Effects of exercise and thyroxine on cholesterol and fatty acid synthesis in aging rats

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Serum and tissue lipid levels increase with age in both humans and the rat (Carlson, 1960; Brody and Carlson, 1962; Kritchevsky and Tepper, 1964b); and Carlson et al., 1968). Elevated serum and tissue lipid levels have been correlated with an increased incidence of cardiovascular disease (Stamler, 1967).

Two factors, of interest because of their influence on lipid metabolism, are the thyroid gland and exercise. Thyroid hormone influences all aspects of cholesterol metabolism (Kritchevsky, 1960) and lipid metabolism in general (O'Hara et al., 1966). The general trend being that hyperthyroidism increases the rate of metabolic processes involved in lipid metabolism, while hypothyroidism decreases these rates. The catabolic processes appear to be influenced to a greater extent, resulting in decreased lipid levels in the hyperthyroid state, and elevated lipid levels in the hypothyroid state. A possible factor in the age related slowing of lipid metabolism is decreased thyroid secretion rate in old animals (Grad and Hoffman, 1955). The sensitivity of cholesterol metabolism to thyroxine is also decreased in old animals (Hruza, 1971b).

High levels of physical activity have been associated with decreased lipid levels and a decreased incidence of atherosclerosis in man (Golding, 1961; Holloszy et al., 1964). Although results of animal studies have not been as conclusive as with human studies, a

similar effect of exercise has been observed in experimental animals (Jones et al., 1964; Gollnick and Simmons, 1967). There is evidence that the effects of exercise may be mediated by the thyroid gland. Exercise has been found to increase thyroid secretion rates (Irvine, 1967) or at least prevent the age related decrease in thyroid function (Story, 1972).

This study was designed to investigate the effects of exercise and thyroxine on lipogenesis in the aging rat. Both liver and intestine contribute significant amounts of cholesterol to the blood cholesterol pool and the liver is a major site of fatty acid synthesis in the rat. For these reasons, these tissues were chosen to investigate the in vitro synthesis of cholesterol and fatty acids from ^{14}C-acetate. Few reports concerning the effect of exercise on hepatic cholesterolgenesis and fatty acid synthesis have been published. No studies concerning the effects of aging, exercise, or the thyroid gland on intestinal lipogenesis were found. It is hoped the results of this investigation will provide additional information on these aspects of lipid metabolism.

REVIEW OF LITERATURE

The volume of literature related to cholesterol and fatty acid metabolism is extensive and increasing at a rapid rate. Because of the amount of information available, arbitrary limits were placed on the literature to be reviewed. Emphasis is placed on papers concerning the effects of age, exercise, and thyroid status on cholesterol and fatty acid metabolism.

Cholesterol Metabolism

An outline of cholesterol metabolism is presented in Figure 1. Only the rate limiting steps are included. Experimental evidence suggesting that the rate limiting step for the synthesis of cholesterol from acetate is the conversion of β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) to mevalonic acid by HMG-CoA reductase has been reported by Bucher et al. (1959) and Siperstein and Guest (1960). Ninety percent of the HMG-CoA reductase activity was found in the microsomal fraction of cell homogenates. Under some conditions the conversion of acetoacetyl-CoA to HMG-CoA by HMG-CoA synthetase has been suggested to be rate limiting (Dugan et al., 1972; White and Rudney, 1970). Gould and Swyryd (1966) have reported reactions beyond the formation of mevalonate whose rates were altered by dietary factors. However, inhibition developed more slowly and to a lesser degree indicating they were not rate limiting.

The principle products of cholesterol catabolism are the bile acids. In the rat, cholic acid (80%) and chenodeoxycholic acid

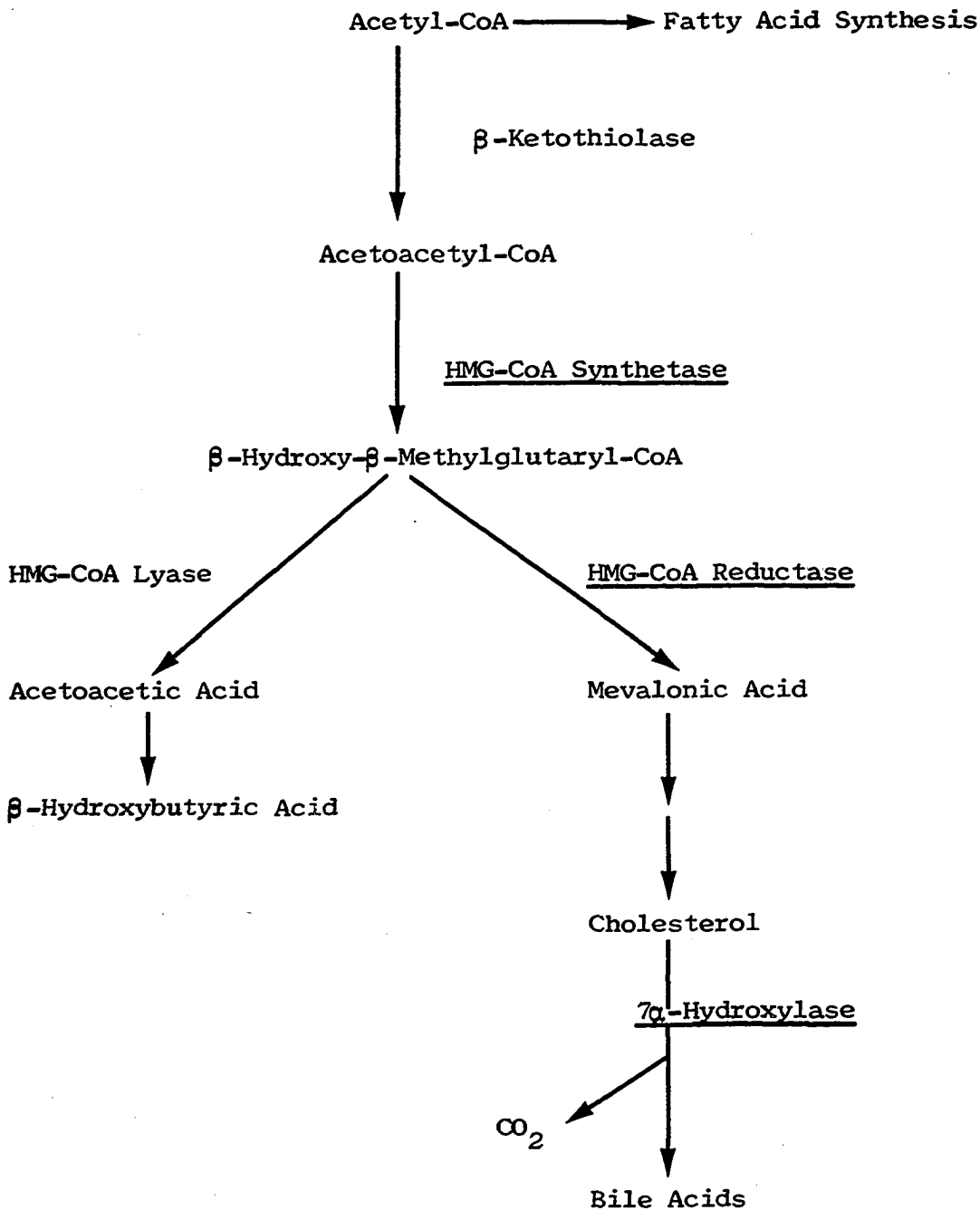

Figure 1. Pathway of hepatic cholesterol metabolism

(Enzymes which may be rate limiting are underlined)

(10%) are the principle bile acids. The rate limiting steps for their formation is catalyzed by cholesterol 7α-hydroxylase (Danielsson et al., 1967). This enzyme has also been localized in the microsomal fraction of the cell homogenate.

The existence of a diurnal variation in the rate of cholesterol metabolism has been demonstrated. Both cholesterol synthesis and reductase activity follow a diurnal rhythm (Back et al., 1969). In animals on a normal day-night schedule, fed ad libitum, cholesterolgenesis was at a minimum during the morning and afternoon. Peak activity was observed at midnight, 3.8 times greater than basal activity during the day. Danielsson (1972) has shown that the diurnal variation in 7α-hydroxylase activity closely parallels changes in HMG-CoA reductase activity indicating a close coupling of the synthetic and catabolic aspects of cholesterol metabolism.

Regulation of cholesterolgenesis by nonhormonal factors

Many experiments involving dietary manipulations have been conducted to provide a better understanding of cholesterol metabolism. Only a few of these studies are discussed here.

Both fasting, and feeding high-cholesterol diets inhibit hepatic cholesterolgenesis. Dietschy and Siperstein (1967) reported that feeding a 5% cholesterol diet reduced hepatic cholesterolgenesis to 1% of that observed in control animals. Fasting reduced hepatic cholesterolgenesis to 10% of that found in control animals. Feeding bile acids at the level of .5-1.0% of the diet also inhibited

hepatic cholesterolgenesis (Back et al., 1969; Shefer et al., 1973b). Dietary manipulations which increased hepatic cholesterolgenesis include the feeding of high fat diets, bile acid sequestering agents, and plant sterols such as sitosterol (DuPont et al., 1972; Goldfarb and Pitot, 1972; and Shefer et al., 1973b).

Two surgical procedures, canulation of the bile duct and ligation of the bile duct, also increase hepatic cholesterolgenesis. The physiological effects of these two procedures are similar to those of the bile acid sequestering agents. All prevent the normal cycling of bile acids through the enterohepatic circulation. This results in a greatly reduced uptake of cholesterol, and greatly increased rates of cholesterolgenesis (Weis and Dietschy, 1969; Harry et al., 1973).

It was by a combination of the experimental procedures discussed above, that first the biochemical pathways and later the factors controlling these pathways were elucidated. A complete understanding of the factors involved in the regulation of hepatic cholesterol metabolism has not been achieved. Representative papers are presented to explain what has been found to the present time.

Cholesterol feeding, and fasting (24-48 hrs) greatly reduced the incorporation of ^{14}C-acetate into cholesterol, no reduction of synthesis occurred when mevalonate was used as the sterol precursor (Back et al., 1969). This led to the postulation that the rate limiting step was prior to the formation of mevalonate. Ketogenesis, which also has HMG-CoA as an intermediate, was not inhibited by

cholesterol feeding (Siperstein and Guest, 1960). This suggested that conversion of HMG-CoA to mevalonate by HMG-CoA reductase was the rate limiting step in hepatic cholesterolgenesis. Siperstein and Fagan (1966) confirmed that feeding dietary cholesterol reduced the synthesis of mevalonate from HMG-CoA. It has since been demonstrated by many investigators that HMG-CoA reductase is the rate limiting enzyme for the synthesis of cholesterol.

There are reports indicating that HMG-CoA reductase may not always be rate limiting (White and Rudney, 1970). Fasting decreased HMG-CoA reductase activity, while cholesterol feeding reduced both reductase and HMG-CoA synthetase activity. HMG-CoA reductase activity was reduced to a greater degree, but the authors felt that under certain physiological conditions, substrate and cofactor availability, and HMG-CoA synthetase activity could be rate limiting. Dugan et al. (1972) observed that although cholesterolgenesis remained at basal levels in fasted rats, HMG-CoA reductase activity continued to follow a normal diurnal rhythm of reduced magnitude. Subba Rao and Ramasarma (1971) verified the existence of the diurnal rhythm in vivo. They observed a three-fold increase in cholesterolgenesis, somewhat less than the ten-fold increase in reductase activity observed in vitro (Shapiro and Rodwell, 1969). This suggests that at the time of peak reductase activity, other factors may be rate limiting. In summary, there is strong experimental evidence that HMG-CoA reductase activity is rate limiting, although experimental evidence indicates this may not always be true.

The precise mechanisms by which dietary manipulations and alterations in the enterohepatic circulation of bile acids influence the rates of hepatic cholesterolgenesis are not clearly defined. The effect of fasting will be discussed first.

Fasting for 5 hours reduced HMG-CoA reductase activity 50%. A 24-hour fast essentially abolished reductase activity. In refeeding experiments, reductase activity returned to normal more slowly that it disappeared (Regen et al., 1966). The results of Dugan et al. (1972) conflict with those of Regen and coworkers. The diurnal variation in reductase activity was observed, but cholesterolgenesis was inhibited in fasted animals. It was suggested that perhaps fasting resulted in the inhibition of reaction(s) beyond mevalonate synthesis. White and Rudney (1970) obtained results similar to those of Regen et al. (1966), fasting decreased reductase activity. They also noted that fasting did not alter HMG-CoA synthetase activity. Fasting increased the production of ketone bodies, indicating HMG-CoA availability was not rate limiting.

There seems to be no clear consensus as to how fasting inhibits cholesterolgenesis. Whether there is a specific inhibitor of some step in cholesterolgenesis, or a general reduction in enzyme levels has not been clearly demonstrated up to the present time.

The mechanisms by which high-cholesterol diets inhibit cholesterolgenesis have been the subject of many investigations. There are a variety of opinions as to the nature of the control exerted. It was initially thought that a product inhibition type

of feedback control was being exhibited. There are several studies such as one by Shapiro and Rodwell (1971) where adding cholesterol to the reaction media did not inhibit HMG-CoA reductase activity. Thus it is not cholesterol *per se* which inhibits cholesterolgenesis. Siperstein (1970), in a review article, stated that only cholesterol which has been absorbed by the intestine will inhibit cholesterolgenesis. Bortz (1973), also in a review article, cited evidence that both endogenous and exogenous cholesterol inhibit synthesis. However, as Siperstein pointed out, endogenous cholesterol soon becomes exogenous cholesterol due to the enterohepatic recycling of cholesterol. Another factor which has made a final resolution of the problem difficult is that inhibition occurs prior to any observable increase in total hepatic cholesterol concentration. Harry *et al.* (1973) reported cholesterol feeding greatly increased hepatic and microsomal cholesterol ester concentration. No changes in hepatic free cholesterol concentrations were observed. When cholesterol feeding was stopped, the concentration of microsomal cholesterol esters dropped, and the rate of hepatic cholesterolgenesis increased. As the enzymes for cholesterol synthesis are also found in the microsomes, this can be regarded as circumstantial evidence that it is the concentration of microsomal cholesterol esters that regulates cholesterolgenesis in cholesterol-fed animals.

The amount and type of fat fed also affects cholesterol metabolism. High-fat diets increase cholesterolgenesis, polyunsaturated fats (PUFA's) having the greatest effect (Diller and

Harvey, 1964). Goldfarb and Pitot (1972) found that a 20% corn oil (high in PUFA's) diet increased both the magnitude and the duration of peak HMG-CoA reductase activity as compared to the diurnal rhythm of animals on a low-fat diet. PUFA's were found to have a similar effect on cholesterolgenesis *in vivo*. Diets containing equivalent amounts of saturated fat did not increase synthesis *in vivo* (Dupont *et al.*, 1972).

Feeding a 24% corn oil diet increased liver cholesterol and fecal excretion of cholesterol significantly. Similar amounts of coconut oil had no effect on these parameters (Gollnick and Taylor, 1969). It is generally assumed that fat feeding increases serum cholesterol, but the above studies did not strongly support this, at least in the rat.

Fat feeding increased HMG-CoA activity independent of any caloric effect (Goldfarb and Pitot, 1972). The increase in rates of cholesterolgenesis appeared to be due to the synthesis of new enzyme protein. Gollnick and Taylor's work indicates increased catabolic rates. Experimental evidence suggests fat feeding, especially PUFA's, stimulate increased cholesterol turnover rates.

The role of bile acids in the regulation of cholesterolgenesis is also an area of active research. Feeding bile salts inhibited the *in vivo* synthesis of cholesterol (Beher and Baker, 1958). Finognari and Rodwell (1965) found that bile salts inhibited the *in vitro* synthesis of mevalonate when added to the incubation media. This type of experimental evidence led to the hypothesis that bile

acids directly inhibited cholesterolgenesis. Data of Liersch et al. (1973) suggest bile acids are not allosteric inhibitors of cholesterolgenesis. Perfusing rat livers in situ with bile salts did not decrease cholesterolgenesis, even in livers where cholesterolgenesis had been stimulated by pretreatment with cholestryamine, a bile acid sequestering agent.

Weis and Dietschy (1969) observed that biliary diversion, which decreased hepatic bile acid concentration, and biliary obstruction, which increased hepatic bile acid levels, both increased hepatic cholesterolgenesis. The authors concluded that bile acids had no direct effect on hepatic cholesterolgenesis in vivo. Inhibition was thought to be due to impaired uptake of cholesterol from the gut. They believed that both cholesterol feeding and bile salt feeding worked via the same feedback mechanism. Increased flux of cholesterol in the enterohepatic circulation was proposed to inhibit cholesterolgenesis. The in vitro action of bile acids was thought to be due to the detergent action of these agents which disrupted the microsomal membranes and the enzymes associated with them.

There is experimental evidence which contradicts the views of Weis and Dietschy (1969). Back et al. (1969) observed that feeding bile acids inhibited cholesterolgenesis within 4 hours, while it takes much longer for cholesterol feeding to induce a similar degree of inhibition. Harry et al. (1973) found that biliary diversion increased cholesterolgenesis before any decrease in microsomal cholesterol concentration was observed. Cycloheximide, an inhibitor

of m RNA synthesis, blocked the increase in cholesterolgenesis. Goldfarb and Pitot (1972) reported a 40-fold increase in HMG-CoA reductase activity in cholestryamine fed rats. These results indicate that bile acids regulate HMG-CoA reductase activity in a direct way independent of the effect they have on the flux of cholesterol through the enterohepatic circulation. Bile acids suppress the synthesis of HMG-CoA, while the absence of bile acids increase the rate of enzyme synthesis.

There is preliminary evidence that the physiological demand for bile acids may control hepatic cholesterolgenesis. Danielsson (1972) followed the diurnal variation of both HMG-CoA reductase activity and 7α-hydroxylase activity. The 7α-hydroxylase enzyme reached its peak activity 2 to 4 hours before HMG-CoA reductase activity was at a maximum. This raises the possibility that cholesterolgenesis increased because of increased demands for bile acids. Such a time would be prior to a meal when bile acid synthesis is increased in preparation for ingestion of dietary lipids (Chevallier, 1967).

The factors discussed above indicate that HMG-CoA reductase activity is regulated by changes in the amount of enzyme present rather than the activation of preformed enzyme or allosteric inhibition. Dugan et al. (1972) estimated the rate constants for both synthesis and degradation of HMG-CoA reductase enzyme. An eight-fold increase in the rate of HMG-CoA synthesis was reported during the rising phase of the dirunal rhythm. There was no change

in rate of degradation. Higgins et al. (1971) found that ^{3}H-leucine was incorporated into reductase for 6 hours during the rising phase of the diurnal variation followed by a 15-hour period of no incorporation. Puromycin blocked the increase in leucine incorporation.

Other factors have been reported to affect the diurnal rhythm of reductase activity. Time of feeding appears to control the time of day when the increase in activity occurs. Reversing the time of feeding so food is consumed during the day reversed the timing of the diurnal rhythm. This also reversed the diurnal rhythm of 7α-hydroxylase activity (Danielsson, 1972). Altering lighting patterns did not change the timing of the rhythm (Huber et al., 1973).

Regulation of cholesterolgenesis by hormones

The experiments discussed above describe the effects external stimuli have on cholesterol metabolism. The means by which cholesterol metabolism responds to these external stimuli are not well defined. It quite possible that these responses are mediated by endogenous hormonal factors.

Hormones suggested to play a role in some aspect of cholesterol metabolism include glucocorticoids, catecholamines, glucagon, growth hormone (GH), insulin, and the thyroid hormones. Because of the many hormones involved, there is much controversy concerning the role of individual hormones. It is the purpose of this discussion to present some current concepts of hormonal regulation of cholesterol metabolism.

Hepatic cholesterolgenesis has been investigated more extensively than other aspects of cholesterol metabolism so it will be discussed first.

Two hormones which have been investigated most extensively are the thyroid hormones and the adrenal hormones, especially the glucocorticoids. Some type of antagonism has long been suspected to exist between the thyroid hormones and the glucocorticoids. Bogoroch and Timiras (1951) found that many kinds of stress, including short-term exercise reduced ^{131}I uptake by the thyroid gland in rats. Brown-Grant et al. (1954) reported that treatment with adrenalin, corticotropin (ACTH) or glucocorticoids reduced thyroid function. The results indicated that decreased thryrotropin (TSH) release caused the decrease in thyroid function. Ducommin et al., (1966) reported results supporting the existence of a common precursor for ACTH and TSH. Retiene et al. (1968) reported an inverse relationship between ACTH and TSH concentrations in the pituitary of male rats.

Parallel circadian rhythms for adrenal and plasma corticoids, and pituitary and plasma ACTH have been reported. Wilber and Utiger (1969) found that infusion of dexamethasone, a synthetic glucocorticoid, reduced plasma TSH levels by 50% in one hour. If thyroid releasing factor (TRF) and dexamethasone were added simultaneously, no decrease in TSH was observed. This was interpreted to mean glucocorticoids block the release of TRF. They did not feel that peripheral utilization of TSH was increased.

No increase in the peripheral utilization of thyroid hormone was reported following treatment with ACTH or glucocorticoids (Ingbar and Frenkel, 1965).

Because of the antagonism between the two hormones and the inverse relationship of their diurnal rhythms, it has been suggested that this could be a mechanism for the regulation of the diurnal rhythm of cholesterolgenesis.

Thyroxine increases cholesterolgenesis (Fletcher and Myant, 1958; Ellefson and Mason, 1962; and Perin and Comolli, 1962). Thyroidectomy had no effect or decreased cholesterolgenesis (Dayton et al., 1960). Nejad and Chaikoff (1964) investigated the effects of both thyroxine (L-T_4) and glucocorticoids on cholesterolgenesis in rat liver slices. L-T_4 stimulated a seven-fold increase in the rate of incorporation of ^{14}C-acetate into cholesterol by liver slices from hypophysectomized rats. Adding glucocorticoids to liver slices of hypophysectomized rats further decreased synthesis. Glucocorticoids also blocked the L-T_4 stimulated increase in cholesterolgenesis.

Guder et al. (1968) reported that thyroidectomy reduced HMG-CoA reductase activity by 50%. A 50 μg injection of triiodothyronine (L-T_3) returned reductase levels to normal after a 30-hour latent period. Reductase activity remained elevated for 72 hours. L-T_3 had a similar effect on hypophysectomized rats (Ness et al., 1973). When L-T_3 was given to intact controls, it abolished the diurnal rhythm. Cholesterolgenesis occurred at a

uniformly high rate. Hypophysectomy reduced HMG-CoA reductase activity to near zero, indicating hormones in addition to thyroxine were important in the regulation of hepatic cholesterolgenesis.

The effects of adrenalectomy are subject to considerable debate. Hickman et al. (1972) reported adrenalectomy abolished the diurnal rhythm and hepatic cholesterolgenesis proceeded at a uniformly high rate in liver slices. An earlier study by Willmer and Foster (1960) also reported that adrenalectomy increased cholesterolgenesis in liver slices. They also reported that exogenous corticosteroids increased cholesterolgenesis in adrenalectomized animals.

Edwards et al. (1972) reported quite different results in in vivo studies. Adrenalectomy did not increase cholesterolgenesis to a uniformly high rate. There was no difference in the rates found in intact and adrenalectomized rats at midday. Hypophysectomy did reduce cholesterolgenesis to very low levels. No response to epinephrine, norepinephrine, or cortisone plus epinephrine was observed in the adrenalectomized or hypophysectomized rats. In intact animals, epinephrine increased HMG-CoA reductase activity 200 to 400%. This increase was blocked by cycloheximide. Cortisone had no effect. Subba Rao and Ramasarma (1971) also observed no effect of exogenous hydrocortisone on the in vivo synthesis of cholesterol in intact animals. Huber et al. (1972) reported that adrenalectomy had no effect on the circadian rhythm of cholesterolgenesis.

Ness et al. (1973) observed that hypophysectomized rats were

much more sensitive to exogenous L-T_3 than were intact control animals. Giving hydrocortisone and L-T_3 simultaneously to hypophysectomized animals reduced the increase in HMG-CoA reductase activity to levels observed in control animals. This suggested that hydrocortisone antagonized the action of L-T_3 in some way. No mechanism of action was proposed, but the 30-hour delay before seeing a response suggests T_3 stimulates the formation of new mRNA. Hydrocortisone could act in some way to block this increase in protein synthesis.

The same investigators have also studied the role of insulin and glucagon in the regulation of hepatic cholesterolgenesis (Lakshmanan *et al.*, 1973). Insulin increased both HMG-CoA reductase activity and hepatic cholesterolgenesis. The response time to insulin was only 2 hours. Glucagon blocked the response to insulin. Chattopadhyay and Martin (1969) reported a three-fold increase in cholesterolgenesis in liver slices from rats given insulin.

Dugan *et al.* (1974) have attempted to integrate the various hormonal influences on hepatic cholesterolgenesis into a coherent pattern. In hypophysectomized-diabetic rats, both insulin and T_3 are required to increase HMG-CoA reductase activity. This increase in activity can be blocked by either glucagon or hydrocortisone. It was further observed that exogenous cortisone and glucagon blocked the normal diurnal rise in reductase activity.

Combining the information presented by Bortz (1973), Dugan

et al. (1974) and other papers discussed here, the following picture of the regulation of hepatic cholesterolgenesis emerges. In intact rats, on a normal lighting schedule, fed ad libitum a low-fat, low-cholesterol diet, hepatic cholesterolgenesis may be regulated in the following manner. Increased feeding activity increases insulin levels, which rapidly increases reductase activity. During the day when animals are not feeding, glucagon levels are increased, depressing HMG-CoA reductase activity. As indicated earlier, fasting inhibits cholesterolgenesis, but a greatly reduced diurnal rhythm of reductase activity is still present. This may represent the effect of the diurnal variation of T_4 and adrenal corticoids.

Bortz (1973) said that any factor which increases fatty acids in the blood, increases cholesterolgenesis because of the increased demand for lipoproteins to transport the fatty acids. The effect of hypophysectomy indicates other pituitary hormones such as growth hormone (GH) are also important. The conflicting reports on adrenal hormones must still be resolved.

Experimental evidence indicates that both cholesterol feeding, and alterations in the enterohepatic circulation of bile may operate independent of hormonal mediators. The mechanism(s) by which HMG-CoA reductase activity is regulated are not yet known. The very short half-life of HMG-CoA reductase (2 to 4 hours) makes regulation at the level of protein synthesis a strong possibility. The stimulatory effect of many experimental treatments is blocked by inhibitors of protein synthesis. Pretreatment of animals with

cycloheximide, which increases the half-life of HMG-CoA reductase, delays the inhibition of cholesterolgenesis by dietary manipulations.

Regulation of cholesterol turnover and excretion

It is well established that bile acids are the end products of cholesterol metabolism (Block et al., 1943). The thyroid hormones have been shown to greatly influence the turnover and catabolism of cholesterol. Kritchevsky (1960) concluded that thyroxine has a greater influence on the catabolism of cholesterol than on its synthesis. Beher et al. (1963) studied cholesterol metabolism in hypophysectomized rats. There was a 50% reduction in the fecal excretion of ^{14}C-sterols. The greatest reduction was in the bile acid fraction. In a second study, Beher et al. (1964) found that thyroidectomy also reduced total sterol excretion, but not to the same degree as hypophysectomy. Administering thyroxine to hypophysectomized rats increased steroid excretion, but did not restore it to normal. This suggested the involvement of pituitary hormones. Strand (1963) reported that the administration of L-T_3 or D-T_3 to intact rats greatly increased bile acid production. Kritchevsky and Tepper (1964a) suggested that the site of action may be the 7α-hydroxylase enzyme. Suzuki et al. (1969) have reported increased concentrations of cholesterol in liver cell mitochondria, the site of cholesterol side-chain oxidation. Side-chain oxidation is the final step in the synthesis of bile acids from cholesterol. Byers and Friedman (1973) reported that the most significant effect of

thyroidectomy was a 40% decrease in bile flow over a 6-hour time period. The concentration of bile acids in the bile was not affected. If the thyroidectomized animals were given thyroid extract, bile flow returned to normal levels.

Beher et al. (1964) suggested that other hormones were involved in bile acid metabolism. Gielen et al. (1970) have found that the diurnal rhythm of 7α-hydroxylase is influenced by glucocorticoids. Growth hormone appears to play an important role (Byers et al., 1970; Friedman et al., 1970).

Marx et al. (1950) observed that hyperthyroid rats have reduced serum cholesterol levels, and elevated hepatic levels. Rats made hypothyroid with thiouracil had greatly elevated serum levels and somewhat elevated hepatic concentrations of cholesterol. Thyroid hormone prevented any significant rise in serum and hepatic cholesterol levels due to cholesterol feeding. In the hypothyroid animals, cholesterol feeding increased serum cholesterol concentrations four-fold. However, it has since been shown that thiouracil increases mean serum cholesterol levels and lowers hepatic cholesterol concentrations independent of its anti-thyroid action (Best and Duncan, 1958). Many of the early studies must be evaluated with this in mind.

Ellefson and Mason (1962) found that hypothyroidism increased serum cholesterol (133 vs. 97 mg/100ml), while hyperthyroidism decreased serum cholesterol (77 vs. 97 mg/100ml) as compared to values found in control animals. Hypothyroidism had little effect

on hepatic cholesterol levels, but hyperthyroidism greatly increased hepatic cholesterol (9.69 mg/liver vs. 7.90 mg/liver). Sterol excretion was greatly increased in the hyperthyroid animals. A study by Miettinen (1968) of myxedematous humans demonstrated that thyroid therapy greatly reduced the hypercholesterolemia usually associated with hypothyroidism. Little increase in rates of synthesis was noted while excretion rates were greatly increased. These studies indicate that the hypocholesterolemic effect of thyroxine is due to an elevated cholesterol excretion rate.

Friedman et al. (1970), Byers et al. (1970), reported that growth hormone (GH) prevented hypercholesterolemia in hypophysectomized rats. Thyroid extract alone did not prevent the increase in serum cholesterol in cholesterol-fed, hypophysectomized rats. A combination of thyroid extract and GH did prevent the increase in serum cholesterol levels. ACTH had no effect on serum cholesterol levels in hypophysectomized rats.

Aging and cholesterol metabolism

Increased serum and tissue lipid levels have been reported in both aging man (Brody and Carlson, 1962; Carlson, 1960) and rat (Carlson et al., 1968; Dupont et al., 1972).

Carlson et al. (1968) followed changes in tissue lipid content of male rats of the Sprague-Dawley strain ranging in age from 1 to 18 months. A three-fold increase in plasma cholesterol was observed. Plasma phospholipids and triglycerides also increased significantly

with age. In the liver, only the cholesterol concentration increased, phospholipids decreased, and triglycerides were unchanged. Only triglycerides increased in the skeletal muscle. The net effect of aging was to increase tissue lipids, probably due to decreased rates of turnover and utilization.

Several investigators have investigated the effects of aging on various aspects of cholesterol metabolism. Most have reported decreased rates of cholesterolgenesis (Rosenman and Shibota, 1952; Trout et al., 1962; and Yamamoto and Yamamura, 1971). Dupont et al. (1972) reported aging increased rates of cholesterolgenesis, but differences in ways of expressing data account for some of the discrepancy.

Yamamoto and Yamamura (1971) investigated four aspects of cholesterol metabolism: cholesterolgenesis, biliary excretion, fecal excretion, and uptake of cholesterol from the gut. All of these parameters were found to decrease with age, suggesting a slowdown of cholesterol metabolism in old animals.

Cholesterol turnover in aging rats has been extensively studied by Hruza and coworkers. Hruza (1971a) found that 72 hours after injection of ^{3}H-cholesterol, the level of radioactivity in serum of old animals was twice that found in young animals. There are two reasons for the decreased removal of cholesterol from the serum of the old animals. One is a much slower uptake of cholesterol by tissues of the old animals, the other is a reduced rate of excretion.

The factors responsible for the reduced uptake of cholesterol

by tissues of old animals are not known. Incubation of any tissue with serum from old rats decreased the uptake of ^{3}H-cholesterol by that tissue. A tighter chemical bonding between cholesterol and serum proteins in old animals was suggested. In parabiosis experiments where the circulatory systems of young and old rats were connected, the turnover rate of serum cholesterol and the uptake of cholesterol by the muscle and aorta was increased in the old animals to values similar to those found in the young animals.

The parabiosis experiments raised the possibility that hormonal factors might be involved. Hypophysectomy decreased turnover in both young (6 weeks) and old (13 months) rats. Uptake of cholesterol by the aorta of young animals was decreased, but no change was seen in the old animals. The effect of thyroidectomy was similar to that of hypophysectomy in young animals, but had no effect in old animals. Insulin and thyroxine increased serum cholesterol turnover and cholesterol uptake by the aorta in young animals. Insulin and thyroxine had no effect in the old animals indicating decreased sensitivity to circulating thyroxine and insulin in old animals. It may be that a lack of GH in the hypophysectomized rats accounted for the decreased turnover in the old animals (Hruza, 1971b).

A decreased excretion rate of cholesterol and its metabolites in old animals is well documented (Dupont et al., 1972; Hruza and Zbuzkova, 1973; Yamamoto and Yamamura, 1971). Hruza and Zbuzkova (1973) reported a 70% decrease in the rate of cholesterol excretion in old rats. Story and Kritchevsky (1974) compared the activity of

7α-hydroxylase enzyme, and the rates of oxidation of 26-^{14}C-cholesterol to $^{14}CO_2$ in young and old animals. These reactions represent the first and last steps in the conversion of cholesterol to bile acids. Using liver preparations from animals 2 months and 18 months old, they found that the rates of both reactions were reduced approximately 70% in the old animals.

Exercise and cholesterol metabolism

The effects of exercise on cholesterolgenesis, serum and tissue concentrations, and excretion of cholesterol will be reviewed. Effects of exercise on cholesterolgenesis are considered first. Only one paper directly investigating the effect of exercise on cholesterolgenesis was found. Rodionov and Yakubovska (1968) studied the effects of exercise on the incorporation of ^{14}C-acetate into cholesterol *in vivo*. Rats, trained and untrained, were subjected to acute bouts of exercise to determine the effects of both short-term and long-term exercise on liver cholesterolgenesis. In untrained rats forced to swim for 2 hours, run 2 hours, or run 10 hours, the specific activity of both the liver and blood cholesterol was much greater than the values found in unexercised animals. As the severity of the exercise increased, the specific activity also increased.

In trained animals, a similar increase in liver and blood specific activities in response to exercise was observed. However, the liver and blood specific activities of trained control animals

were only 60% of those found in the untrained controls. Even after running 2 hours, the specific activities found in the trained animals were still lower than those found in the untrained controls. In the untrained animals ran for 2 hours, the specific activity of liver cholesterol increased 60%, that of the blood, 50%. In the trained rats, the specific activity of the liver cholesterol increased 40%, that of the blood remained unchanged.

In summary the immediate effect of exercise was to increase cholesterolgenesis, the long-term effect was to decrease cholesterolgenesis. Two factors which could explain the short-term increase in cholesterolgenesis are the elevated serum cholesterol levels observed immediately after an exercise period, and the increased rate of cholesterol catabolism observed in exercised animals. Both of these factors will be discussed later. Reasons for the long-term reduction in cholesterolgenesis are not clear. Alterations in relative amounts of hormones, and/or changes in substrate and co-factor availability are possibilities.

Exercise reduces tissue lipid levels. When the effects of exercise on serum and hepatic cholesterol only are considered, results are much more variable. However, in studies where initial cholesterol levels are high, or the animals are being fed a high-fat, high-cholesterol diet, exercise reduces or prevents the development of elevated serum and hepatic cholesterol levels. In studies where animals are fed a basal diet, very strenuous exercise will decrease serum and hepatic cholesterol levels.

Carlson and Froberg (1969) subjected 12-13 month old rats to an exercise program consisting of running on a treadmill. This exercise program significantly reduced plasma cholesterol, phospholipid and triglyceride levels. In the liver and skeletal muscle, only triglycerides were reduced significantly. In rats fed a high-fat diet, and food intake equalized between the exercised and non-exercised animals, Gollnick and Simmons (1967) reported that swimming rats 15 minutes/day reduced hepatic cholesterol levels significantly. Only animals exercised for 60 minutes/day had significantly reduced serum cholesterol levels. Exercise was found to significantly increase sterol excretion.

Jones et al. (1964) exercised rats fed a basal diet. Strenuous exercise reduces food intake, so an experimental group fed 65% of the control group's food intake was included in this experiment. Exercise prevented the increase in serum cholesterol usually found in aging animals. Total body lipids and liver lipid content were reduced, hepatic cholesterol content was not significantly reduced. Caloric restriction decreased growth, but not total body lipid content, liver lipids, or serum cholesterol. These results suggest that the lipid reducing effects of exercise are not due to reduced caloric intake. Papadopoulos et al. (1969) reported that strenuous exercise would reduce serum cholesterol and triglyceride levels in rats fed a low-fat diet.

Rochelle (1961) observed that an exercise program reduced blood cholesterol levels significantly in men. He noted an 18%

increase in blood cholesterol immediately after the exercise period, but levels soon returned to normal. It was suggested that the elevated cholesterol levels were related to fat mobilization during exercise. Most fats are transported in combination with lipoproteins which have a high cholesterol content.

Tipton et al. (1968) and Barnard et al. (1968) suggested that thyroxine was necessary for exercise to reduce cholesterol levels. Training reduced hepatic cholesterol levels in normal and hypophysectomized rats given thyroxine. No decrease was reported in thyroidectomized rats.

Hebbelinck and Casier (1966) used mice to determine the effects of exercise on 4-^{14}C-cholesterol metabolism. Exercise increased bile acids, and decreased cholesterol found in the feces. In the liver, sterol concentrations decreased, and bile acid concentrations increased. Results indicate that exercise stimulates the conversion of cholesterol to bile acids in the liver.

Malinow and coworkers (1969, 1970) investigated the effects of muscular exercise on rates of cholesterol degradation. Muscular exercise increased $^{14}CO_2$ production from 26-^{14}C-cholesterol. Adrenalectomy reduced oxidation rates by 20%, suggesting most of the increased catabolism is in other tissues (Malinow et al., 1969). Later work showed that hepatectomy plus adrenalectomy abolished the increased excretion of $^{14}CO_2$ (Malinow et al., 1970). The decreased amounts of cholesterol in the feces, increased side-chain oxidation, and increased amounts of bile acids in the feces all

suggest increased formation of bile acids as a result of exercise.

Results of experiments designed to prove this point have been contradictory. In a study designed to determine the effect of short-term exercise on cholesterol degradation, Simko and Chorvathova (1968) cannulated the bile duct of rats that had been exercised for 2 hours. Over a 24-hour period they observed a significant reduction in bile flow and cholesterol excretion in the exercised animals as compared to non-exercised control animals. No significant differences in bile acid excretion were observed. In a similarly designed experiment, the effects of swimming for 105 days, 1 hour/day, were determined (Simko et al., 1970). The same results were obtained in this long-term experiment, as in the short-term study. Changes in fat pad composition due to exercise were also determined in the long-term study. Unsaturated fatty acids appeared to be preferentially mobilized. This could help account for the increased fecal excretion of bile acids in exercised rats.

Using a different experimental approach, Malinow et al. (1971) have observed significantly increased bile flow and bile acid excretion during muscular exercise. Reasons for the negative results of Simko and coworkers may include the fact that animals were fasted during the 24-hour collection period, and bile was not collected until after the exercise period.

Intestinal cholesterolgenesis

Sere et al. (1950) demonstrated the presence of cholesterolgenic capability in nearly every tissue, including the small intestine, of the rat. Few additional studies concerning intestinal cholesterolgenesis were published until recently. The work of Lindsey and Wilson (1965) demonstrating that the intestine may contribute significant amounts of cholesterol to the circulating cholesterol pool has stimulated new interest in the intestine and its role in cholesterol metabolism.

Dietschy and Siperstein (1965) found that the ileal portion of the small intestine was the site of greatest cholesterolgenic activity. Within the intestinal wall, the crypt cells were found to be responsible for 95% of cholesterol synthesis from ^{14}C-acetate.

The cholesterolgenic pathway of the intestinal mucosa appears to be similar to that of the liver. The enzymes involved are associated with the microsomal fraction of the cell, and HMG-CoA reductase catalyzes the rate limiting step (McNamara and Rodwell, 1972). Edwards et al. (1972) have demonstrated that intestinal cholesterolgenesis follows a diurnal rhythm similar to that of the liver, but of smaller amplitude. As in the liver, timing of the rhythm was dependent on when food was eaten, and independent of the lighting schedule.

Although there are many similarities between intestinal and hepatic cholesterolgenesis, there appears to be some differences in the control mechanisms involved. Dietschy and Siperstein (1965)

reported that fasting and cholesterol feeding have little influence on intestinal cholesterolgenesis, even though hepatic cholesterolgenesis is suppressed by both treatments. In a later study, they again reported that feeding high cholesterol diets had no effect on intestinal cholesterolgenesis. Fasting reduced intestinal cholesterol synthesis by 40% (Dietschy and Siperstein, 1967).

Dietschy and Siperstein (1965) observed that biliary fistula greatly increased intestinal cholesterolgenesis. A series of experiments indicated that the concentration of bile acids in the intestinal lumen, or the flux of bile acids across the mucosa regulated the rate of intestinal cholesterolgenesis. Since the ileal portion of the small intestine would be exposed to the lowest concentrations of bile acids, this could explain the higher rates of cholesterolgenesis found there. The authors admitted that cholesterol being transported could be the regulatory agent, but felt that results of the cholesterol feeding experiments argued against this.

The effects of fasting and high cholesterol diets were also investigated by Cayen and Black (1969). They found that a 24-hour fast reduced both hepatic and intestinal cholesterol synthesis by 98%. The reason for the conflict between their results, and those of Dietschy and Siperstein are not clear. Cholestryamine feeding increased both hepatic and intestinal cholesterolgenesis. Feeding levels of cholestryamine, that did not reduce cholesterol absorption, stimulated a five-fold increase in intestinal cholesterolgenesis.

Feeding a 2% cholesterol diet abolished this increase. This suggested that both bile acids and cholesterol are involved in the regulation of intestinal cholesterolgenesis.

Shefer et al. (1973a) investigated the effects of bile acids and dietary sterols on intestinal HMG-CoA reductase. They found that feeding excessive amounts of bile salts had no effect on basal rates of intestinal cholesterolgenesis, however, the diurnal variation was abolished. If rates of cholesterolgenesis were first stimulated by bile fistula, infusion of bile salts reduced rates of synthesis back to basal levels.

Feeding a 2% cholesterol diet failed to reduce rates of intestinal cholesterolgenesis or alter its diurnal rhythm. A combination of a 2% cholesterol, 1% bile salt diet reduced the rate of cholesterolgenesis 60% below basal rates found in control animals.

When the cholesterol content of the ileal crypt cells was determined, cells of animals fed the combination cholesterol-bile salt diet had 50% more cholesterol in them, than did animals from other dietary treatment groups. It was suggested that the elevated intracellular cholesterol concentrations may inhibit cholesterolgenesis. McIntyre and Isselbacher (1973) have also suggested cholesterol may have a direct role in the regulation of intestinal cholesterolgenesis. Cholesterol uptake is maximal in the jejunum, where cholesterolgenesis is minimal. This can be regarded as circumstantial evidence that the amount of cholesterol entering the

cell regulates cholesterolgenesis.

In summary, experimental evidence indicates that both bile acids and cholesterol have a role in the regulation of intestinal cholesterolgenesis.

Since most cholesterolgenesis occurs in the crypt cells, which give rise to the intestinal mucosal cells, cholesterol may be synthesized primarily for structural purposes (Dietschy and Siperstein, 1965). However, Wilson and Reinke (1968) have shown that cholesterol synthesized in the intestine is transported by the lymphatic circulation to the blood. The processes involved in passing from the crypt cell to the lymph are not known. Apparently the cholesterol does not have to be excreted into the intestinal lumen, but passes directly from the cell to the lymph. The amount of cholesterol contributed to the blood cholesterol pool has not been determined, but may be considerable in fasted animals, or animals fed high-cholesterol diets.

The role hormones play in the regulation of cholesterol synthesis in the intestine has not been determined, but the presence of the diurnal rhythm and the influence of feeding time on the diurnal rhythm suggest the possibility of hormonal regulation.

Thyroxine and growth hormone have been shown to have a significant influence on the rate of mitosis in the crypt cells. Leblond and Carriere (1955) have reported that thyroidectomy or hypophysectomy reduced mitotic rates. Thyroxine restored mitotic rates to normal in thyroidectomized rats, but both growth

hormone and thyroxine were required in the hypophysectomized rats. Carriere (1966) confirmed the effect of thyroxine on mitotic rates, but observed no effect after castration or testosterone administration to castrated animals. Levin and Smyth (1963) observed that thyroid status affected the weight of the intestine. The most obvious feature was the greatly thickened mucosal layer in hyperthyroid rats, and the very thin intestinal mucosa found in hypothyroid rats. Intestinal weights of hypothyroid animals were 75% of those found in control animals. In hyperthyroid animals, the intestinal weights were 175% of those found in control animals.

Fatty Acid Metabolism

As in the review of cholesterol metabolism, emphasis is placed on reports related to variables examined in this investigation: age, exercise, and thyroid status.

Fatty acid synthesis: Hormonal and dietary regulation

A brief outline of fatty acid synthesis is presented in Figure 2. This discussion will focus on the reactions catalyzed by acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS). ACC is found in the cytoplasm of the cell where it exists in two forms, an inactive protomer, and an active polymer. ACC is usually considered the rate limiting enzyme for fatty acid synthesis (Ganguly, 1960). ACC activity is regulated by short-term and long-term mechanisms. Short-term regulation is mediated via allosteric inhibition and activation. Citrate allosterically activates ACC by promoting the

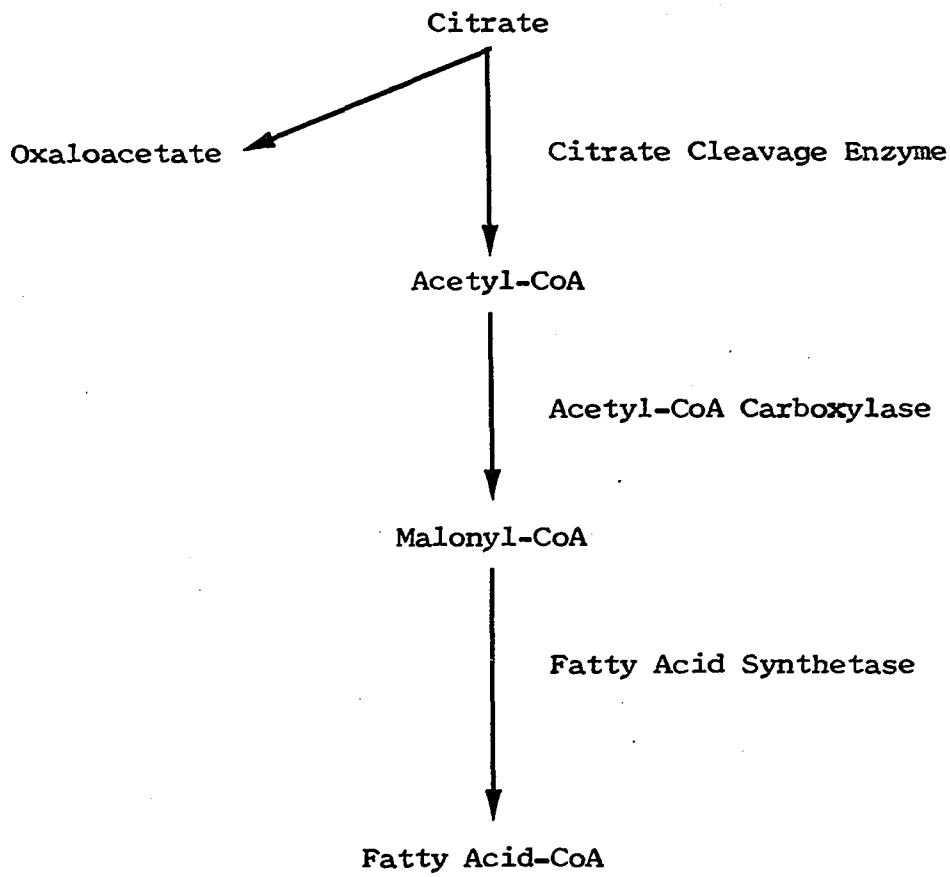

Figure 2. Fatty acid synthesis in the cytoplasm

(Reactions occurring in the cytoplasm only are shown)

formation of the active polymer. Palmityl-CoA, the major end product of fatty acid synthesis acts as an allosteric inhibitor. This type of regulatory system permits rapid change in rates of fatty acid synthesis in response to changing physiological conditions. Long-term regulation is achieved by changes in cellular ACC concentration. Changes in both rates of synthesis and degradation are responsible for changes in the levels of enzyme present.

The second enzyme to be considered is fatty acid synthetase (FAS). It is actually a multi-enzyme complex. Allosteric regulators have not been conclusively demonstrated. Volpe and Kishimoto (1972) suggested that in some cases, FAS activity may limit the rate of fatty acid synthesis. Additional information concerning ACC and FAS is found in a recent review by Volpe and Vagelos (1973).

Rates of fatty acid synthesis have been shown to follow a diurnal rhythm in both the liver (Bortz and Steele, 1973) and the intestine (Edwards et al., 1972). The timing of the rhythm appears to be related to the feeding schedule as peak activity occurs soon after eating.

Intestinal fatty acid synthesis occurs at a slow rate. Romsos et al. (1971) reported that in the pig, the intestine synthesized from 1-6% of the total fatty acids. Tame and Dils (1967) have suggested that intestinal fatty acid synthesis may be concerned with the structural needs of the cell, or possibly play a role in supplying specific fatty acids required for ester synthesis. Fatty acid synthesis in the liver has been studied much more

extensively and further discussion is limited to hepatic fatty acid synthesis.

Fatty acid synthesis in the liver is a major metabolic pathway. Dayton et al. (1960) found that 22% of an administered dose of ^{14}C-acetate was incorporated into fatty acids, as compared to only 1% of the label being incorporated into cholesterol. These results were from an in vivo study using rats fed a low-fat diet ad libitum.

Fatty acid synthesis is greatly influenced by dietary manipulation. Fasting and high-fat diets inhibit fatty acid synthesis. Feeding high carbohydrate, low-fat diets enhances fatty acid synthesis. These changes in rates of fatty acid synthesis are mediated via hormonal and nonhormonal means. Only the hormonal means of control are reviewed.

Meikle et al. (1973) investigated the effects of glucagon, epinephrine, and cyclic adenosine -3', 5'-monophosphate (cAMP) on fatty acid synthesis in fed, fasted, and fasted-refed animals. All treatments inhibited fatty acid synthesis in liver slices from fed animals. In fasted animals, where fatty acid synthesis is inhibited, no further reduction occurred when glucagon, epinephrine, or cAMP were added to the incubation media. In fasted-refed animals, fatty acid synthesis occurred at a rapid rate. Adding glucagon or cAMP reduced synthesis to levels found in fasted rats. Epinephrine reduced synthesis rates, but not to the same degree as glucagon or cAMP. The authors suggested that glucagon inhibited fatty acid synthesis

by raising cAMP levels in the liver.

The effects of insulin on fatty acid synthesis have been investigated by Chattopadhyay and Martin (1969). Liver slices from control rats incubated with ^{14}C-acetate incorporated three times as much acetate into fatty acids as did liver slices from diabetic rats. When insulin was added to the media, rates of fatty acid synthesis were increased seven-fold in liver slices from control animals. A 30-fold increase was observed in the slices from diabetic animals. Results indicated insulin has a powerful lipogenic effect.

Alloxan-induced diabetes decreased the level of ACC activity in the liver, probably by reducing rates of synthesis (Nakanishi and Numa, 1970). However, most reports in the literature discuss the effects of insulin on FAS activity. Whether this lack of information represents a lack of positive results concerning the hormonal regulation of ACC is not known.

Lakshmanan et al. (1972) reported that insulin restored FAS activity to normal levels in diabetic rats, stimulating a 20-fold increase in the rate of FAS synthesis. Glucagon and cAMP reduced FAS activity in fasted-refed rats. The authors proposed that FAS activity is regulated by insulin and glucagon levels in the liver. Insulin decreased cAMP levels which stimulated FAS activity. In contrast, glucagon increased cAMP levels which inhibited FAS activity. Changes in rates of synthesis of FAS was proposed as a possible mechanism.

Volpe and Vagelos (1974) reported that long-term fructose feeding restored normal FAS activity in diabetic rats. This suggested that intermediates of carbohydrate metabolism (citrate, acetyl-CoA) induced the synthesis of FAS independent of hormonal (cAMP) influences. This suggested the existence of at least two mechanisms for controlling the rate of FAS synthesis.

Glucocorticoids depressed fatty acid synthesis (Eisenstein, 1973). FAS activity was reduced in suckling rats by daily injections of hydrocortisone. In diabetic rats, Brady et al. (1951) observed that adrenalectomy restored fatty acid synthesis to normal levels. Cortisol treatments again inhibited synthesis of fatty acids.

Studies on the role of thyroid hormones have produced conflicting results. In an in vitro study, Fletcher and Myant (1958) reported that pharmacological amounts of T_4 reduced fatty acid synthesis, but results for hypothyroid, control, and physiologically hyperthyroid animals were inconsistent.

Gompertz and Greenbaum (1966) also studied fatty acid synthesis in liver slices. Exogenous L-T_4 (10 μg/day or 100 μg/day) greatly increased fatty acid synthesis, but 500 μg L-T_4/day inhibited it. They also reported that hyperthyroid animals synthesized more unsaturated fatty acids. Faas et al. (1972) have reported that L-T_4 added in vitro (directly to the media) produced similar increases in unsaturated fatty acid synthesis.

In vivo studies have provided more consistent results. Dayton *et al.* (1960) observed a small decrease in ^{14}C-acetate incorporation in hypothyroid animals as compared to controls. Hyperthyroidism increased incorporation of ^{14}C-acetate 50-70%. Ellefson and Mason (1964) also reported that thyroidectomy had little effect on fatty acid synthesis. Hyperthyroid animals (30 µg L-T_4/day) had rates of synthesis 2.5 times greater than control animals.

Recent studies have determined the effect of thyroid hormones on enzyme concentrations. Volpe and Kishimoto (1972) reported exogenous L-T_3 increased FAS levels 90% in suckling rats. Hypothyroidism reduced FAS levels to 60% of those found in control animals. Hypothyroidism also reduced the response to a fat-free diet by 50% in weaned rats.

Diamant *et al.* (1972) determined the effect of L-T_3 (20 µg/day) on a number of enzymes involved in fatty acid metabolism. Liver tissue was collected from both fed and fasted animals. In fed animals, L-T_3 increased 1-^{14}C-acetyl-CoA incorporation in fatty acids four-fold. In fasted animals, although rates of incorporation were much lower, L-T_3 treatment increased incorporation five-fold. The effect of L-T_3 on individual enzyme activities was determined. ACC activity was increased, both in the presence and absence of citrate. FAS, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, NADP-malate dehydrogenase, and ATP citrate lyase activities were all increased significantly in fed and fasted animals. Increased synthesis of the enzymes involved was suggested

as the mode of action.

L-T_3 doubled the rate of fatty acid oxidation by liver slices from both fed and fasted rats. The authors suggested that the increase in both synthesis and oxidation of fatty acids represents a cycling of metabolites through the fatty acid metabolic pathways. This would increase O_2 consumption and decrease ATP production. This "cycling" could be an important factor in the thermogenic effect of thyroid hormones.

Thyroxine and lipolysis

Felt et al. (1962) reported a decreased lipolytic response to adrenaline in athyroid animals. Serum free fatty acid (FFA) levels increased in control animals given an equal amount of adrenalin. Goodman and Bray (1965) reported reduced lipolytic responses to thyrotropin (TSH) and the catecholamines in hypothyroid animals, no lipolytic response to glucagon or corticotropin (ACTH) was observed. If large amounts of epinephrine were given, the hypothyroid animals mobilized normal amounts of fatty acids. Pretreatment of the hypothyroid animals with L-T_3 restored lipolytic activity within 3 hours. Rosenquist (1972) suggested that the decreased response to catecholamines was due to changes in the relative numbers of α and β adenergic receptors. In hypothyroid animals, α receptors are stimulated to a greater extent, resulting in decreased cAMP levels, which results in decreased rates of lipolysis. He suggested that decreased lipolysis in hypothyroidism was not due to decreased amounts of lipolytic enzymes.

Exercise and fatty acid metabolism

Exercise elevates circulating levels of catecholamines. Most of the increase is due to elevated norepinephrine levels. There is disagreement in the literature concerning the effects of exercise on epinephrine levels (Banister and Griffiths, 1972; Chin and Evonuk, 1971). Serum fatty acid concentrations were also elevated. Pre-treatment with L-T_3 caused even greater increases during exercise (Kaciuba-Uscilko and Brzezinska, 1973).

It is accepted that fatty acids are a major source of energy for muscular exercise. The source of fatty acids, whether adipose tissue or intramuscular, is still being debated.

Therriault et al. (1973) reported that strenuous exercise reduced intramuscular triglyceride stores by 50%. The authors assumed intramuscular triglycerides were mobilized by a hormone-sensitive lipase similar to the one found in adipose tissue. They also reported that muscle glycogen was reduced 75% indicating much of the ATP needed for muscular work was derived from intramuscular sources.

Ahlborg et al. (1974) reported that during a 4-hour exercise period, glucose mobilized from hepatic glycogen stores and fatty acids mobilized from adipose tissue provided up to 90% of the energy needs of exercising muscle. During the exercise period, insulin levels declined by 50%, while glucagon concentrations were elevated 500%. Rates of hepatic gluconeogenesis were increased during exercise in an attempt by the body to keep blood glucose levels at near normal levels despite increased peripheral utilization by exercising

muscle.

Mole et al. (1971, 1973) have investigated enzymatic adaptations to exercise in muscle of trained rats. Mitochondrial protein was increased 60%. Activities of enzymes involved in the β-oxidation of fatty acids were doubled. Enzyme systems which generate Krebs-cycle intermediates needed for the oxidation of fatty acids were also doubled.

In summary, the energy generating processes of trained muscle are adapted to use fatty acids as their major energy source. Kraus and Kinne (1970) have reported that thyroid hormones play an important role in these metabolic adaptations to exercise.

Aging and fatty acid metabolism

Yamamoto and Yamamura (1971) reported decreased rates of fatty acid synthesis on a per gram liver basis in old rats. However, if total liver synthesis is calculated, old rats synthesized more fatty acids than young rats. Dupont et al. (1972) have reported increased fatty acid synthesis in old rats. Increased amounts of "carcass" lipid were also reported in the old rats.

Carlson et al. (1968) have also reported increased lipid in old rats. The authors suggested that the increased blood and liver lipid levels in old rats could be due to decreased rates of lipid clearance from the blood. Decreased lipo-protein lipase activity was suggested as a possible explanation.

The lipolytic response to catecholamines was decreased with

age (Jelinkova et al., 1972). In rats 3, 6, and 24 months of age, a 50 μg injection of adrenalin stimulated 70, 40, and 30% increases in serum FFA levels respectively.

Livers of old rats were more susceptible to fatty liver (Szamosi, 1972). Hyperlipemia was induced in rats 4 to 6 months and 24 to 26 months of age. The younger animals were able to adapt metabolically to the hyperlipemia and recovered rapidly. In the old animals, recovery was much slower. In the young animals, the ability of the mitochondria to oxidize fatty acids was increased, and fatty acid synthesis reduced. Mitochondria from the old animals were less able to oxidize fatty acids, and there was no change in fatty acid synthesis.

METHODS AND MATERIALS

Treatment of Animals

Male rats of the Sprague-Dawley-Rolfsmeyer strain were used in this study. Animals were kept in wire mesh cages, six to eight rats per cage. Wayne Lab Blox and water were provided ad libitum. Animals were housed in a temperature controlled (25 ±3°C), artificially lighted room (8 A.M. to 10 P.M.).

Animals were obtained from the supplier at 50 days of age. Animals to be placed in the young experimental groups were allowed 1 week to adjust to the surroundings. They were then thyroidectomized and allowed 1 week to recover before assignment to experimental groups. Animals to be used in the mature experimental groups were maintained at the described conditions until thyroidectomized when 190 days old. The animals were allowed 1 week to recover before assignment to mature experimental groups.

Experimental Design

A two by two by three factorial design was used in this study. Animals were divided into two age groups: a young group 70 days of age, and a mature group 200 days of age at the beginning of the experimental period. Each age group was subdivided into exercise and nonexercise subgroups. Each of these four groups was further divided into three thyroid treatment groups.

1. Control - Thyroid gland intact, received no exogenous L-thyroxine.

2. Athyroid - Surgically thyroidectomized (Appendix A), received no exogenous L-thyroxine.

3. Hyperthyroid - Surgically thyroidectomized, given 7.0 μg L-thyroxine per 100 g body weight per day (Appendix B).

Experimental Procedures

Body weight

All animals were weighed weekly to the nearest gram. Body weights were used to determine exercise loads and the dosage of L-thyroxine to be given.

L-Thyroxine administration

The hyperthyroid animals were given exogenous thyroxine subcutaneously for 70 consecutive days. The control and athyroid animals were given an equivalent volume of placebo to equalize any effects the injection procedure might have had across all experimental groups.

Exercise procedures

The exercise program consisted of swimming the animals 5 days per week for 10 weeks. Animals were swum in large plastic tanks containing 2 feet of water ($35 \pm 2^{o}C$). A wetting agent, which had been used by previous investigators in this laboratory (Craig, 1972; Story, 1972) was omitted. Animals were thoroughly wetted within a few seconds after being placed in the water without the wetting agent.

Two things were done to promote vigorous swimming activity: 1) three rats were placed in each tank at a time and 2) a lead weight equal to 4% of the body weight was attached to the rat's tail.

During the first 3 days of the exercise period the animals were placed in the water without weights to allow the animals to become accustomed to being placed in the water. The next 2 days, a weight equal to 2% of the animal's body weight was taped to the tail. For the remaining 9 weeks, a weight equal to 4% of the body weight was taped to the tail. During the first week, animals were not allowed to swim longer than 10 minutes. During the last 9 weeks animals were swum to exhaustion or for 1 hour. Animals were considered to be exhausted when they were unable to return to the surface within 10 seconds (Dawson and Horvath, 1970).

Collection of tissues

Since it is well established that a diurnal rhythm exists in regard to cholesterol synthesis in both liver and intestine, the animals were sacrificed between 12 PM and 4 PM. Cholesterolgenesis is at a minimum rate during this time period.

Animals were not exercised on the day of sacrifice to eliminate any short-term effects of exercise. Animals were allowed access to food and water until time of sacrifice. Animals were stunned and decapitated. The livers were removed, rinsed in cold Krebs-Ringer bicarbonate solution, weighed, and placed in cold Krebs-Ringer bicarbonate solution until needed. The ileal portion of the small intestine was removed, and rinsed by forcing cold Krebs-Ringer

bicarbonate solution through it using a 25 ml syringe. This rinsing process was repeated several times until all intestinal contents were flushed out. The intestinal tissue was then placed in cold Krebs-Ringer bicarbonate solution until used.

Incubation procedures

Liver was sliced into thin slices free hand using an apparatus similar to one described by Cohen (1949). The ileum was slit open and cut into small squares. About 400 mg of each tissue was used in each flask. The tissue was blotted and carefully weighed before being placed in a Warburg flask. The incubation media contained 1 μCi of 1-^{14}C-acetate, 2 mmole cold acetate, and .5 mmole glucose in 5 ml of Krebs-Ringer bicarbonate. The amounts of acetate and glucose were those used by Hillyard and Entenman (1973). Procedures for preparation of the media are in Appendix C.

Tissues were incubated for 2 hours in a Warburg apparatus. It had been previously determined that the rate of product formation was essentially linear over the 2-hour incubation period. Substrate availability was not limiting under the described conditions. Duplicate incubations were carried out for each tissue. A blank incubation was also carried out under the same conditions, except that the ^{14}C-acetate was omitted.

Analytical procedures

The separation of the sterol and fatty acid fractions was a modification of the method of Kyd and Bouchier (1972). Following separation, each fraction was dissolved in a toluene scintillation fluid, and the amount of activity determined with a Beckman model LS-250 scintillation counter. See Appendix D for a complete description of procedures.

The amounts of protein in both the intestine and liver were determined by the method of Lowry *et al.* (1951).

Analysis of data

All data analyses were carried out using the *Statistical Analysis System* (Barr and Goodnight, 1971). The MEANS procedure was used to calculate the means and the standard errors. The ANOVA procedure was used to calculate the analysis of variance. Least significant differences (lsd) were calculated using the error mean square as the best estimate of the variance (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

The results of this investigation will be divided into two main areas for purposes of discussion. The effects of age, exercise, and thyroid status on final body weight (FBW) and liver weight will be considered first. Discussion of the effects of age, exercise, and thyroid status on hepatic and intestinal lipogenesis will follow.

Final Body Weight and Organ Weight

Final body weight

Weekly body weights for each experimental group are given in Tables 1 and 2. Means, standard errors of these means, least significant difference (lsd), and the analysis of variance for the final body weight (FBW) are presented in Table 3. All three parameters, age, exercise, and thyroid status, had highly significant effects ($p < .005$) on FBW. That the effect of age would be significant is not surprising in view of the age differences in the experimental groups at the beginning of the experimental period (70 days vs. 200 days). At the beginning of the experimental period, the young animals were still growing at a fairly rapid rate. The young-nonexercised-control animals gained 36 g the first week. By the 10th week the growth rate had declined to 15 g per week. The mature-nonexercised-control animals gained very slowly (2 g/week), indicating their rate of growth had reached a plateau.

The effect of thyroid status on FBW was dramatic. The young-nonexercised-athyroid animals did not grow over the 10-week

Table 1. Weekly body weights for young animals

Group	Number of rats	Week of experiment											
		0[a]	1	2	3	4	5	6	7	8	9	10	11[b]
		Weight to the nearest gram											
Control													
Ex	8	268	293	307	309	315	327	328	337	336	353	366	367
Nex	15	252	288	306	327	344	357	369	369	380	358	387	401
Athyroid													
Ex	13	287	285	286	288	295	302	303	304	297	299	301	300
Nex	15	283	283	282	284	285	288	288	288	281	282	283	281
Hyperthyroid													
Ex	9	286	271	289	298	302	307	324	321	326	335	338	338
Nex	11	294	292	310	322	322	330	338	343	342	354	356	356

[a]Weight of animals at beginning of the experimental period.

[b]Weight of animals on the day of autopsy.

Table 2. Weekly body weights for mature animals

Group	Number of rats	Week of experiment											
		0[a]	1	2	3	4	5	6	7	8	9	10	11[b]
		Weight to the nearest gram											
Control													
Ex	14	493	474	473	472	472	475	479	475	474	478	474	466
Nex	15	489	493	497	504	505	508	510	502	508	504	513	508
Athyroid													
Ex	9	468	450	444	430	428	426	424	416	419	414	415	413
Nex	17	485	475	469	464	459	459	451	449	438	429	427	429
Hyperthyroid													
Ex	11	444	411	410	408	404	408	405	394	401	389	385	385
Nex	14	444	434	436	434	438	438	439	427	418	418	426	431

[a]Weight of animals at beginning of the experimental period.

[b]Weight of animals on day of autopsy.

Table 3. Means, standard errors of these means, least significant difference, and analysis of variance for the final body weight (FBW)

	Young		Mature	
	Ex	Nex	Ex	Nex
	g	g	g	g
Control	368.3 ±8.5[a]	401.1 ±9.6	466.1 ±9.4	507.7 ±9.5
Athyroid	300.9 ±14.9	281.1 ±6.1	413.1 ±20.6	429.2 ±4.4
Hyperthyroid	338.1 ±13.3	356.3 ±8.1	398.3 ±11.7	430.5 ±12.5

Least significant difference at $p < .05 = 30.1$

Analysis of variance (all animals)

Source	d.f.	F. value	Prob > F
Age	1	294.23	.001
Exercise	1	10.56	.005
Treatment	2	76.33	.001
Age*Ex*Trt	2	4.24	.025
Residual	138		

Analysis of variance (young animals)

Source	d.f.	F value	Prob > F
Exercise	1	3.00	.100
Treatment	2	48.02	.005
Ex*Treat	2	2.27	.100
Residual	65		

Analysis of variance (mature animals)

Source	d.f.	F value	Prob > F
Exercise	1	7.25	.010
Treatment	2	27.09	.001
Ex*Treat	2	2.91	.100
Residual	73		

[a]Mean ± standard error of the mean.

experimental period. This is in accord with the known requirements of thyroid hormone for growth in mammals (Shellabarger, 1964). In the mature- nonexercised-athyroid animals, a loss of weight occurred over the 10-week experimental period (485 vs. 440 g).

Part of the reason for the lack of growth in the young athyroid animals and a loss of weight in the mature athyroid animals was decreased food intake (Naito, 1971). Scow (1951) has shown that decreased food intake is not the only factor involved. He force fed thyroidectomized rats so that they achieved near normal weight gain. At the end of the experiment, carcass analysis demonstrated that the weight gain was mainly due to increased adipose tissue. The lean body mass of the force-fed thyroidectomized rats was not any different than that found in thyroidectomized rats fed ad libitum, although their final body weights were much less than those of the force-fed rats. This study demonstrates the important role thyroid hormone plays in achieving normal protein deposition in growing rats.

The hyperthyroid state (7 μg L-T_4/100 g bw/day) decreased the weight gain in the young animals resulting in significantly lower final body weights for both the exercised and nonexercised hyperthyroid animals as compared to their respective control groups. The major reason for the decreased FBW was the absence of fat stores. Carcasses of the hyperthyroid animals were nearly devoid of the peri-renal as well as other visceral adipose stores. Food intake was not monitored in this study, but other investigators have

found that hyperthyroidism increased food intake (Grossie and Turner, 1961; Naito, 1971). The greatly increased metabolic rate is more than adequate to prevent the deposition of adipose tissue due to the increase in food intake.

In the mature animals, the hyperthyroid condition resulted in a moderate weight loss over the 10-week experimental period. This resulted in FBW's being significantly smaller ($p < .05$) in the hyperthyroid animals when compared with the control animals.

Considered *in toto*, exercise significantly reduced the FBW ($p < .05$). There were exceptions to this general trend. Exercise did not significantly lower the FBW in either the young or the mature athyroid animals.

In the young animals, the athyroid exercised animals had a larger FBW than did their nonexercised counterparts (300 g vs. 281 g). This difference was not significant at the $p < .05$ level. Examination of the data indicated this growth was due to large increases in weight of two of the animals in this experimental group. When the FBW was calculated omitting these two animals, the mean FBW for the young-exercised-athyroid animals is 283 g. Except for weight gain, the animals appeared to be athyroid, and no thyroid tissue was found at the time of autopsy, so the animals were not omitted from the experimental results.

A similar lack of effect of exercise on the FBW of athyroid animals was reported previously by Naito (1971). These results are in conflict with those of Tipton *et al.* (1968). Running on a

treadmill caused a significant decrease in FBW. However, his athyroid animals gained weight steadily throughout the exercise period, although other metabolic parameters indicated these animals were athyroid.

Exercise did not significantly reduce FBW in the young hyperthyroid animals.

The failure of exercise to significantly reduce the FBW in the above groups are probably due to several reasons. In the case of the young hyperthyroid rats, the lack of significant fat depots may have been the major reason. The loss of weight due to exercise is mostly due to a loss of adipose tissue (Babirak et al., 1974; Jones et al., 1964). The failure of exercise to significantly reduce FBW in the young animals as a group may have been due to the smaller amounts of adipose tissue in these animals. In the mature animals sizable fat stores were accumulated prior to the experimental period, so exercise did reduce the FBW significantly ($p < .05$) in the mature animals.

In the athyroid animals, a greatly reduced capacity for work may be an important factor. There was also a decrease in the intensity of an athyroid rat's swimming motions. The athyroid animals swam in "slow motion." The inability of skeletal muscle to contract rapidly is characteristic of myxedema. The exact nature of the defect is not clear. Conduction rate of the nerve impulse is normal (Lambert, 1951), so the impairment is apparently within the muscle itself. Fanburg (1969) has observed that hypothyroidism

greatly reduced Ca^{++} transport by rat muscle sarcoplasmic reticulum. This could account for greatly increased contraction and relaxation times of skeletal muscle in hypothyroid rats.

Because of the animals inability to swim effectively, the swim times are greatly reduced (Long and Griffith, 1972). This reduced swim time reduced the amount of work performed, and the amount of energy utilized to do the work.

The significant ($p < .025$) age*treatment*exercise interaction on FBW was due to factors already discussed. The different response of the young and mature animals to exercise, and the effect thyroid status had on the response to exercise accounts for the significant interaction.

Organ weight

It is not possible to completely separate the discussion of body weight and organ weight due to the high degree of correlation between them. Discussion will be concerned primarily with the liver, but other organs will be discussed where appropriate. Means, standard errors of these means, lsd ($p < .05$), and analysis of variance for liver weight (LW) are given in Table 4. Age, exercise, and thyroid status all influenced LW significantly ($p < .025$). Liver weight per 100 g body weight (LW/BW) was also calculated. Means, standard errors of the mean, lsd, and analysis of variance for LW/BW are in Table 5. When the liver weight was corrected for variations in body weight, only thyroid status had a significant

Table 4. Means, standard errors of these means, least significant difference, and analysis of variance for liver weight (LW)

	Young		Mature	
	Ex	Nex	Ex	Nex
	g	g	g	g
Control	11.26 ± .35[a]	12.61 ± .52	13.40 ± .46	15.06 ± .52
Athyroid	7.10 ± .53	6.74 ± .23	9.96 ± .78	10.49 ± .44
Hyperthyroid	12.21 ± .63	12.17 ± .57	13.26 ± .50	15.08 ± .83

Least significant difference at $p < .05 = 1.52$

Analysis of variance (all animals)

Source	d.f.	F value	Prob > F
Age	1	87.18	.001
Exercise	1	6.59	.025
Treatment	2	110.82	.001
Residual	138		

Analysis of variance (young animals)

Source	d.f.	F value	Prob > F
Exercise	1	2.26	.250
Treatment	2	83.33	.001
Residual	65		

Analysis of variance (mature animals)

Source	d.f.	F value	Prob > F
Exercise	1	4.11	.050
Treatment	2	32.37	.005
Residual	73		

[a]Mean ± standard error of the mean.

Table 5. Means, standard errors of these means, least significant difference, and analysis of variance for liver weight per 100 g body weight (LW/BW)

	Young		Mature	
	Ex	Nex	Ex	Nex
	g/100 g body weight	g/100 g body weight	g/100 g body weight	g/100 g body weight
Control	3.06 ± .06[a]	3.13 ± .07	2.87 ± .05	2.96 ± .09
Athyroid	2.33 ± .07	2.39 ± .05	2.39 ± .10	2.45 ± .10
Hyperthyroid	3.61 ± .11	3.40 ± .10	3.33 ± .05	3.49 ± .10

Least significant difference at $p < .05 = .23$

Analysis of variance (all animals)

Source	d.f.	F value	Prob > F
Age	1	.08	n.s.[b]
Exercise	1	.73	n.s.
Treatment	2	159.15	.001
Age*Treat	2	3.16	.050
Residual	138		

Analysis of variance (young animals)

Source	d.f.	F value	Prob > F
Exercise	1	.169	n.s.
Treatment	2	120.240	.001
Ex*Treat	2	1.67	.250
Residual	65		

Analysis of variance (mature animals)

Source	d.f.	F value	Prob > F
Exercise	1	.58	n.s.
Treatment	2	58.21	.005
Ex*Treat	2	.87	n.s.
Residual	65		

[a]Mean ± standard error of the mean.

[b]n.s. = not significant at $p < .05$.

effect ($p < .001$). There was also a significant age*treatment interaction ($p < .05$).

The significant effect of both age and exercise on LW was due to the effect these two parameters have on FBW. When the variation in FBW was corrected for, neither of these parameters had a significant effect on LW/BW.

Other organ weights influenced by exercise in intact animals include the heart and the adrenals, whose weights are increased due to exercise and the testes, which are lighter in exercised animals (Tipton et al., 1968).

Jones et al. (1964) also observed that exercise decreased liver weight. Tipton et al. (1968) reported that the athyroid condition prevented any increase in heart and adrenal weight due to exercise. Naito (1971) observed a similar lack of effect of exercise on the adrenal gland in athyroid rats.

The effect of thyroid status on LW and LW/BW was highly significant ($p < .001$) in both young and mature animals. The significant age*treatment interaction ($p < .05$) on LW/BW appears to be due to decreased response to both the absence of L-T_4, and the presence of excess L-T_4 in the mature animals. There is much debate in the literature as to whether animals become hypothyroid with age. There has been considerable discussion as to whether animals are less responsive or more responsive to circulating levels of thyroid hormone (Grad, 1969; Grad and Hoffman, 1955). The data presented here would suggest a decreased responsiveness, but due to

the influence of many other hormones, the answer is probably not that simple. Story (1972) and Frolkis et al. (1973) have reviewed the relationships between age and thyroid function.

Intestine was not weighed, but the difference in gross appearance was striking. The intestines of the athyroid animals were thin walled, with the mucosal layer greatly reduced in thickness as compared to the mucosal layer of intestines of both control and hyperthyroid rats. Leblond and Carriere (1955) and Carriere (1966) have reported that thyroidectomy greatly reduced the rate of mitosis in the Crypts of Lieberkuhn. Replacement therapy with thyroxine restored the mitotic rate to normal.

A visual comparison of the carcasses at autopsy revealed striking differences in gross appearance. In the athyroid groups, the diminished size of the liver, kidney, and the gut were the most obvious features. Adipose tissue was reduced when compared to control animals, but the visceral fat pads were still extensive. In the hyperthyroid animals, the enlarged liver, and the absence of adipose tissue were the most striking features. The net effect of these observations were reflected in the FBW of the respective experimental groups.

Protein determinations

Protein content of the liver and intestine on a per gram basis was determined. None of the experimental treatments had a significant effect on tissue protein levels. Apparently this

approach is not sensitive enough to detect changes in specific enzyme concentrations known to be influenced by varying levels of thyroid function.

Cholesterolgenesis

The rate of incorporation of ^{14}C-acetate into digitonin precipitable sterols (DPS) by liver and intestinal slices was determined. The 3-β-hydroxy sterols were precipitated with digitonin and not further purified. Reports in the literature indicate that about 90% of the DPS is cholesterol (Dayton et al., 1960; Dietschy and Siperstein, 1967; and Dietschy and Weiss, 1971). For purposes of discussion, the terms cholesterol and DPS will be used interchangeably.

Hepatic cholesterolgenesis

Means, standard errors of these means, lsd ($p < .05$), and analysis of variance for synthesis of DPS are in Table 6. Age and thyroid status had highly significant effects ($p < .001$) on cholesterolgenesis. Significant age*treatment ($p < .001$) and age*exercise ($p < .005$) interactions were found to exist. Exercise was found to reduce cholesterolgenesis significantly ($p < .05$) in the young animals.

A reduced rate of cholesterolgenesis in old animals has been reported by many investigators (Block et al., 1946; Trout et al., 1962; and Yamamoto and Yamamura, 1971). The significant effect of thyroid status on cholesterolgenesis has also been widely reported

Table 6. Means, standard errors of these means, least significant difference, and analysis of variance for hepatic synthesis of digitonin precipitable sterols (DPS)

	Young		Mature	
	Ex	Nex	Ex	Nex
	dpm/g of liver/hr	dpm/g of liver/hr	dpm/g of liver/hr	dpm/g of liver/hr
Control	12682 ±636[a]	13770 ±674	11183 ±529	11224 ±460
Athyroid	8933 ±638	9747 ±406	9248 ±460	8050 ±210
Hyperthyroid	20227 ±496	22538 ±876	18118 ±416	18347 ±633

Least significant difference $p < .05 = 1552$

Analysis of variance (all animals)

Source	d.f.	F value	Prob > F
Age	1	26.30	.001
Exercise	1	1.12	n.s.[b]
Treatment	2	380.21	.001
Age*Ex	1	8.28	.005
Age*Treat	2	11.20	.001
Ex*Treat	2	2.15	.250
Residual	138		

Analysis of variance (young animals)

Source	d.f.	F value	Prob > F
Exercise	1	6.25	.050
Treatment	2	176.73	.001
Residual	65		

Analysis of variance (mature animals)

Source	d.f.	F value	Prob > F
Exercise	1	1.87	.250
Treatment	2	218.69	.001
Residual	73		

[a]Mean ± standard error of the mean.

[b]n.s. = not significant at $p < .05$.

in the literature (Dayton et al., 1960; and Kritchevsky, 1960). The level of β-hydroxy-β-methylglutaryl CoA reductase, the regulatory enzyme in the cholesterolgenic pathway, is directly dependent on the presence of thyroid hormone (Guder et al., 1968).

Literature concerning the effects of exercise on hepatic cholesterolgenesis is quite limited. Rodionov and Yakubovska (1968) reported that hepatic cholesterolgenesis increased immediately after a bout of exercise, but the long-term effect of a conditioning program was a reduced rate of cholesterolgenesis. Such a reduction was observed in the young but not the mature animals in this study. It may be that a decreased ability to exercise as well as the age associated decrease in cholesterolgenesis may have prevented any further decrease in the rate of cholesterol synthesis in the mature animals.

Exercise had its greatest effect in the young control and young hyperthyroid groups. While this study does not provide a definitive answer as to the mechanism(s) involved, the following hypothesis is offered as a possible explanation.

Story (1972) reported that exercise increased the thyroid secretion rate (TSR) significantly in young animals and prevented the decrease in TSR associated with aging. However, Winder and Heninger (1971, 1973) reported increased turnover of thyroxine and increased hepatic utilization of thyroxine in exercised young animals as compared with sedentary control animals. Exercise increased the hepatic concentrations of L-T_4 30%, while plasma levels

were reduced 20%. The half-life of exogenous L-T_4 was reduced to 18 hours in exercised animals as compared to a half-life of 24 hours in sedentary animals. It is possible that exercise may increase the utilization of thyroxine in metabolic processes needed to meet the greater energy needs of animals performing physical work. In support of this hypothesis, Kraus and Kinne (1970) have reported increased activity of mitochondrial enzymes in the liver and the heart. Thyroxine was necessary for these increases to occur.

In the mature animals, a decreased ability to adapt to metabolic changes may be responsible. Szamosi (1972) has reported a decreased flexibility in liver metabolic processes.

Since the athyroid rats lack thyroid hormones, cholesterolgenesis is at a basal level and no further reduction due to exercise would be expected.

The significant ($p < .001$) age*treatment interaction was due to the decreased sensitivity of hepatic cholesterolgenesis to changes in thyroid status in the mature animals as compared with the young animals. Hruza (1971b) has reported a similar lack of responsiveness of cholesterol turnover rates to thyroid hormones in old (13 months) rats.

The significant ($p < .005$) age*exercise interaction was due to the significantly reduced rates of hepatic cholesterolgenesis observed in young, exercised rats, but not the old, exercised rats.

Hepatic cholesterolgenesis was also examined in terms of the total synthetic capability of the liver. Total hepatic

cholesterolgenesis was calculated by multiplying the per gram rate of cholesterolgenesis by the liver weight. Means, standard errors of these means, lsd ($p < .05$) and the analysis of variance are given in Table 7. Expressing the data in this way altered the influence of some of the parameters.

The lower rates of cholesterolgenesis on a per gram basis in the mature rats were compensated for by the much larger livers found in the mature animals. This effect was most apparent when comparisons of young and old athyroid animals were made. The mature animals had a much higher rate of synthesis than young animals (84,854 vs. 65,006 dpm/liver/hr).

The effect of exercise was significant ($p < .025$) for the combined groups. This is due to the combining of the trends towards reduced cholesterolgenesis on a per gram basis with the tendency of exercised animals to have smaller livers.

The effect of thyroid status was enhanced in a similar fashion. The increased rate of synthesis on a per gram basis in the hyperthyroid was combined with the significantly larger livers in the hyperthyroid animals. A similar amplification occurred in the athyroid animals. The reduced rate of incorporation on a per gram basis was coupled with reduced liver size to give a greatly reduced rate of cholesterolgenesis in the athyroid rats.

Table 7. Means, standard errors of these means, least significant difference, and analysis of variance for total hepatic synthesis of digitonin precipitable sterols (DPS)

	Young		Mature	
	Ex	Nex	Ex	Nex
	dpm/liver/hr	dpm/liver/hr	dpm/liver/hr	dpm/liver/hr
Control	142,656 ±7714[a]	171,952 ±8934	149,154 ±7554	170,245 ±11363
Athyroid	63,800 ±7226	65,006 ±2357	93,534 ±10512	84,854 ± 4725
Hyperthyroid	246,158 ±12033	273,820 ±15901	240,454 ±11095	276,513 ±16436

Least significant difference at $p < .05 = 28,500$

Analysis of variance (all animals)

Source	d.f.	F value	Prob > F
Age	1	7.06	.025
Exercise	1	6.15	.025
Treatment	2	321.50	.001
Ex*Treat	2	4.18	.025
Residual	138		

Analysis of variance (young animals)

Source	d.f.	F value	Prob > F
Exercise	1	7.09	.025
Treatment	2	219.23	.001
Residual	65		

Analysis of variance (mature animals)

Source	d.f.	F value	Prob > F
Exercise	1	1.11	n.s.[b]
Treatment	2	124.34	.001
Ex*Treat	2	3.14	.05
Residual	73		

[a]Mean ± standard error of the mean.

[b]n.s. = not significant at $p < .05$.

Intestinal cholesterolgenesis

Means, standard errors of these means, lsd ($p < .05$) and analysis of variance for the synthesis of DPS are in Table 8. Neither age nor exercise had significant effects on intestinal cholesterolgenesis. Thyroid status had a highly significant ($p < .005$) effect, with the hyperthyroid groups having higher, and the athyroid groups lower rates of cholesterolgenesis than their respective control groups.

The mode of action of thyroid hormones on intestinal cholesterolgenesis could be direct, indirect, or both. As discussed earlier, thyroxine stimulates mitosis in the crypts of the intestine. Since cholesterol is an integral component of cellular membranes, an increase in mitosis would require increased cholesterolgenesis. It is also possible that thyroid hormone directly influences the level of enzymes in the intestinal mucosal cells. No reports in the literature were found concerning the effects of thyroxine on intestinal cholesterolgenesis, but changes in thyroid status affect the level of other intestinal enzymes (Bronk and Parsons, 1965; Yeh and Moog, 1974).

Fatty Acid Synthesis

Lipogenesis can be influenced by many factors including diet, feeding patterns, and hormones. Because of the many interactions possible, it is often difficult to interpret results. Also some investigators (Clark et al., 1974) feel that rates of lipogenesis

Table 8. Means, standard errors of these means, least significant difference, and analysis of variance for intestinal synthesis of digitonin precipitable sterols (DPS)

	Young		Mature	
	Ex	Nex	Ex	Nex
	dpm/g of intest/hr	dpm/g of intest/hr	dpm/g of intest/hr	dpm/g of intest/hr
Control	8242 ±605[a]	8272 ±312	8277 ±279	7386 ±316
Athyroid	6090 ±495	6616 ±486	6102 ±248	6306 ±537
Hyperthyroid	11221 ±599	10964 ±220	11778 ±349	11756 ±214

Least significant difference at $p < .05 = 1162$

Analysis of variance (all animals)

Source	d.f.	F values	Prob > F
Age	1	.60	n.s.[b]
Exercise	1	.32	n.s.
Treatment	2	152.09	.001
Age*Ex	1	1.60	.250
Age*Treat	2	1.54	.250
Residual	138		

Analysis of variance (young animals)

Source	d.f.	F values	Prob > F
Exercise	1	.24	n.s.
Treatment	2	52.44	.001
Residual	65		

Analysis of variance (mature animals)

Source	d.f.	F values	Prob > F
Exercise	1	1.87	.250
Treatment	2	106.90	.001
Residual	73		

[a]Mean ± standard error of the mean.

[b]n.s. = not significant at $p < .05$.

observed in tissue slices are not representative of what may be occurring *in vivo*. With these reservations in mind, data on hepatic and intestinal lipogenesis will be discussed.

Hepatic fatty acid synthesis

Means, standard errors of these means, lsd ($p < .05$) and analysis of variance for fatty acid synthesis are in Table 9. Age and thyroid status significantly ($p < .001$) influenced rates of fatty acid synthesis. The reduced rates of synthesis in the mature animals are in agreement with results of other investigators (Yamamoto and Yamamura, 1971). The effect of thyroid status on lipogenesis is similar to that observed by Gompertz and Greenbaum (1966). These results support the contention of some investigators that the greatly increased basal metabolic rate observed in hyperthyroidism is partially due to a cycling of carbon through fatty acid metabolic pathways (Diamant *et al.*, 1972).

The athyroid condition does not appear to influence fatty acid synthesis to the same degree that it does cholesterolgenesis. In the young animals, fatty acid synthesis was reduced to 90% of control levels in the athyroid animals. A significant age*treatment interaction ($p < .001$) was found to be present. This was due to the decreased response of the mature animals to the changes in thyroid status as compared to the response of the young animals.

No long-term effects of exercise on fatty acid synthesis were observed. It is quite possible that short-term effects do exist. It is well established that fatty acids provide up to 50% of the

Table 9. Means, standard errors of these means, least significant difference, and analysis of variance for hepatic synthesis of fatty acids

	Young		Mature	
	Ex	Nex	Ex	Nex
	dpm/g of liver/hr	dpm/g of liver/hr	dpm/g of liver/hr	dpm/g of liver/hr
Control	112779 ±1941[a]	111932 ±2479	106384 ±1428	104649 ±1606
Athyroid	105297 ±1939	102923 ±1260	95171 ±2228	96448 ±1381
Hyperthyroid	173658 ±2557	173497 ±2762	144782 ±2219	146734 ±1813

Least significant difference at $p < .05 = 5470$

Analysis of variance (all animals)

Source	d.f.	F value	Prob > F
Age	1	106.09	.001
Exercise	1	1.95	.250
Treatment	2	981.09	.001
Age*Treat	2	49.78	.001
Residual	138		

Analysis of variance (young animals)

Source	d.f.	F value	Prob > F
Exercise	1	2.10	.250
Treatment	2	549.56	.001
Residual	65		

Analysis of variance (mature animals)

Source	d.f.	F value	Prob > F
Exercise	1	.14	n.s.[b]
Treatment	2	451.03	.001
Residual	73		

[a]Mean ± standard error of the mean.

[b]n.s. = not significant at $p < .05$.

energy for exercise (Therriault et al., 1973). On the basis of this data, I would expect no increase in fatty acid synthesis immediately after a bout of exercise.

As with cholesterolgenesis, fatty acid synthesis was calculated for the total liver. Means, standard errors of these means, lsd ($p < .05$), and analysis of variance for total hepatic fatty acid synthesis are in Table 10. Again, as in the case of cholesterolgenesis, calculating fatty acid synthesis in terms of total liver capacity altered the results. The mature animals now synthesized fatty acids at a greater rate ($p < .001$). This was due to the much larger livers found in the mature animals. The smaller livers in exercised animals led to a reduced rate of fatty acid synthesis that approached significance ($p < .10$).

Intestinal fatty acid synthesis

Means, standard errors of these means, lsd ($p < .05$) and the analysis of variance for intestinal fatty acid synthesis are in Table 11. Thyroid status was the only parameter studied that had a significant ($p < .001$) effect on intestinal fatty acid synthesis. The rate of intestinal fatty acid synthesis was much less than the synthetic rate in the liver. Possibly, much of the fatty acid that was synthesized in the mucosa was used for structural purposes. This would account for the increased rates of synthesis observed in the hyperthyroid animals as well as the decreased rates observed in the athyroid animals. The significant ($p < .025$) age*treatment interaction is due to a lack of response to thyroxine in the mature animals.

Table 10. Means, standard errors of these means, least significant difference, and analysis of variance for total hepatic synthesis of fatty acids

	Young		Mature	
	Ex	Nex	Ex	Nex
	dpm/liver/hr	dpm/liver/hr	dpm/liver/hr	dpm/liver/hr
Control	$12.70 \times 10^5 \pm 45893$[a]	$14.10 \times 10^5 \pm 59981$	$14.30 \times 10^5 \pm 51381$	$15.80 \times 10^5 \pm 60914$
Athyroid	$7.53 \times 10^5 \pm 63655$	$6.93 \times 10^5 \pm 24564$	$9.38 \times 10^5 \pm 57784$	$10.10 \times 10^5 \pm 45240$
Hyperthyroid	$21.20 \times 10^5 \pm 111709$	$21.20 \times 10^5 \pm 119764$	$19.20 \times 10^5 \pm 84702$	$22.20 \times 10^5 \pm 116566$

Least significant difference at $p < .05 = 2.08 \times 10^5$

Analysis of variance (all animals)

Source	d.f.	F value	Prob > F
Age	1	20.30	.001
Exercise	1	3.57	>.100
Treatment	2	286.57	.001
Ex*Trt	2	1.76	.250
Age*Ex*Trt	2	2.16	.250
Residual	138		

Analysis of variance (young animals)

Source	d.f.	F value	Prob > F
Exercise	1	.37	n.s.[b]
Treatment	2	181.82	.001
Residual	65		

Analysis of variance (mature animals)

Source	d.f.	F value	Prob > F
Exercise	1	3.84	.100
Treatment	2	108.20	.005
Ex*Trt	2	3.01	.100
Residual	73		

[a]Mean ± standard error of the mean.

[b]n.s. = not significant at $p < .05$.

Table 11. Means, standard errors of these means, least significant difference, and analysis of variance for intestinal synthesis of fatty acids

	Young		Mature	
	Ex	Nex	Ex	Nex
	dpm/g of intest/hr	dpm/g of intest/hr	dpm/g of intest/hr	dpm/g of intest/hr
Control	5922 ±169[a]	5755 ±127	5288 ±120	5581 ±315
Athyroid	4335 ±121	4251 ±149	4906 ±368	4508 ±250
Hyperthyroid	6191 ±203	6259 ±118	5593 ±169	5950 ±253

Least significant difference at $p < .05 = 606$

Analysis of variance (all animals)

Source	d.f.	F value	Prob > F
Age	1	.11	n.s.[b]
Exercise	1	.03	n.s.
Treatment	2	54.70	.001
Age*Treat	2	4.63	.025
Residual	138		

Analysis of variance (young animals)

Source	d.f.	F value	Prob > F
Exercise	1	.04	n.s.
Treatment	2	102.70	.001
Residual	65		

Analysis of variance (mature animals)

Source	d.f.	F value	Prob > F
Exercise	1	.01	n.s.
Treatment	2	10.03	.001
Residual	73		

[a]Mean ± standard error of the mean.

[b]n.s. = not significant at $p < .05$.

Interpretation of Data

Results of this study indicate that both hepatic cholesterolgenesis and fatty acid synthesis decline with age if expressed on a per gram basis. This suggests that the synthetic processes at the cellular level are reduced in the mature animal. However, when the data is expressed on a total liver basis, both cholesterolgenesis and fatty synthesis are significantly greater in the mature animal. When the data of Yamamoto and Yamamura (1971) are expressed in a total liver basis, the mature animals in their study also synthesized more cholesterol and fatty acids than did the young animals.

Dupont et al. (1972) have investigated cholesterolgenesis and fatty acid synthesis in vivo. Using rats 3, 6, 12, and 18 months of age, no differences in rates of cholesterolgenesis in rats 3 months and 18 months were found. They did observe some oscillation in rates of cholesterolgenesis over the 18-month experimental period. Fatty acid synthesis was found to increase steadily during the experimental period.

Does lipogenesis decrease with age? Data from this study and results of other investigators indicate that there is not a simple answer to this question. The answer depends on the interpretation of the data. Experimental evidence strongly suggests that hepatic lipogenesis declines with age when the results are expressed on a per gram basis. The increase in liver size associated with aging compensates for the decreased activity on a per gram basis so that total cholesterolgenesis and fatty acid synthesis are increased

with age.

Dupont et al. (1972) did report a decreased rate of cholesterol turnover and higher serum and hepatic cholesterol levels in old rats. It may be that the decreased rate of cholesterolgenesis on a per gram basis is the result of feedback inhibition due to the elevated serum and hepatic levels of cholesterol. A decreased sensitivity to feedback control combined with the decreased rate of cholesterol turnover could account for the higher serum and hepatic cholesterol levels found in old rats.

In many studies, data is expressed as nmoles of C^{14}-acetate incorporated into cholesterol and fatty acids. To make comparisons of results easier, results from this study are expressed in this manner in Table 12.

Table 12. Means, standard errors of these means, and least significant differences for the incorporation of 1-^{14}C-acetate into digitonin precipitable sterols (DPS) and fatty acids by liver slices and intestinal squares expressed as nmoles of 1-^{14}C-acetate incorporated

	Young		Mature	
	Ex	Nex	Ex	Nex
	nmoles/g tissue/hr	nmoles/g tissue/hr	nmoles/g tissue/hr	nmoles/g tissue/hr
Liver DPS				
Control	114.8 ±5.7	120.5 ±6.1	100.6 ±4.8	101.0 ±4.1
Athyroid	80.5 ±5.7	87.8 ±3.6	83.2 ±4.1	72.5 ±1.8
Hyperthyroid	182.2 ±4.5	202.8 ±7.9	163.1 ±3.7	164.5 ±5.7
Least significant difference at $p < .05$ = 14.0				
Intestinal DPS				
Control	74.2 ±5.5	74.4 ±2.8	74.5 ±2.5	66.5 ±2.8
Athyroid	54.8 ±4.5	59.5 ±4.4	54.9 ±2.2	56.8 ±4.8
Hyperthyroid	101.0 ±5.4	98.7 ±2.0	106.0 ±3.1	105.8 ±1.9
Least significant difference at $p < .05$ = 10.5				

Liver fatty acids

Control	1014.9 ±17.5	100.7 ±22.3	957.5 ±12.9	941.8 ±14.5
Atnyroid	947.7 ±17.5	926.3 ±11.3	856.5 ±20.1	868.0 ±12.4
Hyperthyroid	1562.9 ±23.0	1561.1 ±24.9	1303.0 ±20.0	1320.6 ±16.3

Least significant difference at $p < .05 = 49.2$

Intestinal fatty acids

Control	53.3 ±1.5	51.8 ±1.2	47.6 ±1.1	52.3 ±2.8
Athyroid	39.0 ±1.1	38.3 ±1.3	44.2 ±4.1	40.6 ±2.3
Hyperthyroid	55.7 ±1.8	56.3 ±1.1	50.3 ±1.5	53.6 ±2.3

Least significant difference at $p < .05 = 5.5$

SUMMARY

The effects of age, exercise, and thyroid status on cholesterolgenesis and fatty acid synthesis were investigated in male rats. Rats were divided into young (70 days of age) and mature (200 days of age) age groups. Each age group was subdivided into exercise and nonexercise subgroups. Each of these subgroups was divided into three thyroid treatment groups. The three groups were a control group, composed of intact animals receiving no exogenous L-T_4, an athyroid group composed of thyroidectomized rats receiving no exogenous L-T_4, and a hyperthyroid group composed of thyroidectomized rats receiving 7 μg L-T_4/100 g body weight/day. The exercise program consisted of swimming the animals to a state of exhaustion 5 days/week for 10 weeks.

At the end of the 10-week experimental period, liver and intestinal slices were incubated *in vitro* with 1-^{14}C-acetate. The sterols and fatty acids were then separated and the amount of radioactivity in each fraction determined using liquid scintillation techniques.

Results of this study were as follows:

1. Exercise significantly reduced final body weight. Since most of the weight lost due to exercise is adipose tissue, exercise was most effective in reducing final body weight in the mature animals which have larger fat stores.

2. Thyroid status had a significant effect on both final body

weight and liver weight. Young athyroid animals did not grow, while both control and hyperthyroid young animals gained weight during the experimental period. In the mature group, both the hyperthyroid and athyroid animals lost weight. Liver weight was more sensitive to changes in thyroid status than was the total body weight of the animal. Hyperthyroid animals had significantly larger livers on a per 100 g body weight basis, while the athyroid animals had significantly smaller livers than the control animals.

3. Exercise significantly reduced hepatic cholesterolgenesis in young animals, but not mature animals. Exercise did not alter rates of fatty acid synthesis.

4. The effect of age on hepatic cholesterolgenesis and fatty acid synthesis depends on interpretation of the data. Expressed on a per gram basis, synthesis was reduced in mature animals. However, because of larger livers, mature animals had a greater total capacity for lipid synthesis than did the young animals. Age had no significant effect on intestinal lipogenesis.

5. Thyroid status significantly influenced both hepatic cholesterolgenesis and fatty acid synthesis. Hyperthyroidism significantly increased rates of both synthetic processes above those found in control animals. The rates of synthesis in the athyroid animals were significantly reduced below rates found in control animals.

Thyroid status influenced rates of intestinal cholesterolgenesis and fatty acid synthesis in the same manner as it did

hepatic cholesterolgenesis and fatty acid synthesis. Rates were greater than normal in hyperthyroid rats and less than normal in athyroid rats.

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APPENDIX A: THYROIDECTOMY

Thyroidectomies were performed 1 week prior to the beginning of the experimental time period. Animals were anesthetized and maintained with ether. The ventral region of the neck was shaved and a mid-ventral incision was made. Subcutaneous glands, muscles, and connective tissue were separated, exposing the thyroid gland. The thyroid gland was then dissected free of surrounding tissue and removed. When the bleeding had stopped, connective tissue, muscle, and subcutaneous glands were placed back in their original positions. The incision was closed with wound clips and sprayed with a disinfectant.

APPENDIX B: L-THYROXINE

Procedure

1. Weigh 73 mg L-thyroxine[1] (sodium salt) and place in a 1 liter volumetric flask.

2. Add 300 ml .1 N sodium hydroxide (NaOH) and stir until the L-thyroxine is dissolved.

3. Add 600 ml distilled water. Next add 1 N HCl (drop by drop) until a fine precipitate forms. Adjust pH to 5 with 1 N HCl.

4. Bring the final volume to 1 liter with distilled water. The concentration of the stock solution is 70 μg L-thyroxine/ml. The stock solution may be refrigerated and stored for several months.

5. To prepare L-thyroxine for injection, add 1 N NaOH (drop by drop) until the solution becomes clear. Activated L-thyroxine is unstable and should be discarded after 48 hours.

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[1]General Biochemicals, Chagrin Falls, Ohio.

APPENDIX C: INCUBATION

Preparation of Media

Krebs-Ringer bicarbonate solution was prepared as described by Cohen (1949).

Method

1. Prepare .77 M stock solutions of NaCl, KCl, $CaCl_2$, $KHPO_4$, and $MgSO_4 \cdot 7H_2O$. If refrigerated, these stock solutions may be kept for several months.

2. Prepare $NaHCO_3$ (.154 M) fresh daily. Dissolve $NaHCO_3$ in distilled water and place in an ice bath and bubble for 1 hour with 100% CO_2.

3. Combine stock salt solutions (step 1) in the following amounts: 100 ml NaCl, 4 ml KCl, 3 ml $CaCl_2$, 1 ml KH_2PO_4, and 1 ml $MgSO_4 \cdot 7H_2O$. Take 100 ml of this solution and dilute to 500 ml with distilled water to form a dilute "salt" solution.

4. Mix 84 ml dilute "salt" solution with 16 ml $NaHCO_3$ (step 2) to give 100 ml Krebs-Ringer bicarbonate solution. Adjust pH to 7.4 with 1 N NaOH.

5. Place the Krebs-Ringer bicarbonate solution in an ice bath and bubble with 95% O_2-5% CO_2 gas mixture for 15 minutes.

6. Add glucose and sodium acetate to give concentrations of .125 mmole glucose and .5 mmole sodium acetate per 1 ml Krebs-Ringer bicarbonate solution.

7. Prepare a "hot" Krebs-Ringer bicarbonate solution

1-^{14}C-acetate containing 1 μCi 1-^{14}C-acetate[1] per ml following steps 1-5.

8. In this experiment, 1 ml "hot" Krebs-Ringer bicarbonate solution (step 7) was added to 4 ml "cold" Krebs-Ringer bicarbonate solution containing .125 mmole glucose and .5 mmole sodium acetate per ml (steps 1-6) to give 5 ml incubation media containing .5 mmole glucose, 2 mmole sodium acetate, and 1 μCi 1-^{14}C-acetate.

Incubation of Tissues

Tissues were incubated in Warburg flasks.

Method

1. Add .2 ml KOH to center well of Warburg flask to absorb CO_2. To increase absorption of CO_2, place a 2 cm^2 piece of filter paper in the center well after adding the KOH.

2. Add .5 ml 1 N H_2SO_4 to the side arm of the Warburg flask.

3. Place 400 mg liver slices or 400 mg intestinal "squares" in the Warburg flask and add 4 ml "cold" Krebs-Ringer bicarbonate and 1 ml "hot" Krebs-Ringer bicarbonate solutions.

4. Gas Warburg flask and contents for 30 seconds with 95% O_2-5% CO_2 gas mixture.

5. Incubate for 2 hours at 37°C. At the end of the incubation time period, dump H_2SO_4 from side arm into the reaction compartment to stop the reaction.

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[1]Amersham/Searle Co., Arlington Heights, Illinois.

6. For blank incubation, do not add the 1 ml "hot" Krebs-Ringer bicarbonate solution (step 3). Also dump the .5 ml H_2SO_4 into the reaction compartment before beginning the 2-hour incubation time period.

APPENDIX D: ANALYTICAL PROCEDURES

Cholesterol and Fatty Acid Analysis

Separation of the saponifiable and non-saponifiable lipid fractions was done as described by Kyd and Bouchier (1972). The 3-β-hydroxy-sterols were precipitated with digitonin and recovered as described by Siperstein and Guest (1960).

Reagents

1. 40% aqueous KOH
2. Acetone:ethanol (1:1 by volume)
3. Digitonin solution (.5 mg/ml). Prepare digitonin solution by dissolving 500 mg digitonin[1] in 100 ml ethanol:water (1:1 by volume). Stir and heat gently on a thermomixer to dissolve the digitonin (30-45 minutes).
4. Scintillation fluid. Dissolve 4 g 2,5-diphenyloxazole (PPO) and 50 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 1 liter toluene.

Saponification

1. Empty contents of Warburg flasks (Appendix C) into 50 ml test tube containing 5 ml absolute ethanol and 2 ml 40% KOH.
2. Stopper and place test tubes in hot water bath (70°C) for 1 hour. Store at -20°C.

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[1]Cal Bio-Chem, Elk Grove Village, Illinois.

Separation of cholesterol and fatty acids

1. Place saponified samples in 60 ml separatory funnels and extract 3 times with petroleum ether (B.R. 40-60°C).

2. Pool the petroleum ether extracts and wash twice with 10 ml distilled water. Place extracts in 50 ml centrifuge tubes and save.

3. Save the aqueous fraction for further extraction to recover the fatty acids.

Digitonin separation

1. Evaporate washed petroleum ether extracts to dryness under vacuum.

2. Immediately dissolve residue in 5 ml acetone:ethanol. Add 4 ml digitonin solution and refrigerate overnight.

3. Centrifuge sample and aspirate the supernatant, saving the pellet.

4. Resuspend the digitonin pellet in 3 ml acetone. Centrifuge and aspirate the supernatant.

5. This time resuspend the digitonin pellet in 3 ml diethyl ether. Centrifuge and aspirate the supernatant.

6. Dissolve the digitonin pellet in 3 ml methanol. The digitonin precipitate is not readily soluble and should be allowed to stand 15 minutes before stirring vigorously.

7. Take a 2 ml aliquot of the methanol-digitonin solution and place in a scintillation vial. Add 15 ml scintillation fluid.

Recovery of fatty acids

1. Acidify the aqueous extract (from the separation of cholesterol and fatty acids) with 2 ml 12 NH_2SO_4.

2. Extract 3 times with petroleum ether as described in separation of cholesterol and fatty acids.

3. Wash the pooled extracts with distilled water and place in a scintillation vial.

4. Allow the extract to evaporate to dryness (overnight) in hood. Add 15 ml scintillation fluid to the residue.

Liquid Scintillation Procedure

In this experiment, samples were counted on a Beckman LS-250 liquid scintillation counter. The Beckman counter has an internal standard to determine the degree of quenching in each sample. A standard curve was used to determine the counting efficiency for a given amount of quenching. The counting efficiency was then used to convert counts per minute (cpm) to disintegrations per minute (dpm). The blank incubations (without "hot" Krebs-Ringer bicarbonate solution, Appendix C) were used to correct for any activity not due to the ^{14}C-acetate present in the samples.