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ABSTRACT

The purpose of this study was to explore the control mechanisms and consequences of cholesterol induced hypertriglyceridemia, since, although it has been known that hypercholesterolemia of rabbits is accompanied by hypertriglyceridemia, the mechanism of this altered metabolism has not been elucidated.

Hypertriglyceridemia was produced by feeding rabbits a high cholesterol diet; plasma and tissue lipids were analysed, hepatic lipid synthesis assessed and the lipoprotein lipase activities in plasma, heart and adipose tissue were measured. The time course of changes in plasma lipids and the triglyceride turnovers were studied in hypercholesterolemic and control rabbits.

The experimental results showed that the increase in plasma triglyceride in hypercholesterolemic rabbits was not primarily due to an increase in triglyceride synthesis by the liver, nor to a decrease in tissue lipoprotein lipase. Triglyceride synthesis in the livers of hypercholesterolemic rabbits did not significantly differ from that of control rabbits. The cardiac lipoprotein lipase activity of the experimental group was even higher than that of control when Ediol or normal very low density lipoprotein (VLDL) was used as the substrate. The lipoprotein lipase activity of heart from both control and hypercholesterolemic rabbits was significantly decreased when a large amount of free cholesterol was present in the Ediol or VLDL. The intact rabbit,

with a high plasma concentration of cholesterol, showed a decreased hydrolysis of plasma triglyceride by the post-heparin lipoprotein lipase.

Thus, it was proposed that the free cholesterol causes a decrease in hydrolysis and hence uptake of the plasma triglyceride. This is enzyme inhibition of a competitive nature and eventually produces hypertriglyceridemia in these rabbits.

The cause of the increase in heart lipoprotein lipase activity in hypercholesterolemic rabbits is probably due to an adaptive change in response to a decreased triglyceride uptake by the heart. The latter is a result of inhibition of lipoprotein lipase by plasma cholesterol. Since lipoprotein lipase is believed to be indispensable for triglyceride uptake by extrahepatic tissues, a depression in its activity, through unknown mechanisms, induces new synthesis of the enzyme to maintain energy balance.

Both cardiac and hepatic mitochondria accumulated a large quantity of cholesterol and its esters within a few months of giving a high-cholesterol diet, while the phospholipid and triglyceride content of all the subcellular elements of the heart and liver cells were not affected by the high concentration of these lipids in the plasma. The selective uptake and interference of the efflux of cholesterol from the cell to the plasma may be responsible for the observed increase in cholesterol and its esters in mitochondria.

The interrelationship between cholesterol and triglyceride in plasma and tissues may be explained as follows: in the intravascular space and in blood-tissue interspaces, cholesterol inhibits lipoprotein lipase activities and this results in hypertriglyceridemia. In tissues, cholesterol does not induce triglyceride accumulation. Thus, this finding implies that triglyceride accumulation in the plasma of these rabbits does not aggravate the cholesterol-induced atherosclerotic process. Furthermore, an inhibitory effect on lipoprotein lipase may be found in any conditions where the lipoprotein species contain a large proportion of cholesterol.

# CHAPTER I

## INTRODUCTION

Lipids are one of the major constituents of animal cells, accounting for 7 to 32 per cent of the dry weight of the sub-cellular elements. They are important in the formation of cellular membrane structures, energy supply and storage and in the formation of steroid hormones. The different classes of lipids such as glycerides, phospholipids, cholesterol and its esters are complexed together in the performance of these functions, and their metabolism is closely interrelated. Bloom (1950), Chaikoff (1952) and Bragdon (1956) and their associates found that the dietary lipids enter the circulation in combination as a stable particle - the chylomicron. The protein and the phospholipid of the chylomicron play an important part in stabilizing the various lipids in the particle (Wilkens et al., 1963). The transportation of lipids from the liver to other tissues takes place by means of combining all the lipids and small amounts of protein to yield lipid-protein complexes, the lipoproteins. Korn and Quigley (1957) showed that it is the plasma lipoprotein, not individual plasma lipid, that is metabolized by the lipoprotein lipase.

A change in the metabolism of one of the lipids generally accompanies that of other lipids due to the close interrelation

between lipids. Carroll (1953, 1956 and 1960A) found that the feeding of long-chain mono-unsaturated fatty acids to rats will increase the concentration of cholesterol in the adrenals and liver, and will promote the excretion of fecal cholesterol in the absence of dietary sterol. Haines and Mookerjea (1965) found that a defective ability to synthesize phospholipid may result in a reduced ability to form very low-density lipoprotein and hence triglyceride accumulates in the liver.

On the other hand, there are a number of clinical entities which accompany hypercholesterolemia, hypertriglyceridemia and hyperphosphatidemia (Fredrickson et al., 1967; Pomeranze et al., 1954 and Porte et al., 1966). Is the abnormal lipid metabolism found in these diseases interrelated i.e. does an increase in one of these lipids cause an increase in others? If so, by what mechanism? May it be due to the stimulation of intestinal absorption, hepatic synthesis of lipids or due to interference with transportation or utilization of these lipids? Is lipid disposal at fault, or is there a defect in enzymes which catalyse its utilization? Hypercholesterolemia is one of the major factors in the development of atherosclerosis (Keys, 1963; Stamler, 1963). If this is so, then what is the effect of the associated hypertriglyceridemia and hyperphosphatidemia on atherosclerotic processes or lipid deposition in tissues?

It is therefore essential to study the patho-physiology

of bodily lipid metabolism as a mutually interacting group of substances rather than a single entity. Such interrelated metabolic changes have been produced in a variety of animals. For example, when rabbits are fed a diet containing a high cholesterol content, not only does the plasma concentration of cholesterol increase, but an increase in plasma phospholipid and triglyceride levels also occurs (Weinhouse and Hirsch, 1940; Wang et al., 1954; Horlick and Duff, 1954; Kritchevsky et al., 1954 and Silver et al., 1965). Animal experiments are well suited for studying such an interrelationship of lipids. Animals can be fed with one kind of lipid and then the synthesis, transportation and utilization of other lipids can be examined. At the same time the mechanisms and secondary effects of any changes can be effectively studied.

Before describing the experiments, it seems justifiable to review both the background information and the achievements of previous research, particularly those aspects which deal with the interrelationship of lipids, in order to bring this study into proper perspective.

## A. HISTORICAL REVIEW

### 1. EARLY HISTORY

As early as 1908, Ignatowski (1908 and 1909) and Saltykow (1908) found that rabbits develop atherosclerotic lesions when fed on meat, milk and eggs. Hueck (1913), but especially

Anitschow and Chalatow (1913) showed conclusively that the atherosclerosis was due, not to protein, but to the cholesterol which was ingested.

## 2. THE DISCOVERY OF LIPOPROTEIN AND ITS IMPORTANCE

Macheboeuf (1929) first described a lipoprotein of constant composition from horse serum. The importance of the interrelationships between various lipids, such as phospholipids, triglycerides, cholesterol and its esters in plasma, became evident at this time. Amphiphilic phospholipid can be bound to nonpolar lipids such as triglyceride and cholesterol and at the same time the polar region of its molecules can further combine with protein, producing a water-soluble complex. This is an important process for the transportation of lipids in the blood. Recknagel and Ghoshal (1966) showed elegantly this interaction in their experiments; they produced damage in the endoplasmic reticulum of the liver cell by $CCl_4$ which promotes peroxidation of the lipids of this membranous structure. This was followed by a defect in the ability of the endoplasmic reticulum to synthesize the protein moieties of lipoprotein, resulting in a greatly impaired transportation of lipids in the form of lipoprotein.

Green et al. (1966) showed that most biological membranes, which subdivide the mammalian organism into functional compartments and thus regulate the translocation of chemical substances between compartments, are composed of lipoprotein subunits. Kates et al.

(1961) found that a defect of phospholipid metabolism in the red cell membrane may cause spherocytosis. They found a partial block in the enzymic system for conversion of lysophosphatidyl ethanolamine to phosphatidyl ethanolamine in the red cell membrane of the patients with hereditary spherocytosis. Wolken (1966) showed that photoreception is also dependent on the lipoprotein structure of the retinal rod and cone cells.

3. THE RELATIONSHIPS BETWEEN FAT, FATTY ACIDS AND CHOLESTEROL METABOLISM

From controlled dietary experiments on man, Keys (1964) concluded that dietary saturated fatty acids cause a rise in blood cholesterol level and that polyunsaturated fatty acids have a considerably less effect in the opposite direction. Dietary cholesterol has some effect on the blood cholesterol level of man, though this is much less than in some animal species. Epidemiological studies showed that the risk of developing atherosclerosis is directly related to the concentration of cholesterol in the serum (Keys, 1963; Stamler, 1963). These findings agree well with the results of the study of Groen (1968) about the epidemiology of atherosclerosis. He found that the type of nutrition which produces the obesity appears to be of importance. If the obesity is due to overconsumption of saturated fat and sugar, it is generally accompanied by hypercholesterolemia and thereby with an increased risk of atherosclerosis. In contrast, when the obesity

is the result of the over-eating of bread, peas and beans, as in the Middle East, it is accompanied by a low serum cholesterol and it carries no extra risk for the development of coronary heart disease.

The mechanisms of the fat or fatty acid induced metabolic change in cholesterol have been studied in animal experiments. Swell et al. (1955B) found that addition of fat or fatty acids to the diet usually increases cholesterol absorption by stimulating the flow of bile and by providing fatty acid for cholesterol esterification during absorption. Hofmann et al. (1962) showed that fat increases cholesterol absorption by improving micellar solubilization of cholesterol. Carroll studied the effects of dietary fat on both acetate incorporation into rat liver cholesterol and the plasma cholesterol level of rabbits. He demonstrated that feeding fats in a commercial diet rather than semisynthetic diet, or addition of erucic acid or nervonic acid enhanced acetate incorporation into rat-liver cholesterol (Carroll, 1960B; 1962). On the other hand, addition of casein into butter or corn oil-containing diet depressed acetate incorporation into cholesterol (Carroll, 1967). He found further that rabbit plasma cholesterol was more elevated on butter diets than on corn oil diets. Further elevation of plasma cholesterol was observed when casein was added to the butter diet but not when it was added to the corn oil diet (Carroll, 1967). These findings by Carroll strongly suggest that

a diet containing little cholesterol can dramatically influence cholesterol metabolism.

4. THE MECHANISMS OF PHOSPHOLIPID INDUCED HYPERCHOLESTEROLEMIA

Friedman et al. (1965) found that the infusion of phospholipids could induce a hypercholesterolemia. Almost all phospholipids, when infused in animals at a sufficiently rapid rate to induce a sustained high plasma level, led to hypercholesterolemia. This phospholipid induced hypercholesterolemia is not dependent upon a primary change in the rate of cholesterol synthesis, catabolism, intestinal absorption or excretion of cholesterol. Byers and Friedman (1969) recently gave additional evidence by applying continuous infusion of lecithin into the rats which were in the isotopic steady state with respect to $C^{14}$ -cholesterol. They found that plasma accumulation of cholesterol during lecithin infusion derived from both cholesterol pre-existing prior to infusion and from that newly synthesized after the start of infusion, and that about one third of this cholesterol of mixed origins was supplied from the liver. However, there was no increase in the rate of cholesterol synthesis in the lecithin-infused rats. This new study confirms the hypothesis of Friedman et al. (1965) that the increase in plasma cholesterol is caused by the phospholipid which changes the partition of cholesterol between plasma and the tissue. A portion of the cholesterol normally discharged into plasma and then later taken up again by the liver and other

tissues is "sequestrated " in plasma by the physicochemical effect of excess phospholipid. It is noted that total amount of plasma cholesterol of normal rabbits is in order of 50 mg whereas that in the liver 300 mg. A minute off-balance in the latter could produce a great change in the plasma cholesterol level.

A lipid suspension derived from intestinal lymph possesses the ability to extract cholesterol existing in soluble lipoprotein of hypercholesterolemic serum (Minari and Zilversmit, 1963). This fact, too, gives support to the theory proposed by Friedman and his associates.

5. THE MECHANISMS OF CHOLESTEROL INDUCED HYPERPHOSPHATIDEMIA

McCandless and Zilversmit (1956) studied the cholesterol induced hyperphosphatidemia by feeding rabbits with Purina rabbit chow to which 1% (w/w) cholesterol in fat had been added. At the end of 5 months, both control and cholesterol-fed rabbits received an intravenous injection of 1 mc $P^{32}$. Six hours later, blood samples were taken. Plasma concentrations of non-choline-containing phosphatides, lecithin and sphingomyelin in the experimental animals were 5, 9 and 12 times the control values respectively. The net synthesis of each phosphatide per gram of liver was unchanged in the cholesterol fed animals. The liver, however, doubled in weight and to this extent the turnover of each fraction was increased by a factor of two. Occasionally hepatic phospholipid synthesis in the rabbit was observed to be increased on a

per gram basis also. More significant than the changes in hepatic phosphatides were those produced in plasma, in which concentration increments of 5- to 12-fold occurred in the various phosphatides, with only a 2-fold decrease in specific activities. These studies indicate a greatly accelerated rate of supply of newly formed phospholipids by the liver.

6. INTER-ACTION BETWEEN LIPIDS

Boyd (1937) demonstrated that phospholipids played an important part in maintaining the stability of the serum lipid emulsion, a fact subsequently confirmed by Ahrens and Kunkel (1949). Wilkens and Krut (1963) added a very small amount of total serum lipid to a supersaturated cholesterol solution in triglyceride oil, and found that the serum lipid markedly depressed the rate of crystallisation of cholesterol. Burns and Rothblat (1969) found that the excretion of free cholesterol by prelabelled cultured cells was enhanced by the addition of phospholipids to the culture medium.

Ansell et al. (1964) and Masoro (1968) explained the relationship between the phospholipids and other lipids on the basis of the polarity of the lipids. Plasma is an aqueous solution. Water is a polar compound, i.e. water molecules in the electric field tend to orient with the hydrogen atoms toward the negative pole and the oxygen atom toward the positive pole and such

polar molecules tend to attract each other. Most lipids are nonpolar compounds; the regions of marked localization of positive or negative charge are not found in these molecules. Nonpolar compounds are insoluble in water because there is no charge on the molecule, and therefore no strong attraction for the water molecule. Also water molecules have a relatively strong attraction for each other, which prevents simple mixing or intermingling of water with nonpolar molecules. Phospholipids are often called polar lipids. They have a polar region at one end of the molecule and a nonpolar region at the other and thus can work as a bridge to connect the nonpolar lipids such as triglyceride and cholesterol with the polar compounds such as protein and water. This is an important action for the solubilization and stabilization of the plasma lipids.

7. LIPOPROTEIN LIPASE AND FAT TRANSPORT

Hahn (1943), while engaged in an investigation of the mass of circulating red cells, discovered that the infusion of blood, to which heparin had been added as an anticoagulant, into lipomic dogs caused a rapid clearing of the lipemia. Since then the clearing reaction has been investigated extensively. Anderson and Fawcett (1950) reported that the plasma of an animal which received heparin contained a factor able to clear lipemic plasma in vitro. Anfinsen, Boyle and Brown (1952) introduced the term "clearing factor" for this factor. Korn (1955A and 1955B) proposed the term

"lipoprotein lipase" , instead of clearing factor, because he found that the clearing factor lipase attacks only lipoproteins or the triglyceride emulsions which have been activated by a high density lipoprotein from plasma. Jeffries (1954) perfused the isolated hind limbs and liver. He found that this lipase entered the circulation from the hind limb and was destroyed by the liver.

The lipoprotein lipase (LP-lipase) has been demonstrated in extracts of heart (Korn, 1955A and 1955B) and adipose tissue (Korn and Quigley, 1957). The products of the reaction catalyzed by LP-lipase have been studied by Robinson and French (1953) and Korn (1959). A complete hydrolysis of triglyceride and monoglyceride by this enzyme was demonstrated by these investigators. From these findings Robinson and French (1960) concluded that LP-lipase catalyzes the hydrolysis of the triglyceride moiety of chylomicrons and certain other low density lipoproteins. The liberated free fatty acid (FFA) combines with plasma albumin, and since the complex formed is soluble and the chylomicrons disappear, the plasma becomes clear.

The mechanism of exogenous heparin in inducing LP-lipase activity was studied by Robinson et al. (1959B). They suggested that heparin releases the enzyme from its site, and this reaction appears to depend on the negative charge associated with the heparin molecule. Korn (1955A and 1957) showed that heparin may form an

integral part of the lipase, since the enzyme in extracts of adipose tissue is progressively inactivated by a bacterial heparinase preparation. It is also inhibited by pyrophosphate and this inhibition can be reversed by heparin.

The importance of the LP-lipase in fat transport was shown by the following experiments. Bragdon and Havel (1954) demonstrated that protamine sulfate, a known inhibitor of LP-lipase, increased the concentration of triglyceride in the plasma of rats and decreased the rate of removal from the blood of intravenously injected chylomicrons. Havel and Gordon (1960) found that the inheritable deficiency of LP-lipase is the cause of the decrease in hydrolysis of glyceride at sites of removal in Type I hyperlipoproteinemia. These experiments showed that LP-lipase is important for the removal of plasma glycerides.

The mechanism of the incorporation of plasma triglyceride fatty acids (TGFA) into adipose tissue was investigated by Bezman et al. (1962A). Slices of adipose tissue from rabbits in different nutritional states were incubated under various conditions with plasma very low density lipoprotein (VLDL), in which TGFA had been biologically labelled with palmitate-1-$C^{14}$. LP-lipase activity, released into a heparin-containing medium, was assayed in the same tissues. Their results show that the incorporation of TGFA into the slices is dependent on the nutritional state of the animal and is positively correlated with the LP-lipase activity released

from the tissue under the influence of heparin, which in turn correlates with the total LP-lipase activity of the tissue.

Garfinkel et al. (1967) showed that LP-lipase activity in epididymal adipose tissue of a fasted rat can be increased significantly within a relatively short period by administration of glucose or by treatment of the rat with actinomycin D. The antibiotic actinomycin D has been used to explore a mode of regulation of LP-lipase activity that probably differs from that of an enhanced carbohydrate metabolism. The mechanism of actinomycin action involves the inhibition of an unstable, actinomycin-sensitive form of ribonucleic acid (RNA), which destroys the stable RNA template. Thus more of the stable RNA templates are present for the synthesis of LP-lipase (Eagle and Robinson, 1964). The rapid re-esterification of FFA that accompanies glucose refeeding is absent from the tissues of fasted rats injected with actinomycin. These experiments demonstrated that LP-lipase regulates triglyceride uptake by fat tissue. Garfinkel and his associates observed that in almost all actinomycin-treated rats which responded with increased LP-lipase activity, triglyceride uptake was greatly enhanced. This was not due to the enhancement of re-esterification as occurred after glucose refeeding. On the other hand, in up to

50% of the animals, the actinomycin injection failed to increase the LP-lipase activity and, as well, no increase in triglyceride uptake was found in these animals, thus providing evidence that the uptake of lipoprotein triglyceride by epididymal adipose tissue is critically related to the level of the enzyme LP-lipase in the tissue.

8. STUDIES ON TURNOVER OF TRIGLYCERIDE

Havel and Fredrickson (1956) studied the rate of disappearance of intravenously injected chylomicrons in anesthetized dogs. They observed that over 90% of the palmitate-1-$C^{14}$ labelled triglycerides disappeared from the blood at an exponential rate. Furthermore, the greater the load, the slower the fractional rate of disappearance of plasma triglyceride (Fredrickson et al. 1958A). Formation and fate of endogenous triglycerides in plasma of rabbits were studied by Havel et al. (1962). Specific activity-time curves of triglyceride fatty acid in the liver and plasma VLDL was compared. There was a precursor-product relationship between the triglyceride fatty acid of the liver and plasma VLDL. In addition, they observed two components in the specific activity-time curves after intravenous injection of labelled VLDL, indicating the existence of a component representing the transfer of triglyceride fatty acid from plasma to liver and that for

extrahepatic removal.

Farquhar et al. (1965) found that the kinetic behaviour of radioactive plasma triglyceride was more complex when palmitate was used than when glycerol served as a labelled precursor, probably because glycerol recycled less than did palmitate. For both glycerol-2-$H^3$ and palmitate-1-$C^{14}$, most of the labels incorporated into triglyceride were in the $S_f > 20$ fraction. Analysis of the hydrolysate of $S_f > 20$ triglyceride demonstrated that essentially all the $H^3$ was in the glycerol moiety, and all the $C^{14}$ in the fatty acid portion. The specific activity - time curve of palmitate-labelled $S_f > 20$ triglyceride was clearly no longer linear by 24 hours, whereas the disappearance of glycerol-labelled molecules remained first-order up to 48 hours. Thus, the use of glycerol provides a more valid estimate of plasma triglyceride turnover than does fatty acid.

Reaven et al. (1965) found that higher triglyceride concentrations were associated with higher turnover rate of plasma triglyceride in the human being.

## B. CHOICE OF EXPERIMENTAL ANIMAL AND DIET

Experiments on animals have been very important in advancing the knowledge of lipid metabolism, as related to human disease, but the results should be interpreted with caution. There are a number of advantages in using the animal as an experimental model. For instance, when studying the metabolic interrelationship of lipids, we can simplify the results by feeding a simple, controlled diet, such as a cholesterol enriched diet to the animals. Then the primary factor is this controlled diet, all the resulting metabolic changes are secondary to it. An additional advantage is that tissue samples can easily be taken from the animal but not from the human being. Consequently, many laboratory animals, such as the chicken, pigeon, monkey and pig, have been used for different aims in lipid research (Hartroft et al., 1963). However, there are certain limitations using the animal model in answering the problems of human disease; the species difference in metabolism, the difference in dietary habit and the life span all have to be considered. The effect of diet on blood lipids is widely different among different species. For example, oleic acid in the diet has little or no effect on the plasma cholesterol in man (Keys et al., 1958), while in the rat it appears to be as productive of hypercholesterolemia as saturated fatty acid (Hegsted et al. 1959).

When large amounts of cholesterol are fed to the experimental animals, the homeostatic mechanisms of the body react to maintain a constant concentration of plasma cholesterol. The body has three options; absorption may be decreased, endogenous synthesis may be inhibited or the disposal of cholesterol may be accelerated. Friedman and Byers (1954) found that the homeostatic mechanisms in rabbits were different from those in rats and dogs in that the rabbit usually absorbed more cholesterol by the intestine than the rat when an equivalent amount of cholesterol per body weight was given. The liver of the rabbit is unable to remove the dietary excess of cholesterol from the plasma as rapidly as the rat (Hartroft er al., 1963). Gould (1951) found that the liver of the rabbit was apparently less able to compensate by means of a decrease in synthesis of cholesterol than the livers of the dog and the rat. As a result, the rabbit quickly develops hypercholesterolemia when fed with cholesterol. Therefore the rabbit is an ideal animal in which to induce hypercholesterolemia and thus to study the change in triglyceride metabolism related to hypercholesterolemia.

In order to induce hypercholesterolemia in rabbits, many varieties of diet have been fed; some examples are given below:

| First author of the reference | | Amount of cholesterol in diet | | Other addition |
|---|---|---|---|---|
| Page | (1935) | 0.2 | g/day | olive oil |
| Weinhouse | (1940) | 1 | g/day | |
| Kritchevsky | (1954) | 3 | % (w/w) | |
| Horlick | (1954) | 0.75 | % (w/w) | |
| Hartroft | (1963) | 1 | g/day | margarine |
| Kwaan | (1964) | 1 | g/day | peanut inhibitor |
| Maier | (1965) | 0.6 | % (w/w) | |
| Silver | (1965) | 1 | % (w/w) | |
| Parker | (1966) | 4 | % (w/w) | |
| Carroll | (1967) | | | 15% butter or corn oil, casein or dextrose |

## C. STATEMENT OF THE PROBLEMS

The preceeding review of previous work in the field of lipid research indicates the importance of studies dealing with the inter-relationships between the lipids of plasma and tissues. All lipids are complexed with protein to form chylomicrons or lipoproteins during the processes of absorption, transportation and utilization. The change in quantity or quality of one kind of lipid in the diet or in plasma is followed by the change in metabolism of other lipids. Fat and fatty acids affect the cholesterol metabolism by stimulating its absorption by the intestine as well as its synthesis by the liver. Physicochemical sequestration may be one of the mechanisms by which the intravenously injected phospholipid could induce hypercholesterolemia. A high cholesterol diet can in turn induce the elevation of plasma phospholipid by accelerating the hepatic output of phospholipid. The elevation of cholesterol ester in the plasma of the animal fed free cholesterol is due to an increase in the esterifying activity of the enzymes existing in the intestinal mucosa.

However, the mechanism of cholesterol induced hypertriglyceridemia has not been elucidated. What are the control factors regulating a plasma triglyceride level? What are metabolic patterns in rabbits fed a high cholesterol diet in terms of intestinal absorption, hepatic synthesis, transportation and utilization of triglyceride? What consequences do combined

hypercholesterolemia, hypertriglyceridemia and hyperphosphatidemia produce ? An attempt was made to answer some of these questions with respect to normal and abnormal lipid metabolism.

## D. THE AIM AND THE DIRECTIONS OF THE PROJECT

The aim of this study was to find possible mechanisms for and secondary effects of cholesterol induced hypertriglyceridemia.

### 1. THE POSSIBLE MECHANISMS

a. Physicochemical sequestration-- Friedman et al. (1965) postulated a physicochemical relation between the triglyceride and cholesterol. According to the hypothesis, the injected triglyceride traps a large amount of cholesterol in the plasma by a mutual attraction. The increase in plasma triglyceride in the hypercholesterolemic condition may also be due to a mechanism similar to that proposed by Friedman et al. (1965). Conversely, the increased plasma cholesterol itself could trap a large amount of triglyceride in the plasma. Unfortunately, this physicochemical mechanism was not proved by direct evidence in Friedman's work, but was postulated indirectly by the exclusion of other possibilities. Thus, this mechanism cannot be proposed as an answer to my question and therefore further studies were necessary.

b. Increased entry of triglyceride into the plasma - There are two sources of plasma triglyceride; intestinal absorption and hepatic secretion; the latter including an increase in hepatic fatty acid synthesis and/or a secondary increase due to an increased sypply of FFA from

the adipose tissue. The increase in plasma triglyceride in rabbits on a high-cholesterol diet could therefore originate from any one of these three sources. In order to find the mechanisms underlying the increase in plasma triglyceride, the participation of these organs in the plasma triglyceride turnover has to be evaluated in the hypercholesterolemic state.

c. Decreased disposal of triglyceride from the plasma - The transport of triglycerides from the plasma into extrahepatic tissues is mostly accomplished by their hydrolysis to fatty acid and glycerol. This hydrolysis is catalyzed by lipoprotein lipase (LP-lipase). In fact, Bezman (1962A) and Garfinkel (1967) and their associates concluded from their experimental results that LP-lipase is necessary for triglyceride uptake by the extrahepatic tissues. Therefore, the determination of LP-lipase activity should prove to be important if the increased plasma triglyceride found in rabbits on a high-cholesterol diet is secondary to the decreased disposal of triglyceride from the plasma.

2. THE POSSIBLE EFFECTS OF CHOLESTEROL INDUCED HYPERTRIGLYCERIDEMIA

To find the controlling factors of pathogenesis of atherosclerosis is one of the final goals in studying metabolic changes of lipid in the rabbits. It is well known that a high plasma cholesterol level, if sustained long enough, damages arteries in all species and leads to atheromatosis (Gould et al., 1963). The question then is what kind of role will increased triglyceride

have with regard to hypercholesterolemia? Can the cholesterol esters, phospholipid and triglyceride in tissues also be influenced by the elevation of cholesterol and other lipids in plasma? Do these biochemical changes in tissues influence the function of the subcellular elements?

It is the purpose of this thesis to examine these questions by a subcellular fractionation study of two target organs, the heart and liver; lipid composition and functional changes of these elements will be analyzed.

## E. THE RELEVANCE OF THIS STUDY TO MEDICINE

As described above, by using the animal as an experimental model, the synthesis, transportation and utilization of triglyceride under the influence of cholesterol will be examined. An hypothesis will be proposed to explain the mechanisms and possible effects of cholesterol induced hypertriglyceridemia. The clinical implications of such information follows:

### 1. THE PATHOGENESIS OF VARIOUS DISEASES

There are many diseases, the manifestation of which includes hypercholesterolemia and hypertriglyceridemia. In patients with ischemic heart disease, hypercholesterolemia (Doyle et al., 1959) and sustained hypertriglyceridemia are common (Pomeranze et al., 1954; Woldow et al., 1954; Albrink et al., 1961). Hypertriglyceridemia may be found in the patients with myxedema with hypercholesterolemia (Porte et al., 1966). Finally, some primary

hyperlipemic patients may also have hypercholesterolemia (Klein, Lever and Fekete, 1959; Fredrickson et al., 1967). The relationship between hypercholesterolemia and hypertriglyceridemia may be similar to that which will be proposed here for rabbits. There are limitations in human experimentation i.e. we cannot give certain diets or chemicals to human beings to induce a metabolic change which, as the primary change, may induce secondary effects. Tissue specimens from the human being are hard to obtain. Therefore, animal experiments must be used to supplement investigations on humans.

As will be described here, a simple cholesterol enriched diet, as the primary inducer, may be administered to an animal to bring about hypercholesterolemia, and secondary changes in lipid synthesis, turnover and LP-lipase activities could be investigated in detail. It is my hope that the information obtained will be useful in understanding the pathogenesis of certain metabolic changes in human disease.

## 2. POSSIBLE EFFECTS OF CHANGES IN TRIGLYCERIDE METABOLISM IN THE ABOVE DISEASE

The cause and effects of hypercholesterolemia in myxedema, ischemic heart disease and other atherosclerotic diseases have been extensively studied, while studies of the associated hypertriglyceridemia in these diseases were scarce. It is as yet unanswered whether the elevated plasma triglyceride exaggerates the pathological processes caused by the cholesterol. A further

question is whether plasma cholesterol in some idiopathic hyperlipemic patients aggravate the triglyceridemia. The analysis of the lipid compositions of tissues and of the function of subcellular elements, as will be described here, implies that secondary metabolic alterations in tissues are minute.

# CHAPTER II

## MATERIALS AND METHODS

### A. DIET

New Zealand albino male rabbits, (from the Canadian Breeding Farm, Montreal, Canada) with body weights ranging from 1.9 to 2.2 kg were divided into two groups. One group was fed with ordinary rabbit pellets (from the Maple Leaf Mills Co. Montreal, Canada) *, while the other group was fed with the same pellets with 3% cholesterol added. The cholesterol (U.S.P. powder) was dusted onto the pellets and mixed as thoroughly as possible. The residual pellets and cholesterol powder in the food holder were not discarded so that all the cholesterol was finally ingested by the rabbits. Although the residual food may have contained more cholesterol powder than the top pellets, sooner or later the rabbits consumed all the given food and thus all the cholesterol.

Judging from the plasma cholesterol changes, this method of feeding was found to be a simple, effective method for rabbits fed on this diet for more than one week. For those rabbits fed on this diet for less than one week, the cholesterol was dissolved in ethyl ether, then mixed with the pellets and the ether was allowed to evaporate.

The daily food intake was 104 ± 52 g (mean ± S.E.M.) in the control rabbits and 137 ± 106 g in the experimental rabbits and the body

* Triglyceride content was found to be 1% (w/w).

weight at the time of sacrifice (ranging 2-6 months) was from 2.7 to 3.2 kg in the control and 2.3 to 3.2 kg in the experimental group.

All the rabbits were fasted over-night before the experiment.

B. ANESTHESIA

For the anesthesia, 20% urethan (ethyl carbamate, w/v) and 2% chloralose (w/v) in water was injected intraperitoneally in the dose of 1.2 ml per pound of body weight. Chloralose was dissolved in the warm urethan solution (55-65°C) and was kept in an incubator to prevent re-crystallization. Animals anesthetized with chloralose do not react to painful procedures but exhibit exaggerated responses to auditory and tactile stimuli. Urethan has a rather weak hypnotic action in man but produces fairly rapid and profound narcosis in animals without significantly affecting respiration, circulation or the spinal reflexes (Shideman, 1958).

In the experiments in which the heart, liver and arteries were needed only for the subfractionation, or for examination of atherosclerosic lesions, rabbits were killed by a blow on the head.

C. SAMPLING

In long term experiments (more than one month), rabbits were examined at monthly intervals for cholesterol depository lesions on iris, ears and skin, and the plasma protein, cholesterol, cholesterol ester, triglyceride and phospholipid levels were

measured. The blood was taken from the ear vein or cental ear artery for these experiments. The syringe was first wet with heparin solution (1000 u/ml water).

For the experiment in which the blood had to be taken several times at short intervals a polyethylene cannula was inserted into the femoral artery.

## D. PROTEIN DETERMINATION

The plasma or tissue proteins were measured by the method described by Lowry et al. (1951) because of its high sensitivity and simplicity. There are two distinct steps which lead to the final color with protein: 1. reaction with copper in alkali, and 2. reduction of the phosphomolybdic-phosphotungstic reagent by the copper-treated protein. Since the relation of color to protein concentration is not quite linear, care was taken to dilute the protein sample to an appropriate concentration range.

In order to avoid the deterioration of the reagents, $CuSO_4 .5H_2O$ and potassium tartrate solution was prepared as a separate, doubly-concentrated stock solution.

## E. LIPID EXTRACTION AND DETERMINATION

### 1. LIPID EXTRACTION

The plasma or tissue lipids were extracted according to the method of Folch et al. (1957). This is a simple method for the preparation of extracts of total lipids from plasma or tissue. The plasma or tissue homogenate was mixed with chloroform-methanol (2:1, v/v) mixture. The clear lipid extract obtained by filtration

was then washed free of non-lipid contaminants by shaking it with 0.2 its volume of 0.58% (w/v) NaCl in water. The washed clear lipid extract was quantitatively transfered to the test tubes and evaporated to dryness under nitrogen gas at 55°C for the determination of various lipids.

2. CHOLESTEROL DETERMINATION

Cholesterol and its esters were determined according to the method of Searcy and Bergquist (1960). Free cholesterol was separated from the ester by digitonin precipitation and washed once with acetone-ether. An orange color was formed when the total or free cholesterol and a solution of $FeSO_4$ in glacial acetic acid were treated with $H_2SO_4$. The color was quantitated at 490 mμ, a wave-length less susceptible to interfering substances. The amount of esterified cholesterol was calculated by subtraction. A rapid protein precipitation was obtained when serum was treated with acetone-ethanol and this extract could be treated directly with the $FeSO_4$ reagent to produce new color reaction. Maximum color intensity was reached in 8 to 15 min and was relatively constant during the period. It was possible to measure accurately cholesterol concentrations below 100 μg.

Liebermann-Burchard method was widely used for serum cholesterol estimation. Many modifications of this method have been necessary since the color reaction is dependent on temperature, is sensitive to light and lacks stability. Zlatkis et al. (1953)

have produced a stable chromogen by treating cholesterol with a $FeCl_3$ reagent. However, this reaction lacks specificity. Protein, tryptophan, bilirubin, bromine, vitamin A, unsaturated fatty acids, hemoglobin and steroids formed interfering color complexes, many of which absorb significantly in the spectral region suggested by Zlatkis et al. for cholesterol quantitation. In order to remove interfering proteins Henly (1957) or Chiamori and Henry (1959) modified the Zlatkis technique by allowing serum to stand for at least 30 min with the $FeCl_3$-acetic acid or by heating the samples.

As compared with these methods of cholesterol determination the method of Searcy and Bergquist has the definite advantages of stability, specificity, simplicity and lack of temperature and light sensitivity. Digitonin developed color when mixed with $FeCl_3$, acetic acid and $H_2SO_4$; but this color contributed minimal interference in the wave-lengths near 490 mμ which were used in this experiment.

3. PHOSPHOLIPID-PHOSPHORUS DETERMINATION

Phospholipid-phosphorus was determined by the method of Hurst (1964). The determination of orthophosphate by the reduction of the yellow phosphomolybdate complex to molybdenum blue is a standard analytical procedure. Hydrazine sulphate is added to the stannous chloride solution to stabilize its reducing ability. After the phospholipid (PL) was digested with 2 ml of 15 N sulfuric

acid, 3 drops of concentrated perchloric acid, instead of hydrogen peroxide and urea were added to the digested phospholipid solution. The amount of phospholipid was calculated from the value of phosphorus assuming that the molecular weight of phosphorus is 1/25 of the average molecular weight of phospholipids.

4. TRIGLYCERIDE (TG) DETERMINATION

The method of determination of triglyceride was based on the principles proposed by Van Handel and Zilversmit (1957), and also by Carlson and Wadstrom (1959) and modified by Jagannathan (1964). The principle of the methods is adsorption of phospholipids on Zeolite or silicic acid, saponification of the phospholipid-free components to yield glycerol, oxidation of glycerol to formaldehyde with periodate, reduction of the excess periodate with sodium bisulfite and colorimetric determination of the formaldehyde by its reaction with chromotropic acid. After saponification of the triglyceride, one drop of 40% acetic acid was added. No loss of glycerol occurred when acetic acid instead of sulfuric acid was added (Jagannathan, 1964). Thiourea solution was added prior to photometric measurements to minimize the development of a red color contributed by the reagents.

In this experiment, Florisil (magnesium silicate) instead of Zeolite was used to adsorb phospholipids. This modification has an advantage of quantitatively removing phospholipids and allowing good recoveries of the glycerides (Carroll, 1961).

5. FREE FATTY ACID (FFA) DETERMINATION

For the determination of triglyceride synthesis by the liver and the assay of lipoprotein lipase (LP-lipase) activity, the FFA were measured by method of Goss and Lein (1967). The FFA were extracted once with the mixture containing the same amount of isopropyl alcohol and heptane but twice the amount of 1 N sulfuric acid as in Dole's extraction mixture. The two-phase titration of Dole's method (Dole and Meinertz, 1960) was unsatisfactory in that reliable end points using the suggested indicators (thymol blue and Nile blue) were difficult to detect. In this modification the standard base, 0.1 N sodium ethoxide, is miscible with heptane and consequently obviates the need for a two-phase system. A solution of phenolphthalein in absolute ethanol serves as the indicator and provides an easily observable end point. The recovery data for palmitic acid dissolved in heptane over a concentration range of 29-591 µEq/1. using a visual end point were 98.6 ± 1.6% (S.E.M.).

F. TRIGLYCERIDE SYNTHESIS BY THE LIVER

1. ESTERIFICATION OF TRIGLYCERIDE IN THE LIVER OF CONTROL AND HYPERCHOLESTEROLEMIC RABBITS.

a. Incubation - Liver homogenates and radioactive fatty acid were incubated according to the method of Tietz and Shapiro (1956). Palmitic-1-$C^{14}$ acid in benzene was diluted with KCl-Tris buffer and neutralized with KOH (pH 7.5). One tenth microcuries (0.04 µM) of potassium-1-$C^{14}$ palmitate and 10 µM of ATP (adenosine

triphosphate) were put into each sample for incubation, and the protein concentration of this sample was determined before incubation. Six samples of this same liver homogenate were incubated simultaneously. After 5, 10, 20, 40, 60 and 90 min of incubation, one of these samples was taken out for lipid extraction as described above (Folch et al., 1957). The amount of lower chloroform phase of the lipid extract was recorded. One half ml of this extract was pipetted into 10 ml 0.4% (w/v) Omnifluor* in toluene solution and counted in liquid scintillation counter for the measurement of the total activity of potassium-1-$C^{14}$ palmitate presented in the incubation mixture. Another 10 ml of this extract was evaporated to dryness under nitrogen gas at 55°C, then dissolved in 0.5 ml of chloroform for thin layer chromatography.

The preparation of thin layer chromatography was carried out according to the methods of Christophe and Natthijs (1967). A slurry of 30 g silica gel G (with $CaSO_4$) in 60 ml distilled water was spread out on four of 20 X 20 cm glass plates to a thickness of 0.25 mm. The plates were activated for 30 min at 160°C. The developer was a mixture of ether-petroleum ether-glacial acetic acid (20:80:2, v/v/v). The proportion of acetic acid was increased from 1 part (Hofmann, 1963) to 2 parts in order to improve the

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*Omnifluor - New England Nuclear Corp.
Contents: A blend of 98% PPO and 2% Bis - MSB

separation of FFA. The plates were air-dried. The spots were detected with iodine vapor (Skipski et al., 1964). A greater amount of starting material can be applied without sacrificing resolution with thin-layer chromatography compared to paper chromatography, and hence sufficient material may be recovered to perform various chemical tests, e.g. radio-activity can be measured and gas-liquid-chromatographic analysis of fatty acids can be performed. Quantitative thin-layer chromatography possesses an advantage over single column chromatography in that a more complete separation of the majority of individual components can be attained at ease with greater efficiency.

The separated triglyceride with silica gel G was suspended in 4% (w/v) Cab-O-Sil* and 0.4% (w/v) Omnifluor in toluence solution and counted by two different channels simultaneously in liquid scintillation counter.

b. Statistical considerations for counting - Radioactive decay is a random phenomenon, i.e. the distribution of a sufficiently large number of cpm (counts per minute) follows the Poisson distribution curve. Because of this situation, one obviously cannot know the "true" value of the result of a given counting operation. The accuracy of any individual measurement (n) is

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*Cab-O-Sil: thixotropic gel powder.

gauged by the magnitude of its deviation from the mean value.

In radiotracer assay, however, it is common to make only one or two activity determinations per sample. In this case there is no mean value from which the standard deviation may be calculated. Because of the nature of the Poisson distribution, however, the standard deviation of a single determination of magnitude (n) may be taken as $\sqrt{n}$ , that is, the square root of the total number of counts collected. A related unit, the relative standard deviation is defined as $\frac{\text{standard deviation}}{\text{total number of counts}} = \frac{\sqrt{n}}{n}$ thus the larger the value of n, the smaller the relative standard deviation, the higher the reliability of the counts. Radiotracer measurements are usually made so that the relative standard deviation is equivalent to 1% of the total counts (Wang et al., 1965). In order to achieve this, samples were counted for 10-40 minutes to collect at least 10,000 counts, since when

$$\frac{\sqrt{n}}{n} = 1\% = \frac{1}{100}, \quad \frac{n}{n^2} = \frac{1}{(100)^2}, \quad n = 10{,}000$$

There is a 68.3% probability that the true total counts are within the range of 10,000 $\pm$ 100 counts.

c. Determination of the counting efficiency - There are two purely instrumental methods for determining counting efficiency which do not require preparation of multiple samples or permanent alteration of the samples by addition of internal standard: the "external standard" method and the "channels ratio" method. Two

sample countings are required for external standardization with and without exposure to the external gamma source, while the "channels ratio" method only requires a single count in two different channels (Bush, 1963). The latter method was chosen in these experiments. The average efficiency was 66% for $C^{14}$ and 30% for $H^3$.

In order to compare samples of various efficiencies with one another, the counts per minute (cpm) of the samples were converted to disintegrations per minute (dpm) according to the efficiency.

$$dpm_{sample} = \frac{\text{(count rate of sample) - (count rate of background)}}{\text{efficiency}}$$

The percentage of FFA in liver which is esterified to new triglyceride is equal to the percentage of total radioactivity of potassium-1-$C^{14}$ palmitate in the sample which is incorporated into triglyceride. Liver was weighed and calculated as g per kg of body weight. The FFA content of the liver homogenate was determined and then the amount of FFA which was esterified to new triglyceride in the liver per kg of body weight was calculated. Although the FFA determination of the liver may be influenced by the presence of acidic phospholipids, the original method by Goss and Lein (1967) was used without modification to extract FFA, since the change in phospholipid content in these experiments was negligible.

2. FATTY ACID SYNTHESIS, ESTERIFICATION AND CHOLESTEROL SYNTHESIS

The method described by Longmore et al. (1967) was used for the determination of acetate-1-$C^{14}$ incorporation into triglyceride,

fatty acid and cholesterol in rabbit liver slices. 5 μc sodium acetate-1,2-$C^{14}$ and 10 μM sodium acetate in 0.5 ml water were added to 5 ml of medium containing intracellular cation. The incubation medium was gassed with 95% oxygen and 5% carbon dioxide for a minimum of 20 minutes before tissues were added. The 25 ml flasks were covered with Parafilm and placed in Dubnoff shaker for 2 hours at 37°C, then the total lipids were extracted and separated. 0.5 ml of upper aqueous phase was dissolved in 10 ml of Bray's solution (Bray, 1960) and counted. 0.5 ml of lower chloroform phase of the lipid extract was dissolved in 10 ml of Omnifluor toluene solution and counted. The amount of acetate being used for the synthesis of different lipids by the liver was calculated from the percentage of total added radioactivity incorporated into individual lipids.

Most metabolic studies on liver in vitro have been carried in an extracellular type of medium. It has long been known that, on incubation, liver cells rapidly exchange their intracellular potassium for extracellular sodium, thereby providing a highly abnormal intracellular environment. In this experiment, the effect of cationic exchange was obviated by providing only potassium ions in the extracellular environment. It was demonstrated that the decrease in fatty acid synthesis in the extracellular type of medium was attributable primarily to its low magnesium concentration and secondarily to its high sodium concentration.

An increase in $HCO_3$-concentration from 10 to 40 mM at constant pH produced a two- to fivefold increase in acetate incorporated into fatty acids (Longmore et al., 1967).

## G. LIPOPROTEIN LIPASE (LP-LIPASE) ACTIVITIES

### 1. GENERAL CONSIDERATIONS

There are numerous methods for the determination of LP-lipase activity. The most simple one is to follow the decrease in turbidity of lipemic plasma, a suspension of chylomicrons or a triglyceride emulsion in vivo or in vitro. A somewhat more complicated one is to determine the difference in the ultra-centrifugal pattern or electrophoretic distribution of the serum lipoprotein after the injection of heparin in vivo or incubation with LP-lipase in vitro. LP-lipase catalyzes the hydrolysis of the triglyceride moiety of lipoprotein, therefore the LP-lipase activity can also be assayed by incubating the enzyme with a suitable substrate and titrating the FFA release or determining glycerol. Chylomicrons (Robinson et al., 1953), soluble lipoproteins of $S_f$ greater than 10 (Korn, 1955B), and egg yolk lipoproteins (Nichols et al., 1954) are all the suitable substrates. No detectable hydrolysis of the triglyceride moiety of plasma lipoproteins with $S_f$ smaller than 10 occurs (Shore, 1955). Simple triglyceride emulsions make good substrates in the presence of plasma lipoproteins, especially $\alpha$- lipoproteins (Korn, 1955B). Commercial emulsions of coconut oil, olive oil, sesame oil and

soybean oil are all suitable.

Under carefully controlled conditions the turbidity method may also be used as a quantitative enzyme assay, but special care must be taken. Since only the largest particles scatter light, the decrease in optical density indicates only the hydrolysis of chylomicrons or triglyceride emulsions. If soluble lipoproteins are also present in the incubation mixture, their hydrolysis is not detected and, as they are competitive substrates, the enzyme activity, when measured by a decrease in turbidity, will be underestimated. Therefore, the turbidity method is of no use if soluble lipoproteins are to be used as substrate. Further, the turbidity of a given suspension of chylomicrons is profoundly affected by temperature and addition of other components such as enzyme inhibitors (Korn, 1959). The turbidity of the suspending medium may change as a result of protein or fatty acid precipitation (Robinson and French, 1960).

For the assay of the action of heparin in vivo, particular precautions are necessary, especially in acute experiements. As heparin is rapidly destroyed, its level in the circulating plasma, and hence the degree of alteration of the lipoproteins, at any instant, is a function of both the amount of heparin injected and the time of withdrawal of the sample. Determination of the concentration of the FFA and glycerol, although they may be elevated, is difficult to relate to enzymatic activity in vivo,

as these compounds are rapidly metabolized.

2. IN VITRO EXPERIMENTS

a. Ediol* as the substrate - The plasma LP-lipase, heart and adipose LP-lipase were determined by the methods of Muir (1967), Alousi and Mallov (1964) and Hollenberg (1959) respectively. To unify the procedures the following modifications were adopted. In all these experiments, 5% Ediol in Krebs-Ringer phosphate (KRP) buffer (Umbreit et al., 1964) was used. The pH of Ediol was adjusted to the optimal condition (8.5) for this enzyme with $NH_4OH$ (Korn et al., 1957). Bovine serum albumin (fraction V) was added as the FFA acceptor. A molecule of plasma albumin can bind as many as 25-30 molecules of FFA (Masoro, 1968). Approximately 3 micromoles of albumin were added to the incubation medium. Therefore, this amount of albumin can accept at least 21 micromoles of FFA. In most of our experiments about 15 micromoles of FFA were released by the LP-lipase i.e. FFA acceptor was sufficiently available. Heparin was added in the experiments for tissue LP-lipase at the level of 30 μg per ml of incubation medium, which gives an optimal concentration. At a high level, heparin itself inhibits the action of LP-lipase (Korn, 1955A, Gartner et al., 1966). For the plasma post-heparin LP-lipase assay, 1 mg

*Ediol: 50% emulsion of coconut oil containing 12.5% sucrose, 1.5% glyceryl monostearate, and 2.0% polyoxyethylene sorbitan monostearate. Calbiochem.

of heparin per kg of body weight was injected intravenously and blood was taken before and 10 min after injection for LP-lipase determination.

Another modification was the method of homogenization of the tissues. The heart was blotted on the Whatman's filter paper immediately after being taken from the animal. The left ventricle without visible fat tissue was cut off , weighed and washed with ice-cold KRP buffer (pH 8.5). Then it was cut into fine pieces and homogenized using a Teflon pestle in 5 volumes of KRP buffer (w/v). The final volume of the heart homogenate was recorded, and 0.5 ml of this homogenate was added to the incubation medium. The LP-lipase of the epididymal fat tissue was measured in a similar manner. In this instance, in order to avoid difficulties in obtaining an even homogenate in ice cold medium, the weighed epididymal fat tissue (100 mg) was thrown directly into the medium and cut into fine pieces and incubated.

One ml of the mixture of tissue and medium was taken before and after one hour of incubation at 37°C for the determination of FFA by the method described above (Goss and Lein, 1967). The activities were expressed as microequivalents (μEq) of FFA formed during 1 hr of incubation per 1 g of tissue or 1 ml of plasma corrected for μEq FA present before incubation. The FFA release was linear after an initial lag period of about 15 min, during the period 20 min to 60 min (Fig. 1). It was assumed that the lag period was due to the fact that the homogenate was added ice-cold, and that the lag period was constant for all samples.

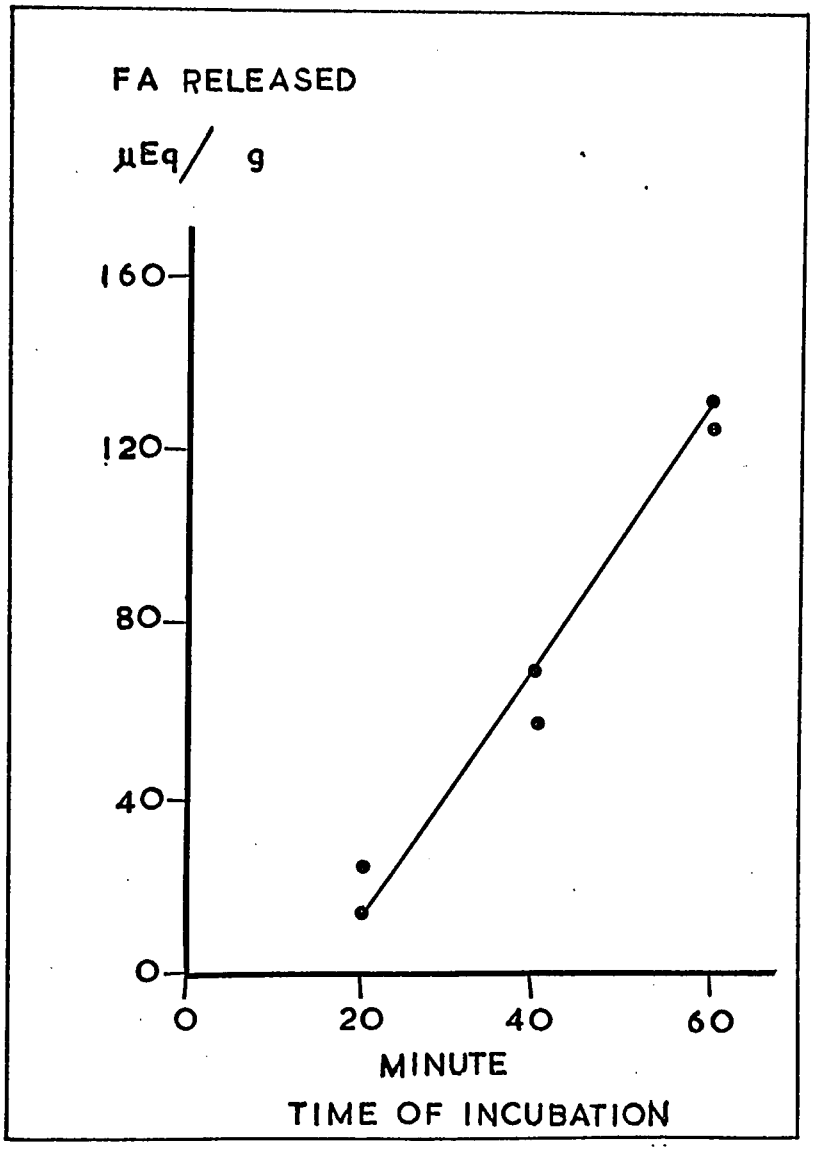


Fig. 1. The Rate of FA Release by Heart LP-lipase during Incubation.

Normal heart homogenate was used as a source of the enzyme and incubated at 37°C in the presence of substrate, Ediol. The number of experiments are two and the results are expressed as µEq FA per g of heart (ordinate). Abscissa indicates time in minutes.

b. Very low density lipoprotein (VLDL. $d < 1.006$) as the substrate - This lipoprotein was isolated from the control and hypercholesterolemic rabbits according to the method of Havel et al. (1962). The serum was centrifuged for 15 hr at 115,000 x g at 10°C. The $d < 1.006$ lipoprotein (LP) contained in the top 1 cm of the tube, was aspirated out. Six-tenth ml of the mixture of different proportions of these LP was used as the substrate for heart LP-lipase of the control or hypercholesterolemic rabbit.

The serum infranate was brought to $d = 1.019$ by mixing with $d = 1.085$ saline according to the following equation:

$$A \times Y + B \times Z = (A + B)W$$

where A is the volume of the serum infranate, B is the volume of salt solution, Y is the density of the serum infranate ($d = 1.006$), Z is the density of salt solution (1.085), W is the desired density of the mixture. It was centrifuged as before, the LP in the top 1 cm of the tube was harvested as LP of $d = 1.006$-1.019, while the final infranate was the LP of $d > 1.019$. (Havel et al., 1955).

The protein, cholesterol, cholesterol ester, phospholipid and triglyceride content (mg/ml) of above lipoproteins were measured. The values were calculated as the percentage of individual lipid in the total. The lipids in the VLDL were also calculated as mg of lipid in 1 ml of VLDL.

c. Cholesterol or cholesterol ester enriched Ediol as the substrate - 27, 18 or 9 mg of cholesterol or 27 mg of cholesterol acetate or stearate was dissolved in 1 ml of acetone. 3 ml of 5% Ediol in KRP buffer (pH 8.5) was added and was shaken at 37°C for 2 hr to evaporate the acetone, although the physical and metabolic properties of the cholesterol and of Ediol were reported not to change by the presence of acetone (Quarfordt et al., 1966). 0.6 ml of this mixture of free or esterified cholesterol and Ediol was used as the substrate instead of Ediol to determine their effect on the heart LP-lipase activity of the control rabbit. The control which was treated similarly with acetone but without cholesterol showed no significant deviation in the activity of LP-lipase (86.1 ± 14.2, n = 7, acetone-treated, vs. 92.2 ± 13.1, n = 11, non-treated).

d. Phospholipid and Ediol mixture as the substrate - 27 mg of lecithin, β, γ-dipalmitoyl-DL-α-lecithin*, was mixed with acetone and Ediol and prepared as above.

e. A mixture of control and hypercholesterolemic VLDL - Control heart homogenate was incubated with 0.6 ml of substrate which is composed of 0.6, 0.4, 0.2 or 0 ml of control VLDL and 0, 0.2, 0.4 or 0.6 ml of hypercholesterolemic VLDL, respectively. The lipid and protein composition of these VLDL was calculated from the known composition of each component.

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* Synthetic, Sigma Chemical Company

f. The kinetics of enzyme inhibition - Since factors other than LP-lipase existing in the heart homogenate might influence the results, the LP-lipase from rabbit plasma was purified and the inhibitory effect of cholesterol was tested using this enzyme.

The plasma LP-lipase was purified according to the methods of Fielding (1969). The cold post-heparin plasma was mixed with 1/50 vol of 25% Ediol and was gently shaken in a water bath at 37°C for 7 min to activate the emulsion and allow the formation of the enzyme-substrate complex which was purified by flotation. The purified complex was solubilized by sodium deoxycholate. 0.5 mM of potassium oleate was added to the sodium deoxycholate solution to stabilize the enzyme. A further purification was obtained by the adsorption of the solubilized enzyme on calcium phosphate gel. The enzyme was eluted with the sodium deoxycholate-buffer solution containing 0.05 M sodium citrate.

The inhibitory effect of cholesterol and the kinetics of the inhibition were tested with this purified enzyme according to the Lineweaver-Burk method (Webb, 1963; Lineweaver and Burk , 1934). 5.4 mg of free cholesterol was put into each of three 25 ml Erlenmeyer flasks. 0.8 ml acetone was added to each flask to dissolve the cholesterol. 15, 30 or 45 mg of triglyceride (in the form of 5% Ediol) was added to each of these flasks. In some experiments the amount of triglyceride was 20, 30, 40, 50 or

60 mg (Ediol was used here). The volume of the mixture of Ediol and cholesterol was adjusted to 1.2 ml with KRP buffer. The acetone was evaporated by shaking the flasks at 37°C for 2 hours. The control with different concentrations of Ediol was prepared in the same way but without cholesterol. One ml of the diluted purified plasma LP-lipase was added to each of these flasks and incubated according to the method of Muir (1967). For comparison, 4 mg protamine sulfate with various concentrations of Ediol were also incubated. Although in this experiment the control was not treated with acetone, the experiments with tissue homogenates indicated that the effect of remaining acetone was nil. 1/v was plotted against 1/s in the presence of Ediol alone, Ediol plus cholesterol and Ediol plus protamine sulfate, respectively (Lineweaver and Burk , 1934). v signifies μEq FA released per hour per mg enzyme protein, s signifies mg TG present in the incubation medium.

g. Experiments with cholesterol, palmitic acid and Ediol mixture - 27 mg of cholesterol and 30 μEq of palmitic acid were dissolved in 1 ml of acetone and then mixed with 3 ml of Ediol and shaken as described above. 0.6 ml of this mixture was used as the substrate for an incubation with homogenate of the control heart. The recovery rate of this added fatty acid was calculated to see the effect of the presence of cholesterol.

3. IN VIVO EXPERIMENTS

Both control and hypercholesterolemic rabbits were given heparin intravenously (1 mg/kg body weight) to release the tissue LP-lipase into the blood. Blood was taken through an arterial cannula just before and 10 min after the injection. The plasma cholesterol and its ester, phospholipid, triglyceride and FFA levels were determined to observe the effect of released LP-lipase on these lipids in vivo. The 10 min interval was selected because plasma LP-lipase activity reaches a maximum about 1 min after the injection of heparin and remains elevated at least 10 min (Robinson and Harris, 1959A).

## H. THE TIME COURSE CHANGES OF PLASMA CHOLESTEROL LEVEL, TRIGLYCERIDE LEVEL AND TRIGLYCERIDE TURNOVER

This section dealt with the determination of plasma triglyceride turnover and plasma lipid level during high cholesterol diet of various durations

### 1. INTRAVENOUS INJECTION OF GLYCEROL-2-$H^3$

Rabbits, after 0, 1.5, 7, 14, 42 or 70 days of high-cholesterol diet, were fasted overnight and the femoral artery was cannulated. 80 μC of glycerol-2-$H^3$ was injected through the ear vein. Blood samples were taken through the cannula 30, 60, 90, 150, 210, 270 and 330 min after injection. Plasma cholesterol, phospholipid and triglyceride levels were determined. The triglyceride was separated and counted as above. The activity of the triglyceride was expressed as dpm per ml of plasma after correcting the effect due to individual variations in injected doses. This was done by multiplying a factor which was the ratio of $10^8$ dpm to dpm of the injected glycerol-2-$H^3$ per kg of body weight. The radioactivity of the triglyceride was plotted on semi-logarithmic paper.

The liver takes up glycerol-2-$H^3$, which is incorporated into triglyceride and secreted into the blood as lipoprotein . The labelled triglyceride is constantly removed from the circulation and replaced by the non-labelled triglyceride. Consequently, the specific activity of the plasma triglyceride increases, then

decreases continually and the rate of decrease at a given time (t) depends upon the size of the blood triglyceride compartment (B), the rate of blood triglyceride turnover (q), and the amount of labelled triglyceride present at the time in question (B*). Mathematically one may represent this relation as

$$\frac{dS_b}{dt} = -\frac{qS_b}{B}$$

in which $S_b$ is the specific activity of B. In words this equation reads as follows: the change of specific activity per infinitesimal unit of time is equal to the rate of triglyceride turnover, times the specific activity of triglyceride, divided by the size of the triglyceride pool. The minus sign indicates that the specific activity decreases. If we substitute $K_2$ for q/B (fractional turnover rate) we obtain

$$\frac{dS_b}{dt} = -K_2 S_b$$

or its integrated form

$$S_b = S_b^o \, e^{-K_2 t}$$

in which $S_b^o$ is the specific activity of B at zero time.

$$K_2 t = \text{In} \frac{S_b^o}{S_b}$$

At $t = t_{\frac{1}{2}}$, $K_2 t_{\frac{1}{2}} = \text{In } 2 = 0.693$, then fractional turnover rate

$$K_2 = \frac{0.693}{t_{\frac{1}{2}}} \quad \text{and} \quad t_t = \frac{1}{K_2} = \frac{t_{\frac{1}{2}}}{0.693} = 1.44\ t_{\frac{1}{2}}$$

(Zilversmit, 1960).

If the specific activities of the triglyceride are plotted on semilogarithmic paper, we can obtain $S_b^o$ and $t_{\frac{1}{2}}$. $K_2$ and $t_t$ are derived from these values by using above equations.

In rabbit the average plasma volume is 3.88% of the body weight (Armin et al., 1952). From this, the plasma triglyceride pool size was assumed to be:

$$B = TG \times BW \times 0.0388$$

in which TG is the plasma triglyceride level in mg/ml and BW is the body weight in g (Reaven et al., 1965). However, the volume distribution of an injected isotope was much larger than the pool size calculated as above (e.g. see Fig. 15). Question regarding validity of using the plasma compartment as the pool of TG will be discussed in a later section (Discussion). Plasma triglyceride turnover rate q was obtained by multiplying the fractional turnover rate by the size of the pool.

2. INTRAVENOUS INJECTION OF TRITIUM LABELLED VLDL ($d < 1.006$)

500 μc of glycerol-2-$H^3$ in 10 ml of normal saline was injected intravenously into a normal fed rabbit. The rabbit was bled 1 hr later. VLDL was isolated from this blood as above. The activity of 0.1 ml aliquot of this LP was determined in Bray's solution. Approximately $19 \times 10^5$ dpm of this LP was injected

into fasted rabbits after 0, 1.5, 7, 14, 21 or 49 days of either high cholesterol or normal diet. Blood was taken 5, 30, 60, 90, 150, 210 or 270 min after injection for the determination of plasma lipid and triglyceride turnover. The specific activity of the plasma triglyceride was expressed as dpm per ml of plasma and corrected to a dose of $10^8$ dpm per kg body weight as above. It was then plotted on semilogarithmic paper. The curve of disappearance of radioactivity from the plasma triglyceride after intravenous injection of the VLDL contained two components. The second component was extrapolated to zero time to obtain the specific activity at zero time. The half turnover time $t_{\frac{1}{2}}$ was indicated by the line drawn vertically to the time axis from the cross point of half radioactivity and disappearance curve. The turnover time $t_t$ is 1.44 $t_{\frac{1}{2}}$. The fractional turnover rate and turnover rate were derived as described above.

## I. LIPID COMPOSITION OF THE SUBCELLULAR ELEMENTS AND MITOCHONDRIAL RESPIRATION OF THE HEART AND LIVER

The heart and liver were homogenized and fractionated for the determination of lipid composition and mitochondrial respiration (Huang and Kako, 1968). This was carried out as follows: the heart was immediately soaked in an ice-cold medium composed of 0.25 M sucrose and 0.002 M EDTA (pH 7.4). The ventricles with no visible fat were cut into fine pieces and any adhering blood washed out with the same medium. It was then

passed through a stainless steel tissue press and suspended in the medium (1:10 w/v). A glass-reinforced Teflon pestle was used for homogenization at a speed of 600-1000 rpm. The tube was moved rapidly up and down three times. The homogenate was then transferred into a tighter homogenizing tube and was given one stroke with a glass-reinforced Teflon pestle and two strokes with an ordinary Teflon pestle. The suspension was then centrifuged at 500 x g for 5 min, and the myofibril fraction was prepared from the sediment according to the procedure of Wheeldon et al. (1965). The supernatant was centrifuged at 12,800 x g for 10 min, and the mitochondria thus sedimented were washed once with the homogenizing medium and centrifuged at 8,200 x g for 10 min. The washed pellet was suspended in the medium to give a final concentration of about 10 mg protein/ml. The microsomal and supernatant fractions were prepared by centrifuging the post-mitochondrial supernatant. This last centrifugation was done at 78,000 x g for 120 min. The microsomal fraction was then suspended in the medium to give a concentration of approximately 10 mg protein/ml. For the liver the same medium as that for the heart was used for washing and homogenization using an ordinary Teflon pestle (four strokes in the loose tube and two strokes in the tighter tube). A centrifugal force of 800 x g for 5 min was used to spin down the nuclei and cell debris, 8,200 x g for 10 min to precipitate the mitochondria, and 78,000 x g for 120 min to separate the microsomal fraction

and the supernatant. The mitochondria were washed once with the homogenizing medium and centrifuged at 8,200 x g for 10 min. They were then suspended in the same medium to produce a concentration of about 10 mg protein/ml.

Mitochondrial respiration was measured with an oxygen cathode in a medium containing 15 mM KCl, 5 mM $MgCl_2$, 10 mM EDTA, 20 mM glucose, 30 mM phosphate buffer and 25 mM Tris (pH 7.4). Either 3 mM pyruvate plus 3 mM malate, 3 mM L-glutamate, 3 mM oxoglutarate, or 8 mM succinate was added as the substrate. ADP, 0.2 mM, was used as the phosphate acceptor. The final volume was 2 ml, and the temperature was 25°C. In each experiment, 0.1 ml of the suspension of the subcellular elements was pipetted into the chamber containing the above mixture. The respiratory control ratio, P/O ratio, and oxygen consumption were calculated according to the method of Chance and Williams (1955A).

EDTA was added to the medium for both heart and liver to remove $Ca^{++}$ or other contaminating metal ions. This appears to be essential in order to obtain a low ATPase activity. Sucrose is valuable because it prevents aggregation of mitochondria and other subcellular particles and also the cytological properties of mitochondria are preserved (Schneider, 1959).

An aliquot of the suspension of the subcellular elements was taken for protein determination and the extraction and determination of the lipids according to the method described in the

appropriate section above. The lipid content of the subcellular elements or the respiratory function of mitochondria was expressed on the basis of per mg of protein.

J. STATISTICAL ANALYSIS OF THE RESULTS

The standard error of the mean and the significance of difference in means were calculated according to t-test for small samples (Bancroft, 1957).

# CHAPTER III

## RESULTS

Approximately two months after the high cholesterol diet was started, seven out of nine rabbits developed xanthomatosis and yellowish plaques along the central ear arteries. The plaques were situated at the site of bifurcation of the arteries and at the sites of trauma due to blood aspiration. The xanthomas were 1-5 mm in diameter and firm (Fig. 2).

### A. CHANGES IN PLASMA

There was no significant change in the plasma protein concentration between the two groups (8.3 ± 1.3 g% in the rabbits on a high cholesterol diet, duration of diet average 60 days, n = 28; 7.9 ± 0.6 g% in the control, n = 27).

The plasma cholesterol and its esters, phospholipid and triglyceride in the experimental group were significantly increased (Table I). These values were obtained from animals fed cholesterol for an average of 60 days. The time course of changes during feeding was also studied and it was found that the cholesterol and its ester and phospholipid increased rapidly while the triglyceride increased slowly (Fig. 3).

Although both total and ester cholesterol were increased, the ester/total cholesterol ratio changes from 0.57 in the control rabbits to 0.30 in the rabbits on a high cholesterol diet,

indicating that esterification of cholesterol cannot increase proportionately. The ratio of total cholesterol/phospholipid was found to be 0.55 (control) and 1.46 (hypercholesterolemic), i.e. phospholipid synthesis did not increase in parallel to the cholesterol level. These results suggest that the composition of lipoprotein species must have been dramatically changed.

There was no significant difference in FFA content of the plasma or liver (Fig. 4) between the fasting control and hypercholesterolemic rabbits.

B. FATTY ACID ESTERIFICATION AND ACETATE INCORPORATION INTO LIPIDS

One of the possible mechanisms responsible for an elevated level of plasma triglyceride in the hypercholesterolemic condition is the increase in esterification and/or fatty acid synthesis in the liver of these rabbits. Therefore, the extent of fatty acid esterification to triglyceride was examined in the liver of control and hypercholesterolemic rabbits. The results showed no significant difference, namely, as indicated in Fig. 5 the fatty acid esterified into triglyceride was 85.6 $\pm$ 13.1 μEq/kg of body weight in the control and 81.2 $\pm$ 9.3 μEq/kg in experimental rabbits during 90 min incubation. The number of experiments (control, 4; hypercholesterolemic, 8) was small. But this result was confirmed by a second experiment, in which sodium acetate-1, 2-$C^{14}$ was used to assess synthesis of triglyceride by the liver. A half gram

of liver slices was incubated in the medium which contained sodium acetate-1,2-$C^{14}$ and 2 mM sodium acetate. The latter concentration is approximately three times that of the rabbit plasma (Annison, 1954) and the volume of the incubation medium is large as compared to that of liver and therefore individual variations in acetate contents in the liver can be disregarded. For this reason, it was assumed that each sample contained the same specific activity of sodium acetate-1,2-$C^{14}$. There was again no increase in synthesis of triglyceride in the liver of the hypercholesterolemic rabbit. The amount of acetate being used for the synthesis of triglyceride by the liver showed no significant difference (Table II).

C. LIPOPROTEIN LIPASE ACTIVITES

The above experiments indicated that the increase in plasma triglyceride in the hypercholesterolemic rabbit was not due to an increase in its synthesis by the liver. Thus, a second possibility, a decrease in uptake of plasma triglyceride by extra-hepatic tissue was examined. This may be due to the decrease in tissue LP-lipase activity or due to the physicochemical effect of cholesterol (Friedman and Byers,1965) which sequestrates the triglyceride in the plasma.

LP-lipase is obligatory for triglyceride uptake by the extra-hepatic tissues, (Bezman, 1962A; Garfinkel, 1967) and therefore a decrease in tissue LP-lipase activities can cause a decrease in uptake of triglyceride by the extra-hepatic tissues, thereby

causing an accumulation of triglyceride in the plasma. Such a decrease could be due to a decrease in quantity or quality of the enzyme or to the presence of inhibitors.

It was found that the heart LP-lipase activities did not decrease in the hypercholesterolemic rabbits when coconut oil emulsion (Ediol) or VLDL was used as the substrate. Fig. 6 shows that the heart LP-lipase activity in the hypercholesterolemic rabbits was increased when Ediol was used as the substrate (89.8 $\pm$ 9.4 $\mu$Eq/hr.g in control and 168.7 $\pm$ 14.9 $\mu$Eq/hr.g in experimental rabbits). This was also true when normal VLDL instead of Ediol was used as the substrate (white bars in Fig. 7). The activities were lower with the VLDL than those with Ediol, but, by using the same substrate, VLDL, difference between the two groups persisted, namely, the LP-lipase activity of the hypercholesterolemic heart was significantly higher than that of the control heart (71.0 $\pm$ 8.0 $\mu$Eq/hr.g and 44.2 $\pm$ 4.3 $\mu$Eq/hr.g, respectively). This indicates that the cause of triglyceridemia was not a result of the decrease in quantity of the heart LP-lipase.

When LP-lipase from the same source was tested, the apparent activity measured by using VLDL from the hypercholesterolemic rabbit as the substrate was found to be lower than that using normal VLDL (Fig. 7 black vs. white bars). The LP-lipase activities were depressed to 33-44%. This depression of LP-lipase activity must be caused by some factors existing in the VLDL from

the hypercholesterolemic rabbits. The difference in lipid composition between these two VLDL was thought to be responsible in causing a depression in LP-lipase activity. The lipid content of the VLDL was then analyzed. Table III shows that cholesterol and its ester and phospholipid are significantly higher in hypercholesterolemic VLDL ($p < 0.01$), and that there was no significant difference in the triglyceride content of these two lipoproteins ($p > 0.01$), despite rather a large difference in percentage of composition (Table IV). It is possible, however, the lipoprotein fraction obtained from hypercholesterolemic rabbits may have a heavier density than that from normocholesterolemic rabbits, since the density of triglyceride and cholesterol is 0.92 and 1.06 (g/ml), respectively.

A possible cause of the difference in LP-lipase activities may be that one of these lipids acts as an inhibitor of the enzymic action. Therefore various lipid components were added to the Ediol individually (Fig. 8). It was found that 5.4 mg of free cholesterol inhibits LP-lipase activity, while the cholesterol acetate has no effect (Fig. 8). The inhibition was 79% (89.8 ± 9.4 μEq/hr.g, control vs. 18.9 ± 5.6 μEq/hr.g cholesterol-added). Fig. 9 shows that phospholipid and cholesterol stearate have no inhibitory effect. It is inferred from these results that the inhibition of LP-lipase by the VLDL from the hypercholesterolemic rabbit is due to its high free cholesterol content. This is followed by a

decrease in triglyceride uptake by the tissue, resulting in hypertriglyceridemia.

The inhibitory effect of free cholesterol is dosage dependent, the more free cholesterol exists in the incubation medium, the larger is the inhibitory effect. This is shown in Fig. 10 and 11. Increasingly larger amounts of free cholesterol in an emulsion form (Fig. 10) or in lipoprotein (Fig. 11) caused proportionally greater inhibition of LP-lipase activity.

For a study of the kinetics of inhibition, the purified post-heparin plasma LP-lipase was prepared according to the method by Fielding (1969). Extent of purification of the enzyme is summarized in Table V, which indicates that the enzyme was purified by 816-fold.

The kinetics of inhibition of LP-lipase by the free cholesterol were of the competitive nature. This is shown in Fig. 12, where the rates of the reaction in the presence and absence of inhibitors were plotted against substrate concentrations in the manner of Lineweaver and Burk . The three lines appear to converge on the vertical axis indicating inhibition of competitive nature by protamine sulfate as well as by cholesterol. $-1/K_m$ was found directly by extending the line to intersect with the 1/S axis. It was -0.005, -0.003, and -0.0015 mg for the substrate of Ediol only, Ediol plus protamine and Ediol plus free cholesterol respectively. $K_m$ values are therefore, 248, 412 and 825 μM trigly-

ceride for Ediol, Ediol plus protamine and Ediol plus free cholesterol, respectively. This well known inhibitor of LP-lipase, protamine, produced inhibition similar in magnitude to those reported by previous workers (Korn, 1955A).

It was thought at this point that there might be a possibility in which free cholesterol present in the incubation mixture is esterified or combines with FFA released from the triglyceride. This hypothesis was tested by incubating control heart homogenates in the presence of cholesterol, Ediol and palmitic acid; 92.1% of the added palmitic acid was titrated ($n = 3$). There was no change in amount of esterified cholesterol before and after incubation (44 μg vs. 41 μg per 1 ml of the incubation mixture, $n = 3$). From these observations, it was concluded that the decreased amount of titrable acid found after incubation in the presence of cholesterol is not due to the adsorption or esterification of FFA but due to the real decrease in hydrolysis of triglyceride.

The inhibitory effect of cholesterol upon LP-lipase activity was also shown in an in vivo experiment. Fig. 13 shows the plasma lipid level following the injection of heparin. The upper panel shows the changes of plasma lipids in normocholesterolemic animals. The triglyceride level is 114.7 ± 9.5 mg% before LP-lipase is released by heparin. The level is significantly decreased by the action of LP-lipase (74.7 ± 9.5 mg%). The total cholesterol

and phospholipid are not affected by the LP-lipase. The lower panel shows the changes observed in hypercholesterolemic rabbits. There is no significant hydrolysis of triglyceride upon heparin administration. This decreased hydrolytic activity of LP-lipase supports the idea that the LP-lipase is inhibited by the high concentration of cholesterol. Significant changes in FFA concentration after heparin injection will be discussed in the "Discussion" (Chapter IV).

### D. INTERRETATIONSHIP BETWEEN TRIGLYCERIDE TURNOVER AND PLASMA LIPIDS

The experiments so far performed showed that the increase in level of free cholesterol in the plasma caused inhibition of tissue LP-lipase activities and the accumulation of triglyceride in the plasma. This cause-effect relationship between the plasma free cholesterol and triglyceride was further confirmed by the determination of time course changes in plasma cholesterol level, triglyceride level and triglyceride turnover rate. The increase in plasma free cholesterol is accompanied by an initial decrease in plasma triglyceride turnover rate (Fig. 14, 15). Thereafter, the plasma triglyceride was gradually increased. The plasma free cholesterol (panel A) increased from 25.0 ± 1.7 mg% to 35.7 ± 12.3 mg% after 36 hr of a high cholesterol diet, and 137.7 ± 62.7 mg% at the end of the first week of this diet. It reached 481.0 ± 60.2 mg% after two months of a high cholesterol diet and then stayed at a high level with some fluctuations. The fractional

turnover rate (panel C), determined by injecting tritium-labelled VLDL, decreased rapidly from 58.2% to 33.5% per hr after 36 hr of a high cholesterol diet. It remained at the range of 24.3% - 36.6% per hr while the plasma free cholesterol stayed at a high level. The decrease in fractional turnover rate of triglyceride was followed by the gradual increase in plasma triglyceride level (panel B). It increased from 91.0 ± 8.9 mg% to 155.6 ± 46.3 mg% at the end of the second week of cholesterol diet and reached 259.4 ± 81.5 mg% four months later. The elevation of plasma triglyceride level, i.e. enlarged triglyceride pool size, should accompany a decrease of triglyceride fractional turnover rate, unless there is a corresponding increase in turnover rate. Because the free cholesterol in plasma remained high in the experimental rabbits and therefore tissue LP-lipase activities were continuously inhibited, the hydrolysis of triglyceride by the enzyme could not increase to keep pace with the increase in triglyceride pool size. This imbalance between the triglyceride hydrolysis and pool size would cause a further decrease in triglyceride fractional turnover rate. However, there was no progressive decrease in the fractional turnover rate, indicating that the hydrolysis of plasma triglyceride did increase even though the plasma free cholesterol was still high. The increase in hydrolysis of triglyceride at a later period should be interpreted as the compensatory increase in LP-lipase activity in hypercholesterolemic rabbit (Fig. 6 and 7).

A return towards control values of the once decreased turnover rate (panel D, decreased from the control value of 24.1 mg/hr to 16.4 mg/hr after 36 hr of high cholesterol diet) is a manifestation of this compensation, although a mass action effect owing to an increased level of substrate for the lipase may be in part responsible for this increase in triglyceride turnover. Hence, the fractional turnover rate was maintained at a relatively constant level.

The turnover rate measured in the parallel-fed control rabbits at the 5th, 21st and 28th day showed no significant deviation as compared to the 0 day-control value.

The plasma triglyceride turnover rate, measured by the injection o: glycerol-2-$H^3$, agreed well with the results with VLDL-TG-$H^3$ described abov (Fig. 14 and 15). The glycerol-2-$H^3$ serves as a precursor of plasma VLDL. With this method, the fractional turnover rate decreased from 51.8 ± 6.5% per hr (control) to 14.0% per hr (36 hr of high cholesterol diet). It remained in the range of 3-14% per hr throughout all the experimental peri The turnover rate similarly decreased (from 36.3 ± 7.2 mg/hr to 5.0 mg/hr after 36 hr of cholesterol diet) but it gradually returned towards the con The results obtained by using the latter method should be interpreted with reservation, since the low values found appear to be grossly underestimate In contrast to the 1st method (VLDL method), the turnover rate did not exceed the control at a later period of the experiment (40-70 days). This corresponds to the results of hepatic triglyceride synthesis which was not increased in hypercholesterolemic rabbits.

Fig. 16 is a semilog plot showing the second component of

the disappearance curves of radioactivity. The results were taken from experiments using tritium labeled VLDL in one control and rabbits fed high cholesterol diet of 1.5, 7, 14, 21 or 49 days. The turnover time of hypercholesterolemic rabbits was all about 169 min, while that of the control was 112 min.

## E. INTER-RELATIONSHIP BETWEEN THE PLASMA CHOLESTEROL AND TISSUE LIPIDS

This section deals with the analysis of lipid in the subcellular elements of the heart and liver. In addition, the respiratory function of the mitochondria of heart and liver was assessed.

An electron micrograph of the heart mitochondria prepared from a control rabbit is shown in Fig. 17. There is little damage of the mitochondria. Contamination by the other subcellular elements cannot be observed. Since the mitochondria align in rows in the narrow clefts between the tough myofibrils of cardiac muscle (Fawcett, 1967), a glass-reinforced Teflon pestle had to be used for homogenization. However, the damage appears to be very little. The respiratory control ratio of the heart and liver mitochondria are within normal limits (Table VI). The normal respiratory control ratio also indicates the intactness of mitochondria structure (Sobel et al., 1966). There was no significant difference in the respiratory control ratio, P/O ratio, or oxygen consumption between the heart mitochondria with normal and with high cholesterol

content ($p > 0.1$). In these experiments, L-glutamate, oxoglutarate or pyruvate and malate were used as substrates. Liver mitochondria of the hypercholesterolemic rabbits likewise showed no significant impairment of respiratory function when succinate was used as the substrate. The change in oxygen consumption of hepatic mitochondria was of borderline significance ($0.025 > p > 0.01$).

Fig. 18 to 21 show the contents of total and esterified cholesterol in the subcellular fractions of the heart and liver. The elevation of plasma free and esterified cholesterol was accompanied by a significant increase in these two lipids in the mitochondria and supernatant fractions of the heart and in the mitochondria, microsomes and supernatant fractions of the liver, while the contents in heart microsomes and myofibrils were not affected. The total cholesterol and cholesterol ester in heart mitochondria increase 2-fold, in the heart supernatant 8-fold, in the liver mitochondria 5-fold, in the microsomes 3-to 4-fold, and in the supernatant 17-fold. The changes were all significant ($p < 0.01$). The phospholipid and triglyceride of all the subcellular elements of both heart and liver remained unchanged (Fig. 22 to 25). The livers of the hypercholesterolemic rabbits weighed an average of 129.1 g and were 30% heavier than the control livers.. Therefore, the total quantities of hepatic phospholipid and triglyceride were increased in the hypercholesterolemic rabbits.

Fig. 5. The Ear of a Hypercholesterolemic Rabbit.

Note the yellowish plaques along the central ear artery and xanthomatosis. This rabbit was fed a high-cholesterol diet for two months.

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TABLE I

PLASMA LIPIDS OF CONTROL RABBITS AND OF RABBITS ON A HIGH CHOLESTEROL DIET

The high cholesterol diet consisted of 3% cholesterol (w/w) dusted onto rabbit pellets similar to those fed to the control rabbits. The values are expressed as mean ± S.E.M. The experimental rabbits were fed a high cholesterol diet for an average of two months. The number of observations of the control includes; (1) the samples from the control rabbits at the beginning of the experimental period; (2) those from the experimental rabbits before the high cholesterol diet was initiated; and (3) the samples from the control rabbits at the end of the experimental period, i.e., after they have been kept on the diet without added cholesterol for the same period of time as the experimental rabbits. There was no difference among values for these three sets.

| | | Control | | Experimental | | |
|---|---|---|---|---|---|---|
| | | No. | mg% | No. | mg% | p |
| Cholesterol: | Total | 30 | 59.4 ± 5.1 | 10 | 533.9 ± 61.5 | <0.001 |
| | Ester | 30 | 34.4 ± 3.4 | 10 | 162.4 ± 19.9 | <0.001 |
| Phospholipid: | | 30 | 109.5 ± 6.3 | 10 | 365.4 ± 43.6 | <0.001 |
| Triglyceride: | | 30 | 91.0 ± 8.9 | 10 | 168.8 ± 35.7 | 0.001< p <0.01 |

Fig. 3 THE TIME COURSE OF CHANGES IN PLASMA LIPIDS DURING CHOLESTEROL FEEDING

The high cholesterol diet consisted of 3% cholesterol (w/w), dissolved in ethyl ether, then mixed with the rabbit pellets or dusted directly onto the pellets. The means of 30 observations of the control and 5 observations of the experimental rabbits are shown. Abbreviations are: CHOL. (cholesterol), P.L. (phospholipid), T.G. (triglyceride), WKS (weeks) and mg/100 ml of plasma.

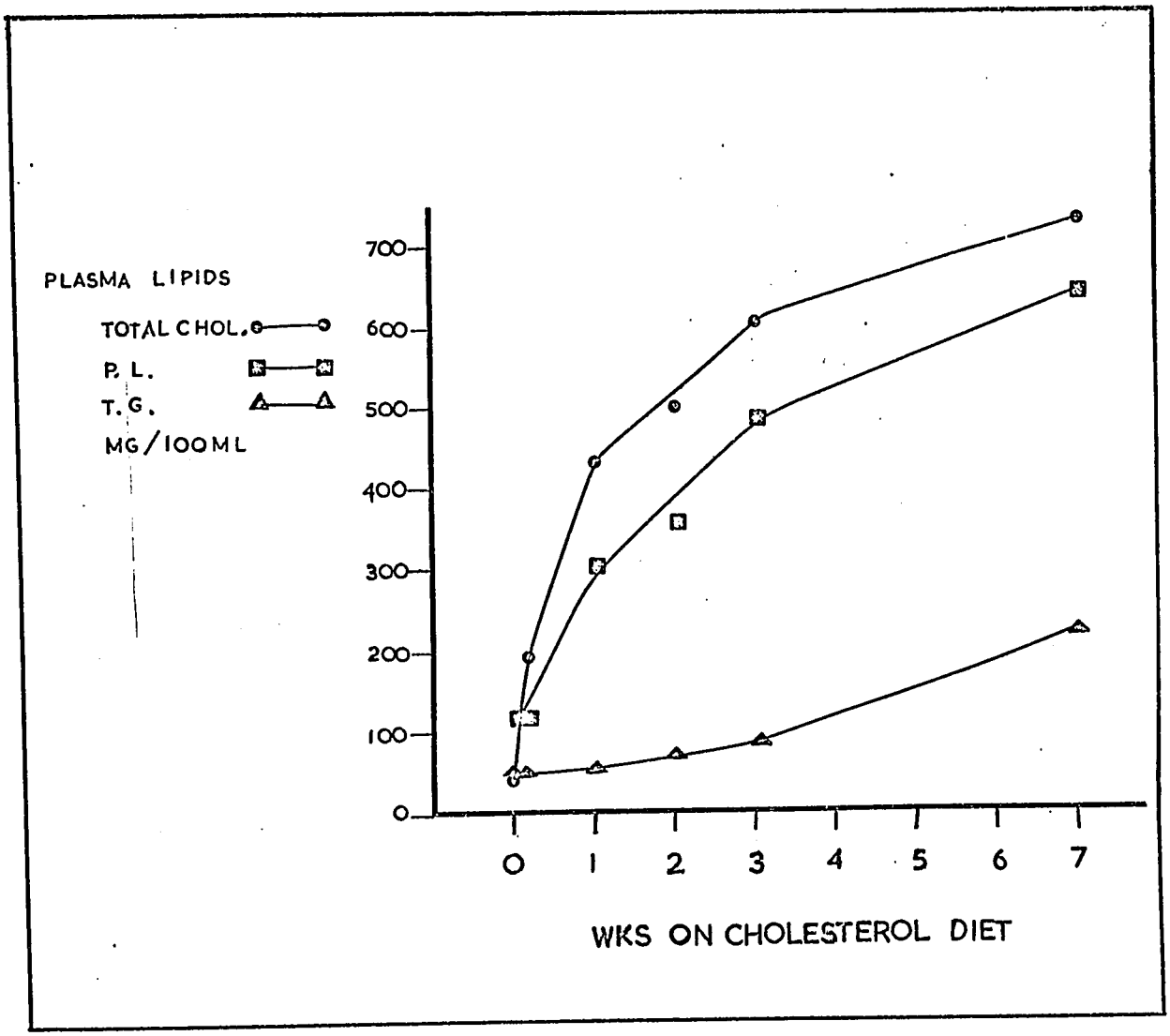



Fig. 3. The Time Course of Changes in Plasma Lipids during Cholesterol Feeding.

Fig. 4. FREE FATTY ACID LEVEL IN THE LIVER AND PLASMA OF OVERNIGHT FASTED RABBITS

The FFA were extracted from 1 ml of liver homogenate or plasma with 5 ml of isopropanol-heptane-$H_2SO_4$. The FFA in heptane phase were titrated with 0.1 N sodium ethoxide. A 0.02 % (w/v) solution of phenolphthalein in absolute ethanol served as the indicator. The content of FFA is expressed as μEq per gram wet weight of liver, and as μEq per ml of plasma (ordinate). The results are shown by the white (control) and black (hypercholesterolemic rabbits) columns. They are expressed as mean ± S.E.M. The animals were on a high cholesterol or control diet for an average of 44 days before samples were taken.

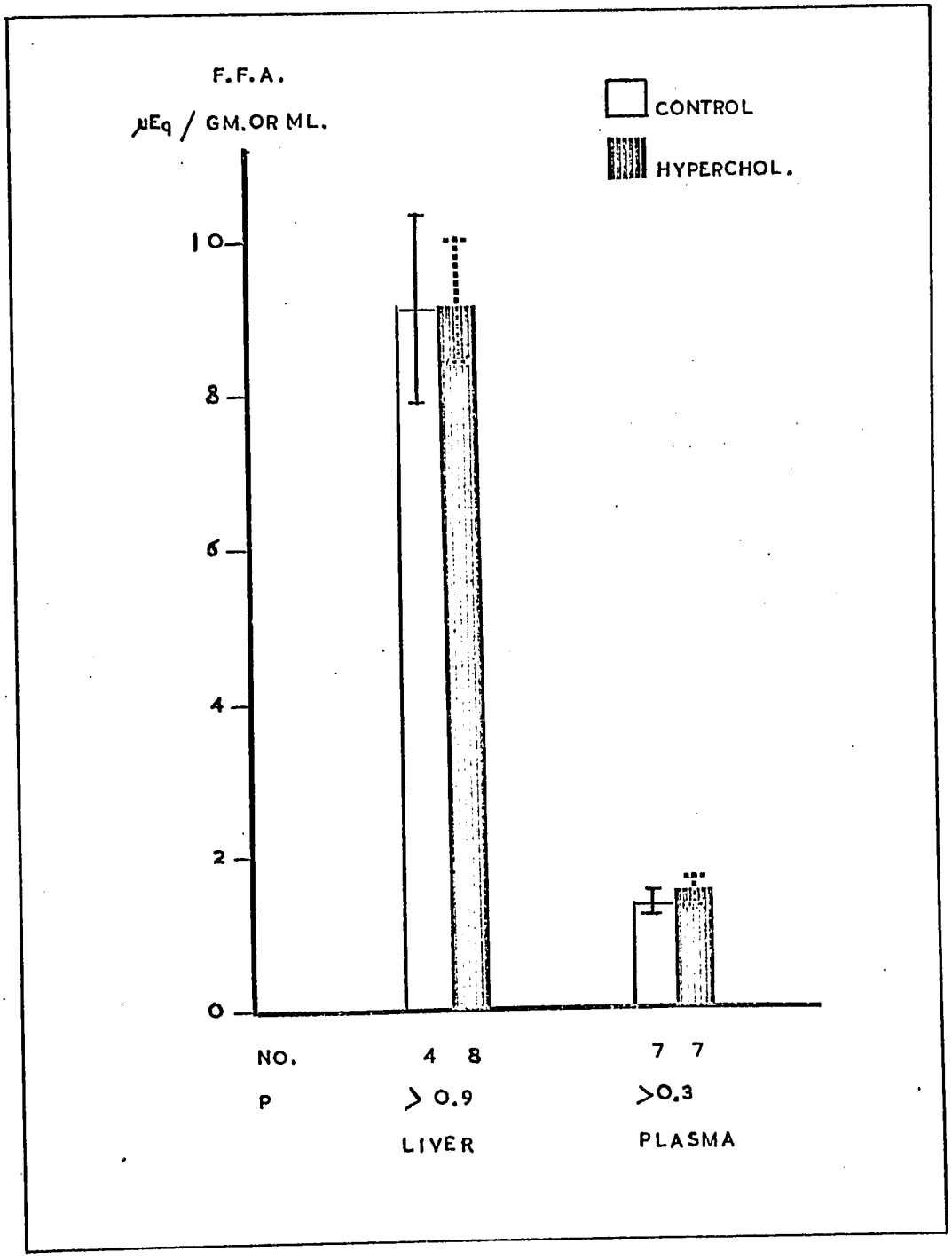



Fig. 4. Free Fatty Acid Level in the Liver and Plasma of Over-Night Fasted Rabbits.

Fig. 5 FATTY ACID ESTERIFICATION INTO TRIGLYCERIDE IN LIVER HOMOGENATES

Ten grams of liver were homogenized in 30 ml of KCL Tris buffer (9 parts of 0.154 M KCL and 1 part of Tris buffer, 0.5 M pH 7.5) and centrifuged at low speed. Two ml of supernatant were incubated in 1 ml of medium containing 0.1 microcuries (0.04 μM) of potassium-1-$C^{14}$-palmitate and 10 μM ATP, 20 μM potassium phosphate buffer (pH 7.5) and 10 μM $MgCl_2$ at 37°C for 90 min. The results are expressed as mean ± S.E.M. μEq of FA incorporated per kg of body weight over an incubation period of 90 min (ordinate) on the assumption that the specific activity of the palmitate would remain constant and would represent that of FA. The liver of experimental rabbits was excised after a high cholesterol regimen of an average of 2 months duration.

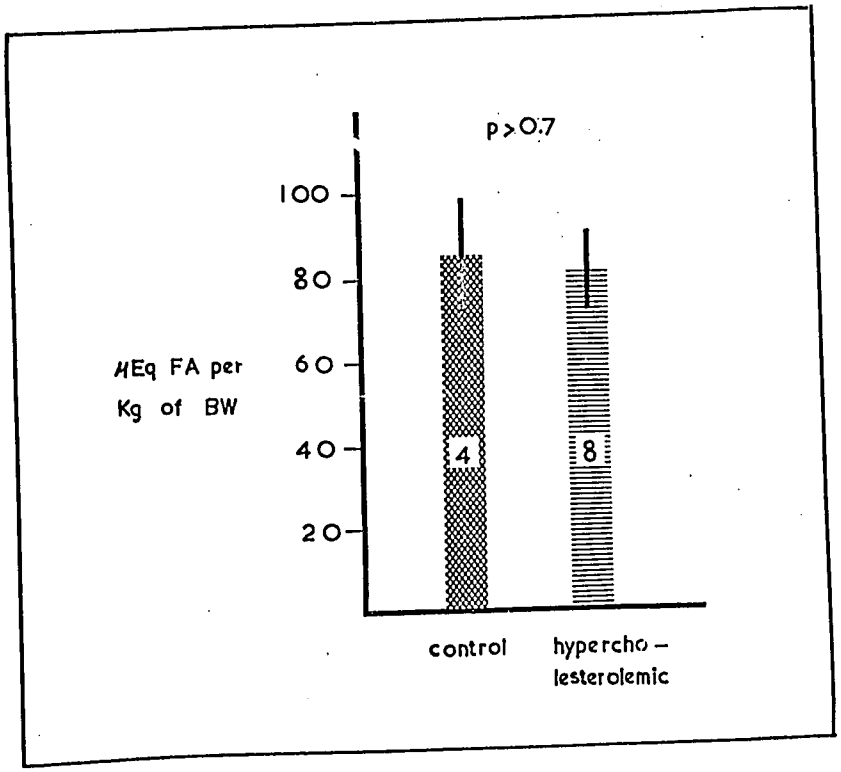



Fig. 5. Fatty Acid Esterification into Triglyceride in Liver Homogenates.

TABLE II

INCORPORATION OF ACETATE-1,2-$C^{14}$ INTO THE LIPIDS OF THE LIVER

0.5 g of liver slices were incubated in 5 ml of intracellular cation type medium in the presence of 5 microcuries (2mM) sodium acetate-1,2-$C^{14}$ at 37°C for 2 hours. The medium composition was 120 mM $K^+$; 20 mM $Mg^{++}$; 2.5 mM $Ca^{++}$; 100 mM $Cl^-$; 20 mM $HCO_3^-$, and it was gassed with 95% $O_2$ and 5% $CO_2$ for a minimum of 20 min before tissues were added. The values were calculated as mμ moles of sodium acetate incorporated into the lipids per gram of liver (mean ± S.E.M.). The liver of experimental rabbits was excised after an average of two month high cholesterol regimen.

| Diet | No. | Lipids | | | |
|---|---|---|---|---|---|
| | | FFA | TG | Cholesterol | |
| | | | | free | ester |
| Normal | 11 | 2.0 ± 0.2 | 1.1 ± 0.2 | 5.2 ± 0.9 | 0.1 |
| 3% cholesterol | 10 | 2.4 ± 0.6 | 0.8 ± 0.2 | 7.6 ± 1.2 | 0.1 |
| | p | > 0.5 | > 0.2 | > 0.1 | |

Fig. 6 LIPOPROTEIN LIPASE ACTIVITIES OF RABBIT HEART HOMOGENATES, PLASMA AND ADIPOSE TISSUE

100 mg of the tissue in 0.5 ml of Krebs-Ringer-phosphate buffer (pH 8.5) was added to the incubation medium which consisted of albumin (Fraction V), serum, 5% Ediol, and heparin. For the post-heparin plasma, heparin was omitted and Tris buffer (pH 8.5) was used. The fatty acids released were determined at zero time and after 1 hour of incubation at 37°C. Enzyme activities are expressed as μEq per 1 ml of plasma or 1 g of heart or adipose tissue (ordinate) in the control (white columns) or hypercholesterolemic rabbits (black columns) (mean ± S.E.M.). The animals were on a control or high-cholesterol diet for an average of 44 days before samples were taken.

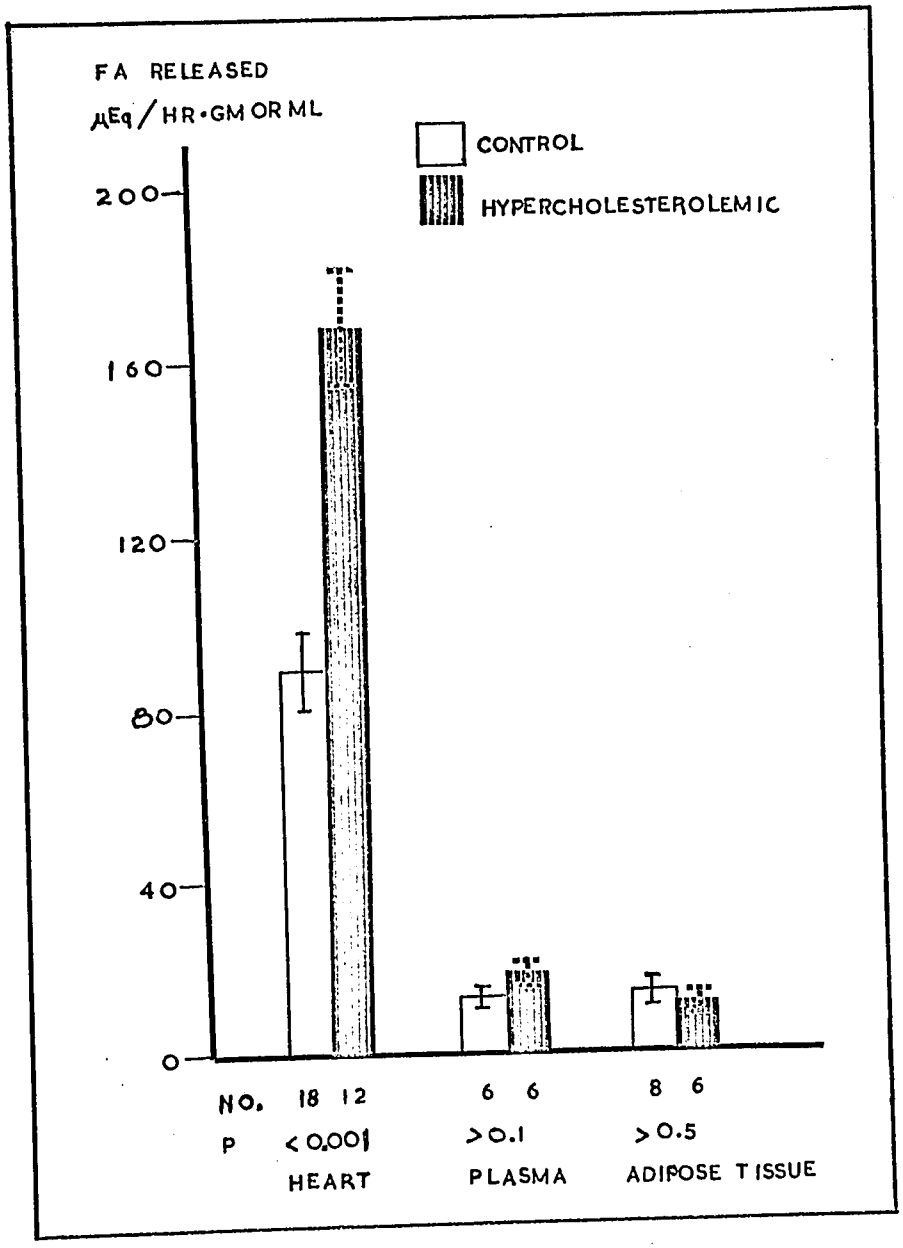



Fig. 6. LP-lipase Activities of Rabbit Heart Homogenates, Plasma and the Adipose Tissue.

Fig. 7 LIPOPROTEIN LIPASE ACTIVITIES OF RABBIT HEART HOMOGENATES WITH VERY LOW DENSITY LIPOPROTEIN (VLDL) AS THE SUBSTRATE

0.6 ml of VLDL ($d < 1.006$) from the control (white columns) or hypercholesterolemic rabbits (black columns) was used as the substrate. The results obtained by using control heart enzyme (left side bars) are shown in contrast to those obtained from hypercholesterolemic heart enzyme (right side bars). For further details see legend to Fig. 6.

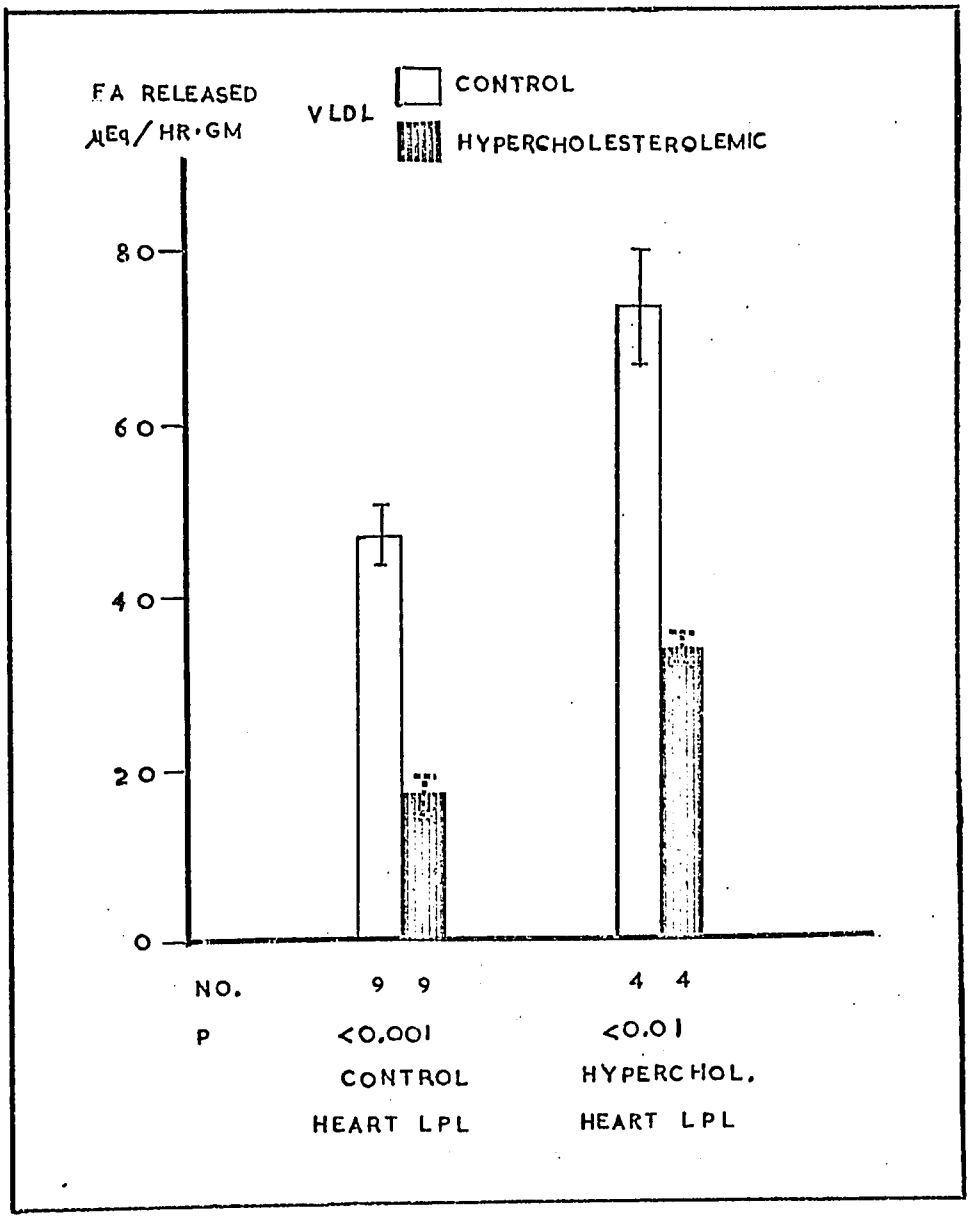



Fig. 7. LP-lipase Activities of Rabbit Heart Homogenates with Very Low Density Lipoprotein as the Substrate.

TABLE III

THE CONCENTRATION OF LIPIDS IN VLDL ($d < 1.006$)

The serum was centrifuged for 15 hr at 115,000 x g at 10°C., the lipoprotein (LP) contained in the top 1 cm of the tube was aspirated ($d < 1.006$) for the determination of the protein, cholesterol, cholesterol esters, phospholipid and triglyceride content. The values are expressed as the mean ± S.E.M. Samples were taken from the rabbits after an average of 30 days of control or high cholesterol diet.

| Diet | No. | Lipids (mg/ml) | | | |
|---|---|---|---|---|---|
| | | Cholesterol | | phospholipid | triglyceride |
| | | free | esterified | | |
| control | 7 | 0.24 ± 0.05 | 0.51 ± 0.06 | 1.53 ± 0.28 | 4.42 ± 0.66 |
| 3% cholesterol | 5 | 2.51 ± 0.71 | 4.71 ± 1.26 | 5.46 ± 1.45 | 1.99 ± 0.33 |
| | p | <0.01 | <0.01 | <0.01 | 0.02> p> 0.01 |

TABLE IV

LIPID COMPOSITIONS OF THE LIPOPROTEINS

The serum was centrifuged for 15 hr at 115,000 x G at 10°C. The lipoprotein (LP) contained in the top 1 cm of the tube was aspirated ($d < 1.006$). The remaining lower portion was brought to $d = 1.019$ by mixing with salt solution and centrifuging as before The LP in the top 1 cm of the tube was harvested as LP of $d = 1.006$-1.019. The remaining part was the LP of $d > 1.019$. The protein, cholesterol, cholesterol esters, phospholipid and triglyceride content of these LP were measured. The values are expressed as the mean of three samples which were taken from the rabbits after an average of 30 days of control or high cholesterol diet.

| Density | Diet | Lipids (%) | | | |
|---|---|---|---|---|---|
| | | Cholesterol | | phospholipid | triglyceride |
| | | free | ester | | |
| <1.006 | control | 6.1 | 4.9 | 23.0 | 66.1 |
| | 3% cholesterol | 37.6 | 17.2 | 36.6 | 8.6 |
| 1.006-1.019 | control | 8.4 | 4.9 | 25.1 | 61.7 |
| | 3% cholesterol | 23.0 | 29.1 | 42.9 | 5.1 |
| >1.019 | control | 42.4 | 35.8 | 9.8 | 12.1 |
| | 3% cholesterol | 19.1 | 38.6 | 39.1 | 3.3 |

Fig. 8 LP-LIPASE ACTIVITIES OF THE HEART HOMOGENATES IN THE PRESENCE OF CHOLESTEROL

Ediol in the presence and in the absence of cholesterol or cholesteryl acetate was used as the substrate in these experiments. 5.4 mg of cholesterol or cholesteryl acetate was suspended in 0.6 ml of Ediol(5%, pH 8.5). 100 mg of control heart homogenate was incubated in the medium which consisted of 5% albumin (fraction V), 2.7 ml; serum,2.7 ml and heparin, 0.5 ml (210 μg). FFA was determined at 0 and after 1 hr of incubation at 37°C. Enzyme activities are expressed as μEq FA released per g of heart during 1 hr of incubation (ordinate) (mean ± S.E.M.).

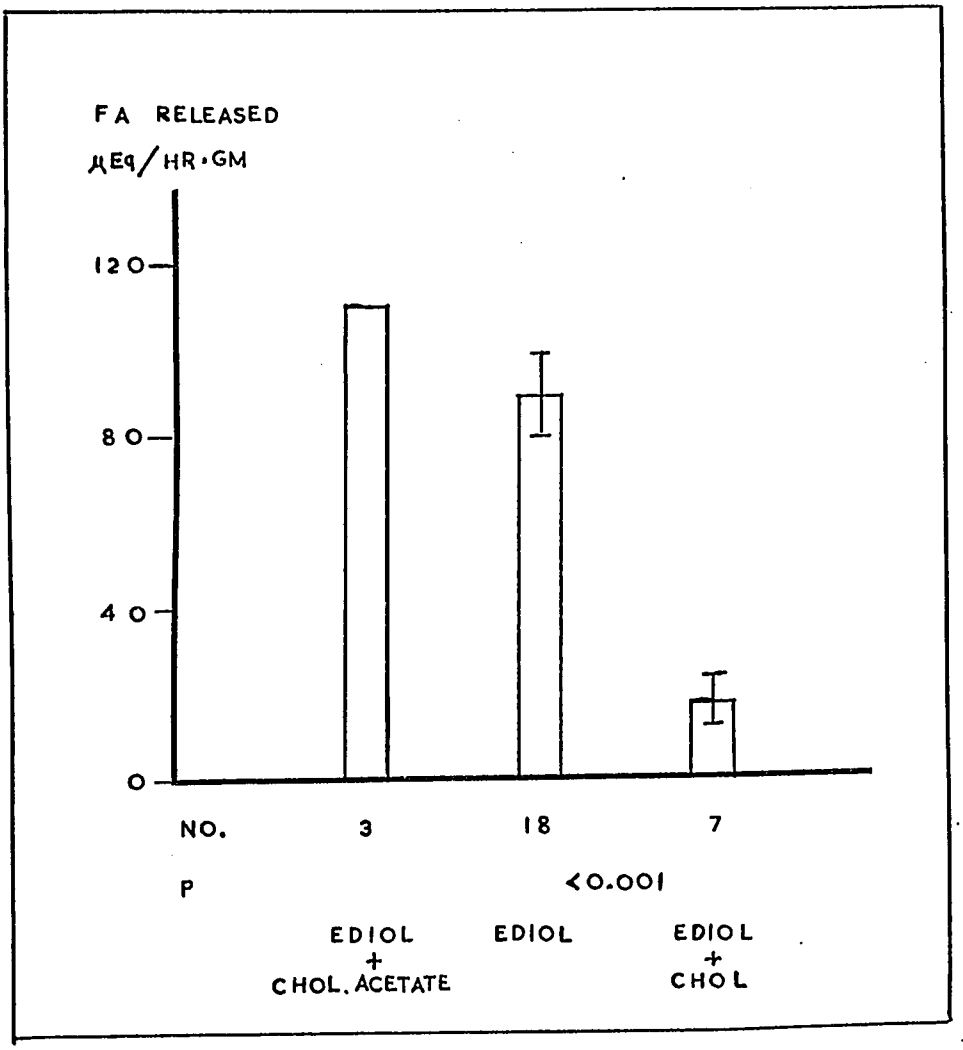



Fig. 8. LP-lipase Activities of the Heart Homogenates in the Presence of Cholesterol.

Fig. 9 LP-LIPASE ACTIVITIES IN THE PRESENCE OF CHOLESTERYL STEARATE OR LECITHIN

5.4 mg of cholesteryl stearate or lecithin was suspended in 0.6 ml of Ediol (5% pH 8.5) and incubated together with the control heart homogenate. For further details see previous Fig. 8.

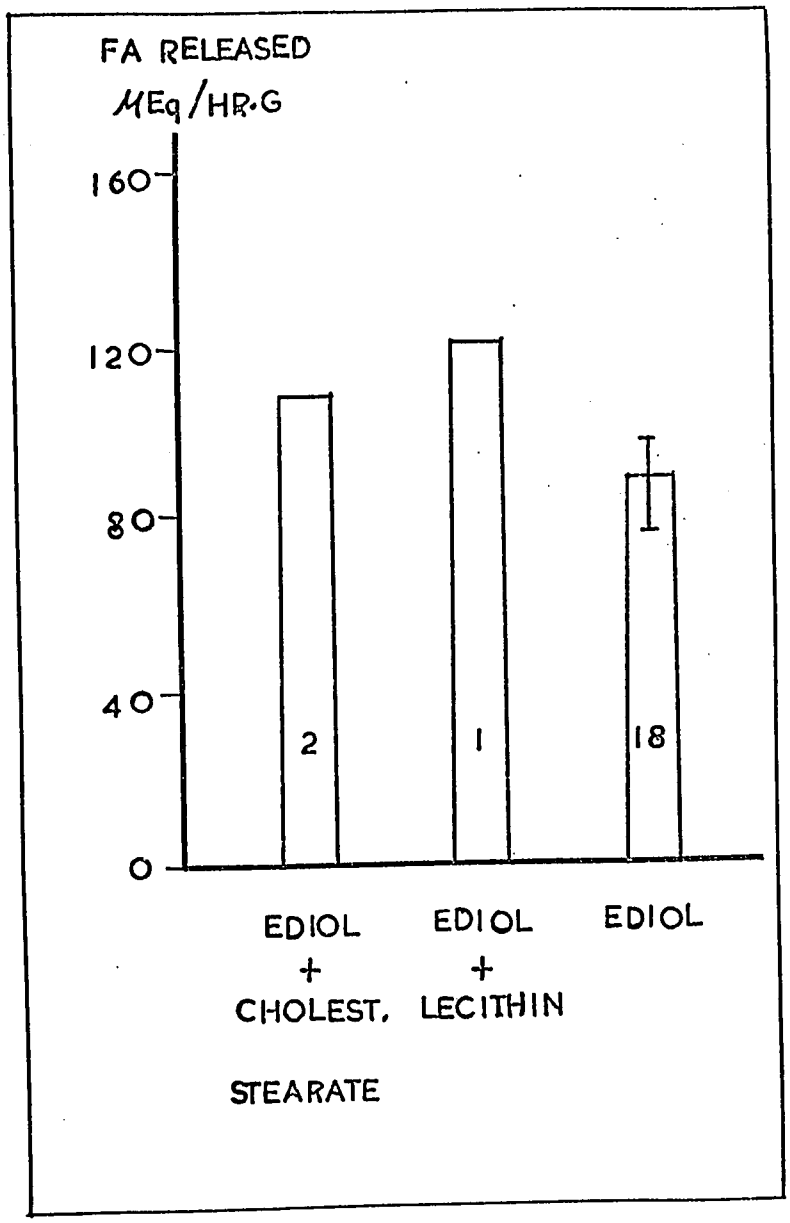



Fig. 9. LP-lipase Acitivities in the Presence of Cholesteryl Stearate or Lecithin.

Fig. 10 LP-LIPASE ACTIVITIES OF THE CONTROL RABBIT HEART HOMOGENATES MEASURED WITH EDIOL AS THE SUBSTRATE IN THE PRESENCE OF DIFFERENT AMOUNTS OF FREE CHOLESTEROL

1.8, 3.6 or 5.4 mg of free cholesterol was suspended in 0.6 ml of Ediol (5%, pH 8.5). The method of incubation and the further details see the legend to Fig. 8.

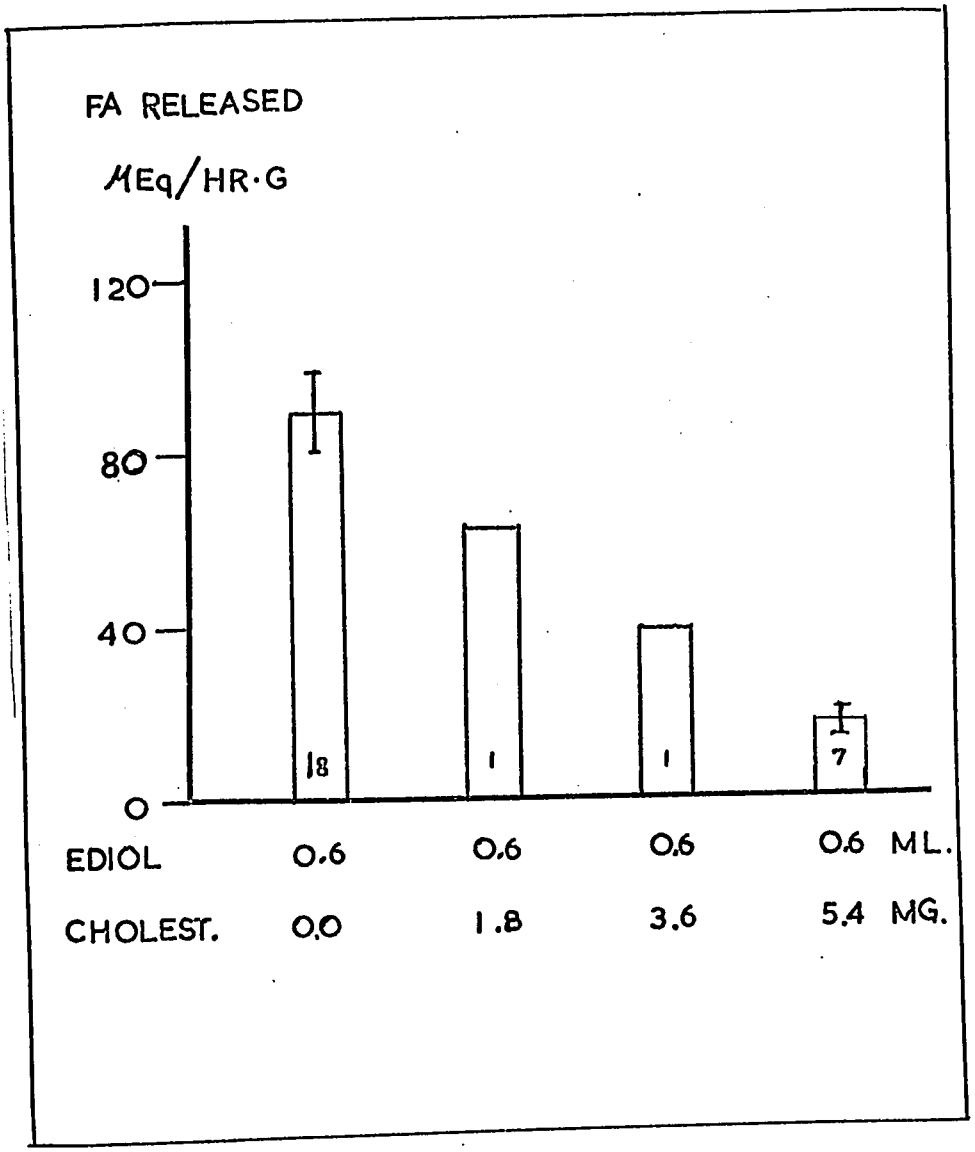
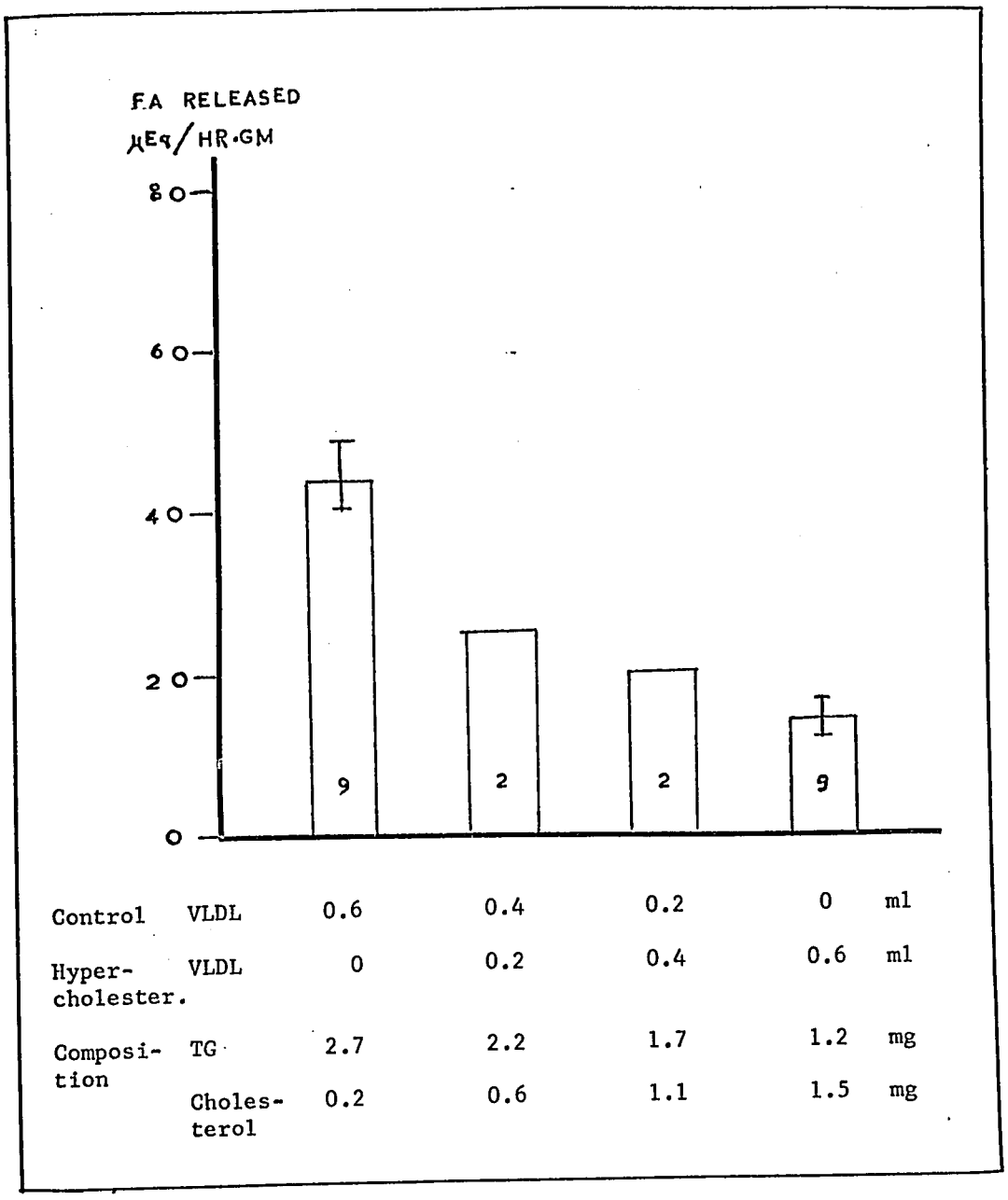



Fig. 10. LP-lipase Activities of the Control Rabbit Heart Homogenates Measured with Ediol as the Substrate in the Presence of Different Amounts of Free Cholesterol.

Fig. 11 LP-LIPASE ACTIVITIES OF CONTROL RABBIT HEART HOMOGENATES WITH VARIOUS VLDL MIXTURES AS SUBSTRATES

100 mg of the homogenate from control heart was incubated with 0.6 ml of substrate which consisted of 0.6, 0.4, 0.2 or 0 ml of the control VLDL and 0, 0.2, 0.4 or 0.6 ml of the hypercholesterolemic VLDL, respectively. The triglyceride (TG) and cholesterol (CHOL) content of these substrates are shown under individual columns. For the method of incubation and other details see the legend to Fig. 6.




| | | | | | | |
|---|---|---|---|---|---|---|
| Control | VLDL | 0.6 | 0.4 | 0.2 | 0 | ml |
| Hyper-cholester. | VLDL | 0 | 0.2 | 0.4 | 0.6 | ml |
| Composi-tion | TG | 2.7 | 2.2 | 1.7 | 1.2 | mg |
| | Choles-terol | 0.2 | 0.6 | 1.1 | 1.5 | mg |

Fig. 11. LP-lipase Activities of Control Rabbit Heart Homogenates with Various Very Low Density Lipoprotein Mixtures as Substrate.

TABLE V

THE COMPARISON BETWEEN RABBIT POST-HEPARIN PLASMA LP-LIPASE ACTIVITIES AND THE ACTIVITIES OF THE SAME ENZYME AFTER PURIFICATION

The enzyme was purified by flotation of the enzyme-substrate complex and its solubilization with deoxycholate. 1 ml of post-heparin plasma or purified enzyme was incubated with 2 ml of 5% bovine albumin (fraction V), 1 ml of 0.1 M Tris (pH 8.5), 1 ml of 5% Ediol (in Krebs-Ringer phosphate buffer, pH 8.5).

| | Volume (ml) | Total unit* | Total protein (mg) | Specific activity (unit/mg protein) | Yield (%) | Purification |
|---|---|---|---|---|---|---|
| Crude plasma | 60 | 604 | 2932 | 0.21 | | |
| Calcium phosphate eluate | 1 | 36 | 0.21 | 171.43 | 6.0 | x 816 |

* 1 unit catalyzes the release of 1 μEq FA/h from the lipoprotein in assay medium at 37°C.

Fig. 12 KINETICS OF THE INHIBITION OF LP-LIPASE BY THE FREE CHOLESTEROL

Post-heparin plasma LP-lipase was purified by flotation of the enzyme-substrate complex and its solubilization with deoxycholate. The purified enzyme was incubated with different concentrations of Ediol (contained 15, 30, 40, 45, 50 or 60 mg of triglyceride) in the medium which consisted of 5% albumin (fraction V), 0.1 M Tris (pH 8.5) and heparin. The final volume was adjusted to 5 ml with Krebs-Ringer phosphate buffer. FFA was determined at 0 and 1 hr of incubation at 37°C. 5.4 mg of free cholesterol or 4 mg of protamine sulfate was added to each of the incubation flasks for inhibition studies. The reciprocal of V (μEq FA released by the enzyme with 1 mg of protein in 1 hr) (ordinate) was plotted against 1/S (S = mg triglyceride in the incubation medium)(abscissa). Each point represents the average of 3 experimental results.

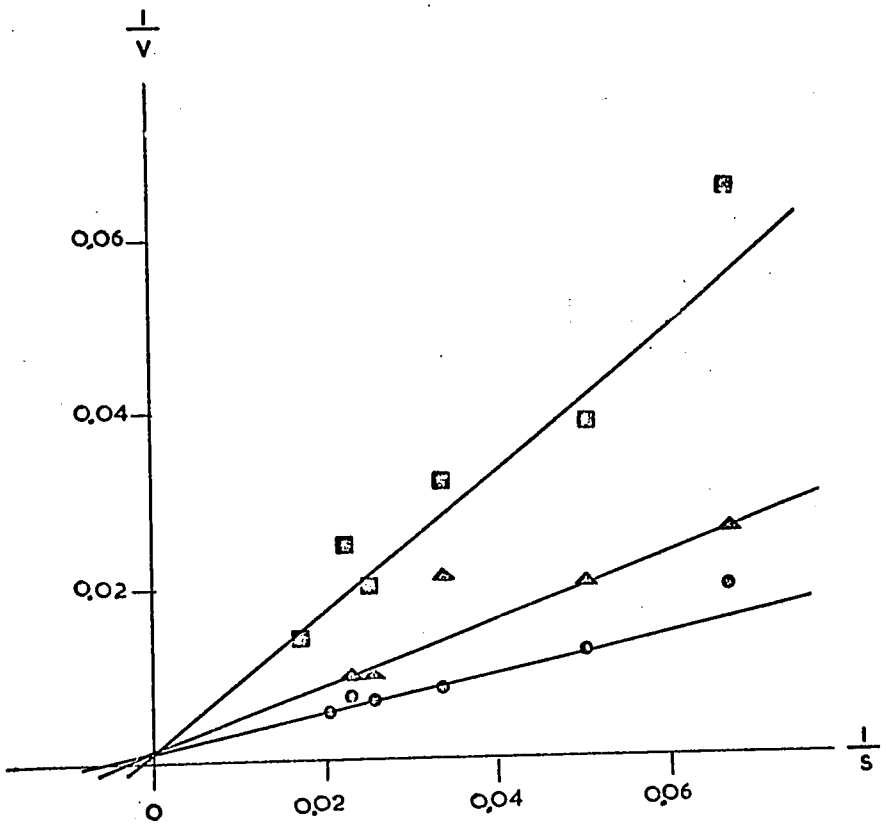



Fig. 12. Kinetics of the Inhibition of LP-Lipase by the Free Cholesterol.

$$\frac{1}{V} = \frac{1}{\mu Eq\ FA/\ HR \cdot MG\ PROTEIN}$$

$$\frac{1}{S} = \frac{1}{MG\ TG}$$

5.4 MG CHOLESTEROL

4.0 MG PROTAMINE

CONTROL

Fig. 13 THE CHANGES IN THE PLASMA LIPID LEVEL OF THE CONTROL AND HYPERCHOLESTEROLEMIC RABBITS IN RESPONSE TO HEPARIN-ADMINISTRATION

Injection of heparin (1 mg/kg BW) causes release of LP-lipase. Lipids were analyzed before (crossed bars) and after (black bars) the injection of heparin. The upper panel shows the results from control rabbits, the lower panel, hypercholesterolemic rabbits. The ordinate indicates mg% of total cholesterol (Cholest), phospholipid (PL) and triglyceride (TG), or μEq FFA/100 ml. Mean ± S.E.M. and p values are also shown.

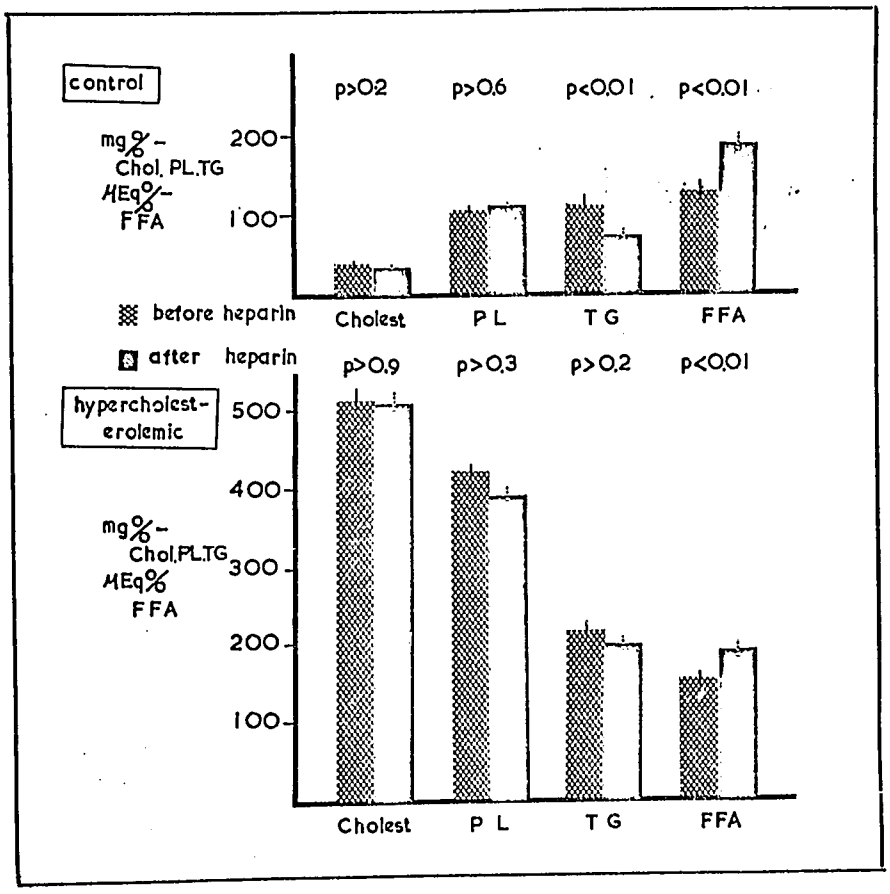



Fig. 13. The Changes in the Plasma Lipid Level of the Control and Hypercholesterolemic Rabbits in Response to Heparin-administration.

Fig. 14 THE TIME COURSE RELATIONSHIPS BETWEEN THE CHANGES IN PLASMA CHOLESTEROL LEVEL, PLASMA TRIGLYCERIDE LEVEL AND TRIGLYCERIDE TURNOVER RATE DURING CHOLESTEROL FEEDING

500 μc glycerol-2-$H^3$ was intravenously injected into a normal rabbit and the VLDL was prepared from the plasma. The known amount of radioactivity (about $19 \times 10^5$ dpm) of this VLDL was injected intravenously into rabbits on 0, 1.5, 7, 14, 21 or 49 days of high-cholesterol regimen. The fractional turnover and turnover rate were calculated from the rate of disappearance of radioactivity. The abscissa indicates the days on cholesterol diet. Plasma total cholesterol ●——● , free cholesterol ■——■ , phospholipid ▲——▲ , and triglyceride ●——● are shown in A and B. The values are expressed in mean ± S.E.M. The number of experiments was 30 at 0 days and the average of 5 in the rabbits after different periods of cholesterol diet. C, fractional turnover rate, n = 2. D, turnover rate of triglyceride in mg/hr, n = 2.

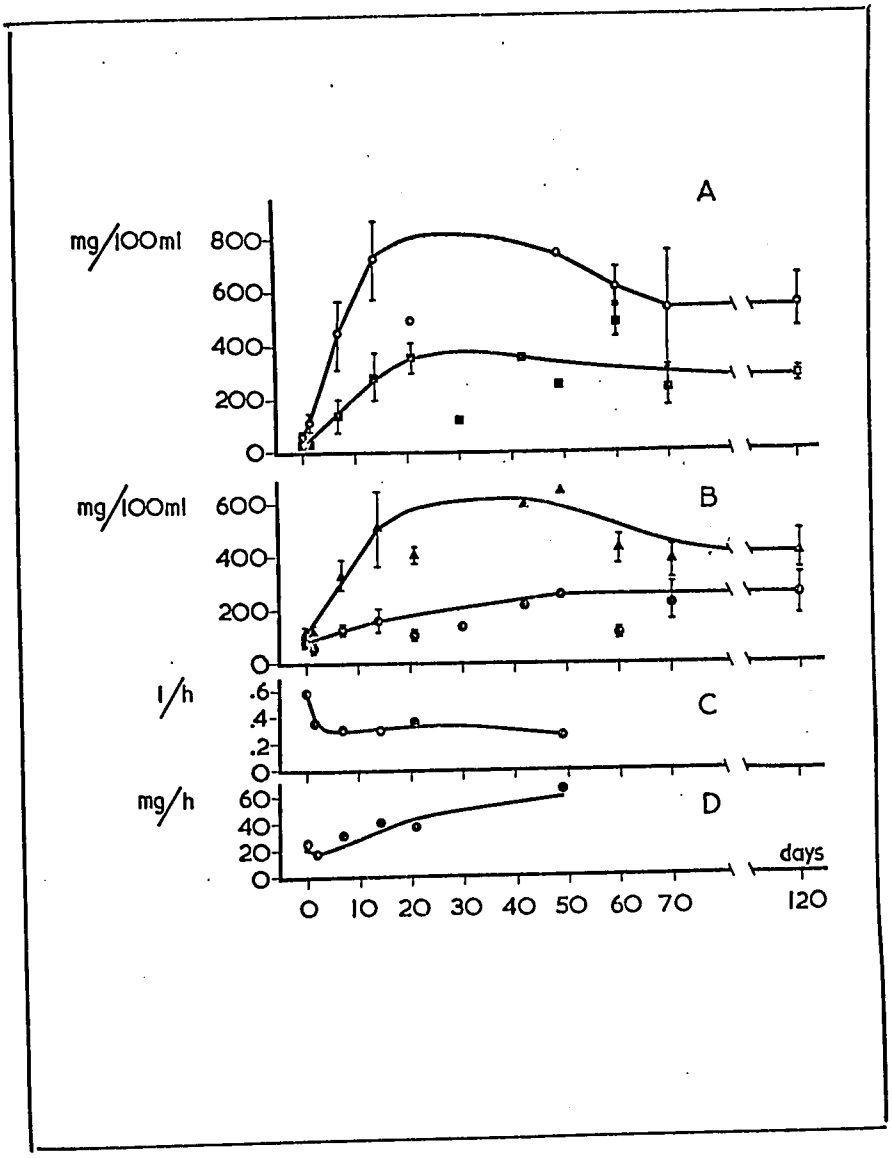



Fig. 14. The Time Course Relationships between the Changes in Plasma Cholesterol Level, Plasma Triglyceride Level and Triglyceride Turnover Rate during Cholesterol Feeding.

Fig. 15 THE TIME COURSE OF CHANGES IN TURNOVER RATE OF PLASMA TRIGLYCERIDE MEASURED BY USING GLYCEROL-2-$H^3$

80 μc of glycerol-2-$H^3$ was injected intravenously into rabbits on 0, 1.5, 7, 14, 42 or 70 days of high-cholesterol diet and turnover rate determined according to the method by Farquhar et al. (1965). E, fractional turnover rate, and F, turnover rate of plasma triglyceride in mg/hr. $n = 4$.

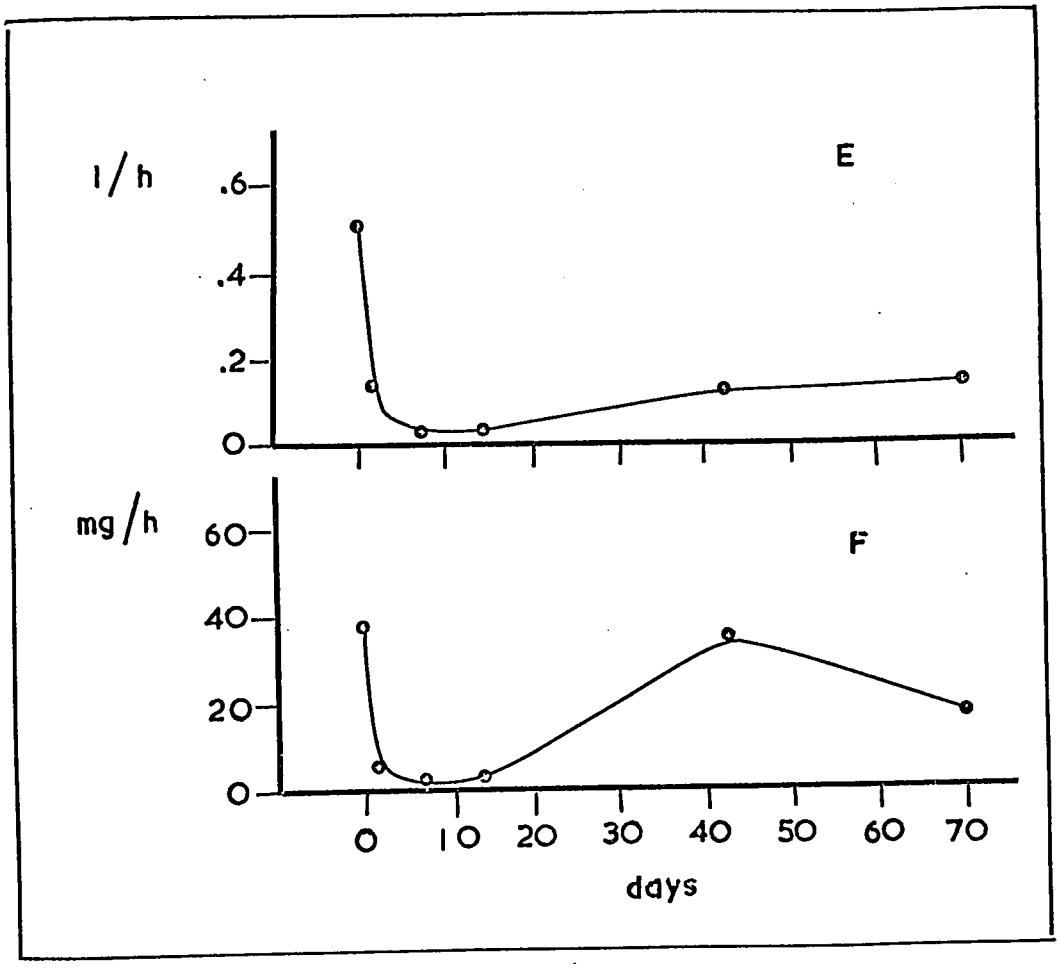



Fig. 15. The Time Course of Changes in Turnover Rate of Plasma Triglyceride Measured by Using Glycerol-2-$H^3$.

Fig. 16 DISAPPEARANCE OF TG-$H^3$ FROM THE PLASMA AFTER INTRAVENOUS INJECTION OF VLDL-$H^3$ IN CONTROL AND HYPERCHOLESTEROLEMIC RABBITS

The ordinate shows labelled triglyceride concentration in plasma (dpm/ml) on a log scale, and abscissa shows hours after injection. Solid lines are the second component of the disappearance curves which indicate largely the transfer of TG-$H^3$ from plasma to extrahepatic tissues. The dotted lines are the extrapolation to 0 time, $t_{\frac{1}{2}}$ can then be read on the graph, and the turnover time be calculated ($t_t = 1.44 \times t_{\frac{1}{2}}$).

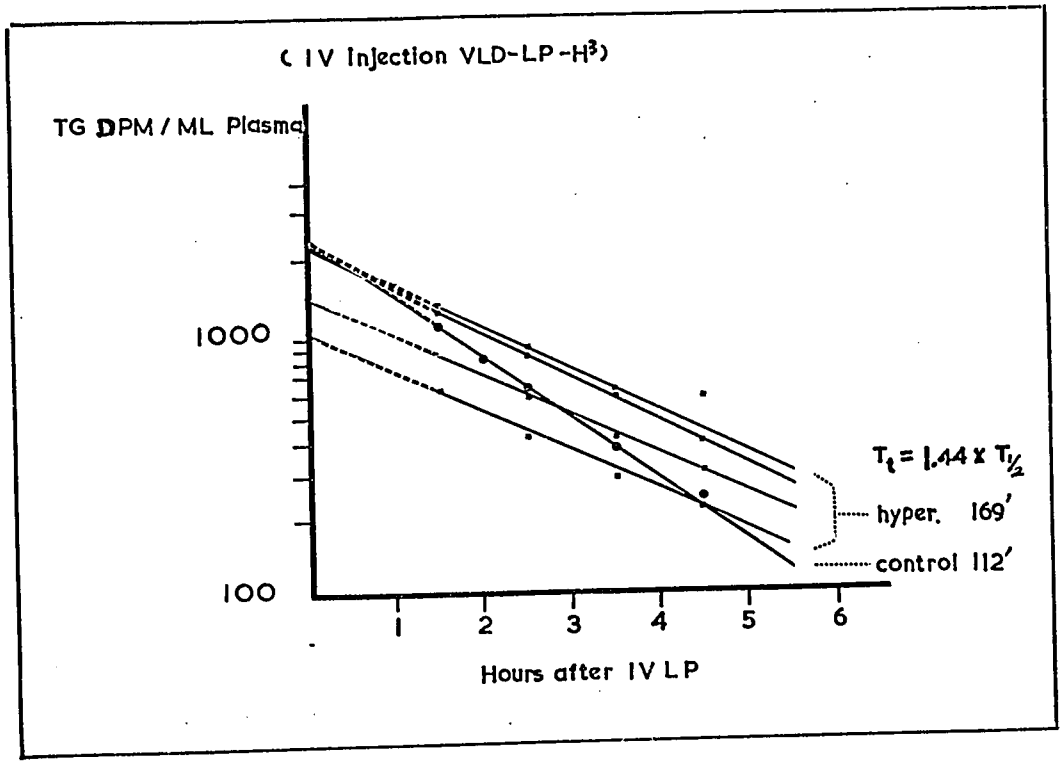



Fig. 16. Disappearance of TG-$H^3$ from the Plasma after Intravenous Injection of VLDL-$H^3$ in Control and Hypercholesterolemic Rabbits.

Fig. 17 ELECTRON MICROGRAPH OF MITOCHONDRIA OF A CONTROL RABBIT HEART

The mitochondria pellet was fixed with 1% $OsO_4$ in phosphate buffer (pH 7.4), embedded in Vestopal and sectioned on Reichert Ultramicrotome with a glass knife. Magnification - 78,000 x, Electron microscope - Phillips 100B, 60 KV.

There is no marked contamination with other subcellular elements. The mitochondria are intact.

* The author is deeply obliged for Dr. J. Metuzals and Mr. V. Redmond who prepared this picture.

Fig. 17. Electron Micrograph of Mitochondria of a Control Rabbit Heart.

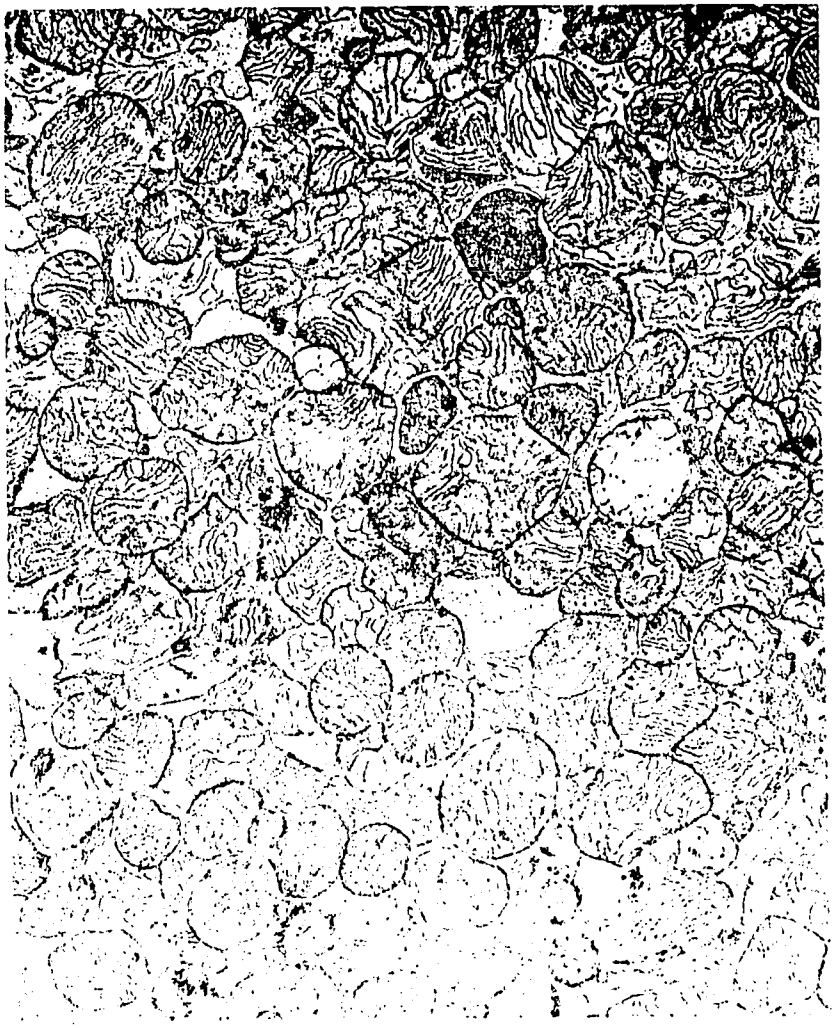


Fig. 17. Electron Micrograph of Mitochondria of a Control Rabbit Heart.

TABLE VI

RESPIRATORY FUNCTION OF RABBIT MITOCHONDRIA

The mitochondria were isolated by the method described in Figs. 18 and 20. The mitochondrial respiration was measured polarographically in a medium containing 15 mM KCl, 5 mM $MgCl_2$, 10 mM EDTA, 20 mM glucose, 30 mM phosphate buffer and 25 mM Tris (pH 7.4). Either 3 mM pyruvate plus 3 mM L-glutamate, 3 mM oxoglutarate or 8 mM succinate was added as a substrate. 0.1 ml (10 mg protein/ml) of the suspension of the subcellular elements was pipetted into the polarographic chamber containing the above mixture. As the phosphate acceptor, 0.2 mM ADP was used. The final volume was 2 ml and the temperature 25°C.

| | | Rabbit Heart | |
|---|---|---|---|
| | Substrate | Normal | Hypercholesterolemic |
| Respiratory control ratio | glutamate | 3.4 ± 0.1 | 3.8 ± 0.4 |
| | oxoglutarate | 2.8 ± 0.3 | 3.0 ± 0.3 |
| | pyruvate + malate | 3.3 ± 0.5 | 3.4 ± 0.3 |
| $QO_2$ (m microatom $O_2$ /mg protein/min) | glutamate | 90.7 ± 12.2 | 124.8 ± 13.7 |
| | oxoglutarate | 98.5 ± 10.9 | 125.4 ± 14.0 |
| | pyruvate + malate | 124.0 ± 11.4 | 144.4 ± 15.5 |
| P : O ratio | glutamate | 2.9 ± 0.2 | 2.7 ± 0.2 |
| | oxoglutarate | 2.6 ± 0.2 | 2.8 ± 0.1 |
| | pyruvate + malate | 2.8 ± 0.2 | 2.8 ± 0.2 |

| | | Rabbit Liver | |
|---|---|---|---|
| | Substrate | Normal | Hypercholesterolemic |
| Respiratory control ratio | succinate | 4.2 ± 0.2 | 5.8 ± 0.5 |
| $QO_2$ (m microatom $O_2$ /mg protein/min) | succinate | 66.0 ± 5.7 | 86.3 ± 4.9 |
| P : O ratio | succinate | 1.9 ± 0.6 | 2.1 ± 0.1 |

n = 6 (normal) and 10 (hypercholesterolemic). The values are expressed as mean ± S.E.M. p values of differences between two groups are all larger than 0.05, except that of the hepatic $QO_2$ which is $0.025 > p > 0.01$.

Fig. 18 CHANGES IN THE TOTAL CHOLESTEROL OF THE HEART SUBCELLULAR FRACTIONS IN RABBITS ON A HIGH-CHOLESTEROL DIET

The ventricles were cut into small pieces, passed through a stainless tissue press and homogenized in 10 volumes of 0.25 M sucrose with 0.002 M EDTA (pH 7.4). The nuclei, cell debris and myofibrils were separated from the mitochondria and microsomes by centrifuging at 500 x g for 5 min. The lower 2/3 of the precipitate was used as a source of myofibrils. The precipitate was rehomogenized and washed 3 times with KCl-borate-EDTA solution. Mitochondria were isolated by centrifuging the supernatant at 12,800 x g for 10 min and washed with the same medium. The microsomal and supernatant fractions were separated by centrifuging the post-mitochondrial supernatant for 120 min at 78,000 x g. All the fractions were suspended in the medium to give a concentration of approximately 10 mg protein/ml. Lipids were extracted with chloroform-methanol. The total cholesterol of control (white bars) and hypercholesterolemic (dark bars) hearts is expressed as μg/mg protein (ordinate) (mean ± S.E.M.).

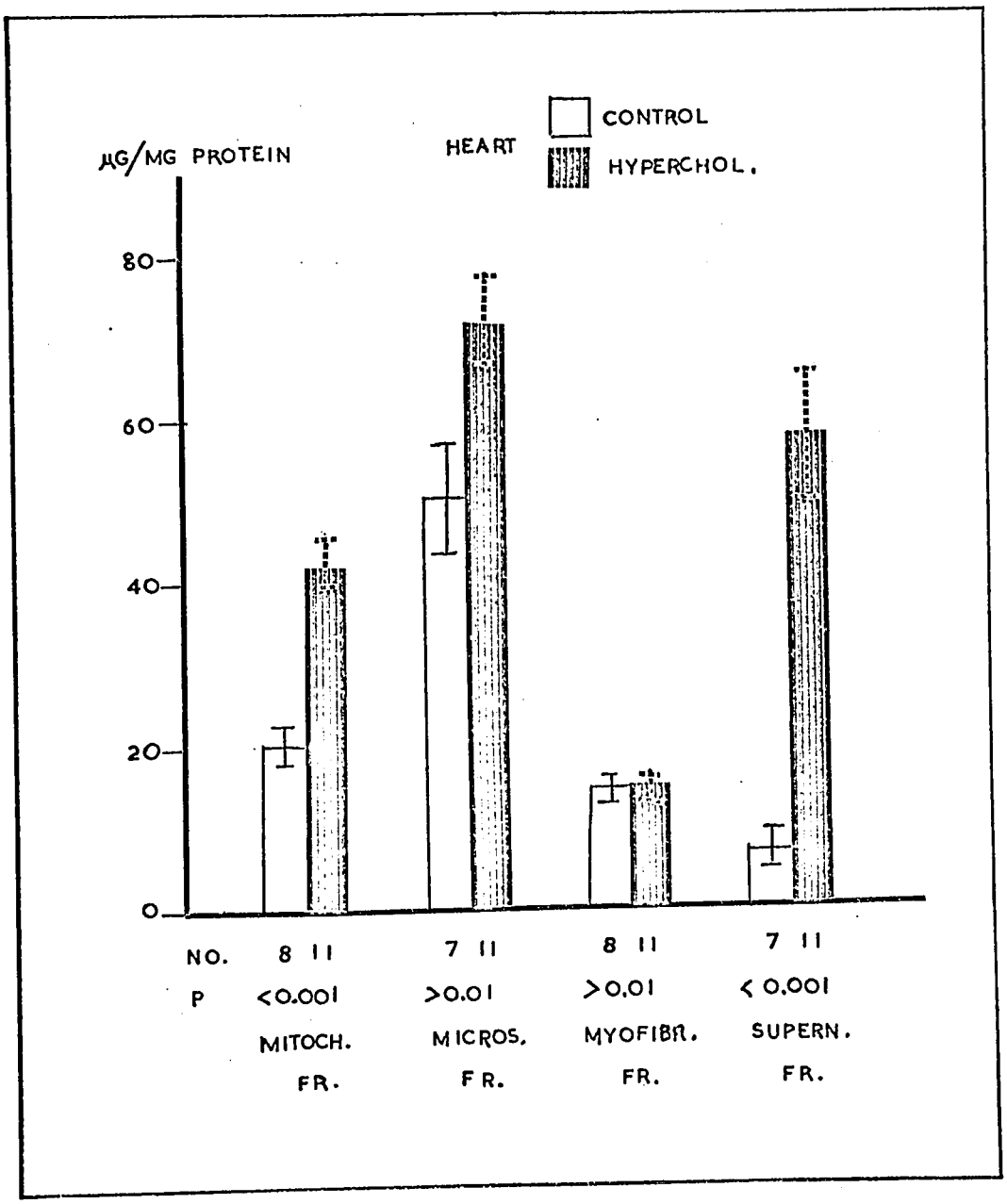



Fig. 18. Changes in the Total Cholesterol of the Heart Subcellular Fractions in Rabbits on a High-Cholesterol diet.

Fig. 19 CHANGES IN THE CHOLESTERYL ESTER OF THE HEART SUBCELLULAR FRACTIONS IN RABBITS ON A HIGH-CHOLESTEROL DIET

Free cholesterol was separated from the ester by digitonin precipitation and the cholesteryl ester content was calculated by subtraction (μg/mg protein, ordinate). For further details see legend for Fig. 18.

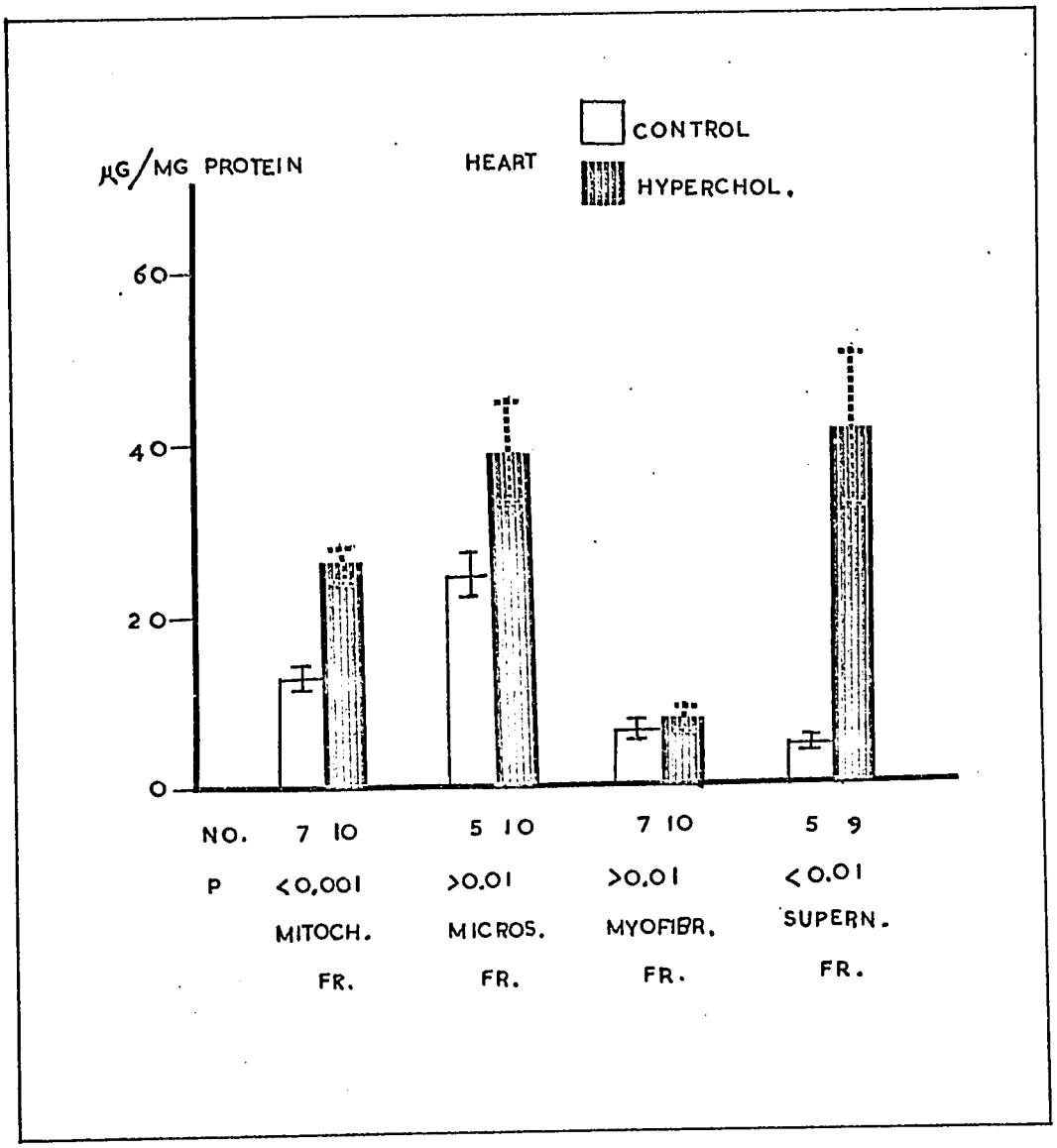



Fig. 19. Changes in the Cholesteryl Ester of the Heart Subcellular Fractions in Rabbits on a High-Cholesterol Diet.

Fig. 20 CHANGES IN THE TOTAL CHOLESTEROL CONTENT OF THE SUBCELLULAR FRACTIONS OF LIVER IN RABBITS ON A HIGH-CHOLESTEROL DIET

Liver was homogenized in 10 vol of the medium containing 0.25 M sucrose and 0.002 M EDTA (pH 7.4). A centrifugal force of 800 x g for 5 min was used to spin down the nuclei and cell debris, 8,200 x g for 10 min to precipitate the mitochondria, and 78,000 x g for 120 min to separate the microsomal fraction and the supernatant. The mitochondria were washed once with the homogenizing medium, then suspended in the same medium (10 mg protein/ml). Total cholesterol was determined according to Searcy and Bergquist (1960). The values are expressed as mean ± S.E.M. μg/mg protein (ordinate) for the control (white bars) and and hypercholesterolemic (dark bars) rabbits.

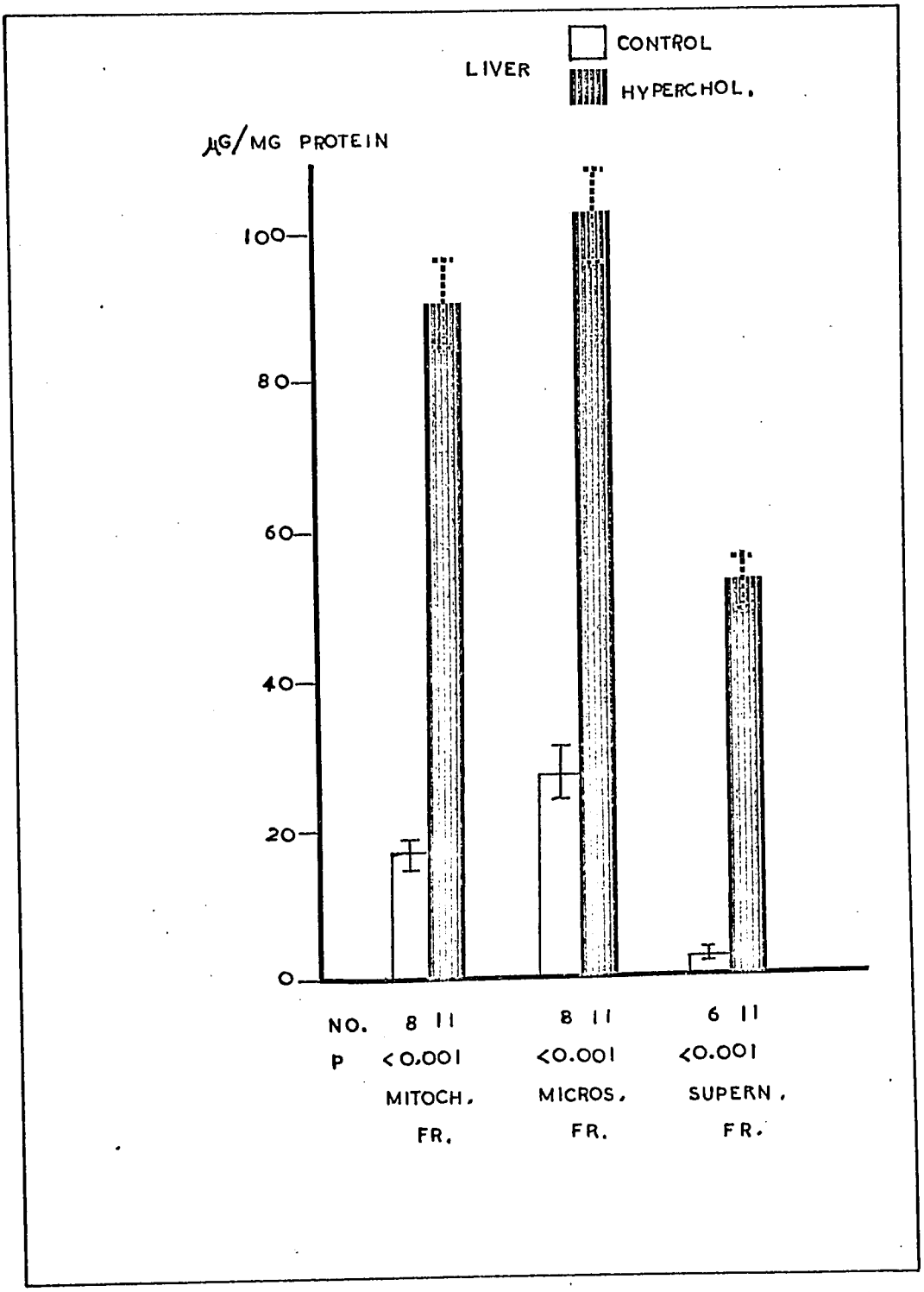



Fig. 20. Changes in the Total Cholesterol Content of the Subcellular Fractions of Liver in Rabbits on a High-Cholesterol Diet.

Fig. 21 CHANGES IN THE CHOLESTERYL ESTER CONTENT OF THE SUBCELLULAR FRACTIONS OF LIVER IN RABBITS ON A HIGH-CHOLESTEROL DIET

For explanation see Fig. 20.

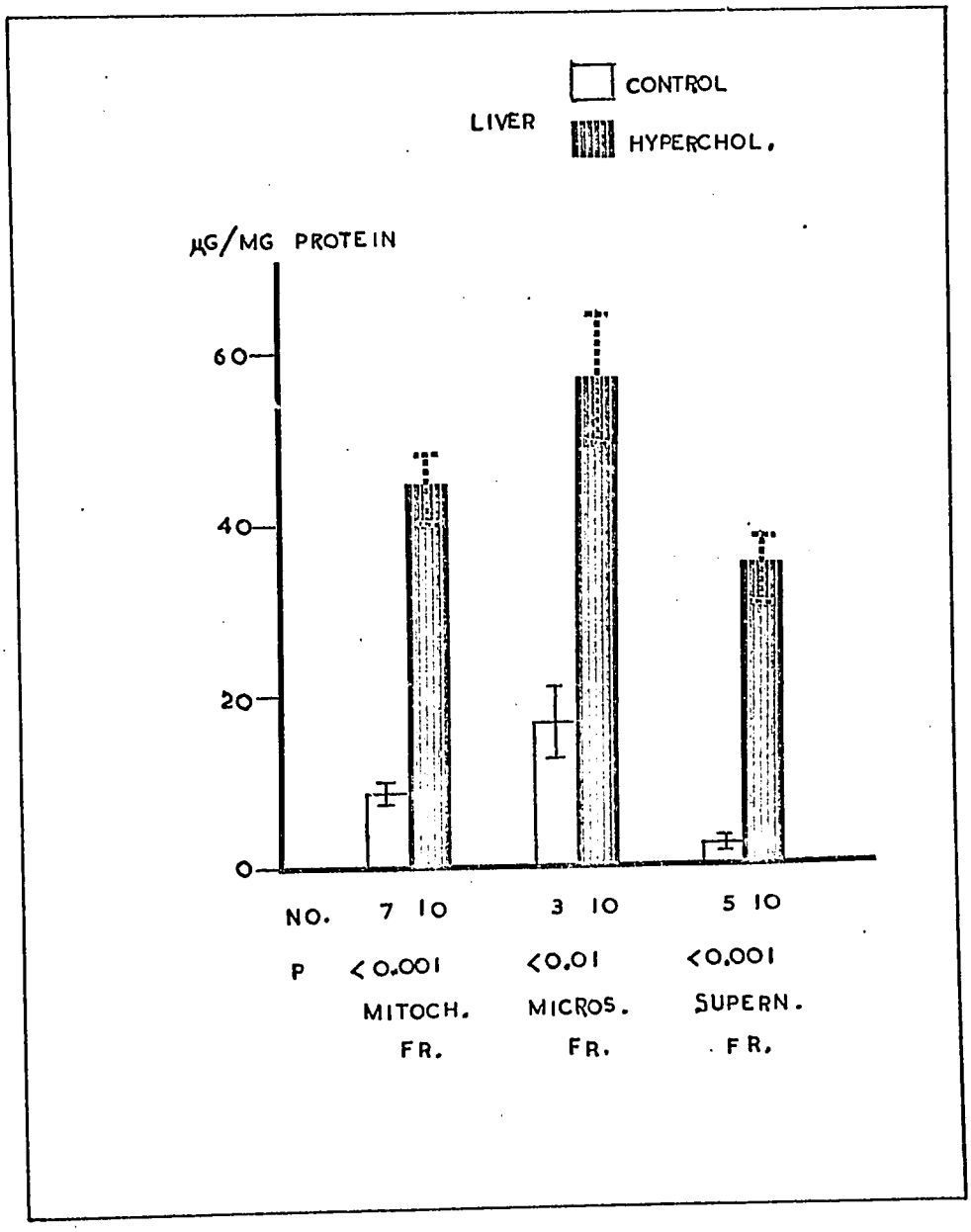



Fig. 21. Changes in the Cholesteryl Ester Content of the Subcellular Fractions of Liver in Rabbits on a High-Cholesterol Diet.

Fig. 22 COMPARISON OF PHOSPHOLIPID-P CONTENTS OF THE HEART BETWEEN SUBCELLULAR FRACTIONS IN CONTROL AND HYPERCHOLESTEROLEMIC RABBITS

The phospholipid was measured as orthophosphate in the lipid extract and expressed as phospholipid-P in μg/mg protein (ordinate) (mean ± S.E.M.). For further detail see Fig. 18.

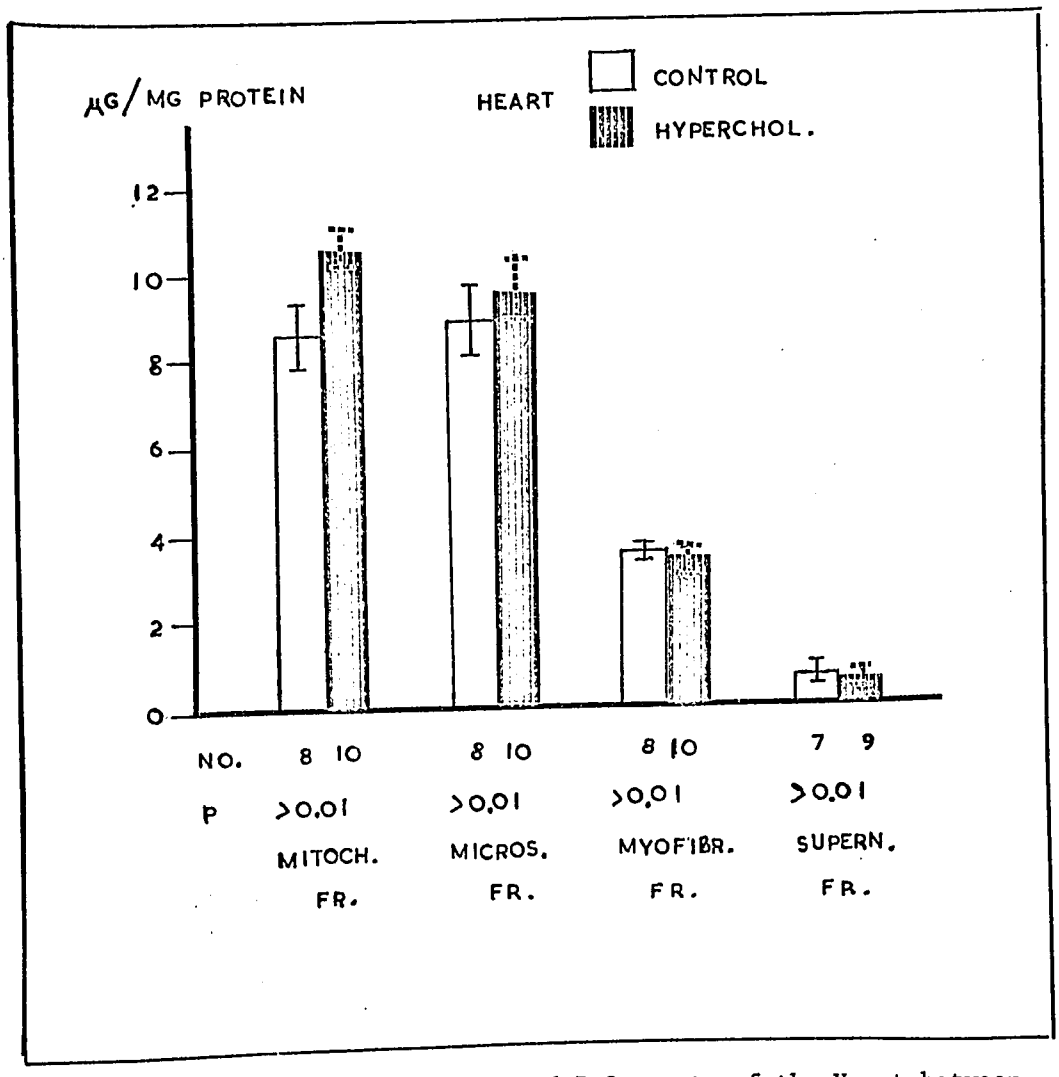



Fig. 22. Comparison of Phospholipid-P Contents of the Heart between Subcellular Fractions in Control and Hypercholesterolemic Rabbits.

Fig. 23 TRIGLYCERIDE CONTENTS OF CARDIAC SUBCELLULAR FRACTIONS IN CONTROL AND HYPERCHOLESTEROLEMIC RABBITS

Triglyceride was separated from phospholipid by the absorption with Florisil then determined colorimetrically. The values were expressed as μEq/mg protein (mean ± S.E.M.). For further explanation see Fig. 18

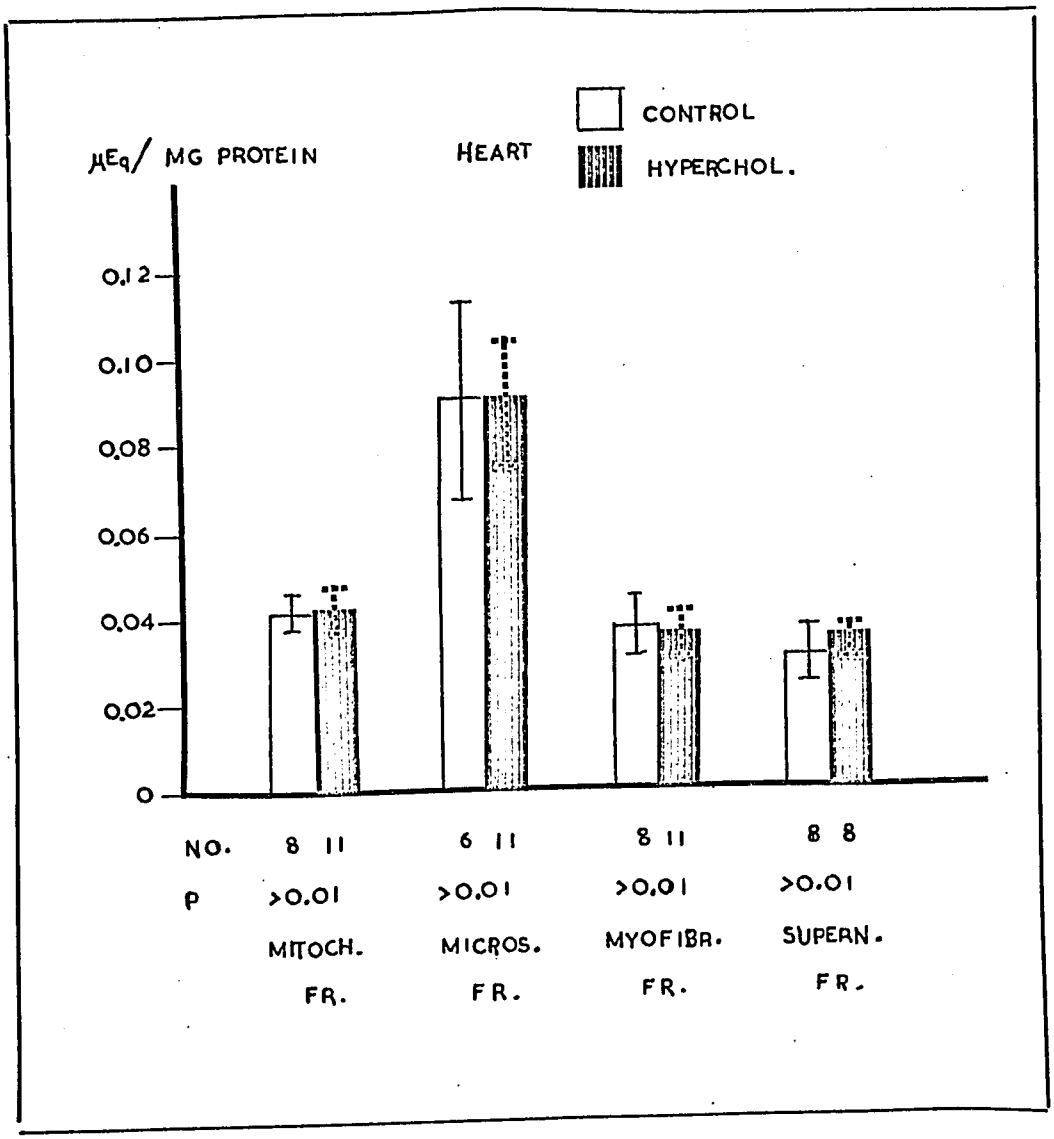



Fig. 23. Triglyceride Contents of Cardiac Subcellular Fractions in Control and Hypercholesterolemic Rabbits.

Fig. 24. COMPARISON OF THE PHOSPHOLIPID-P CONTENTS BETWEEN SUBCELLULAR FRACTIONS OF THE LIVER OF CONTROL AND HYPERCHOLESTEROLEMIC RABBITS

The phopholipid was measured as orthophosphate in the lipid extract and expressed as phospholipid-P in μg/mg protein (ordinate) (mean ± S.E.M.). For further detail see Fig. 20.

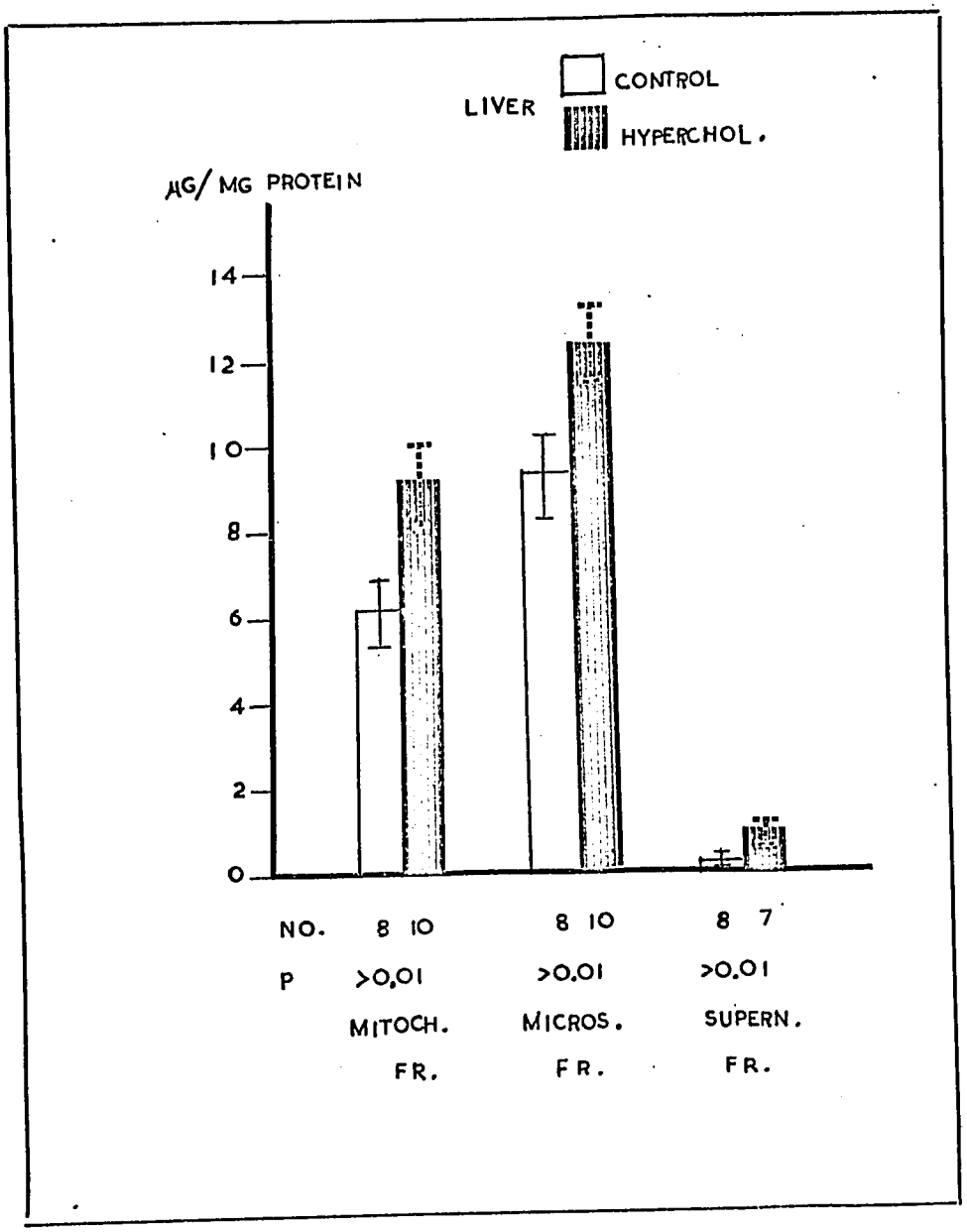



Fig. 24. Comparison of the Phospholipid-P contents between Subcellular Fractions of the Liver of Control and Hypercholesterolemic Rabbits.

Fig. 25 TRIGLYCERIDE CONTENTS OF HEPATIC SUBCELLULAR FRACTIONS IN CONTROL AND HYPERCHOLESTEROLEMIC RABBITS

Triglyceride was separated from phospholipid by the absorption with Florisil then determined colorimetrically. The values were expressed as μEq/mg protein (mean ± S.E.M.). For further explanation see Fig. 20.

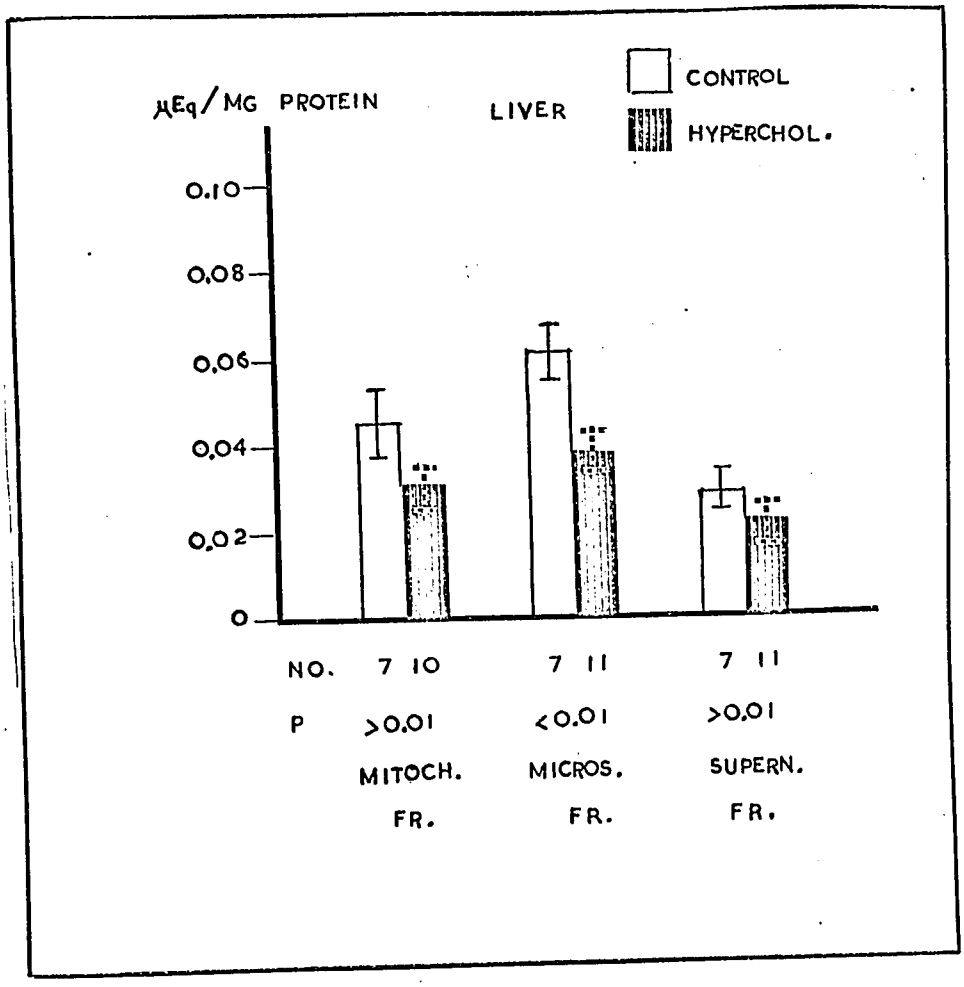



Fig. 25. Triglyceride Contents of Hepatic Subcellular Fractions in Control and Hypercholesterolemic Rabbits.

# CHAPTER IV

## DISCUSSION

This study was an attempt to elucidate the mechanism of changes in triglyceride metabolism which is accompanied by hypercholesterolemia of rabbits fed a high cholesterol diet. The results will be discussed under five major subsections: the change in plasma lipid levels, the change of lipid synthesis in liver, LP-lipase activities, plasma triglyceride turnover and the lipid composition of subcellular fractions.

### A. THE CHANGE IN PLASMA LIPID LEVEL

The plasma level of cholesterol, phospholipid and triglyceride in the normal rabbits used in this study are within the ranges reported previously (Boyd, 1942; VanHandel et al., 1957). Plasma FFA measured by a modified Dole's procedure showed large variations (from 196 μM/l to 1369 μM/l) (Trout et al., 1960). This is in part due to the difficulty in reaching a reliable end point during titration. The method of Goss and Lein (1967) was used in my experiments to measure the plasma and tissue level of FFA and therefore reliable end points were obtained. The plasma FFA of the fasting control rabbits showed a small standard error (1331 ± 130 μM/l).

Cholesterol fed rabbits showed, in two months, a significant increase in plasma cholesterol and its esters, phospholipid and

triglyceride (Table I).

1. EXCESS DIETARY CHOLESTEROL AS THE PRIMARY CAUSE OF OTHER METABOLIC CHANGES

A high cholesterol diet was the only difference between our experimental and control rabbits. No vegetable oil or other form of fat was added to the rabbit pellets. The experimental rabbits consumed no more food than the control group, therefore the plasma lipid changes are a direct consequence of the extra amount of cholesterol in the diet.

As mentioned in the "Historical Review", many varieties of diet have been used by previous investigators to induce hypercholesterolemia in rabbits. When cholesterol was included in the diet, the content of cholesterol was also variable; for example, Scebat et al.(1961) reported that, when rabbits were fed only 0.25 g of cholesterol per day, the magnitude of atherosclerotic changes was almost as severe as when 1 g of cholesterol per day was fed, even though the level of serum cholesterol was only half as high. Nothing but cholesterol was given in my experiments in order to avoid complicating the results by feeding oil. Three per cent (w/w) cholesterol was added to the rabbit pellets, in order to obtain an acute effect on both plasma and tissues.

The plasma level of neutral fat has been reported to be higher than 600 mg% after one month of cholesterol feeding in

rabbits (Weinhouse and Hirsch, 1940; Wang et al., 1954). Fatty acids of neutral fat in plasma and the amount of $S_f$ 100-400 LP were also reported to increase in these rabbits (Horlick and Lyman, 1954; Kritchevsky, et al., 1954). In this study the elevation of plasma triglyceride level was statistically significant (Table I), although it was not as high as levels reported by Weinhouse and Hirsch (1940) and Wang et al. (1954).

2. THE MECHANISMS OF CHOLESTEROL INDUCED HYPERTRIGLYCERIDEMIA

An increased plasma level of triglyceride is either due to an increase in the rate of entry of triglyceride into the plasma or to a decrease in the rate of exit of triglyceride from the plasma. The triglyceride enters the blood circulation from three sources; intestinal absorption, hydrolysis of adipose triglyceride and their subsequent esterification in the liver, and synthesis by the liver. An increase in any one of these sources may cause hypertriglyceridemia. A decrease in the rate of exit of triglyceride can be due to a physicochemical effect between the lipids in the plasma or due to a decrease in triglyceride uptake by the tissues. The high cholesterol diet and the subsequent elevation of plasma cholesterol can induce hypertriglyceridemia through one or more of these mechanisms.

## B. THE RATE OF ENTRY OF TRIGLYCERIDE INTO THE PLASMA

### 1. TRIGLYCERIDE SYNTHESIS, ESTERIFICATION AND SECRETION BY THE LIVER

The liver is the major source of endogenous plasma triglyceride. The latter is released into the blood stream in the form of LP (Borgstrom and Olivecrona, 1961; Havel et al., 1962). Therefore, increased plasma triglyceride in hypercholesterolemic rabbits could be due to an increased secretion of LP, increased synthesis of LP, increased fatty acid esterification or increased fatty acid uptake or synthesis in this organ.

There was no significant difference in esterification of palmitate-1-$C^{14}$ by control or hypercholesterolemic rabbit liver in my experiments. Also there was no difference in triglyceride synthesis from acetate-1,2-$C^{14}$. These results suggest that over-production of triglyceride by the liver of the rabbits with hypercholesterolemia is unlikely.

The incorporation of palmitate-1-$C^{14}$ into the triglyceride of the liver (21.4-37.7%) was comparable to that reported by Tietz and Shapiro (1956). On the other hand, these experiments were performed by the use of liver homogenates or slices and not by the use of liver perfusion. Therefore, the imbalance between the triglyceride synthesis and the secretion by the liver cannot be detected. Indeed, there are conditions where the liver may synthesize the normal amount of triglyceride but may not secrete enough of this into the blood (fatty liver formation). However,

there has been, to my knowledge, no evidence showing that the unchanged liver synthesis of triglyceride is accompanied by a disproportionately high secretion into the blood. Unless the capacity of hepatic synthesis measured by conventional methods is far in excess of the normal rate of hepatic triglyceride output, there would be no room for regulation of triglyceride synthesis by triglyceride output by the liver. This is supported by the fact that the turnover rate of triglyceride in the liver differs among species: that of man is much faster while that of dog and rabbit is much slower than the corresponding plasma triglyceride turnover (Farquhar et al., 1965). In my experiments, triglyceride synthesis in the liver of hypercholesterolemic rabbits was within the normal range. The triglyceride content of the liver and Plasma FFA levels also showed no change. Therefore it is unlikely that the increase in plasma triglyceride is due to an increase in secretion by the liver.

The results of triglyceride turnover study showed an increase in turnover rate in the later period of the experiment (Fig. 14). This increase signifies an increase in both triglyceride removal out of and triglyceride entry into the plasma. Since the major source of triglyceride in the fasting state is the liver, the above result suggests that an increase in triglyceride synthesis by the liver is a possibility.

The discrepancy between these two results, the hepatic

triglyceride synthesis and plasma triglyceride turnover, may be due to the difference between the in vitro and in vivo experiment. The homogenates or slices of the liver were used for the determination of the capacity of hepatic triglyceride synthesis. As discussed above, in vitro experiments indicate maximal capacity of a tissue; i.e. in this instance, the highest synthesis activity, whereas the turnover study indicates an actual rate of synthesis. This would be much smaller than the former. The supply of FFA to the liver and the hepatic triglyceride content, although they individually did not show great change, might have influenced the results collectively.

2. INTESTINAL ABSORPTION OF TRIGLYCERIDE IN RABBITS ON A HIGH CHOLESTEROL DIET

The second source of plasma triglyceride is by intestinal absorption. The absorbed lipids react with a small amount of protein to form chylomicron in the intestinal mucosal cell then go via the thoracic lymphatic system to the blood stream. The triglyceride in the chylomicron is rapidly taken up by the liver and most of the extrahepatic tissues with a half time of 5 to 15 min (Dole and Hamlin, 1962; Hartroft et al., 1963; Bragdon and Gordon, 1958). Therefore, in the post-prandial state, the plasma triglyceride level can be influenced by the fat content of the diet and the rate of uptake of triglyceride by the tissues (Havel and Gordon, 1960). If the elevated triglyceride in the plasma of the cholesterol fed rabbits comes from the intestine, either the fat content of the diet must have been high or the uptake of the

chylomicron triglyceride by the tissues must have been low. The experimental rabbits did not receive extra dietary fat (triglyceride content 1%, w/w) and there was no evidence that cholesterol feeding might promote triglyceride absorption. Therefore, the former factor can be ruled out. However, the latter possibility, the decrease in the uptake of chylomicron triglyceride by the tissues, has been examined in this study by the determination of LP-lipase activities of the extrahepatic tissues. This was done because LP-lipase was found to be important for the uptake of chylomicron triglyceride by extrahepatic tissues (Havel and Gordon, 1960). The experimental results showed that free cholesterol may inhibit LP-lipase activity and thus the decrease in uptake of chylomicron triglyceride by extrahepatic tissues could be one of the mechanisms of cholesterol induced hypertriglyceridemia. The same mechanism has been described in TypeI hyperlipoproteinemia by Fredrickson et al. (1967) in the human being. The uptake of chylomicron triglyceride by the liver will be discussed in the section on the hepatic uptake of plasma triglyceride.

A high-carbohydrate diet could have increased the plasma triglyceride level, since the response of practically all subjects to high-carbohydrate feeding is to convert much of the carbohydrate to triglyceride in the liver and eventually release it into the plasma as pre-β LP to increase the plasma triglyceride level (Fredrickson et al., 1967). This is the mechanism responsible for carbohydrate-induced (Type IV) hyperlipoproteinemia. However, this could not have occurred

in the experimental rabbits because they consumed no more food than did the control animals.

3. THE HYDROLYSIS OF ADIPOSE TISSUE TRIGLYCERIDE IN THE HYPERCHOLESTEROLEMIC CONDITION

There is a possibility that the increased plasma triglyceride is secondary to an increased hydrolysis of adipose tissue triglyceride. According to Reshef et al. (1958), there is little evidence for the direct release of triglyceride from isolated adipose tissue in vitro. The transformation and release of adipose tissue triglyceride to plasma triglyceride occurs by way of the liver (Duncan, 1964). Fat mobilized from the storage tissues circulates as FFA and is taken up by liver and muscle (Bragdon and Gordon, 1958; Laurell, 1959). The amount of FFA extracted by the liver is proportional to the concentration of FFA in the portal blood (McElroy et al., 1960). An increased FFA uptake leads to increased triglyceride synthesis in the liver, (Gidez et al., 1962; Nestel et al., 1963A). Therefore, if the increase in plasma triglyceride in hypercholesterolemic rabbits was due to an increase in hydrolysis of adipose tissue triglyceride, there would be an increase in plasma and hepatic FFA. However, in my experiments there was no increase in FFA in the plasma or liver of the hypercholesterolemic rabbits (Fig. 4). This argues against the possibility that the increased plasma triglyceride in these rabbits originated from the adipose tissue.

## C. THE RATE OF REMOVAL OF TRIGLYCERIDE FROM PLASMA

The plasma triglyceride is removed by the liver and the extrahepatic tissues. Since triglyceride input into the plasma compartment appears unchanged in the hypercholesterolemic rabbit, the increase in plasma triglyceride must be due to a decrease in the rate of removal of triglyceride from the plasma by these tissues.

### 1. THE UPTAKE OF PLASMA TRIGLYCERIDE BY EXTRAHEPATIC TISSUES IN HYPERCHOLESTEROLEMIC RABBITS

Bezman (1962A), Bragdon (1954), Garfinkel (1967) and their associates provided evidence that the LP-lipase is essential for the hydrolysis and uptake of plasma triglyceride by the extrahepatic tissues. For this reason, LP-lipase activities of the heart, adipose tissue and plasma were determined. Heart homogenates were used as sources of LP-lipase in most of my experiments, because the LP-lipase activity in the heart is high (Korn, 1955A; Cherkes et al., 1960) and hence its depression was easy to assess.

a. The possible factors which may change the LP-lipase activities - The LP-lipase was assayed by using mixed substrates, i.e., a commercial triglyceride emulsion containing monoglyceride, or VLDL. Thus, the activity expressed in the results is the activity of LP-lipase composed of tri-, di- and monoglyceridases (Greten et al., 1969). The activity of other lipases reported to exist in heart tissue should not be of great influence because of the

unfavourable assay condition for them (pH=8.5) (Borensztajn et al., 1970).

The principle of the enzyme assay is to measure the FFA released from triglyceride by the hydrolytic action of this enzyme. The change of activity can be due to the change of quantity or quality of the enzyme or due to the change of the characteristics of the substrate. For example, simple triglyceride emulsions are hydrolyzed at an extremely slow rate by LP-lipase prepared from tissues but become readily available as substrate in the presence of plasma LP (Korn, 1955B). This was shown by incubating α-LP together with triglyceride emulsions. The resultant complex was rapidly hydrolyzed by the enzyme (Korn and Quigley, 1957). This specificity was apparently due to the formation of an enzyme-substrate complex, LP-lipase forming a firm complex with both chylomicrons and activated triglyceride emulsions but not with simple triglyceride emulsions.

The change of LP-lipase activity may also be due to the presence of an enzyme-inhibitor. An inhibitor has been reported to be present in normal plasma and has been partially purified (Hollett and Meng, 1957). An increase in platelets, leukocytes or heparin may also inhibit LP-lipase activity (Scanu, 1965).

b. Unchanged LP-lipase activity with Ediol or normal VLDL as the substrate - Heart LP-lipase was determined by the method of Alousi and Mallov (1964). The heart was homogenized in KRP

buffer (pH 8.5). Mallov and Cerra (1967) showed that in control rats the cardiac LP-lipase activity prepared in Krebs-Ringer bicarbonate buffer was 354 ± 49 μEq FFA/g of dry tissue per 1 hr of incubation, while in Krebs-Ringer phosphate buffer it was 349 ± 35 μEq FFA/g. Thus, phosphate appeared not to inhibit LP-lipase activity contrary to the view of Korn (1955A). Inhibition studies by Mallov and Cerra (1967) indicated that this assay method determines the activity of lipase in heart that is largely, if not entirely, LP-lipase. It should be noted that, as mentioned above, LP-lipase is defined here to include mono-, di- and triglyceride lipase.

When Ediol or normal VLDL was used as the substrate, the heart LP-lipase activity of the hypercholesterolemic rabbits was significantly higher than that of the control. These results suggest that the increase in plasma triglyceride in the hypercholesterolemic rabbits is not due to the decrease in triglyceride disposal by inactive or a low level of LP-lipase.

c. Decrease in LP-lipase activity measured with hypercholesterolemic VLDL as the substrate - LP-lipase either of normal or of the hypercholesterolemic heart showed significantly depressed activity when hypercholesterolemic VLDL was used as the substrate. It appears that the hypercholesterolemic VLDL has an inhibitory effect on the hydrolytic action of the enzyme or has a different structure which resists the hydrolytic action of enzyme.

The naturally occurring LP-lipase inhibitors such as platelets and leukocytes were not determined in control and hypercholesterolemic VLDL, since these particles would not be likely to appear in the same zone as VLDL after high speed centrifugation.

In my experiment, optimal amounts of heparin were added to each incubation medium (Korn, 1955A) in order to release the LP-lipase from the tissue into the incubation medium and to stabilize the enzyme (Korn, 1955A; Cherkes and Gordon, 1959). A high concentration of heparin has been reported to inhibit LP-lipase activity (Gartner and Vahouny, 1966). However, the optimal amount of heparin used does not cause the inhibition of LP-lipase activity (Gartner and Vahouny, 1966).

Although numerous other inhibitors of LP-lipase have been described, such as a high concentration of NaCl or divalent cations, protamine sulfate, diisopropyl fluorophosphate or tetraethyl pyrophosphate, they are not naturally occurring substances and could not have caused inhibition in my experiments.

d. The differences between VLDL of hypercholesterolemic and normocholesterolemic rabbits - The lipid composition of these two lipoproteins is quite different. The LP from the rabbits on a high cholesterol diet contained significantly greater amounts of free and esterified cholesterol and phospholipid than did the control. Thus, the depressed activity of LP-lipase could be due to the

presence of the high concentration of these lipids in the incubation medium.

e. Mechanism of the inhibitory effect of lipids - Free cholesterol, cholesterol acetate, cholesterol stearate or lecithin were added individually to the substrate of LP-lipase to test which of these lipids caused the decrease in enzyme activity. Ediol was used as the substrate in the presence of relatively large amount of one of these lipids. It was found that free cholesterol was the only lipid to cause significant depression in LP-lipase activity (Fig. 8 and 9). There was sufficient triglyceride in Ediol for hydrolysis during the incubation lasting one hour. Thus, it seems reasonable to conclude that the observed decrease in LP-lipase activity was due to the high concentration of free cholesterol in the plasma of the rabbit fed a high cholesterol diet. This would have been followed by both a decrease in hydrolysis of triglyceride and a decreased uptake by the tissues, resulting in an accumulation of triglyceride in the plasma.

Acetone was used to dissolve the lipid in aqueous Ediol. Although the properties of Ediol and LP-lipase would not be changed by the acetone according to the data of Quarfordt et al. (1966) and Korn (1959), an attempt was made to evaporate the acetone prior to the addition of enzyme. Therefore, the decrease in LP-lipase activity observed in the presence of free cholesterol was

not due to the effect of acetone. Furthermore, the cholesterol acetate and stearate prepared in the same way, did not have an inhibitory effect.

f. Relationship between the extent of inhibition and the amount of cholesterol - In my experiments, various amounts of free cholesterol were suspended in the same amount of Ediol. In addition, hypercholesterolemic and control LP were mixed in various proportions and used as the substrate. It was found that the inhibitory effect was proportional to the amount of free cholesterol present. The higher the free cholesterol content of the mixture, the lower were apparent activities of LP-lipase. When the amount of enzyme was high (e.g. in the heart of hypercholesterolemic rabbits), the activity was relatively high in the presence of a given amount of cholesterol, as compared to situations when the amount of enzyme was low (e.g. in the heart of normocholesterolemic rabbits). In other words, there appears to be interdependence between the enzyme (LP-lipase), substrate (triglyceride) and inhibitor (cholesterol).

g. The kinetics of cholesterol inhibition of LP-lipase - The mode of inhibition of LP-lipase by free cholesterol was studied by measuring the rate of the enzyme catalysed reaction in the presence of inhibitors. There are three types of enzyme inhibitors, competitive, noncompetitive and mixed. These can be differentiated by studying the kinetics of the reaction.

The Lineweaver-Burk plot shown in Fig. 26 has been widely

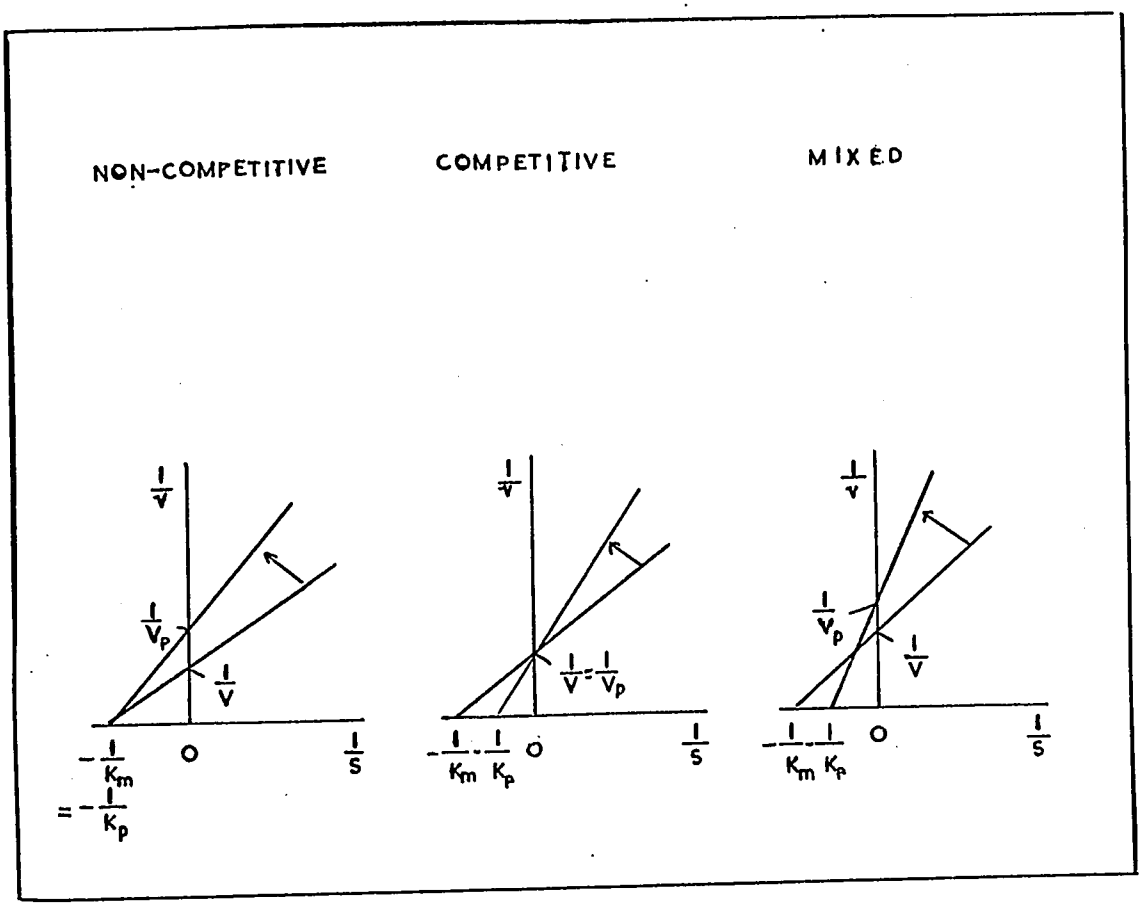



Fig. 26. Effect of Inhibitors of Various Types on the Lineweaver-Burk Plot Relating Substrate Concentration and Reaction Velocity. (From Dixon, M. and E.C. Webb: Enzymes. London, W. 1, Longmans, Green and Co., 2nd edition, 1964, p. 326).

used for distinguishing the type of inhibition. With fully competitive inhibitors, the lines intersect on the vertical axis, whereas with fully non-competitive inhibitors they intersect on the base line; with mixed types the point of intersection is between the two axes.

To test this point, the plasma LP-lipase was purified by the extracting the enzyme from the post-heparin plasma with substrate, then solubilizing the enzyme-substrate complex with sodium deoxycholate. There was difficulty with this second procedure. Only a small amount of LP-lipase was solubilized from the enzyme-substrate complex. The enzyme activity was completely inhibited when we increased the concentration of deoxycholate to improve the solubilization. Therefore the original concentration of deoxycholate was used although this meant that the yield of the enzyme was much lower than that reported by Fielding (1969). The enzyme activity was improved by increasing the heparin concentration in the $NH_4OH$-$NH_4Cl$ buffer-sucrose medium from 5 μg/ml to 30 μg/ml. Using this purified enzyme, the rate of hydrolysis and substrate concentration in the presence and in the absence of cholesterol were examined. The results demonstrated that both cholesterol and protamine inhibited lines intersected with the line of the non-inhibited enzyme on the vertical axis (see Fig. 12). This indicates that both free cholesterol and protamine are competitive inhibitors of the LP-lipase. The points of the

cholesterol inhibited enzyme reaction were scattered. It is possible that purified plasma LP-lipase was still contaminated by other enzymes and that a lag period during the first 15 min of incubation (Fig. 1) resulted in a certain error. Therefore it remains possible that free cholesterol can be the inhibitor of the mixed type.

h. A comparison with the experiments of other investigators - French, Robinson and Harris (1955) reported that when chyle was added to the plasma of an animal previously injected with heparin there was a progressive fall in the turbidity of the mixture due to the disappearance of chylomicra. This clearing was due to a lipolytic process in which the triglyceride fraction of the chylomicron was broken down by hydrolysis. When cholesterol was added to this mixture, the turbidity of the cholesterol suspension was also cleared.

In another experiment (Robinson et al., 1956) they found that when the added cholesterol was more than 0.6 mg per ml of incubation mixture, there was a residual turbidity which increased with the amount of cholesterol present. They also found that the plasma from the cholesterol-fed rabbits had a marked inhibitory effect on the rate of clearing chyle by heparinized plasma of the rabbit.

These results are in agreement with mine in that the plasma from the cholesterol-fed rabbits has a marked inhibitory effect on LP-lipase. But the similarity ends at this point. They found that the hydrolysis of triglyceride by LP-lipase was uninfluenced by an

added cholesterol suspension. Accordingly, they explained that cholesterol-containing LP rather than cholesterol itself had the inhibitory effect on the LP-lipase activities. In contrast, my experiments showed that cholesterol, both in suspension and LP form, had a significant inhibitory effect on LP-lipase activities. Neither cholesterol esters nor lecithin has any inhibitory effect on this enzyme. These findings favor the proposition that cholesterol itself, rather than LP or other lipids in the LP, has an inhibitory effect on the LP-lipase activities.

The cause of the disparity between these experiments is the difference in the amount of cholesterol added or present in the incubation medium. Cholesterol suspension 16.2 mg or lipoprotein-cholesterol 1.8-4.5 mg was added for the inhibition of 30 units* of LP-lipase in my experiments, while in the experiments of Robinson et al. (1956), only 0.5 mg or less of cholesterol was added to 1 ml of post-heparin plasma (containing approximately 30 units of LP-lipase) for incubation and this did not cause inhibition. Since 0.5 mg of free cholesterol per ml of plasma is still within the normal limit of rabbit plasma cholesterol levels, normal LP-lipase activities are expected. Therefore, if this quantity of cholesterol is added to the incubation mixture it cannot conclusively prove its inhibitory effect on the LP-lipase activities. They neglected one of their own results, which was that there was a residual turbidity which increased when more than 0.6 mg cholesterol was added to the

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* 1 unit catalyses the release of 1 μEq FA/h from the triglyceride.

incubation mixture. This indicates that cholesterol itself has an inhibitory effect on the rate of clearing of chyle by heparinized plasma of rabbits.

The purpose of my experiments was to study the mechanism of the increase in plasma triglyceride level in the hypercholesterolemic condition. If cholesterol is indeed involved in the development of this hypertriglyceridemia, the amount present should be higher than the normal value. Therefore a large amount of cholesterol was added in my in vitro experiments to make a condition equivalent to that existing in the experimental hypercholesterolemic rabbits.

Robinson and French (1960) proposed another possibility that the cholesterol present might be redistributed in association with the FFA released during hydrolysis by clearing factor lipase. When cholesterol was added to a mixture of post-heparin plasma and chyle, the turbidity of the cholesterol suspension disappeared. They explained that the clearing of cholesterol suspensions was dependent on the release of fatty acid during the clearing of the chyle, and that the reaction between the cholesterol and the fatty acid was analogous to the effect of soap solutions on the solubility of cholesterol. Thus it could be possible that the decrease in titratable fatty acid observed in the presence of hypercholesterolemic VLDL or Ediol containing cholesterol is not due to the decreased hydrolysis but rather to the association of cholesterol with the released fatty acid, resulting an underestimation of fatty acid by

titration. This possibility was excluded by one of my experiments, in which, palmitic acid, 6 μEq, was added into the cholesterol-Ediol mixture and incubated with a control heart homogenate. 92.1% of this acid was recovered by titration after one hour's incubation, i.e. the titration detected most of the fatty acid present in the cholesterol enriched incubation mixture. This check unfortunately does not indicate the possibility of the existence of a weak reaction between cholesterol and FFA, since it is likely that the heptane-isopropanol-$H_2SO_4$ mixture would destroy such a binding. Nevertheless, it appears that the decrease in titratable acid in the medium containing a large amount of cholesterol is due to the decrease of hydrolysis of triglyceride i.e. due to the inhibition of LP-lipase activities.

Klein, Lever and Fekete (1959) found a relatively high level of antilipolytic activity in hyperlipemic serum. It was associated with the supernatant fatty layer obtained after high-speed centrifugation. The cholesterol concentration of these hyperlipemic sera was around 520 mg%. Accordingly, it can be inferred that inhibition of LP-lipase by cholesterol is again the mechanism of this antilipolytic activity.

i. Possible mechanisms for the increase in LP-lipase activities in the hypercholesterolemic heart - Heart LP-lipase activities were found to be significantly higher in the hypercholesterolemic condition than in the control. Heart tissue is not the only tissue to possess LP-lipase activity, but heart and adipose tissue

contain the largest amount of this enzyme. The enzyme is also present in kidney, spleen and aorta (Korn, 1955A) and the isolated hind limb, skin, lung and intestine (Jeffries, 1954). However, since the heart and adipose tissue contain the largest amounts of LP-lipase, these organs were chosen as models which might represent the effect of free cholesterol on the tissue LP-lipase activity. This study shows that the tissues could respond to the inhibitory effect of free cholesterol by an increase in enzyme activities.

Bezman, Felts and Havel (1962A) and Garfinkel et al. (1967) showed that LP-lipase is necessary for triglyceride uptake by extrahepatic tissues. Therefore inhibition of LP-lipase activity by free cholesterol should be followed by a decrease in triglyceride uptake by the extrahepatic tissues. Most et al. (1969) demonstrated a positive uptake of triglyceride by the human heart. This was shown by utilizing labelled triglyceride in order to avoid difficulties inherent in chemical methods, by which a number of ambiguous results had previously been obtained. (Bing, et al., 1954). This new finding supports an earlier thesis (Ballard, et al., 1960) that the triglyceride is utilized by the fasting human heart. It is possible, therefore, that the decrease in triglyceride uptake by the extrahepatic tissue, through inhibition of LP-lipase, changes the substrate availability of the tissue. This may in turn, cause an increased synthesis of LP-lipase.

Alousi and Mallov (1964) found a significant increase in

heart LP-lipase activity in rats after prolonged fasting, while a significant fall in cardiac LP-lipase activity was observed in rats on the high-carbohydrate or high fat diets. In contrast, the adipose tissue LP-lipase activities increased in rats after the feeding of pure glucose or fat loads (Pokrajac and Lossow, 1967). This indicates that tissue level of LP-lipase can be affected by the nutritional state of the animals. In-vivo experiments, in which triglycerides labelled in both the glycerol and fatty acid moieties were administered to rats, indicate that rapid cleavage of the triglyceride molecule occurs in tissues (Reiser et al., 1960). The specificity of LP-lipase for LP-triglyceride substrates suggests that it may regulate this lipolytic reaction and may thus determine the rate of triglyceride assimilation by the various tissues that contain this enzyme. This suggestion draws support from the fact that in vitro incorporation of triglyceride into adipose tissue of fed, fasted and refed rabbits is linearly related to the LP-lipase activity of the tissues (Bezman et al., 1962A and B). The alterations in rat heart and adipose tissue LP-lipase activity, produced by fasting, are in keeping with a regulatory role of this nature. These changes are such that the activity of the enzyme in adipose tissue is greatest under conditions favoring fat deposition such as in the fed animals, while in a fasting state, when the peripheral tissues require fat as an energy source, the LP-lipase activity falls in adipose tissue and rises in heart muscle.

It is inferred from these findings that the increase in heart LP-lipase is a compensation to maintain an adequate supply of energy in the form of triglyceride. This may represent an adaptation of the peripheral tissues to the inhibition of LP-lipase activity by cholesterol, in order to counteract a resultant decrease in triglyceride uptake by these tissues. Unfortunately, this hypothesis is descriptive and gives no explanation of the mediating mechanisms. The level of plasma triglyceride itself may induce the enzyme. Whether or not new biosynthesis of the enzyme is involved is also unanswered.

Such a compensatory increase may not occur in adipose tissue LP-lipase. As discussed above, LP-lipase in this tissue generally behaves differently to that of other tissues. In addition, since the rabbits in my experiments were fasted overnight, the adipose tissue LP-lipase activity was decreased (Bezman et al., 1962A). This was so in both control and hypercholesterolemic rabbits (Fig. 6), although the mean value in the latter group showed a tendency to decrease.

j. In vivo experiment - Inhibition of LP-lipase was also found in the in vivo experiments, namely, there was less intravascular hydrolysis of plasma triglyceride in the hypercholesterolemic rabbits than in the normal controls by the post-heparin LP-lipase. There was no significant decrease in the plasma lipids other than triglyceride following the injection of heparin in my in vivo

experiments. It has been well documented that a decrease in triglyceride after the injection of heparin to normal rabbit was due to the hydrolytic action of LP-lipase which was released into the plasma by this drug (Shore et al., 1953). The plasma triglyceride level remained unchanged after the injection of heparin in hypercholesterolemic rabbits, suggesting that the action of LP-lipase was inhibited. A change in heparin-activation of LP-lipase (Robinson, 1956) or a change in quantity or rate of release could also be a factor responsible for the above observation. In addition, many factors present in the plasma, including platelets, white blood cells and bile salts may inhibit LP-lipase activity (Fekete et al., 1958; Korn, 1957). It is not known whether these factors are involved in the hypercholesterolemic rabbit. From my experiments, utilizing various technics, it appears reasonable to interpret all my results by a unified hypothesis of cholesterol-inhibition of LP-lipase. Since the time course of changes in post-heparin lipolysis during cholesterol feeding was not studied, an effect of the compensatory change in enzyme activity, which was discussed above, upon intravascular hydrolysis observed cannot be evaluated at this point.

The FFA increased significantly after the injection of heparin in both control and hypercholesterolemic rabbits. However, there are a number of factors which may influence the plasma FFA level. In addition to the direct effect of heparin, there are the rate of transfer of triglyceride from the liver, rate of hydrolysis of

triglyceride, rate of utilization of FFA and the responsiveness of adipose tissue to exogenous fat (Fredrickson and Gordon, 1958). These factors may cause the fluctuation of the plasma FFA level after the injection of heparin in both control and hypercholesterolemic rabbits. The same fluctuation occurred in the lipemic animals of Ballard, et al. (1960); i.e. following injections of heparin into 7 lipemic animals, the arterial levels of FFA rose in 3, fell in 3 and remained constant in 1 animal.

k. LP-lipase and lipoproteins other than VLDL (i.e. $S_f < 10$) - Shore (1955) found that the LP-lipase could not hydrolyze the triglyceride moiety of plasma LP with a density of 1.019-1.063. According to the results of lipid compositions of the different fractions of LP in Table IV, the free cholesterol content of $d > 1.019$ LP of the control rabbits was as high as that of the VLDL ($d < 1.006$) of hypercholesterolemic rabbits. The latter was used as the substrate for the assay for LP-lipase of heart homogenates, and this resulted in an apparent inhibition of LP-lipase. The results support the hypothesis that the absence of hydrolysis of the triglyceride moiety of plasma LP with $S_f < 10$ could be due to the fact that this portion of LP contains a large quantity of free cholesterol which is inhibitory to the activity of LP-lipase.

2. THE UPTAKE OF PLASMA TRIGLYCERIDE BY LIVER

An alternative mechanism for the increase in plasma triglyceride in hypercholesterolemic rabbits may be a decrease in triglyceride uptake by the liver. My experiments with liver

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slices and homogenates indicate only a net triglyceride output. But my triglyceride turnover study indicates that the uptake of plasma triglyceride by the extrahepatic tissues and liver decreased when plasma triglyceride was increasing during cholesterol feeding. This result could mean that the increase in plasma triglyceride in hypercholesterolemic rabbits was due to a decrease in triglyceride uptake by the liver. However, Havel et al. (1962) injected triglyceride fatty acid labelled VLDL to rabbits and found this LP predominantly in the liver in the fasting state, while most was found in the adipose tissue in the fed state. Therefore only in the fasting state can the liver take up a significant amount of triglyceride from the plasma. Friedberg and Estes (1964) in contrast, pointed out that in fasting state the role of the liver in removing endogenous glycerides seems unimportant, because in the fasting animal much of the triglyceride in the VLDL of the plasma is merely recycling between the liver and blood (Olivecrona, 1962; Robinson, 1964). These findings indicate that a decrease in triglyceride uptake by the liver is not the main factor in the cause of hypertriglyceridemia in hypercholesterolemic rabbits.

3. PHYSICOCHEMICAL SEQUESTRATION

Friedman et al. (1965) proposed that the effect of physicochemical sequestration was the cause of triglyceride induced hypercholesterolemia. A reversal of this effect, i.e. physicochemical sequestration of triglyceride by a raised plasma cholesterol level

might be a possibility. With respect to such an interaction between triglyceride and cholesterol, Parker and Bhaskar (1968) studied the self-association of cholesterol and its interaction with triglyceride. The self-association of cholesterol through hydrogen bonds has been studied by infrared measurements of its OH-stretching band. At concentrations below 0.014 M in $CCl_4$, cholesterol exists only as a monomer. As the concentration is increased, it forms a dimer and a higher aggregate, the latter becoming predominant at a concentration of 0.2 M.

The proton-donating OH group of cholesterol suggests hydrogen bonding as a possible mechanism of its interaction with triglyceride, which has proton-accepting carbonyl groups. Parker and Bhaskar provided evidence of formation of a 1:1 hydrogen-bonded complex in the mixed solutions of cholesterol and the triglyceride. Although it is unknown whether or not similar interactions could exist in non-lipid solvents, these types of studies are of great help in elucidating the mechanism of cholesterol-inhibition of LP-lipase activity and the physico-chemical sequestration process.

### D. THE TIME COURSE OF CHANGES IN PLASMA CHOLESTEROL, PLASMA TRIGLYCERIDE AND TRIGLYCERIDE TURNOVER RATE

The inter-relationship between the increase in plasma cholesterol, plasma triglyceride and LP-lipase activity was studied by the simultaneous determination of plasma lipid levels

and the triglyceride turnover rate, to supplement the information obtained from other studies described above.

The triglyceride turnover study indicates the rate of renewal of plasma triglyceride i.e., the combined results of triglyceride synthesis and transport into the plasma as well as its breakdown and transport out of the plasma (Wolf, 1964). In the study of metabolism and transport of body constituents, the turnover is determined when the entry and exit of materials within a given pool are in a steady state; in other words, in a situation in which the rate of entry of triglyceride equals the rate of exit, the plasma triglyceride concentration remains constant (Zilversmit, 1957). Although the plasma triglyceride of the hypercholesterolemic rabbits was increasing in terms of days and weeks in my experiments, this increase was very slow when compared to the rate of triglyceride turnover and hence the condition can be treated as a steady state (Zilversmit, 1960).

The plasma triglyceride turnover rate was determined by two methods: the intravenous injection of tritium labelled very low density lipoprotein (VLDL-$H^3$) and that of glycerol-2-$H^3$. The injected VLDL-$H^3$ mixes with the unlabelled VLDL present in the circulation, resulting in dilution of radioactivity, its specific activity becomes lowered. With time, the specific activity of VLDL-$H^3$ is further lowered by nonradioactive VLDL entering the circulation from the liver. At the same time, VLDL leaves the circulation by

hydrolysis through the action of LP-lipase. However, since there is no discrimination between labelled and unlabelled VLDL in these processes, the specific activity will not be affected by the exit of VLDL, but by that entering the circulation. In steady state, the entry and exit of plasma VLDL of a particular rabbit during the period of test remains equal and the turnover of plasma VLDL is calculated from the rate of decrease in specific activity of the injected VLDL-$H^3$ (Wolf, 1964). In my experiments the specific activity of plasma triglyceride was measured for the following reasons: plasma lipoprotein is composed of many subclasses having various turnover rates (Havel et al., 1962); plasma lipid levels are influenced mainly by lipid fractions of the highest turnover rate; furthermore, the largest portion of plasma triglyceride is localized in the VLDL fraction, the triglyceride of which, as stated above, turns over most rapidly; and finally radioactive triglyceride is almost exclusively found in the VLDL fraction (Farquhar et al., 1965). A disadvantage of this simplification of using triglyceride is that the linearity of wash-out curves gradually deviates due to the influence of slow components of triglyceride turnover. However, this method avoided prolonged centrifugation and cross contamination involved in separating VLDL. Also, the latter technic requires a large sample of blood, which is deleterious to prolonged experimentation.

In the experiments in which glycerol-2-$H^3$ was injected, the

glycerol-2-$H^3$ acted as a precursor of hepatic glycerophosphate which in turn served as the precursor of hepatic triglyceride. In fasting animal, the hepatic triglyceride is the immediate precursor of plasma triglyceride (Havel et al., 1962). Glycerol-2-$H^3$ that is intravenously injected is transformed to hepatic glycerophosphate-$H^3$ and then tritium labelled triglyceride (TG-$H^3$). Therefore, TG-$H^3$ in liver rises, as more radioactive TG-$H^3$ becomes mixed with nonradioactive triglyceride. The specific activity of liver TG-$H^3$ finally reaches its maximum. This is reflected by the specific activity of plasma triglyceride, since newly formed triglyceride is being transported into the plasma. At the moment at which the specific activity of hepatic triglyceride equals that of plasma triglyceride, the plasma triglyceride reaches its maximum specific activity and it then declines, since obviously the specific activity of the product cannot be higher than that of the precursor (Wolf, 1964).

To determine the rate at which triglyceride is synthesized and delivered to plasma, one must obtain the specific activity-time relations of all the precursors in liver as well as several points on the plasma triglyceride specific activity-time curve. This may be accomplished by repeated liver biopsy (Farquhar et al., 1965). In order to study bodily triglyceride turnover in detail liver biopsy is necessay for defining the compartments and the behavior of precursors. The rabbit, in contrast to man, apparently shows a high plasma

triglyceride ($S_f > 20$) turnover rate which exceeds the hepatic triglyceride turnover rate (Farquhar et al., 1965). However, the declining portion of specific activity-time curves of plasma and liver triglyceride are virtually parallel, making hepatic biopsy unnecessary (Reaven, et al., 1965; Gross et al., 1967). Thus the turnover rate was estimated by measuring the rate of disappearance of TG-$H^3$ from the plasma. The primary advantage of this procedure is that recycling of the glycerol-$H^3$ is not great. There was another problem in defining the triglyceride "pool"; generally, bodily triglyceride pool includes some hepatic and plasma compartments where VLDL rapidly recycles. In my calculation, the pool size was assumed to be equal to the size of plasma volume which might have resulted in some errors in my calculation of turnover rate.

The plasma triglyceride turnover rate in rabbit was previously studied by Havel, Felts and Van Duyne (1962). They found two components in the specific activity-time curves of triglyceride fatty acid of VLDL after the intravenous injection of labelled VLDL into the fasting rabbits. They suggested that these components resulted largely from a combination of transfer of triglyceride from plasma to liver and extrahepatic removal. The half-time for the second component was about 50 min, which corresponds well to the 66-78 min of my experiments despite a minute difference in techniques (glycerol-$H^3$ vs. palmitate-$C^{14}$, plasma triglyceride vs. VLDL). In other studies, the turnover

rate of plasma $S_f > 20$ triglyceride was reported to be 9.7 mg per kg per hr in the human being (Farquhar et al., 1965) and 9.3 mg per kg per hr in the dog (Gross et al., 1967). Despite the difference in species, these values are comparable to the turnover rate in my study, namely the triglyceride rate in the control rabbits was 10.7-12.9 mg per kg per hr, measured by the injection of VLDL-$H^3$ and 6.4-21.9 mg per kg per hr measured by the injection of glycerol-2-$H^3$. In view of the fact that lipoproteins are mixtures of triglyceride with widely different turnover rates, the agreement is surprisingly good.

The results of the triglyceride turnover measurement give additional support to the hypothesis that cholesterol induced hypertriglyceridemia is due to the inhibition of LP-lipase. When plasma cholesterol started to rise after 36 hr of high cholesterol diet, the plasma triglyceride turnover rate rapidly decreased. This decrease in triglyceride turnover rate is interpreted as an inhibition of LP-lipase activity by the elevated plasma cholesterol followed by a decrease in triglyceride uptake by the tissues. This temporal imbalance between triglyceride removal from the plasma and entry into the plasma caused an elevation of plasma triglyceride level. Such inhibition of the enzyme activity was shown by a direct assay of LP-lipase activity in the presence of free cholesterol. Alternatively, this decrease might, at least in part, be due to a decreased hepatic triglyceride synthesis-secretion, since, as

mentioned above with rabbits, the rate limiting point in triglyceride of the plasma-liver pool is the hepatic turnover. In this case the decreased rate signifies a decreased hepatic synthesis. However, this hypothesis makes the explanation of a simultaneous elevation of plasma triglyceride level difficult.

As discussed above, LP-lipase is also present in kidney, spleen, aorta, skin, lungs and intestine, and the specificity of LP-lipase for lipoprotein-triglyceride substrate suggests that this enzyme may regulate the lipolytic reaction and may determine the rate of triglyceride assimilation by various tissues which contain this enzyme. The LP-lipase activities of the heart from hypercholesterolemic rabbits were significantly higher than that of the normal heart when it was assayed in the absence of cholesterol. The increase in heart LP-lipase indicates that there is a compensatory increase in LP-lipase of the tissue which takes up triglyceride fatty acid in response to the cholesterol inhibition of the enzyme. This hypothesis is supported by the results which showed a gradual rise in turnover rate of triglyceride in the later period (14th to 49th day) of the experiment. It is also possible that the increasing availability of substrate for LP-lipase (mass-action law) is partly responsible for this rise in turnover. The compensatory increase in tissue LP-lipase activity is an adaptive response to the decrease in triglyceride uptake. Therefore, once the uptake becomes sufficient for the tissue requirement, the adaptation process appears to cease. The fact that the plasma

triglyceride is increased in the hypercholesterolemic rabbits suggests that the compensatory increase in enzyme activity does not fully "compensate" the inhibitory effect of sustained hypercholesterolemia.

The turnover time calculated from the rate of disappearance of the TG-$H^3$ from the plasma during cholesterol feeding was longer than the control turnover time, i.e. 112 min vs. 169 min (Fig. 16). The triglyceride turnover times in the rabbits after 36 hr, one week, two weeks, three weeks and seven weeks of high cholesterol diet were almost the same. This is probably due to the compensatory increase in LP-lipase activity which increases the hydrolysis of rapidly accumulating plasma triglyceride, in spite of the fact that the plasma triglyceride pool is expanding at the same time. Therefore, the triglyceride turnover time was maintained at a little longer but a fairly constant level, causing a rather slow elevation of plasma triglyceride under the hypercholesterolemic condition.

The increase in triglyceride turnover rate in the later period of the experiment indicates that there was an increase in entry of triglyceride into the plasma. In the fasting state the triglyceride enters plasma from the liver (Havel, et al., 1962). Therefore there should be an increase in triglyceride secretion and/or synthesis by the liver, but this increase was probably too small to be detected by the in vitro experiment such as the incorporation of radioactive palmitate or acetate to the triglyceride of the liver. It could be argued that the TG secretion by the liver could

increase independently of the hepatic triglyceride synthesis (i.e. esterification and FA synthesis). However, an increased secretion and unchanged triglyceride synthesis, as were found in these experiments, should result in a depletion of hepatic triglyceride. This was not the case. Alternatively, an elevation of plasma FFA, one of the triglyceride precursors, may result in an increased triglyceride output from the liver. This possibility was also ruled out. Finally, a change in liver weight could give rise to a discrepancy between the results of the turnover study and the in vitro study. Data on the weight of the liver and those on homogenate do not support this last possibility.

## E. THE EFFECT OF CHOLESTEROL FEEDING ON THE DISTRIBUTION OF TISSUE LIPIDS

### 1. HYPERCHOLESTEROLEMIA AND THE LIPID COMPOSITION OF THE SUBCELLULAR ELEMENTS

Although the published data on the lipid composition of normal subcellular fractions are variable, my experimental results of the lipid composition of normal cardiac and hepatic subcellular fractions are in general within the range reported (Table VII). The above variation may be due to differences in animal species and experimental techniques. In order to avoid such difficulties, pair - fed rabbits were used for the comparison of the lipid composition of the subcellular fractions. Scattering due to species and experimental techniques was thereby minimized.

TABLE VII

LIPID COMPOSITION OF SUBCELLULAR ELEMENTS (μg/mg protein)

| | Heart | | Liver | |
|---|---|---|---|---|
| | Control | Hyperchol | Control | Hyperchol |
| Mitochondrial fract. | | | | |
| Total cholesterol | 20.3 ± 2.3 | 42.8 ± 3.6 | 16.6 ± 2.4 | 90.8 ± 6.5 |
| | 3.5* 88.5** | | 4.1* | |
| Phospholipid-P | 8.7 ± 0.7 | 10.6 ± 0.6 | 6.1 ± 0.9 | 9.2 ± 0.8 |
| | 11.3* 10.7** | | 5.6* | |
| Triglyceride | 11.0 ± 1.6 | 11.3 ± 1.9 | 12.4 ± 2.1 | 8.3 ± 1.6 |
| | 14.7* 14.8** | | 2.1* | |
| Microsomal fract. | | | | |
| Total cholesterol | 50.5 ± 6.8 | 73.2 ± 5.8 | 27.5 ± 3.6 | 102.3 ± 6.9 |
| | 66.8** | | | |
| Phospholipid-P | 8.9 ± 0.9 | 9.6 ± 0.9 | 9.3 ± 1.0 | 12.4 ± 0.9 |
| | 19.1** | | | |
| Triglyceride | 24.5 ± 7.0 | 24.5 ± 4.0 | 16.7 ± 1.6 | 10.5 ± 1.3 |
| | 64.6** | | | |
| Myofibrillar fract. | | | | |
| Total cholesterol | 14.1 ± 1.7 | 15.3 ± 1.6 | | |
| | 4.3** | | | |
| Phospholipid-P | 3.6 ± 0.3 | 3.4 ± 0.4 | | |
| | 2.3** | | | |
| Triglyceride | 10.2 ± 1.9 | 9.7 ± 1.6 | | |
| | 3.8** | | | |

| | Heart | | Liver | |
|---|---|---|---|---|
| | Control | Hyperchol | Control | Hyperchol |
| Supernatant Fract. | | | | |
| Total cholesterol | 6.9 ± 1.1 | 58.2 ± 8.4 | 3.1 ± 0.4 | 52.7 ± 3.7 |
| Phospholipid-P | 0.7 ± 0.2 | 0.5 ± 0.1 | 0.3 ± 0.1 | 0.8 ± 0.1 |
| Triglyceride | 8.3 ± 1.9 | 9.4 ± 1.1 | 7.8 ± 1.6 | 5.9 ± 1.3 |

References: *Fleischer et al (1967), ** Wheeldon et al. (1965).

In my experiments, both cardiac and hepatic mitochondria accumulated a large quantity of cholesterol and its esters within a few months after the initiation of a high-cholesterol diet. At the same time the phospholipid and triglyceride contents of all the subcellular elements (i.e. mitochondria, microsomes and supernatant of the heart and liver cells, and myofibrils of the heart cells) were not increased in spite of the high concentration of these lipids in the plasma.

Subcellular elements are vulnerable to isolating procedures. An attempt was made to minimize cross-contamination between these elements by gentle homogenization, careful setting of centrifugation speeds and repeated washing. Initially, measurement of cytochrome oxidase and esterase activities was attempted in order to estimate cross-contamination. However, this was unsuccessful and mitochondrial respiration (oxygen consumption-$QO_2$ , P/O ratio and respiratory control ratio) was measured instead. This was because cardiac mitochondria are easily broken during preparation and it is relatively difficult to obtain them in a pure undamaged form. Such damage would be reflected in respiratory control ratios. Contamination of microsomes by other subcellular particles could not be measured, since the proper marker of microsomes for this purpose was not available.

The liver contains only a very limited enzymatic activity for the degradation of cholesterol and its esters. The capacity of the hydrolytic enzymes for chylomicron cholesterol ester may

well be exceeded in the presence of diets containing high levels of cholesterol, resulting in the accumulation of cholesterol esters in the liver (Deykin et al., 1962A).

The high cholesterol concentration may interfere with the efflux of cholesterol from heart cells. Since most extra-hepatic tissues can synthesize cholesterol but are not able to degrade cholesterol appreciably, it seems likely that in normal conditions there is a continuous slow net efflux of cholesterol from the various extra-hepatic sites to the plasma (Masoro, 1968). The high plasma concentration of cholesterol makes the efflux against a greater concentration gradient thereby resulting in less efflux from tissue to plasma.

All tissues, with the possible exception of the adult brain, can synthesize cholesterol (Masoro, 1968). Does the elevated free cholesterol in the plasma stimulate the mitochondria of heart and liver to synthesize more free cholesterol? This is unlikely, because the evidence showed that in the dog (Gould et al., 1953), the monkey (Clarkson, 1963) and the rat (Rao et al., 1967), feeding of cholesterol results in a marked depression of hepatic cholesterol synthesis. This is due to a sensitive feedback inhibition operating to control the enzyme β-hydroxy-β-methylglutaryl CoA reductase, thus limiting the synthesis of mevalonate, a precursor of cholesterol. The liver slices of rabbits showed the same response, although the rate of cholesterol synthesis was slower than that found in dogs

so that the inhibition was also of a smaller magnitude (Gould, 1951). In humans, dietary cholesterol, whether fed in the form of eggs or in a purified state, has little effect on the total rate of endogenous cholesterol synthesis or of cholesterol degradation to bile acid (Wilson, 1965).

It is also possible that the heart and liver cells, like the smooth muscle cells of the aorta, can selectively take up cholesterol and its ester from the plasma. In the atheromatous rabbit aorta, cholesterol influx can be measured by the accumulation of radioactive cholesterol into the atheromatous lesions. In the period up to 60 days after the commencement of cholesterol feeding, this influx was greater than the net accumulation of cholesterol in the thoracic aorta (Newman and Zilversmit, 1966). The entry of radiactive cholesterol into the atheromatous aorta was found to be related to plasma cholesterol concentration and to arterial cholesterol content. The esterified aortic cholesterol is also derived by direct transfer from plasma (Newman and Zilversmit, 1966), while the phosphatide in the atheromatous plaque is derived by a greatly accelerated synthesis of phosphatide by the artery (Newman, Day and Zilversmit, 1966). It is conceivable that net influx of cholesterol into the cell and its organelles increases in hypercholesterolemic rabbits and causes observed accumulation of cholesterol and its ester in the cells.

Some portion of cholesterol and its esters in the subcellular

fraction may have been adsorbed (Jensen, 1967) during the isolation procedure, resulting in false high values. However, the heart microsome, which was in contact with the soluble fraction for a period of time considerably longer during fractionation than was the mitochondrial fraction, showed a lesser increase in these lipids. In addition, the free cholesterol level of cardiac mitochondria did not increase in parallel with that of the supernatant obtained from the same homogenate. Myofibrillar protein did not accumulate any cholesterol from the surrounding soluble fraction with a high cholesterol content, again suggesting that some mechanism other than simple adsorption was operative.

The phospholipids and triglyceride were significantly elevated in the plasma but were not elevated in the cardiac subcellular fractions. This finding suggests that tissue uptake of lipids does not depend on their plasma level. Apparently, esterification of fatty acids and phospholipid synthesis were not influenced by the presence of a large amount of cholesterol and its esters in the myocardial cell. On the other hand, phospholipid synthesis is increased in the liver of hypercholesterolemic rabbits (McCandless and Zilversmit, 1956). Its increased synthesis provides a favourable condition for increased lipoprotein-production and esterification of cholesterol. However, it is not possible to find phospholipid accumulation in the hepatic subcellular fractions.

The normal triglyceride content in all the subcellular elements of both heart and liver agrees well with the results of my other experiments. The increase in plasma triglyceride level is not due to the increase in synthesis and secretion by the liver. It is due to the inhibition of LP-lipase activity by the high plasma cholesterol levels and a consequent decrease in tissue uptake of triglyceride. As a result, peripheral tissues should not contain an excessive amount of triglyceride. The increase in heart LP-lipase indicates that heart can nevertheless hydrolyze triglyceride by this compensatory mechanism, as discussed already.

2. FUNCTION OF THE MITOCHONDRIA WITH LARGE AMOUNT OF CHOLESTEROL AND ITS ESTER

The $QO_2$, P/O ratio and respiratory control ratio of the mitochondria of both control and hypercholesterolemic heart and liver in our experiments were in the same range reported by Sobel et al. (1966), Chance et al. (1955B) Tarjan et al. (1967).

Cholesterol accumulation in the cell organelles might result in changes in structure, polarity, water-binding capacity, etc. of the membranous components. However, the results of our experiments do not indicate derangement of the mitochondrial P/O ratio and respiratory control ratio. The oxygen consumption in the hypercholesterolemic group tended to be higher than that in the control, particularly when succinate oxidation by the hepatic mitochondria was compared ($0.025 > p > 0.01$). Similarly, Whereat

(1967) isolated mitochondria from rabbit aorta, which were capable of carrying out oxidative phosphorylation. P/O ratios were obtained before and after cholesterol feeding. He observed that mitochondria from cholesterol-fed rabbits had about twice the rate of oxygen consumption.

The rate of oxygen consumption with succinate as the substrate was greater than that measured with other Krebs cycle intermediates. This result agrees well with the finding of Chance and Hollunger (1961). They found that the flavin-linked substrate, succinate, has far greater control over the level of pyridine nucleotide than do NAD-linked substrates such as malate, glutamate or α-oxoglutarate. They explained that only part of the pyridine nucleotide is active in oxidative phosphorylation with α-oxoglutarate or β-hydroxybutyrate as substrates. This was shown by the experiments in which a much larger amount of pyridine nucleotide can be reduced by the addition of succinate to heart mitochondria. They also attributed the different effects of succinate and glutamate to a structural compartmentation. The glutamate-and malate-linked material are bound in the cristae of mitochondria, whereas the succinate-linked material is in the matrices and cannot be reduced by the NAD-linked dehydrogenase in aerobiosis.

Whereat (1966) found that mitochondria from atherosclerotic (cholesterol-induced) rabbit aorta incorporated acetate into fatty acids more than four times faster than did mitochondria from

control aorta. It was shown that cholesterol feeding, but not simply the presence of cholesterol in the incubation medium, altered the mitochondrial fatty acid synthetic rate. The mechanism responsible for an increased fatty acid synthesis is as yet unknown, but Whereat et al. (1967) found that the rate of fatty acid synthesis in rabbit heart mitochondria is controlled by the intramitochondrial NADH/$NAD^+$ ratio. In a study of the effects of all the Krebs cycle acids, it was found that only succinate had a very large capacity to stimulate the fatty acid synthetic system (Whereat, 1967). This is understandable from the findings of Chance and Hollenger (1961), in which they demonstrated that succinate possesses unique characteristics to reduce $NAD^+$ by reversed electron flow. However, a link connecting cholesterol excess and enhanced succinate utilization is still obscure. Nevertheless, my results, too, showed an increase in only succinate oxidation in hypercholesterolemic rabbits. Mitochondria oxidation measured with other substrates was unaltered. It is not possible to state whether or not fatty acid synthesis was increased by these mitochondria, but my results with tissue slices suggest that the increase is unlikely, in contrast to the results by Whereat et al. (1967) above.

Kreslov and Mushkacheva (1966) fed rabbits with sunflower oil and 0.2 g cholesterol per kg of body weight for 6-8 months. They observed a decrease in oxygen uptake by the aorta, liver,

heart and kidney. They believed that the reduced ability to oxidize certain substances was due to an enzyme insufficiency which was part of the atherosclerotic process. The reason for the discrepancy between this and above results is unknown.

It was reported that the enzymes of the electron transport system are located in the inner membrane-matrix fraction of the mitochondria (Schnaitmen et al., 1967). If the bulk of cholesterol is accumulated outside the inner membrane of the mitochondria, it is quite possible that the respiratory function of the mitochondria is not greatly affected.

# CHAPTER V

## CONCLUDING REMARKS AND CLINICAL IMPLICATIONS OF THE EXPERIMENTAL FINDINGS

The lipids in plasma and between the plasma and tissues are closely related. The relationship can be physicochemical; for example, (1) the relationship of lipids in the lipoprotein; the phospholipid forms a bridge between the protein and other lipids, or (2) the sequestration of cholesterol in plasma by the phospholipid or triglyceride. The relationship can also be biochemical; one of the lipids may stimulate or inhibit certain enzymes and influence the metabolism of other lipids, such as the stimulation of hepatic synthesis of phospholipid by the cholesterol and the inhibition of lipoprotein lipase by the cholesterol.

Rabbits have a limited capacity for the homeostatic regulation of cholesterol metabolism. After a high cholesterol diet, they rapidly develope hypercholesterolemia. Cholesterol and its esters become deposited in liver, heart and arteries. The elevated cholesterol in plasma induces hyperphosphatidemia and hypertriglyceridemia. Cholesterol causes the liver to synthesize more phospholipid which solubilizes and transesterifies plasma cholesterol. My work demonstrated that the mechanism for the increase in plasma triglyceride in rabbits on a high cholesterol diet is by the inhibition of lipoprotein lipase activity by the large amount of free cholesterol in the plasma. This decreases the hydrolysis

of plasma triglyceride, and hence interferes with its uptake and utilization by extrahepatic tissues, resulting in accumulation of triglyceride in the plasma. Mention was made that lipoprotein lipase is the rate-limiting factor for the transportation of triglyceride from plasma to the extrahepatic tissues (Bezman et al., 1962A; Garfinkel et al., 1967). The inheritable deficiency (Havel and Gordon, 1960) and the inhibition (Bragdon and Havel, 1954) of lipoprotein lipase both can increase the concentration of triglyceride in the plasma. The results presented in this thesis support the above data, namely, the turnover rate measured with tritium labelled very low density lipoprotein was in order of 40 μEq fatty acids/kg.hr, while the measurement of fatty acid esterification into triglyceride by the liver homogenates gave the value of 86 μEq fatty acids/kg.hr, suggesting that the triglyceride disposal is the rate limiting factor.

The lipoprotein lipase activity in the heart homogenate was higher in the rabbit fed on a high cholesterol diet than in the control when the substrate was free of cholesterol. But a significant portion of this enzyme activity in both the experimental and control rabbits was depressed by the presence of free cholesterol in the incubation medium. The increase in heart lipoprotein lipase activity may be an adaptive mechanism in response to the decrease in triglyceride uptake by the heart when its activity is inhibited by the high concentration of cholesterol.

None of the subcellular elements of heart and liver showed an increase in triglyceride, even after the heart mitochondria and supernatant, liver mitochondria, microsomes and supernatant had all

shown a large accumulation of cholesterol and its esters. This is more evidence indicating that, under cholesterol-induced hypertriglyceridemia, there is a defective triglyceride transport between the extra and intracellular spaces and therefore this type of hypertriglyceridemia may not aggravate the severity of atherosclerosis caused by hypercholesterolemia.

There is much controversy concerning the role of hypertriglyceridemia in the development of atherosclerosis. It would be advisable to consider the causes of hypertriglyceridemia before reaching a conclusion. Hypertriglyceridemias of different origins have different relations with cholesterol and atherosclerosis. As was mentioned above, cholesterol induced hypertriglyceridemia may not aggravate atherosclerosis. The hypertriglyceridemia due to over-consumption of saturated fat and sugar is different to that due to over-eating of bread (Groen, 1968). In the former condition, the fat being absorbed by the intestine enhances cholesterol absorption (Fredrikson et al., 1957), resulting in an increase in cholesterol first in the plasma and then in the arteries (Stormby et al., 1963). While in latter condition there is no increase in fat in the intestine and therefore no enhancement of cholesterol absorption. The hypertriglyceridemia is endogenous in origin and may lead to sequestration of excess cholesterol in plasma (Friedman et al., 1965)1965), thus diminishing the filtration of cholesterol into the artery.

According to my results the interrelationship between prolonged postprandial lipemia, hypercholesterolemia and atherosclerosis could be that hypercholesterolemia causes both atherosclerosis and lipemia. The elevated plasma cholesterol inhibits lipoprotein lipase activity and

induces the accumulation of triglyceride.

The cause of the elevation in plasma cholesterol in patients with myxedema has been well studied. It is due to the retardation of catabolism of cholesterol. The elevated plasma cholesterol inhibits lipoprotein lipase activity. It constitutes at least one of the causes of hypertriglyceridemia present in these patients. Porte et al. (1966) found a low level of post-heparin lipolytic activity in four patients with myxedema associated with relative elevation of plasma triglyceride. The presence of a plasma inhibitor of lipolytic activity was suspected and was ruled out by the fact that inhibition was not found after the incubation of postheparin plasma from the myxedematous patient with that from a normal subject. Our results showed that the elevated free cholesterol can be the inhibitor. After the mixing of these two plasma the cholesterol concentration would be diluted and the lipoprotein lipase activity was increased by the addition of normal postheparin plasma. The inhibitory effect was therefore diminished.

Hypercholesterolemia has been noticed in some idiopathic hyperlipemic patients. The inhibition of lipoprotein lipase by the elevated cholesterol may not be the primary cause of this disease, but once the free cholesterol is elevated, it may contribute in some part to the defect in lipemia clearing response to heparin in these idiopathic hyperlipemic patients.

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