactic measure, vitamin E, vitamin B_{12}, choline and/or inositol have commonly been supplemented to laying rations (Couch, 1956; Reed

et al., 1968; Abbott and Couch, 1971). However, their success has been quite variable (Barton, 1967; Deacon, 1968).

The etiology and pathogenesis of FLHS are unclear and beyond the scope of this paper. The objectives of this study were: (1) to characterize the accumulation of liver lipids in hens under commercial production conditions; (2) to examine changes in plasma lipids, lipoproteins, and liver Fatty Acid Synthetase (FAS) activity associated with liver lipid changes; and (3) to determine if supplementation of the normal layer diet with cholesterol and/or lecithin would alleviate or prevent excessive lipid accumulations in the liver.

CHAPTER 2

REVIEW OF LITERATURE

Introduction

Much data have been reported in the last two decades pertaining to the cause of the "Fatty Liver Syndrome" and its impact on commercial egg production. Concurrently, lipogenesis and the effect of dietary modification in the avian have received considerable attention. Yet few studies have investigated the dietary control of lipogenesis under simulated commercial egg production schemes. This review will survey the literature with regard to: (1) the characterization of abnormal liver lipid accumulation as exemplified by "Fatty Liver Syndrome"; (2) the nutritional and environmental factors implicated as predisposing hens to the "Fatty Liver Syndrome"; and (3) the effect of nutritional factors on lipogenesis in the avain.

Fatty Liver Syndrome and Excess Energy Intake

Excessive liver lipid has been associated with reduced egg production and sometimes mortality. In 1956, Couch first described a "Fatty Liver Syndrome" (FLS). Diagnostic signs included a sudden reduction in egg production; a yellow, friable liver high in lipid content; and excessive abdominal fat. Flock mortality was sometimes increased. Nesheim et al. (1969) reported that a sudden mortality in hens, which

were previously laying at a normal rate, was the result of liver hemorrhage. A predisposing condition to hepatic hemorrhages was an increase in liver lipid content (46 to 83% of dry weight). Nesheim and Ivy (1970) described this condition as "Liver Hemorrhage Syndrome." Wolford and Polin (1972) and later Wolford and Murphy (1972a) confirmed the necessary, but not sufficient association of high liver lipid concentrations which preceeded liver hemorrhage. The syndrome has not been diagnosed in hens having low levels of liver fat. However, hemorrhages associated with fatty livers may be related to factors other than liver fat per se (Ivy and Nesheim, 1973). Therefore the term "Fatty Liver-Hemorrhagic Syndrome" (FLHS) was proposed to describe the condition.

Among laying hens, high producing birds are most commonly the victims of FLS (Ringer and Sheppard, 1963). High dietary carbohydrate (Barton, 1967; Duke et al., 1968); and high levels of animal fat (Sunde, 1966; Bragg et al., 1973) are the main nutritional factors responsible for FLS. Excessive caloric intake may also predispose the hen to hepatic fatty metamorphosis (Wolford and Polin, 1971; Polin and Wolford, 1972a). A technique implemented by Michigan State researchers (Wolford and Polin, 1972b; Polin and Wolford, 1973) to experimentally induce FLHS under laboratory conditions has been force-feeding. Restricted feeding decreases the incidence of FLHS (Wolford and Polin, 1972a).

In reference to the previous observations, Wolford and Polin (1972a) proposed that FLHS is directly related to excessive feed intake or a "positive state of energy balance over a long enough period of time". Later work from the same laboratory reinforces this basic concept (Polin and Wolford , 1973b,c, 1976; Wolford and Polin, 1972a, 1974) that FLHS appears to be a consequence of laying hens generally

overeating and becoming excessively obese. Excess carbohydrate and lipid both effectively in induced FLHS (Polin and Wolford, 1976). Low energy diets have been shown to reduce liver fat accumulation (Barton, 1967; Ivy and Nesheim, 1973), but increasing the energy level of a diet by adding a high level of fat does not necessarily increase liver fat content (Barton, 1967; Bragg et al., 1973; Jensen et al., 1974b).

The source of dietary lipid has been shown to influence FLHS. Leveille and Fisher (1958) compared diets high in saturated fat, in the form of animal tallow, to diets high in unsaturated fat, using corn oil. The hens fed the animal tallow had abnormally high fat deposits in liver and adipose tissue, similar to that observed in FLS. Bragg et al. (1973) also showed that increasing the quantity of animal tallow in the diet increased liver size and lipid content of the liver. These livers appeared enlarged and yellowish in color, and some signs of hemorrhage were noted. However, when comparable levels of sunflower or soybean oils were fed, liver lipids were lowered. This suggests that the fatty acid composition of the diet may be more important than energy per se. Further, the dietary level of linoleic acid from sunflower or soybean oils was inversely related to liver size and lipid content. Work by Donaldson and Gordon (1960), Menge (1967), Morton and Horner (1961) showed further that linoleic acid in the diet prevented fat accumulation in the liver of rats and laying chickens.

The exact relationship between surfeit caloric intake and liver lipid concentrations and/or FLHS is yet unclear. Restriction in caloric intake by approximately 10% has successfully prevented the syndrome under certain conditions (Sallmann and Schole, 1973; Wolford and Murphy, 1972). It is possible, therefore, that under these conditions exces-

sive energy intake might be a contributing factor in observed liver lipid metamorphosis.

Management and a Genetic Predisposition for FLS

Certain management practices and genetic susceptibility appear to have an influence on the incidence of FLS. Early work suggested that FLS is more prevalent in birds in cages than in floor operations (Barton, 1967; Deacon, 1968; Bicknell et al., 1969). Griffith et al. (1969) reported lower liver lipid content (30-50%) in hens housed on the floor compared to a caged system with 2 birds per cage. This was confirmed by Hartfiel et al. (1970b). In a subsequent study Hartfiel et al. (1970a) showed that forced exercise reduced liver fat in hens housed in cages. Two theories have been proposed to explain the higher incidence of FLS in caged-layer systems: (1) increased stress due to confinement; (2) decreased activity and reduced energy expenditure.

Nesheim et al. (1969) reported that FLHS mortality usually occurred during the months of April, May and June. They suggested that mechanisms regulating energy consumption were failing to adjust to the temperature change of winter to summer. Barton (1967) also reported that FLHS mortality occurred primarily during periods of warm weather. Further, when birds were exposed to 32 C. for 12 weeks, FLHS mortality was higher than when exposed to 10-15 C. The level of liver fat has been shown to be influenced by environmental temperature (Sunde, 1966; Griffith et al., 1969; Wolford, 1971; Schexmailer and Griffith, 1973). Yet, Lee et al. (1975) reported that increasing the environmental temperature from 12.2 C. to 27.8 C. did not drastically alter total liver lipid or the incidence of FLHS. Likewise, changing the temperature from 22.2 C. to

30.6 C. or from 30.6 C. to 22.2 C. did not significantly alter total liver fat or liver wet weight. However, these were short duration studies (12 weeks) conducted with relatively young pullets (22 to 40 weeks of age). It is possible that over consumption of energy during warmer weather, accompanied by a reduced thyroid output many increase accumulation of fat in the liver (Roberson and Trajillo, 1975).

Nesheim and Ivy (1970) presented evidence which indicated genetic predisposition to the development of excessive liver lipid and death from liver hemorrhage. Garlich et al. (1975) reported average liver lipid values for 20 varieties of 71-week old laying hens managed in 3 confinement systems of the 1972-73 North Carolina Random Sample Laying Test. Large differences in liver lipid were attributed to variety, to confinement system, and to an interaction between these factors. Few scientific reports researching factors that effect liver lipid levels state the genetic variety of hens used. Yet genetic differences may confound the investigation of the effectiveness of certain lipotropic agents or in the magnitude of treatment responses.

Lipotropic Agents and Their Effect on FLS

Before Couch (1956) originally described FLS, the Corn Belt Hatchery, Hope, Arkansas, had successfully treated the condition by adding supplemental vitamin E, vitamin B_{12} and choline to their feed. Subsequently, a flurry of research focused on various lipotropic agents with conflicting results.

In field trials, supplementing rations with 1.1 g. choline chloride, 11.0 I.U. vitamin E and .13 mg. vitamin B_{12}, per kg. of feed, lowered the incidence of FLS (Deacon, 1968; Parker and Deacon, 1968).

Reed et al. (1968) reported that the addition of inositol to the above premix further increased its efficacy in alleviating FLS in field flocks. However, since these trials lacked appropriate controls, the responses might not be attributable to the specific dietary supplementation (Leveille and Bray, 1970).

Experiments by Nesheim et al. (1969) indicated that the hen has a low dietary requirement for choline; yet choline supplementation to choline-deficient diets reduced the percentage of liver fat. Since practical diets contain large amounts of choline from common feed ingredients, it has been difficult to implicate choline deficiency with FLS (Nesheim et al., 1969). In contrast, dietary supplementation of choline (850 mg. per kg. fed) has reduced liver fat in hens fed a basal diet containing approximately 850 mg. choline per kg. fed (Griffith et al., 1969; Schexnailder and Griffith, 1973). Under practical farm conditions, 1100 mg. of choline per kg. of feed is recommended for laying hens (Scott, 1975). In studies which have shown no response to choline supplementation, the control rations had choline levels greater than that recommended by Scott (Bossard and Combs, 1970; Wolford and Murphy, 1972; Couch et al., 1972).

Jensen et al. (1974a) added a combination of choline, inositol, vitamin B_{12} and vitamin E to a corn-soybean meal diet containing no added fat. A reduction in liver fat accumulation was observed. The control diet was calculated to contain 1138 mg. of choline per kilogram. In a second trial, a corn-soybean meal-4% animal tallow diet was again supplemented with the same lipotropic agents but no reduction in liver lipid was recorded. This control ration contained 1185 mg. of choline per kilogram. Two explanations for these results are possible.

(1) Choline present in the first basal diet was not available and therefore, this diet was marginal in its choline level, or (2) the dietary requirement for choline is affected by the carbohydrate content of the diet. The diets in the first study contained higher carbohydrate levels and thus may have required more choline for the transport of de novo synthesized lipid from the liver.

Reed et al. (1968) first reported the effectiveness of supplemental insoitol as a treatment for FLS in laying hens. Other field trials have confirmed the beneficial effects of inositol supplementation (1 g. per kg. of diet) for reducing liver fat content (Bull, 1968; Nelson, 1968). However, these studies did not report the inositol content of their basal diets.

Leveille and Bray (1970) supplemented inositol (1 g. per kg. diet) to a basal diet containing 400 mg. per kg. inositol and 1,070 mg. choline per kilogram. During a 5 month production period, the addition of inositol had no effect on liver lipid level. They concluded that inositol is not a universally effective lipotropic agent. Several other laboratory have found no reduction in liver lipid due to addition of dietary inositol (Bossard and Combs, 1970; Pearce, 1972; Ragland et al., 1970; Wolford and Murphy, 1972; Jensen et al., 1974). The addition of inositol at levels of 1 g., 2 g. and 5 g. per kg. of diet also had no effect on the specific activities of lipogenic enzymes ATP-citrate lyase and malic enzyme in liver extracts (Pearce, 1972).

Griffith et al. (1960) showed that the addition of choline to a diet containing a marginal choline level (850 mg./kg. diet) or a combination of choline, methionine, and vitamin B_{12} lowered hepatic lipid contest. The combination treatment had a greater effect than choline

alone, although methionine and/or vitamin B_{12} had no effect on liver lipids.

Schexnailder et al. (1973) also compared different vitamins as lipotropic agents. These included riboflavin, pantothenic acid, folic acid, pyridoxine, vitamin B_{12}, choline, biotin, inositol, and vitamin E. The only combinations which depressed liver lipid levels were choline and vitamin B_{12}; methionine and vitamin B_{12}; and choline, methionine, and vitamin B_{12}. The control diets were similar to those utilized by Griffith et al. (1969).

Wolford and Murphy (1972) studied the effects of lipotropic agents on liver hemorrhages in the laying hen. The incidence of hemorrhage, liver weight, final body weight and total liver lipids was not reduced by the addition of vitamin B_{12}, vitamin E, choline, inositol, selenium or cobalt. The control diet in this trial was considered nutritionally adequate. Liver lipid was reduced by lowering the caloric density of the ration from 2900 kcal./kg. to 2400 kcal./kg.; no liver hemorrhages were observed on the lower energy diet.

Jensen et al. (1974) observed that the addition of 1 p.p.m. selenium added to a corn-soybean meal ration, with or without added fat, significantly reduced total fat accumulation in hepatic tissue. However, the level of selenium in the control ration was not reported. But a second trial, selenium addition failed to affect liver fat accumulation.

Quisenberry et al. (1967) observed that treatment with iodinated casein lowered "liver scores" which were presumably based on fat content. This effect of iodinated casein was noted by other researchers (Wolford, 1971; Wolford and Polin, 1975).

Consequently, 19 years after the initial recommendation for use of choline, vitamin E, vitamin B_{12} and/or inositol to prevent or alleviate FLHS, the effectiveness of these vitamins as lipotropic agents is still not clear. Vitamin treatment remains as uncertain therapeutic approach for FLHS (Wolford and Polin, 1975).

Aflatoxins and FLS

Aflatoxins have been implicated as a possible cause of FLS in laying hens. Smith (1972) reviewed aflatoxins and some of their effects on poultry. Dietary intake of aflatoxins in broilers has been associated with decreased growth rate and feed efficiency; increased mortality; enlargement of the liver, heart, spleen, and pancreas; and an increase in liver fat. Most of these effects were noted with dietary levels of aflatoxin exceeding 625 parts per billion (p.p.b.). However, marked differences in susceptibility between inbred strains of chickens has been demonstrated (Smith, 1972).

Dietary modifications will influence the severity of aflatoxicosis in broiler chickens (Smith et al., 1971). Increasing the protein content of the diet to 30 percent gave complete protection against 5 parts per million (p.p.m.) of dietary aflatoxin. Increasing the lipid content of the diet from 2% to 16% also reduced mortality; however, the growth rate was not affected unless the dietary lipid was high in unsaturated fatty acids. Aureomycin stimulated gains and feed conversions while decreasing mortality when added to a ration containing added aflatoxin.

The susceptibility of laying hens to dietary aflatoxins is also quite variable (Smith, 1972). Levels below 1 p.p.m. dietary aflatoxin

may be tolerated by the laying hen without any effect on performance. Higher levels decrease egg production, and hatchability, and alter liver composition. Newberne and Butler (1969) pointed out that the liver is usually the first organ affected by aflatoxin. The first sign of damage is fatty infiltration, then enlargement of the organ followed by hepatic hemorrhages and cell necrosis.

Hamilton and Garlich (1971) investigated the effects of graded doses (between 0 and 20 p.p.m. of diet) of dietary aflatoxin on hen performance and liver lipid content. Intakes of aflatoxin greater than 2.5 p.p.m. of the diet reduced egg production in a dose-related relationship. The livers of laying hens receiving the higher doses of aflatoxin were yellowish, enlarged and friable. Aflatoxin increased the lipid content of liver dry matter from the normal 37 percent to above 55 percent. No effect on spleen or pancreas size was noted. The marked increase of lipid in the liver concomitant with decreased egg production suggests that lipid transport may be impaired (Hamilton and Garlich, 1971). However, Hamilton and Garlich (1972) showed that a mixture of choline, inositol, vitamin B_{12} and vitamin E was not effective in preventing the development of or enhancing the recovery from the FLS caused by aflatoxin.

Garlich et al. (1973) studied the effect on laying hens of feeding 20 p.p.m. dietary aflatoxin during a 7-day feeding period. Plasma protein, cholesterol, and triglycerides were decreased and serum alkaline phosphatase increased during the feeding period. Plasma protein and alkaline phosphatase returned to normal by the seventh day of the recovery period, while plasma total lipid and cholesterol approximated control values by the tenth day. Egg production rate began to decline

the first day of the recovery period and reached a minimum seven days later. Egg production returned to the control rate 19 days after the hens began eating the aflatoxin-free diet. These results suggest that aflatoxin impairs egg production by reducing liver synthesis and transport of yolk precursors. These suppositions are supported by previous work which indicated a greatly impaired movement of lipid from the liver to the blood (Tung et al., 1972) and depressed fatty acid synthesis (Donaldson et al., 1972) during aflatoxicosis in young chicks.

Dietary Lipid and Carbohydrate Sources and Hepatic Lipid Level

Dietary fatty acids influence the depot fatty acid composition in most species of animals. This is true, also, in the avian, especially in the fatty acid pattern of the liver (Machlin et al., 1962; Marion and Edwards, 1964). High dietary levels of saturated fatty acids (animal tallow) have been implicated by some research workers (Leveille and Fisher, 1958; Sunde, 1966) as a predisposing factor in FLHS.

Bragg et al. (1973) studied the effect of dietary fat source on liver lipid accumulation. Laying hens were fed five levels (0,1,2,4, and 8%) of dietary animal tallow, soybean oil, and sunflower oil during six periods of 28 days each. Size and lipid content of livers increased with increasing levels of animal tallow, especially at 8% tallow, whereas soybean oil and sunflower oil decreased liver size and lipid accumulation. However, the dietary treatments were not isocaloric.

Sim et al. (1973) observed that a low-fat diet or a diet containing animal tallow decreased linoleic acid, whereas the addition of sunflower and soybean oil increased the lionoleic acid content of the liver. A

positive relationship existed between the linoleic acid content of the diet and the amount present in hepatic tissue. Oleic acid was increased in the liver of birds fed the low-fat and animal tallow diet whereas the vegetable oils decreased the oleic acid content of hepatic tissue. It was suggested that linoleic acid was deposited at the expense of oleic acid with the vegetable oil diets. The animal tallow and low-fat diets were low in linoleic acid content and therefore, oleic acid was deposited in the tissue. Again, the diets were not isocaloric.

Linoleic acid addition has been shown to prevent total fat accumulation in the livers of rats and laying hens (Donaldson and Gordon, 1960; Menge, 1967; Morton and Horner, 1961). Balnave (1975) maintained 70-week-old white, hybrid laying pullets for 30 weeks on either semi-purified, low-linoleate or an isoenergetic, semi-purified, high-linoleate diet. The low-linoleate diet (less than 10 g. linoleic acid per kg. feed) produced larger livers and increased liver lipid concentrations.

Miller (1974) investigated the effect of dietary fat source and level on hapatic lipid content in the laying hen. Two fat sources (soybean oil and animal tallow) and three dietary fat levels (low, intermediate, and high) were fed for six 28-day periods. These rations were isocaloric. No statistical difference was observed in liver lipid concentration due to source or level of dietary fat. However, soybean oil and high dietary fat levels tended to reduce liver weight and total lipids relative to animal tallow and low dietary fat levels. The discrepancy in magnitude of results reported by Miller and by other researchers may be attributable to confounding effects of increased caloric density with increased dietary lipid levels.

The influence of carbohydrate source on liver fat accumulation in laying hens has been recently reviewed by Jensen et al. (1976). When corn and wheat in various proportions were fed, the percent fat and total fat per liver increased as the proportion of corn increased. This effect was not attributable to lower caloric density of wheat because fat addition to the wheat diet to make it isocaloric with the corn diet did not affect liver fat accumulation (Jensen, et al., 1974b; Jensen et al., 1976). They suggested that wheat may contain a higher content of a nutrient necessary for normal lipogenesis or an unrecognized nutritional factor involved in normal lipid metabolism.

Influence of Diet on Cholesterol Metabolism

The usual diet fed to poultry contains little, if any, preformed cholesterol. Yeh and Leveille (1972) estimated in the chick that 64% of all endogenous cholesterol synthesis took place in the liver, 24% in the carcass, and about 6% in the intestine. Feeding cholesterol has been shown to increase liver, blood plasma and egg yolk cholesterol concentration (Weiss et al., 1967a,b; Menge et al., 1974; Kruski and Narayan, 1972; Chung et al., 1970). Lecithin (5% diet) has been suggested to improve the absorbability of dietary cholesterol and thereby accentuate the increase of cholesterol in blood plasma and egg yolk concentrations (Weiss et al., 1967a).

The ability of the avian to adjust to cholesterol diets has been demonstrated. Cockerels fed cholesterol (.25% diet) markedly increased the amount of bile acids excreted, partially compensating for the high positive neutral steroid balance (Bartov et al., 1973). In the laying female, corporeal cholesterol can be eliminated by way of two major

routes in the feces and via yolk formation. Weiss et al. (1967a) accounted for 60% of their added dietary cholesterol (1% of diet) in the egg yolk.

Weiss et al. (1967b) measured acetate incorporation into various lipid fractions in liver tissue in response to dietary fat. Approximately 70% of acetate carbon was incorporated into triglycerides and about 10% was found in cholesterol on a low fat diet (no added fat). A high fat diet (30% fat addition), from either sunflower oil or animal fat, reduced by half incorporation of acetate carbon into triglycerides (to 40%) while more than doubling incorporation into cholesterol to 25-30% of the total. Addition of 1% dietary cholesterol almost completely eliminated acetate incorporation. Feeding fat did not counteract this effect. This indicates a strong feedback mechanism on liver cholesterol synthesis in the chicken.

Chung et al. (1970) demonstrated that the chicken liver response to ingestion of cholesterol differed depending on the type of fat consumed. Cholesterol addition (1% of diet) to a high-fat diet containing either unsaturated (corn oil) or saturated (hydrogenated coconut oil) fat (10% of diet) increased both the cholesterol ester and free cholesterol content of the liver. Free fatty acids were decreased in hepatic tissue. Phospholipids were increased approximately 40% in the liver with the saturated fat diet but were decreased with the unsaturated fat diet. The dietary fat source also affected the proportion of acetate carbon incorporated into hepatic cholesterol and cholesterol esters. The distribution of labeled acetate was higher in free cholesterol component and lower in cholesterol esters when birds were fed corn oil supplemented diets than with the coconut oil supplemented diets. The addition

of cholesterol (1% of diet) decreased endogeneous total cholesterol synthesis and hepatic free cholesterol concentrations. However, an increase in incorporation of labeled acetate into cholesterol esters was observed.

The influence of dietary fats on plasma cholesterol levels is, at best, confusing. Certain dietary fats have been reported to have no influence on blood cholesterol levels (Wheeler et al., 1959; Marion et al., 1960). Cholesterol level decreased upon feeding either sunflower oil (Fisher and Leveille, 1957) or soybean oil at levels of 20% (Edwards and Jones, 1964), but increased with 10% tallow (Leveille and Fisher, 1958) and 5 to 10% animal fat (Weiss and Fisher, 1957). Weiss et al. (1967a) observed that highly unsaturated safflower oil decreased blood plasma cholesterol levels while partially hydrogenated safflower oil increased and coconut oil greatly increased it; all three vegetable oils increased egg yolk cholesterol. They concluded that blood plasma cholesterol is a poor indicator of changes in the body cholesterol pool.

Increased dietary protein intakes in young chicks increased cholesterol synthesis by the liver and intestine but not the carcass. Yet, total plasma cholesterol was decreased by increasing the dietary protein level. Consequently, plasma level was inversely related to the rate of cholesterol synthesis. This suggests that cholesterol turnover was higher in chicks fed the high protein diet (Yeh and Leveille, 1972). Later studies showed that this hypocholesterolemic effect of high protein intake was mediated, at least in part, by a more rapid turnover of cholesterol, resulting in faster removal from the blood and excretion in the feces as cholesterol and bile acids (Yeh and Leveille, 1973b).

Lipogenesis in the Avian

The liver is the primary site of de novo fatty acid synthesis in the avian (Goodridge and Ball, 1966, 1967; O'Hea and Leveille, 1968, 1969; Evans, 1972; Brady et al., 1976). O'Hea and Leveille (1968) reported that isolated chick adipose tissue had a low capacity for fatty acid synthesis measured by incorporation of acetate-1-^{14}C or glucose-U-^{14}C into fatty acids. Later studies (O'Hea and Leveille, 1969) estimated that 90 to 95% of de novo fatty acid synthesis in the chick occurred in the liver, with the adipose tissue functioning primarily for lipid storage. In vivo studies with the pigeon attributed 96% of the de novo fatty acid synthesis to hepatic tissue leaving adipose tissue responsible for no more than 4% (Goodridge and Ball, 1967). Recent work, using acetate-1-^{14}C, indicated that chick muscle, intestine, adipose tissue, heart and skin synthesized fatty acids via chain elongation (Brady et al., 1976).

Fatty acid synthesis occurs in the cytoplasm of the cell. Yet acetyl coenzyme A(acetyl-CoA) from glycolysis is generated within the mitochondria. Acetyl-CoA crosses the mitochondrial membrane as citrate. In the cytoplasm ATP-citrate lyase (EC 4.1.3.8) converts citrate to oxaloacetate and generates acetyl-CoA for use in lipogenesis (Srere, 1959; Spencer and Lowenstein, 1962). In the presence of HCO_3^-, ATP, Mn^{+2}, acetyl-CoA is carboxylated to malonyl coenzyme A(malonyl-CoA) via acetyl-CoA carboxylase (EC 6.4.1.2). Malonyl-CoA is converted into fatty acids (mostly palmitate) by fatty acid synthetase (FAS), which is a multienzyme complex requiring nicotinamide adenine dinucleotide phosphate-reduced (NADPH) as a hydrogen donor (Yun and Hsu, 1927). In

the avian, most of the NADPH required for this process is provided by the malic enzyme system (EC 1.1.1.40) (Goodridge, 1968a,b).

The activities of these lipogenic enzymes have been shown to be closely correlated with the rate of lipogenesis in chicken livers (Goodridge, 1968a,b; Yeh et al., 1970) and the activities of these enzymes have been shown to be greater in the liver than the adipose tissue (Goodridge, 1968a; O'Hea and Leveille, 1968). The correlation between specific activities of the lipogenic enzymes and hepatic lipogenesis could lead to the conclusion that the rate of fatty acid synthesis is dependent on the activities of these enzymes. However, other results (Leveille, 1966; Yeh et al., 1970; Yeh and Leveille, 1970; Silpananta and Goodridge, 1971; Goodridge, 1973a,b) support the suggestion of Foster and Srere (1968) that the activities of these enzymes do not regulate the rate of lipogenesis, but rather respond to changes in the rate of fatty acid synthesis.

Malic enzyme activity appears to respond to changes in fatty acid synthesis rather than control the rate of lipogenesis (Allee et al., 1972; Leveille, 1966; Romos et al., 1971; Yeh and Leveille, 1970; Yeh et al., 1970). In vivo and in vitro rates of hepatic fatty acid synthesis were depressed 5-to 10-fold within one hour after chicks were force fed corn oil (Yeh et al., 1970). During this time malic enzyme activity was unchanged.

Foster and Srere (1968) rejected the hypothesis that ATP-citrate lyase regulated the rate of fatty acid synthesis in rat liver. Changes in fatty acid synthesis have been shown to precede changes in ATP-citrate lyase in chick liver (Goodridge, 1968b; Yeh and Leveille, 1970; Yeh et al., 1970). Smith and Abraham (1970) concluded that ATP-citrate

lyase activity was always maintained at a level that would not limit fatty acid synthesis.

Acetyl-CoA carboxlyase has been implicated as the rate limiting enzyme in the fatty acid synthesis pathway, since initial studies have shown that its activity was much lower than that observed for fatty acid synthetase. However, Chang et al. (1967) have suggested that the low enzyme activity could be explained on the basis of suboptimal assay conditions. Several investigators have subsequently shown that the activities of acetyl-CoA carboxylase and FAS were very similar in rat, mouse, chick liver and in rat adipose tissue (Chang et al., 1967; Craig et al., 1972; Liou and Donaldson, 1973; Chakrabarty and Leveille, 1969). Consequently, it has been suggested that acetyl-CoA carboxylase may not be the rate-limiting enzyme in fatty acid synthesis under all dietary conditions (Guynn et al., 1972; Porter et al., 1971).

Guynn et al. (1972) developed a procedure for the direct measurement of malonyl-CoA levels in rat liver. Malonyl-CoA levels were not directly related to fatty acid synthesis. Guynn et al. (1972) hypothesized that the rate of fatty acid synthesis in rats fed a high carbohydrate diet may be limited by the ability of the liver to utilize malonyl-CoA i.e. by the activity of fatty acid synthetase. Two mechanisms for the control of fatty acid synthesis were proposed: a short term control mediated by acetyl-CoA carboxlyase via its inhibition by long chain acyl-CoA derivatives; a long term mechanism, under the control of FAS which functions through the quantity of the enzyme complex present (Guynn et al., 1972).

The exact cause-effect relationship between enzyme activity and lipogenesis is still speculative. Recent evidence suggests that various

intermediate metabolites may regulate both the rate of fatty acid synthesis and the concentration of lipogenic enzymes (Goodridge, 1973a,b). However, under steady state conditions, measurements of enzyme activities can be monitored as an indicator of lypogenic capacity (Romos and Leveille, 1974).

Dietary Effects and Control of Lipogenesis

Lipid synthesis in the liver is dependent on the dietary regime of the animal. In the avian, as in mammals, hepatic lipogenesis is increased when a high carbohydrate diet is fed (Liou and Donaldson, 1973; Yeh and Leveille, 1969), and is reduced when the animal is starved (Butterworth et al., 1966; Leveille, 1969; Yeh and Leveille, 1971) or fed fat supplemented diets (Balnave and Pearce, 1969; Yeh and Leveille, 1971).

Many researchers have attempted to explain this reduced lipogenic activity, exhibited by animals on high-fat diets, via changes in various lipogenic enzyme activities. Pearce (1971) observed reductions in the specific activities of ATP-citrate lyase and malic enzyme in response to increased dietary fat intakes. Corn oil supplementation (2% diet markedly decreased the activity of acetyl-CoA carboxylase and ATP-citrate lyase (Balnave and Pearce, 1969). The specific activity of FAS has been demonstrated to sharply increase with fat-free feeding and decrease with fasting or feeding a high-fat diet, in studies with rats (Craig et al., 1972). However, an exact cause-effect relationship between lipogenic enzyme activities and de novo fatty acid synthesis has not been demonstrated to date.

Guynn et al. (1972) speculated that two general types of physio-

logical control are operative under *in vivo* conditions of fatty acid biosynthesis--a short and a long term mechanism. The short term control would act before the FAS step and limit the production of malonyl-CoA. The long term mechanism would control the condensation of malonyl-CoA to palmitate.

Guynn *et al.* (1972) demonstrated that in starved or fat-free animals there was an inverse correlation between the concentration of long chain acyl-CoA and malonyl-CoA. Long chain acyl-CoA derivatives have been implicated as potential physiologically important allosteric regulators of acetyl-CoA carboxylase under *in vitro* conditions (Goodrige, 1973c). In carbohydrate-fed animals, the concentration of malonyl-CoA did not predict the rate of fatty acid synthesis. This suggests that the utilization of malonyl-CoA by FAS is limiting (Guynn *et al.*, 1972). The conversion of malonyl-CoA to palmitate could be influenced by either the specific activity and/or the quantity of FAS present. Certain phosphorylated intermediates have been proposed as activators of FAS activity, but only at concentrations exceeding known tissue levels (Guynn *et al.*, 1972). Also, palmityl-CoA has been shown to be inhibitory to FAS activity (Lust and Lynen, 1968) but not at physiological concentrations (Goodridge, 1973c; Liou and Donaldson, 1973). At present, the simplest explanation for the variations of rate of fatty acid synthesis in the carbohydrate-fed states is that the reaction is largely a function of the quantity of the enzyme FAS present.

Yeh and Leveille (1969) reported that a linear depression in fatty acid synthesis occurred when the dietary protein level was increased from 10 to 40 percent. However, the reduction in lipogenesis was not observed until the difference in protein level between groups exceeded

20 percent. In a later study, increasing the dietary protein level from 15 to 35% markedly reduced hepatic lypogenesis (Yeh and Leveille, 1971). The concentration of liver acetyl-CoA, free CoA, and total CoA were similar under both dietary regimes. However, the higher protein level produced a two fold increase in acyl-CoA derivatives. This suggests that the short term control mechanism of fatty acid synthesis as proposed by Guynn et al. (1972) may be involved with depression of hepatic lipogenesis observed with increased dietary protein levels.

CHAPTER 3

RELATIONSHIP OF PLASMA LIPIDS AND LIVER FATTY ACID SYNTHETASE (FAS) TO LIVER LIPID LEVEL IN THE LAYING HEN

Summary

The purpose of this study was to determine if increased liver lipid levels in laying hens are accompanied by changes in plasma lipids and liver Fatty Acid Synthetase (FAS) activity. Plasma and liver samples were taken from a random sample of caged commercial hybrid layer hens which had been in egg production for fifteen months.

Hepatic water, lipid, and non-lipid fractions increased linearly with increased liver weight. The rate of increase with weight was greatest for water and least for the non-lipid fraction. Cytosolic protein increased linearly with the non-lipid fraction. Fatty acid synthetase (FAS) activity increased linearly with all liver parameters.

Plasma total and free cholesterol, triglycerides, and phosphorus increased linearly with increased dry liver weight, lipid, and non-lipid fractions but were related cubically with wet liver weight, water, and cytosolic protein. Cholesterol esters were not related to any liver parameter. These results indicate that plasma lipid components increase in response to increasing liver weight and lipid level.

Introduction

Lipid comprises some 10 to 23% of total liver dry weight in growing chicks, adult cockerels and immature pullets (Balnave, 1971, 1972; Velu et al., 1971). In contrast, the liver lipid content of laying hens can vary from 20 to 83% of dry weight (Nesheim et al., 1969; Nesheim and Ivy, 1970; Wolford and Polin, 1972a; Ivy and Nesheim, 1973). Antecedent to hepatic liposis in the laying female is the secretion of oestrogen by the ovary (Sturkie and Mueller, 1976). However, the cause of the large individual variations is speculative.

The lipid content of livers from laying hens has received considerable attention since Couch (1956) described the "Fatty Liver Syndrome". Subsequent studies have investigated the effects of management, genetic predisposition, and dietary factors on liver lipid accumulation. However, few studies have reported the effects of such factors on the entire liver composition. The objectives of this study were: 1) to characterize the accumulation of liver lipids and non-lipids in hens under simulated commercial production conditions; and 2) to correlate changes in plasma lipids and liver fatty acid synthetase (FAS) activity associated with hepatic lipid level.

Materials and Methods

Five-hundred-seventy-six pullets (H & N 'Nick Chick') were purchased from a commercial hatchery at 20 weeks of age and maintained under simulated commercial production conditions for approximately 72 weeks. Pullets were housed in individual laying cages in a windowless house. The environmental temperature ranged between 16 to 32 C. A

photoperiod of 17 hours per day was provided by means of incandescent lighting. Feed and water were provided ad libitum. A corn-soybean meal diet (Table 3.1) was fed which was calculated to provide 16 g. of protein and 285 kcal. of metabolizable energy per hen per day.

An experimental group of 30 hens which were in active egg production was selected at random. All birds were surgically opened and their livers were classified initially by visual appraisal of lipid concentration (Barton, 1967; Wolford and Polin, 1972a). Hepatic color and texture was utilized as an indicator of fat infiltration. Liver hemorrhages did not influence hepatic scores. Scores were assigned as follows:

	Liver	
Score	Texture	Color
1	Firm	Mahogany
2	Firm	Slightly Yellow
3	Friable	Yellow
4	Very Friable	Very Yellow

Production pramaters were unaffected by the classification procedure. The hens were allowed a 3-week recovery period prior to the first sampling of 6 hens for chemical analysis. Each classification score was represented in each of the five sampling periods. Six hens were sacrificed for analysis at each sampling period.

Blood samples were obtained by heart puncture and the hens were sacrificed by cervical dislocation. The liver was removed, weighed and placed in cold (2 C.) 0.1 M KCL buffer.

An aliquot of each liver was placed in an aluminum weighing boat, weighed, frozen on dry ice, lyophilized and stored at -20 C. From

TABLE 3.1. RATION COMPOSITION

Ingredients	Percent
Yellow corn, ground	55.21
Soybean meal (44% protein)	15.68
Calcium carbonate	7.24
Tallow (feed grade)	6.52
Meat and bone scrap (50% protein)	5.14
Alfalfa meal (17% protein)	2.63
Live yeast culture[1]	1.68
Distillers solubles	1.68
Dried whey	1.68
Dicalcium phosphate	1.47
Salt	0.50
Vitamin-mineral concentrate[2]	0.50
dl-Methionine	0.07
Calculated analysis:	
Protein, crude % (N x 6.25)	16.10
Metabolizable energy, kcal./kg.	2951.
Daily Intake (hen/day)	95-100 g.

[1]Manufactured by Diamond V Mills, Cedar Rapids, Iowa.

[2]See Table 3.2 for composition of vitamin-mineral concentrate.

TABLE 3.2

COMPOSITION OF VITAMIN-MINERAL CONCENTRATE

Vitamins and Minerals	Units	Supplies Per kg. of Finished Ration
Vitamin A	U.S.P.	17,637.0
Vitamin D_3	I.C.U.	2,646.0
Vitamin E	I.U.	13.2
Vitamin K	mg.	6.6
Vitamin B_{12}	mg.	0.018
Riboflavin	mg.	8.8
Niacin	mg.	70.4
Pantothenic Acid	mg.	17.6
Choline Chloride	mg.	1,102.0
Manganese	mg.	60.9
Iodine	mg.	1.9
Cobalt	mg.	1.3
Iron	mg.	48.1
Copper	mg.	3.6
Zinc	mg.	50.0

storage, samples were allowed to equilibrate to room temperature in a desicator. Samples were reweighed and extracted by the Goldfisch procedure (A. O. A. C., 1960) to determine total lipids. The water content was calculated from the weight difference between fresh and lyophilized samples, and fat content by weight loss during ether extraction. Non-fat dry matter was calculated by difference.

Fatty acid synthetase (FAS) activity was measured at 37 C. by a modification (Diamant et al., 1972) of the spectrophotometric method of Lynen (1969). A tissue homogenate (1:3 wt./vol.) was prepared in 0.1 M phosphate-bicarbonate buffer, pH 8.0, containing 1 mmole. dithioerythreitol and 1 mmole. Na_2-EDTA from the fresh liver sample. The supernatant fluid, after centrifugation at 100,000 g for 1 hr. was used to determine FAS activity and total cytosolic protein. FAS activity was calculated based on the disappearance rate of exogenous nicotinamide adenine dinucleotide phosphate-reduced (NADPH) in the presence of excess malonyl coenzyme A (malonyl-CoA). Supernate protein was determined by the method of Lowry et al. (1951) utilizing bovine serum albumin as the standard protein. FAS activity is expressed in units where 1 unit equals 1 nanomole malonyl-CoA incorporated into long-chain fatty acids per min. per mg. protein.

Blood samples were analyzed for plasma triglycerides (Kessler and Lederer, 1966); free and esterified cholesterol (Schoenhiemer and Sperry, 1934); and phosphorus (Fiske and Subbarow, 1925).

All data were analyzed using the computer program entitled Statistical Analysis System (SAS)-regression procedure developed by Barr and Goodnight (1972).

Results and Discussion

Liver Components. The three major liver components--water, total lipid and non-lipid--increased with increasing wet liver weight (Table 3.3). Total lipid, total non-lipid, water, cytosolic protein increased linearly with wet liver weight (Table 3.4), although the relative rates of increase differed (Figure 3.1). Total water increased at the fastest rate (b = 0.50); grams of non-lipid increase was least (b = 0.17), increase in grams of total lipid component was intermediate (b = 0.30). The slope of the line corresponding to dry liver weight (b = 0.53) was not different than that associated with the total grams of water. Previous work, (Thayer et al., 1973) indicated that the increase in total lipids was due to an increase in triglycerides.

Total grams of cytosolic protein increased linearly with the increase in amount of non-lipid fraction (Figure 3.2). In force-fed geese, the increase in liver weight has been associated with an increase in triglycerides and an increase in total protein content (Nitzan et al., 1973). MacDonald and Mallory (1959) observed that fat infiltration into hepatic tissue increased DNA content of the liver. DNA content was considered as an indicator of cell numbers. They concluded that the increase in liver size (weight) was not only a result of increased cellular content or size (hypertrophy), but also increased cell numbers (hyperplasia). Hyperplasia would increase total protein content of the liver and this might explain the observations of Nitzan et al. (1973). In this study, the observed increased in cytosolic protein also might be attributed to hepatic hyperplasia. Cytosolic protein accounted for approximately 23% of the total increase in non-lipid fraction (Figure

TABLE 3.3. ANALYSIS OF LIVER PARAMETERS

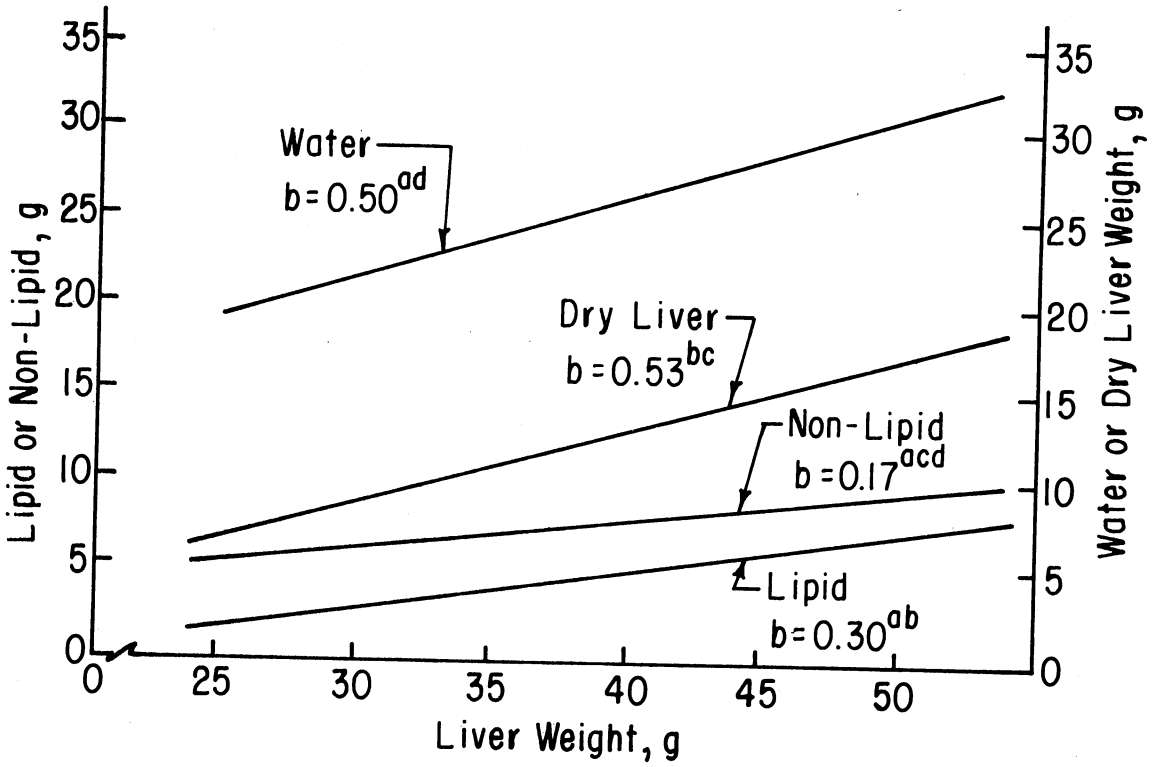
Hen	Liver Score	Liver Weight (g.)	Water (g.)	Lipid (g.)	Non-lipid (g.)	Cytosolic Protein (g.)	FAS[1] Activity (units)
1	1	20.64	15.31	0.95	4.38	1.11	7.42
2	2	29.09	20.86	2.01	6.22	1.63	15.92
3	1	29.81	22.25	1.25	6.31	1.19	8.65
4	1	29.94	21.91	1.60	6.43	1.79	6.76
5	3	32.58	21.93	4.15	6.50	1.57	10.01
6	1	32.66	22.43	3.45	6.78	1.98	18.82
7	1	33.43	23.71	2.62	7.10	1.77	16.11
8	1	34.94	25.94	1.50	7.50	2.05	13.90
9	4	36.23	22.89	6.22	7.12	2.01	16.68
10	1	36.91	26.75	2.02	8.14	1.90	14.05
11	3	37.52	24.44	6.01	7.07	2.28	13.85
12	2	38.98	26.34	3.63	9.01	2.40	9.11
13	4	39.00	24.89	6.23	7.88	1.97	20.42
14	2	39.24	28.44	2.24	8.56	1.90	14.64
15	2	39.54	28.78	2.18	8.58	2.21	11.37
16	2	39.96	28.09	3.28	8.59	2.16	17.53
17	2	40.11	28.09	3.40	8.62	2.08	21.19
18	3	40.94	27.67	4.55	8.72	2.04	11.04
19	4	42.08	26.59	7.76	7.73	2.05	11.97
20	2	42.90	30.96	2.54	9.40	2.34	9.06
21	2	44.82	32.45	2.52	9.85	2.34	16.19
22	3	46.71	30.78	5.99	9.94	2.59	16.66
23	3	46.86	31.15	5.76	9.96	2.40	10.71
24	4	48.75	29.32	10.19	9.24	2.39	14.23
25	4	49.05	33.14	7.77	8.14	2.60	15.61
26	3	49.96	32.51	6.64	10.81	2.64	18.94
27	4	51.27	27.94	15.79	7.54	2.39	16.12
28	4	51.30	31.23	10.51	9.56	2.51	12.84
29	4	51.65	32.58	8.73	10.34	2.62	11.99
30	3	53.15	36.67	5.75	10.73	2.72	18.15

[1]Adjusted of date of sampling and expressed in units where 1 unit equals 1 nanomole malonyl CoA incorporated into long-chain fatty acids/min./mg. protein.

TABLE 3.4. REGRESSION COEFFICIENTS WITH ASSOCIATED STANDARD ERRORS OBTAINED BY REGRESSION OF LIVER COMPONENTS (DEPENDENT VARIABLE) ON WET LIVER WEIGHT (INDEPENDENT VARIABLE)

	Dependent Variables b ± S.E. (P <)			
Independent Variable	Lipid	Non-lipid	Cytosolic Protein	Water
Liver Weight (g.)	.30 ± .05	.17 ± .02	.05 ± .01	.53 ± .04
	(0.001)	(0.001)	(0.001)	(0.001)

FIGURE 3.1. RELATIONSHIP OF LIVER COMPONENTS TO LIVER WEIGHT[1]



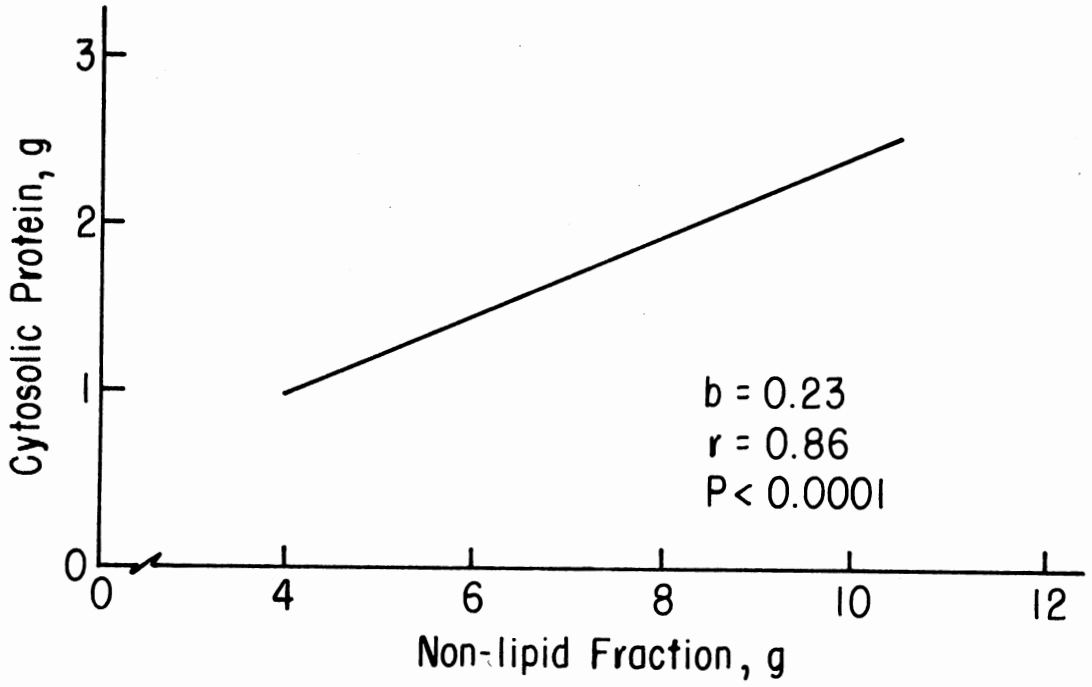
[1]Slopes with same letters differ significantly:

$^{a}P < 0.05$

$^{b}P < 0.01$

$^{c}P < 0.001$

FIGURE 3.2. RELATIONSHIP OF CYTOSOLIC PROTEIN TO NON-LIPID FRACTION



3.2). Correspondingly, cytosolic protein increased linearly with wet liver weight. Approximately 5% of the increase in liver weight was attributable to cytosolic protein.

Fatty Acid Synthetase Activity. FAS activity varied with date of sampling ($P < .001$), accounting for 60% of the total variation in FAS activity. Therefore, FAS activity was regressed within sampling periods pooled. Test of homogeneity of slope showed little evidence that the slopes differed at different sampling times.

Certain liver components were closely associated with FAS activity (Table 3.5). The possible exception to this generalization is total liver lipid. FAS activity increased with all other liver components with the closest relationship to cytosolic protein content. This would be expected because the FAS complex is located in the cytosolic portion of hepatic cells. Because FAS activity increased across all liver parameters, and FAS activity is expressed as a ratio, one might question the ability of hepatic tissue to exert feed back control of fatty acid synthesis.

Plasma Components. Plasma parameters (triglycerides; total, free and esterified cholesterol; and phosphorus) were analyzed relative to the various liver components (Table 3.6). Multiple regression analysis was conducted using the SAS regression procedure for linear, quadratic, cubic and higher polynomial effects (Table 3.7., Figures 3.3., 3.4.).

Plasma lipid components and phosphorus increased cubically with increased wet liver weight. The amount of water present in hepatic tissue parallels this response. Plasma parameters also increased cubically with cytosolic protein content of the liver. The physiological signif-

TABLE 3.5. REGRESSION COEFFICIENTS WITH ASSOCIATED STANDARD ERRORS OBTAINED BY REGRESSING FAS ACTIVITY (DEPENDENT VARIABLE) ON VARIOUS LIVER COMPONENTS (INDEPENDENT VARIABLES)

Independent Variable	FAS Activity b ± S.E.	Significance Level (P <)
Wet Liver Wt. (g.)	.20 ± .10	0.06
Water (g.)	.33 ± .18	0.08
Lipid (g.)	.33 ± .25	0.19
Non-lipid (g.)	.91 ± .54	0.10
Cytosolic Protein (g.)	3.71 ± 1.93	0.06

TABLE 3.6. ANALYSIS OF PLASMA PARAMETERS

Hen	Liver Wt. (g.)	Total Cholesterol (mg./dl.)	Free Cholesterol (mg./dl.)	Cholesterol Esters (mg./dl.)	Triglycerides (mg./dl.)	Phosphorus (mg./dl.)
1	20.64	110	22	149	59	6
2	29.09	63	32	52	483	13
3	29.81	129	91	64	1857	31
4	29.94	70	36	57	605	16
5	32.58	207	141	112	2481	38
6	32.66	140	100	68	2242	40
7	33.43	147	94	90	1734	31
8	34.94	98	51	66	1052	22
9	36.23	134	94	68	1842	32
10	36.91	123	70	90	1362	27
11	37.52	95	46	83	1053	20
12	38.98	208	48	270	378	12
13	39.00	127	85	71	1184	30
14	39.24	126	85	69	1214	29
15	39.54	68	28	68	494	14
16	39.96	108	68	68	1380	27
17	40.11	107	74	56	1578	27
18	40.94	205	152	90	2925	48
19	42.08	69	25	74	438	12
20	42.90	128	74	91	1335	26
21	44.82	93	57	61	1181	24
22	46.71	122	88	57	1841	26
23	46.86	152	115	63	2451	40
24	48.75	176	118	98	1995	32
25	49.05	195	148	79	2491	43
26	49.96	156	107	83	2070	38
27	51.27	280	174	179	3114	41
28	51.30	192	158	57	3155	50
29	51.65	136	96	68	2086	36
30	53.15	352	285	113	5754	78

TABLE 3.7. SUMMARY OF REGRESSION ANALYSIS OF PLASMA DATA ON LIVER COMPONENTS

	PLASMA				
LIVER	Total Cholesterol	Free Cholesterol	Cholesterol Esters	Triglycerides	Phosphorus
Liver Weight	3^{b}	3^{b}	NS	3^{a}	3^{a}
Grams Water	3^{a}	3^{c}	NS	3^{c}	3^{c}
Dry Liver Weight	1^{c}	1^{c}	NS	1^{c}	1^{c}
Grams Lipid	1^{c}	1^{c}	NS	1^{c}	1^{b}
Grams Non-lipid	1^{a}	1^{a}	NS	1^{c}	1^{c}
Cytosolic Protein	3^{b}	3^{b}	NS	3^{b}	3^{b}

Significance Level:

$^{a}P < 0.1$
$^{b}P < 0.05$
$^{c}P < 0.01$
NS = Not significant

Legend:

1 = Linear increase
2 = Quadratic increase
3 = Cubic increase

TABLE 3.8. CORRELATION COEFFICIENTS (r) OF PLASMA DATA WITH RESPECT TO LIVER COMPONENTS

	PLASMA				
LIVER	Total Cholesterol	Free Cholesterol	Cholesterol Esters	Triglycerides	Phosphorus
Grams Lipid	.51[c]	.51[c]	NS	.48[c]	.43[b]
Grams Non-lipid	.33[a]	.41[b]	NS	.45[b]	.48[c]
Dry Liver Weight	.54[b]	.57[c]	NS	.55[c]	.53[c]

Significance Level:

[a] $P < 0.1$
[b] $P < 0.05$
[c] $P < 0.01$
NS = Not significant

FIGURE 3.3. MULTIPLE REGRESSION ANALYSIS OF PLASMA PARAMETERS WITH RESPECT TO INCREASING LIVER WEIGHT

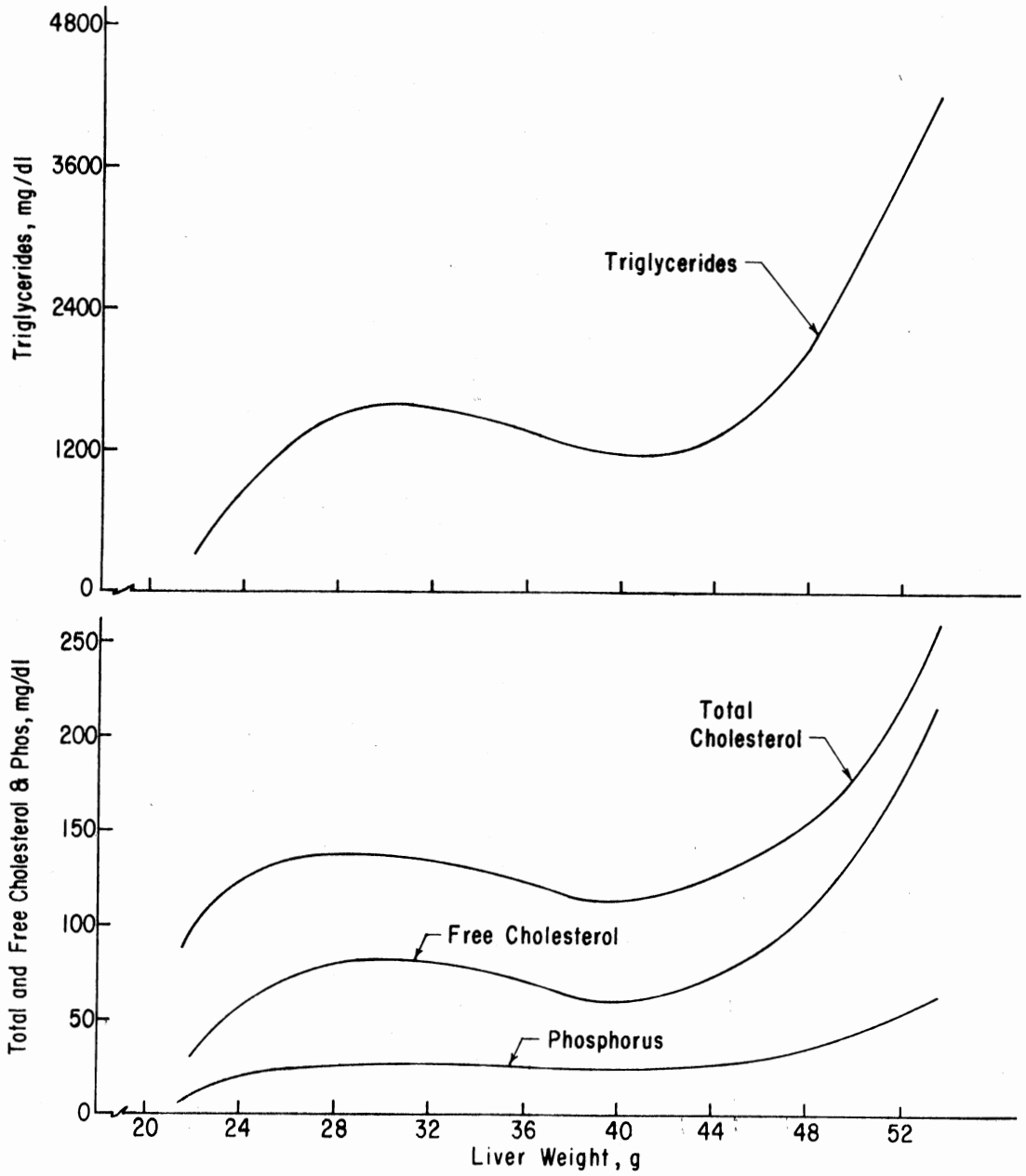
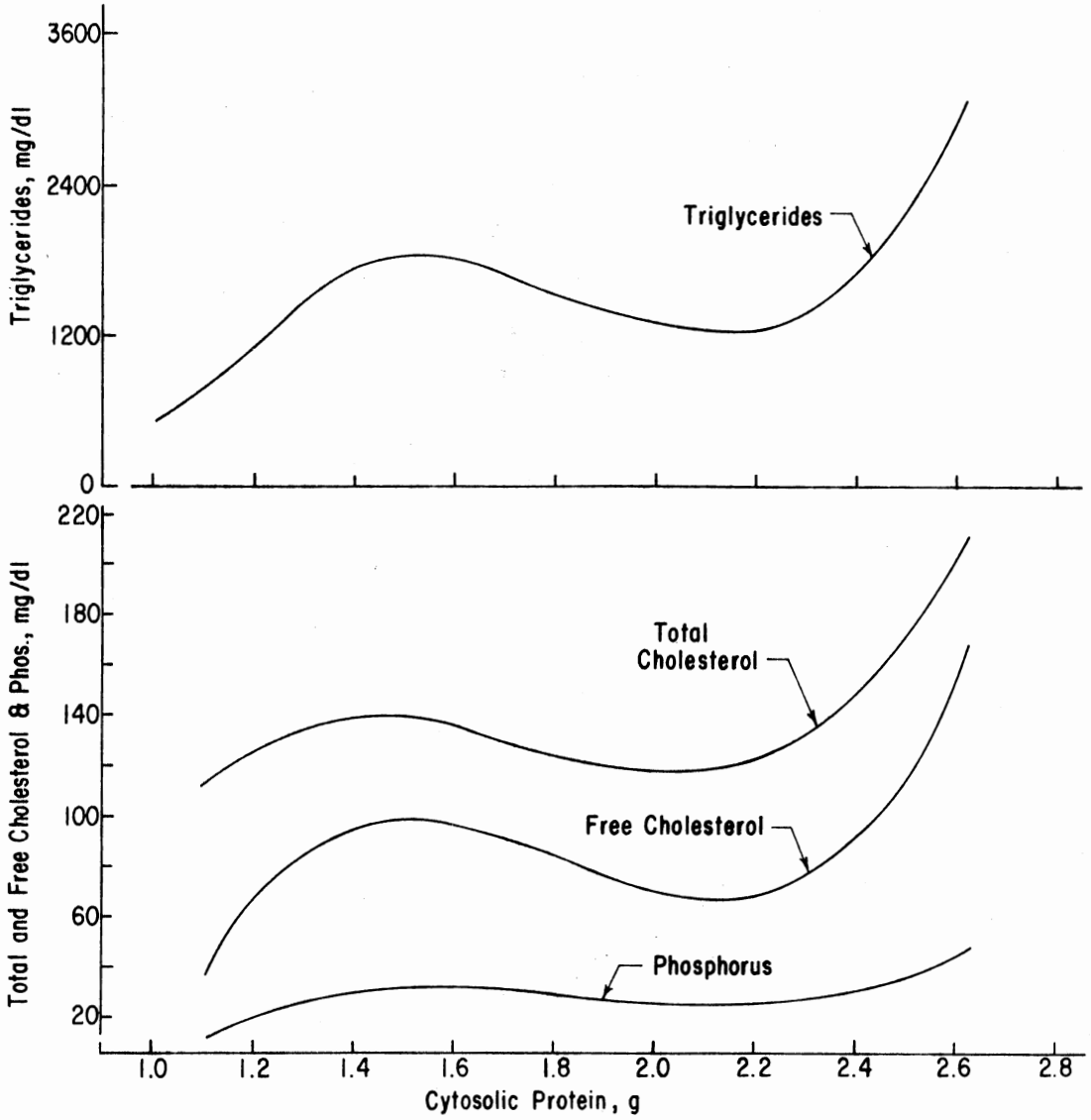


FIGURE 3.4. MULTIPLE REGRESSION ANALYSIS OF PLASMA PARAMETERS WITH RESPECT TO INCREASING CYTOSOLIC PROTEIN



icance of these cubic relationships is unknown; however, this might reflect adaptive characteristics of hepatic tissue. MacDonald and Mallory (1959) observed hyperplasia and hypertrophy associated with lipid infiltration into hepatic tissue. However, no research has ever indicated if these two phenomena occur simultaneously. If fat infiltration first occurs by hypertrophy until pre-existing cell volume is filled, and then hyperplasia is initiated, a cubic relationship between cytosolic protein and plasm lipid components might be exhibited.

Plasma lipids and phosphorus increased linearly with increasing liver lipid and non-lipid fractions. Plasma cholesterol esters do not relate well with any of the measured liver components. The major source of plasma esterified cholesterol is the plasma enzyme lecithin (phosphatidylcholine)--cholesterol acyltransferase (LCAT) which transfers the β fatty acid of lecithin to the hydroxyl group of cholesterol. Because the LCAT enzyme catalyzes a plasma reaction, cholesterol esters would not be expected to respond to any liver component.

CHAPTER 4

EFFECT OF DIETARY CHOLESTEROL AND LECITHIN ON PLASMA AND LIVER LIPIDS IN THE LAYING HEN

Summary

The purpose of this study was to determine if supplementation of the normal layer diet with cholesterol and/or lecithin would alleviate lipid accumulation in the liver of laying hens. Plasma lipids were measured initially and after 4 and 8 weeks of feeding. Liver lipids, liver weight and FAS activity were measured after 8 weeks. Lecithin feeding increased plasma triglycerides relative to control birds but did not change cholesterol or phorphorus levels. Total liver lipids and liver weight were slightly decreased by lecithin and livers were less friable than those on the control diet. Dietary cholesterol increased plasma free and esterified cholesterol and decreased phospholipids and triglycerides. Total liver lipid, cholesterol, friability and weight were increased by cholesterol feeding. These results indicate that cholesterol feeding increased plasma and liver lipids resulting in an enlarged friable liver whereas lecithin feeding slightly increased plasma lipids and decreased liver lipids.

Introduction

The high-producing laying hen must have rapid synthesis and mobilization of lipid for egg yolk formation. This physical stress combined with confinement conditions and selection pressure for high production has precipitated the condition known as "Fatty Liver Syndrome" (Couch, 1956). This syndrome alters lipid metabolism and increases hepatic lipid and reduces egg production. The effect of this syndrome on plasma lipids has not been reported. However, Wagner et al. (1976) observed that increased liver lipids result in a general increase in plasma lipid parameters.

Cholesterol addition at high levels to a ration for hens increases plasma cholesterol concentrations (Davis et al., 1961; Harris and Wilcox, 1963), although Harris and Wilcox observed no additional plasma cholesterol increase when the dietary cholesterol level was increased from 1 to 4%. Incorporation of 3% lecithin to a hypercholesteremic diet decreased plasma total cholesterol, cholesterol esters, phospholipids and lipoproteins in monkeys (Branen and Robbins, 1975). The objective of this study was to determine if added dietary cholesterol and/or lecithin would modify plasma lipids and reduce liver lipid accumulations in the layer.

Materials and Methods

Hybrid pullets (H & N 'Nick Chick) were purchased from a commercial hatchery at 20 weeks of age and maintained under simulated commercial production conditions for approximately 30 weeks. Five-hundred-seventy-six pullets were housed in individual laying cages in a windowless house. The environmental temperature was maintained between 16 and 32

centrigrade. A photoperiod of 17 hours per day was provided by means of incandescent lighting. Feed and water were provided ad libitum. The corn-soybean meal diet (standard layer diet) was calculated to provide 16 g. of protein and 285 kcal. of metabolizable energy per hen per day (Table 4.1.).

An experimental population was selected at random from pullets which were in active egg production. These birds were surgically opened for visual classification of liver lipid concentrations. This classification procedure was described previously (Wagner, 1976). Production parameters were unaffected by the classification procedure. Only pullets which had a "moderate" accumulation (Scores 2 and 3) were utilized in the dietary treatment comparisons of this experiment.

The hens were allowed a 2-week recovery period prior to sampling, and alloted to a 2 X 2 factorial arrangement of dietary treatment. The four groups of 11 individually caged birds were randomly located in the laying house. The four dietary treatments as described in Table 4.1. were: (1) standard layer diet, (2) standard diet with 1.5% cholesterol, (3) standard diet with 2.5% lecithin, (4) standard diet with 1.5% cholesterol and 2.5% lecithin. Dietary cholesterol and lecithin replaced corn grain in the supplemented diets. Diets were fed for 8 weeks.

Blood samples were obtained by heart puncture at 0, 4, and 8 weeks of dietary treatment. The hens were sacrificed by cervical dislocation at 8 weeks of treatment and the livers were removed, weighed and placed in cold (2 C.) 0.1 M KCl buffer.

Hepatic FAS activity, total lipid, non-lipid and water were determined as previously described (Wagner, 1976). An aliquot of hepatic

TABLE 4.1. RATION COMPOSITION

Ingredients	Standard Layer diet %	Lecithin Layer diet %
Lecithin	--	2.50
Yellow corn, ground[1]	55.21	52.71
Soybean meal (44% protein)	15.68	15.68
Calcium carbonate	7.24	7.24
Tallow (feed grade)	6.52	6.52
Meat and bone scrap (50% protein)	5.14	5.14
Alfalfa meal (17% protein)	2.63	2.63
Live yeast culture[2]	1.68	1.68
Distillers solubles	1.68	1.68
Dried whey	1.68	1.68
Dicalcium phosphate	1.47	1.47
Salt	0.50	0.50
Vitamin-mineral concentrate[3]	0.50	0.50
dl-Methionine	0.07	0.07
Calculated analysis:		
Protein, crude % (N X 6.25)	16.10	15.99
Metabolizable energy, (kcal./kg.)	2951.	3087.
Daily Intake (hen/day)	95-100 g.	95-100 g.

[1]Cholesterol replaced 1.5% corn in cholesterol-supplemented diets.

[2]Manufactured by Diamond V. Mills, Cedar Rapids, Iowa.

[3]See Table 3.2. for composition of vitamin-mineral concentrate.

tissue was extracted by a modification of the method of Folch et al. (1957) for the determination of triglycerides, total cholesterol and phospholipids. Approximately 1 g. of liver tissue was weighed and homogenized in 15 ml. of 2:1 choloroform-methanol in an Omni-mixer.[1] The sample was homogenized for 1 min. at high speed and then filtered under a vacuum through a Buchner funnel. The precipitate was re-extracted with 15 ml. and then 9 ml. of the choloroform-methanol solution each for a 1 min. duration. All filtrates were combined and re-filtered to remove any residue present. Exactly 15 ml. of the filtrate was recovered and placed in a glass-stoppered centrifuge tube with 3.3 ml. of 0.15 M NaCl. The solution was mixed and placed in a refrigerator (2 C.) for 1 hr. to aid in the separation of the water and choloroform-methanol phases. The solution was then centrifuged for 10 min. at 2,000 g. after which the aqueous layer was discarded. The remaining choloroform-methanol phase was used for the determination of triglycerides (Van Handel and Zilversmit, 1957); total cholesterol (Hycel, Inc.[2]); and phospholipids (Bartlett, 1959).

Blood samples were analyzed for plasma triglycerides (Kessler and Lederer, 1966); free and esterified cholesterol (Schoenhiemer and Sperry, 1934); phosphorus (Fiske and Subbarow, 1925); and apolipoprotein-B by electroimmunoassay (Laurell, 1966).

All data were analyzed using the computer program entitled Statistical Analysis System developed by Barr and Goodnight (1972).

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[1] Ivan Sorvall, Inc., Norwalk, Conn.

[2] Hycel Cholesterol Kit. Hycel, Inc., Houston, Texas.

Plasma components were analyzed with respect to changes from time zero at 4 and 8 weeks of treatment.

Results and Discussion

Liver Components. Addition of 1.5% cholesterol to the rations increased liver weight ($P < 0.05$) of hens on both the standard and lecithin supplemented diets (Table 4.2). This increase in liver weight was primarily attributable to an increase in water ($P < 0.05$) and non-lipid fraction ($P < 0.10$). A numerical increase was also observed in the lipid fraction ($P > 0.10$). The increase in the lipid fraction was reflected by an increase in total cholesterol ($P < 0.01$) and phospholipids ($P < 0.05$). A numerical increase of approximately 65% ($P > 0.10$) was observed in hepatic triglycerides (Table 4.2).

Tsai and Dyer (1972) and Clarke et al. (1976) utilizing rats found liver enlargement from 1% dietary cholesterol supplementation was associated with an increase in total cholesterol, free fatty acids and total amount of hepatic protein. Changes in the liver lipid composition in male chickens as a result of 1% cholesterol feeding were associated with an increase in cholesterol esters, triglycerides, and total cholesterol with dramatic increases in cholesterol esters (Kruski and Narayan, 1972). Chung et al. (1967) also observed that the increase in cholesterol content of the livers was greatest for the esterified fraction. Liver phospholipids, expressed in mg. per g. wet liver weight, were not affected by the addition of 1% cholesterol (Kruski and Narayan, 1972). This agrees with our data when phospholipids are expressed as a percentage of wet liver weight.

The addition of cholesterol plus lecithin accentuated the responses

TABLE 4.2. EFFECT OF DIETARY LECITHIN AND CHOLESTEROL ON LIVER COMPONENTS[1]

Liver Components	Dietary Treatments[2]				
	Standard Layer	Standard Layer + 1.5% Cholesterol	Lecithin Layer	Lecithin Layer + 1.5% Cholesterol	S.E.M.
	--Grams--				
Liver	43.5	52.1[b]	41.0	57.0[b]	± 2.69
Water	28.5	33.3[b]	27.0	35.6[c]	± 1.17
Lipid	6.4	9.2	5.5	10.6[a]	± 1.62
Triglyceride	5.12	7.04	4.12	8.15	± 1.66
Total cholesterol	0.11	0.75[c]	0.12	0.95[c]	± 0.07
Phospholipid	1.16	1.40[b]	1.23	1.50[c]	± 0.06
Non-lipid	8.6	9.7[a]	8.5	10.8[c]	± 0.41
Cytosolic protein	2.3	2.6	2.2	2.7[a]	± 0.16
FAS-activity[3]	17.30	13.86[a]	15.10	15.16	± 1.32

[1] Data represents treatment means.

[2] Data analyzed by treatment effects with respect to standard layer ration.

[3] One unit equals 1 nmole malonyl CoA incorporated into long chain fatty acids/min.mg. protein.

Significance Level:

[a] $P < 0.1$

[b] $P < 0.05$

[c] $P < 0.01$

noted with the cholesterol-supplemented standard diet (Table 4.2). This effect of lecithin might be due to the "fat-effect" noted by Leveille and Sauberlich (1963). They investigated the effects of cholesterol supplementation (1% diet) with 4 dietary treatments (10% coconut oil, 10% olive oil, 10% corn oil, and no added fat). The fat-cholesterol-supplemented birds had higher hepatic fat and cholesterol levels than those fed the cholesterol-low-fat diet but had lower lipid phosphorus when expressed as a percentage of dry matter. They attributed this increased response to the enhancing effect of dietary fat on cholesterol absorption. Dietary fat may stimulate the flow of bile providing the bile salts necessary for emulsification plus activation of pancreatic and intestinal enzymes involved in cholesterol ester hydrolysis and formation. Ganguley et al. (1972) hypothesized that a prerequisite of lipid entry into the intestinal mucosal cells is water dispersion in the form of micellar aggregates. Lecithin acting as a detergent should enhance micellar formation and thus cholesterol (lipid) absorption.

Plasma Components. Dietary addition of 1.5% cholesterol to the ration increased plasma total ($P < 0.01$), free ($P > 0.10$) and esterified ($P < 0.01$) cholesterol (Tables 4.3, 4.4, and 4.5). Initially, the esterified form of cholesterol increased 140% whereas the free form increased 40%. Plasma free cholesterol accounted for a smaller portion of the increase in plasma total cholesterol at 8 weeks ($P > 0.10$) than observed at 4 weeks ($P < 0.05$) of dietary treatment. Such an elevation of blood cholesterol by cholesterol feeding in chickens has been well documented (Dua et al., 1967; Menge et al., 1975; Kruski and Narayan, 1972). Chung et al. (1967) also observed that cholesterol esters

TABLE 4.3. EFFECT OF TREATMENT ON PLASMA TOTAL CHOLESTEROL[1]

Treatment	Period[2] 0 wk.	4 wks.	8 wks.
		mg./dl.	
Standard layer	167	174[a]	178[a]
Standard layer + 1.5% cholesterol	170	295[e]	287[e]
Lecithin layer	133	131[a]	132[a]
Lecithin layer + 1.5% cholesterol	147	287[e]	297[e]
S.E.M.	±16	±18	±35

[1]Data represents treatment means analyzed with respect to a change from 0-week value.

[2]Periods represent duration of specific dietary treatment.

Values in a column with different superscripts differ in change from 0-week value.

[a,b]$P < 0.10$

[c,d]$P < 0.05$

[e,f]$P < 0.01$

TABLE 4.4. EFFECT OF TREATMENT PLASMA CHOLESTEROL ESTERS[1]

Treatment	Period[2] 0 wk.	4 wks.	8 wks.
		mg./dl.	
Standard layer	100	80[a]	82[a]
Standard layer + 1.5% cholesterol	95	228[e]	270[e]
Lecithin layer	84	73[a]	74[a]
Lecithin layer + 1.5% cholesterol	86	232[e]	252[e]
S.E.M.	±7	±14	±39

[1]Data represents treatment means analyzed with respect to a change from 0-week value.

[2]Periods represent duration of specific dietary treatment.

Values in a column with different superscripts differ in change from 0-week value.

[a,b] $P < 0.10$

[c,d] $P < 0.05$

[e,f] $P < 0.01$

TABLE 4.5. EFFECT OF TREATMENT ON PLASMA FREE CHOLESTEROL[1]

Treatment	Period[2] 0 wk.	4 wks.	8 wks.
		mg./dl.	
Standard layer	108	126[a]	129[a]
Standard layer + 1.5% cholesterol	114	160[c]	138[a]
Lecithin layer	83	88[a]	88[a]
Lecithin layer + 1.5% cholesterol	86	149[c]	148[c]
S.E.M.	±13	±14	±14

[1]Data represents treatment means analyzed with respect to a change from 0-week value.

[2]Periods represent duration of specific dietary treatment.

Values in a column with different superscripts differ in change from 0-week value.

[a,b] $P < 0.10$

[c,d] $P < 0.05$

[e,f] $P < 0.01$

accounted for the major increase in serum cholesterol of hens fed cholesterol containing diets.

Lecithin addition (2.5% diet) to the cholesterol-supplemented diet did not accentuate plasma cholesterol levels. Weiss et al. (1967a) have demonstrated that the addition of 5% lecithin to a layer ration containing 1% cholesterol increased plasma and egg cholesterol levels. This response has also been attributed to an increased absorption of dietary cholesterol. But in their study, the standard diet contained only 1% fat and therefore may have facilitated poor cholesterol absorption. Plasma values of 184 ± 14 mg. per dl. were reported. By comparison, our cholesterol-supplemented diet (6% fat) produced a mean of 287 mg. per dl. of total cholesterol (Table 4.3). Total plasma cholesterol values were comparable on the lecithin-cholesterol diets in our study and the cited work.

Weiss et al. (1967a) and Bartov et al. (1971) have reported variations in blood cholesterol among hens fed a single ration. Our evidence supports such reports and the suggestion of Weiss et al. (1967a) that these variations may be associated with ovulation.

Plasma triglycerides were decreased ($P < 0.10$) by cholesterol supplementation and numerically decreased ($P > 0.10$) by cholesterol plus lecithin supplementation (Table 4.6) at 8 weeks of dietary treatment. Lecithin increased plasma triglycerides ($P > 0.10$) at 8 weeks. Plasma phosphorus decreased on the cholesterol supplemented diets ($P < 0.10$) at 8 weeks of dietary treatment (Table 4.7). Plasa apolipoprotein-B increased on all dietary treatments (Table 4.8).

In the laying hen, very low density lipoproteins (VLDL) comprise approximately 82% of the total plasma lipoproteins with triglycerides

TABLE 4.6. EFFECT OF TREATMENT ON PLASMA TRIGLYCERIDES[1]

Treatment	Period[2] 0 wk.	4 wks.	8 wks.
		mg./dl.	
Standard layer	2631	2693	2466[a]
Standard layer + 1.5% cholesterol	2482	2201	1526[b]
Lecithin layer	1870	1849	1880[a]
Lecithin layer + 1.5% cholesterol	2175	1914	1951[a]
S.E.M.	±271	±264	±215

[1]Data represents treatment means analyzed with respect to a change from 0-week value.

[2]Periods represent duration of specific dietary treatment.

Values in a column with different superscripts differ in change from 0-week value.

$^{a,b}P < 0.10$

$^{c,d}P < 0.05$

$^{e,f}P < 0.01$

TABLE 4.7. EFFECT OF TREATMENT ON PLASMA PHOSPHORUS[1]

Treatment	Period[2]		
	0 wk.	4 wks.	8 wks.
		mg./dl.	
Standard layer	69	64	65[b]
Standard layer + 1.5% cholesterol	68	57	44[a]
Lecithin layer	60	50	45[b]
Lecithin layer + 1.5% cholesterol	67	51	47[a]
S.E.M.	±5	±5	±5

[1]Data represents treatment means analyzed with respect to a change from 0-week value.

[2]Periods represent duration of specific dietary treatment.

Values in a column with different superscripts differ in change from 0-week value.

[a,b]$P < 0.10$

[c,d]$P < 0.05$

[e,f]$P < 0.01$

TABLE 4.8. EFFECT OF TREATMENT ON PLASMA APOLIPOPROTEIN-B[1]

Treatment	Period[2]		
	0 wk.	4 wks.	8 wks.
		mg./dl.	
Standard layer	279	302	365
Standard layer + 1.5% cholesterol	277	302	307
Lecithin layer	242	278	299
Lecithin layer + 1.5% cholesterol	239	314	333
S.E.M.	±29	±21	±21

[1]Data represents treatment means analyzed with respect to a change from 0-week value.

[2]Periods represent duration of specific dietary treatment.

Values in a column with different superscripts differ in change from 0-week value.

$^{a,b}P < 0.10$

$^{c,d}P < 0.05$

$^{e,f}P < 0.01$

providing the majority of the lipid (Gornall and Kuksis, 1973). Kruski and Narayan (1972) observed that cholesterol feeding increased plasma VLDL in cockerels. In the untreated animals the VLDL fraction comprised 1.5% of the total plasma lipoproteins, while in the cholesterol-fed groups the VLDL fraction constituted 73% of the total plasma lipoproteins. Their VLDL fraction contained a low percentage of triglyerides (6%) and a very large amount of total cholesterol (77%). Almost the entire increase in plasma cholesterol was accounted for by this shift in cholesterol content and the elevated VLDL level. The low density lipoproteins (LDL) were only slightly changed. The high density lipoproteins (HDL) and total phospholipids were decreased. Leveille and Sauberlich (1963) also observed a 45% decrease in phospholipid concentration of plasma HDL from chickens fed a cholesterol containing diet. It was postulated that the decrease in HDL hypercholesterolemic birds might be due to either a transfer of protein-phospholipids of HDL or the individual components, protein and phospholipid separately to the VLDL fraction (Kruski and Narayan, 1972).

The observations of this study are consistent with the above hypothesis: The HDL fraction is the phosphorus-rich lipoprotein. The decrease in plasma phosphorus might reflect lower plasma HDL concentrations. Apolipoprotein-B is the major apoprotein in the VLDL fraction. Hence an increase in apolipoprotein-B also would follow a VLDL increase. Finally, the decrease in plasma triglycerides might indicate a shift in the cholesterol content of the VLDL fraction.

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