STUDIES ON THE EFFECT OF STARVATION ON BLOOD AND TOTAL BODY CHOLESTEROL IN ALBINO RATS

By

Samia S. Geha

Submitted in partial fulfillment for the requirements
of the degree of Master of Sciences
in the Biology Department of the
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Beirut, Lebanon
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TO MY MOTHER
AND FATHER

A C K N O W L E D G M E N T

The author is deeply indebted to Professor Levon G. Babikian, who proposed the problem and advised my thesis work all through. Besides his ideas and helpful suggestions, he was of great help to me by his encouragement in my difficult situations.

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INTRODUCTION

Cholesterol has been recognized to be an important substance in the bodies of certain animal phyla. After its discovery in the 18th century, many investigators have been interested in it, and several studies concerning its source, synthesis, location in the body, variations that might occur in its level and the impact of these variations on the well being of humans, were made.

Among the most serious diseases of man are arteriosclerosis and coronary heart disease. The occurence of these has been reported to bear some relationship to the level of the serum cholesterol. This has been a great stimulus for centering the interest of many workers on the different aspects of this substance.

Several factors have been found to effect the level of cholesterol in certain laboratory animals as well as in man. Among these are the dietary, the physical, the environmental, the social, the physiological and the hormonal factors. Stress in various forms, such as cold, heat, emotional disturbance, has been found to cause variations in the serum cholesterol level (Cook, 1958; Deuel, 1955).

In laboratories, cholesterol studies are performed on different animals, and it is found that observations

vary greatly. In this project, the interest is focused on the behaviour of serum cholesterol level and total body cholesterol in rats subjected to various forms of starvation.

HISTORICAL REVIEW

1. Discovery

In 1769, Poulletier de la Salla prepared cholesterol from gallstones. (De Fourcroy, 1789).

This substance has some wax-like properties and De Fourcroy classified it as "Spermaceti" or "Blanc de Baleine". The latter is a waxy substance obtained from marine animals, such as from the head of the sperm whale.

In 1815 Chevreul studied this substance and found it to be unsaponifiable, thus being different from "Spermaceti". In 1816 he termed this substance as cholesterol from the Greek chole, meaning bile, and steros, solid.

II. Occurrence

In 1824 Chevreul found cholesterol to occur in the bile of some animals, as well as man.

In 1838 Lecanu found it in the blood and in 1834 Couerbe foundit in the brain. In 1846, Gobley found it to occur in large amounts in the hen's egg yolk.

It is now known that cholesterol is present in almost all body cells and all body fluids in the form of white or light yellow leaflets or granules (Oza, 1959).

III. Chemistry and Properties

Cholesterol belongs to the group of lipids known as sterols, different from fats in being non-saponifiable.

The basic feature in the structure of cholesterol is that it is a phenanthrene derivative with an isovetyl chain at C_{17} atom of the cyclopentenophenanthrene nucleus. Like other sterols, there are methyl groups attached at positions 10 and 13.

The molecular formula of cholesterol, $C_{27}H_{46}O$ was first suggested by Reinitzer (1888). It is water insoluble, but soluble in organic solvents and hot ethyl alcohol. Its M.P. is 147 - 150°C. It is the main constituent of lipoproteins and may occur both in serum or in tissue in either of the two forms: (1) as the free compound occurring in the gray matter of the nervous system, in the nerve tissue and the blood, or (2) as the esters with fatty acids, occurring in blood plasma, ovaries and adrenal cortex (Oza, 1959).

IV. Sources of body cholesterol

There are two main sources for body cholesterol: (1) exogenous and (2) endogenous. The exogenous one refers to the ingestion and absorption of dietary cholesterol while the endogenous source refers to the biosynthesis of cholesterol in the body itself.

The starting point for the formation of cholesterol by the body is acetate. This was first shown by Bloch and Rittenberg (1942) to occur in rats and mice, using labelled acetate. The main site for cholesterol biosynthesis is the liver (Cook, 1958).

From the several studies starting in 1949, it was established that each carbon atom in the cholesterol molecule is derived from acetate. However, twelve carbons are derived from the carboxyl, while the others (fifteen) from the methyl carbon of acetate (Cook, 1958).

V. Circulating cholesterol

A. Distribution between blood elements and blood plasma

Boyd (1937) studied the distribution of cholesterol between blood cells and blood plasma and found a constancy in the cholesterol content of the erythrocytes, and variations in the plasma cholesterol level attributable to several different factors.

As to the presence of the two forms in the blood, it has been established that the free cholesterol is equally distributed between plasma and blood corpuscles, while the esters are almost absent from the blood corpuscles (Oza, 1959; Byers et al., 1952). The normal serum cholesterol in humans has been estimated to be 150-250 mg/100 ml, and the normal blood cholesterol has been estimated to be 100-200 mg/100 ml.

According to the findings of Boyd (1937, 1942) the free cholesterol to ester cholesterol ratio in human r.b.c. is about 4:1, in w.b.c. about 3:1 and in plasma about 1:3.

B. Physicochemical state

Lipids, including cholesterol are found in the plasma attached to protein molecules, resulting into lipoprotein complexes (Cook, 1958; Byers et al., 1952). The free cholesterol remaining in solution is not abundant. As to the role of the circulating cholesterol, it can give rise to three products: (1) bile acids, (2) sex hormones, (3) corticoids (Favarger, 1957).

C. Species differences

A large number of animal species have shown great difference in the plasma cholesterol levels ranging from as low as 40 mg per 100 ml serum in guinea pigs to as high as 208 - 285 mg per 100 ml in chicken (Cook, 1958).

Boyd in 1942 reported the plasma cholesterol level in the following species to be: 30 mg per cent in pig, 50 mg per cent in albino rats, 110 mg per cent in cow. A good table showing the recording of Boyd's finding in the serum cholesterol level of several normal fasting animals including guinea pig, albino rat, cow, cat, cockerel and man is given by Deuel (1955).

D. Normal variations of blood cholesterol in animals of the same species and in the same individual

There exist great variations in the blood cholesterol as well as in the total blood lipids among individuals of the same species and there is no one precise value for the blood cholesterol level in normal individuals of one species, as was shown from the results of Mayer and Schaeffer (1913), Terroine (1920) and Bloor (1915; 1916) in their work on dogs. Kohn (1950) has found differences as large as 100 per cent in the plasma cholesterol of different strains of rats.

Not only do normal variation in blood lipids occur among individuals of the same species, but also they occur within the same individual (Deuel, 1955).

E. Mechanisms involved in the control and regulation of plasma cholesterol

Plasma cholesterol, according to Byers et al (1952), is the resultant of the interaction of the following four processes (1) absorption, (2) synthesis, (3) excretion, and (4) degradation or conversion of cholesterol.

1. Relation of exogenous cholesterol and its absorption to plasma cholesterol level

Many workers showed that in most animals there is a maximum absorption of 30 per cent of the ingested cholesterol (Biggs et al., 1951; Chaikoff et al., 1952; and Cook and Thomson, 1951).

The range of absorption in rats, as reprted by Biggs et al.(1951), is from 30-50 per cent of the ingested cholesterol.

In cholesterol absorption, the presence or absence of fats together with the ingested cholesterol seems to be of importance (Byers et al., 1952). Usually, the ingested cholesterol is known to be better absorbed when accompanied with fat. However, as was indicated by Pihl (1955), not all fats enhance cholesterol absorption, and the non-easily absorbed fats, such as palmitate and trioleate seem to hinder cholesterol absorption (Lin,1955).

Bile salts, seem to play a very important role in cholesterol absorption. Siperstein et al.(1952) found no cholesterol in the thoracic lymph of rats deprived of their bile, whereas they could recover 48 per cent of the ingested cholesterol in the normal rats.

The pancreatic juice, as reported by Byers and Friedman (1955) has no influence at all on the cholesterol absorption.

Biggs et al.(1951) and Chaikoff et al.(1952) reported that the lymph of the thoracic duct is the major route of the ingested cholesterol to the systemic circulation. Their observations indicate that there is almost no transport by way of hepatic portal system.

As to the effect of cholesterol absorption on the plasma cholesterol lewel, it has been found by Biggs and Kritchevsky (1951) that chronic ingestion of cholesterol causes hypercholesteremia, while short period ingestion has no effect.

2. Synthesis of cholesterol

Except for the adipose and adult brain tissue, it is indicated in the litarature reviewed by Byers et al. (1952), that almost every organ is able to synthesize cholesterol. However, among all these, the liver is the major site of synthesis, supplying considerable amounts of cholesterol to the blood plasma (Byers et al., 1951; Friedman et al., 1951). Also Gould (1951) has found the same thing to occur in dogs. However, although the liver is the main source for cholesterol in the blood, a moderate change in its rate of synthesis would not alter the plasma cholesterol content (Byers et al., 1952; Cook, 1958; and Gould, 1955). Favarger (1957) reported that an increase in cholesterol synthesis does not necessarily imply the occurrence of hypercholesteremia as long as the newly synthesized cholesterol can leave the blood, or as long as the breakdown of cholesterol is itself speeded up.

3. Excretion of cholesterol

Intestinal excfetion seems to be the only way by which cholesterol is eliminated, and that it is partly

influenced by very high plasma cholesterol levels, although high plasma cholesterol levels do not result from decreased intestinal excretion (Byers et al., 1952).

4. Degradation of cholesterol

In 1943, Bloch, Berg, and Rittenberg and later in 1952 Byers and Biggs experimenting with rats, found that cholic acid is one of the end products of cholesterol destruction. Gould in 1950 found after administering labelled cholesterol to rats and mice, that he could recover a good fraction of the isotope in the respired CO_2, and even more in the fecal bile acids. Another product from cholesterol degradation, besides CO_2 and bile acids, is progesterone (Bloch, 1950). Other steroid hormones are also derived from cholesterol (Chaikoff et al., 1952).

5. Role of the liver in regulating plasma cholesterol

The liver is the major site for cholesterol degradation or conversion, and any changes in the fate of this process would cause changes in the plasma cholesterol (Byers et al., 1952; Cook, 1958). Byers et al. (1951) and Byers and Friedman (1952) have demonstrated that biliary obstruction in rats would result in a rise in the plasma cholesterol level.

Normally, hepatic destruction of cholesterol seems to be the major means of regulating plasma cholesterol, and it is only in case of complete inhibition of the hepatic synthesis, such as in case of hepatocellular disease,

that the noticeable change in plasma cholesterol level occurs (Byers et al, 1952).

Experiments done by Fredrickson et al (1954) have shown that preventing the blood cholesterol from reaching the liver would result in an increase in the hepatic cholesterol synthesis in order to compensate for the inhibition.

F. Other factors affecting the serum cholesterol level

A number of factors cause deviations of the blood lipids, including cholesterol from their normal levels. Some of these factors are: age, sex, diet, hormones, and stress.

1. Effect of age on blood cholesterol

The blood cholesterol has been found to be low in early infancy (Deuel, 1955). In newborn infants, the total blood cholesterol is 34 $\pm$ 15 mg per cent where 40 per cent of it is free cholesterol (Boyd, 1936). The blood cholesterol in premature infants does not differ from that of full-term infants, as reported by Whitelaw (1948). It has been shown by several investigators that as the child reaches puberty, the blood cholesterol tends to become stable and in advanced age there is no significant change in the blood lipid level (Deuel, 1955; Cook, 1958).

2. Influence of sex

It has been established that the above is true for both sexes. However, in females, the plasma cholesterol level is later influenced by hormonal factors during the menstrual cycle, pregnancy, and menopause.

There is a regular cyclic change of the plasma cholesterol level, with a rise before menstruation followed by a decrease during or after menstruation. (Cook, 1958).

During pregnancy, a hypercholesteremia is noted in most observations (Cook, 1958). As to the effect of menopause, a postmenopausal increase in the plasma cholesterol has been reported by Muhlbock and Kaufmann in 1938 and later confirmed by Oliver and Boyd (1953) and by Aldersberg et al. (1956).

3. Effect of hormones

The effects of some hormones on the plasma cholesterol levels in several different animal species, including man, have been studied extensively by many investigators. Noticeable effects have been reported, but the mechanism of action is not very clear (Harris et al., 1958; Cook, 1958).

A good review of some of the hormonal influences on the circulating cholesterol is given by Boyd and Oliver (1958), where it is seen that menopause, pregnancy, diabetes, myxedema, bilateral ovariectomy, and androgens cause hypercholesteremia, while thyrotoxicosis, ACTH, cortisone, thyroxine, and estrogens cause hypocholesteremia.

4. Effect of diet

As shown by Joslin et al. (1947) the normal value of blood cholesterol is not always affected by food. However, the kind of diet ingested sometimes seems to play a role in the level of the blood cholesterol. Not all the studies done on the effect of fat administration on the blood cholesterol level agree. In dogs, experiments done in 1914 by Terroine showed a rise in the level upon fat ingestion. Milbradt's results (1930) indicated the same thing in rabbits. On the other hand no such results were obtained by Bang (1918) and Blix (1926) in their experiments on dogs. In rats, dietary fat seems to have less influence on the serum cholesterol than in man (Stare, 1955). In the latter, most of the studies show some relationship between fat ingestion and rise in the plasma cholesterol level (Deuel, 1955).

As to the effect of cholesterol ingestion on the level of plasma cholesterol, experiments done on rats and rabbits resulted in hypercholesteremia (Peters and VanSlyke, 1946).

In man, many conflicting reports have been published, some suggesting a rise in the level of plasma cholesterol, and others a drop upon cholesterol ingestion with the diet. (Reviewed by Deuel, 1955 and Cook, 1958).

In considering the fat ingestion and its effect on plasma cholesterol level, a distinction should be made between animal or saturated fatty acids and vegetable or unsaturated fatty acids. From the review presented by Cook (1958), most evidence shows a decrease in the plasma cholesterol level upon ingestion of unsaturated fatty acids, and a rise upon ingestion of saturated fatty acids. Kinsell et al. (1956) showed a rise in serum cholesterol due to absence of unsaturated fatty acids in the diet. The direct relationship between the amount of unsaturated fatty acids and the decrease in serum cholesterol level was established in 1955 by Friskey et al. and in 1957 by Ahrens et al. Mention should be made of the differences in the hypocholesteremic effects which exist among the different kinds of vegetable fats such as corn oil and sunflower oil, the former being more effective than the latter.

However, according to Beveridge et al. (1956), Jones et al. (1956) and Keys et al. (1957), the ingestion of unsaturated fatty acids is not the only dietary factor that controls the level of plasma cholesterol, but there probably exist some other factors.

5. Effect of stress

Stress is one of the major factors which induces variations in the serum cholesterol. There are some conflicting reports about the effect of stress in its

various forms on the cholesterol level in the blood.

Experimental stress such as inanition in dogs as shown by Mann and White (1953) produced a marked decrease in the plasma cholesterol. A similar effect was demonstrated by the same investigators in rats. Sayer's work (1950) on rats subjected to non-fatal hemorrhage, or injected with ACTH, indicated the same effect. In humans, administration of ACTH to normal individuals caused a decfease in the plasma cholesterol (Conn et al., 1950). The mechanism by which stress influences the serum cholesterol level is not clear. Mann and White (1953) have suggested that reduction of the total serum cholesterol is a physiological response to stress, being mediated through the adrenal gland. However, administration of cortisone showed no such effect.

Recently, in 1961, Joyner et al. conducted a study on cockerels, subjecting them to conditionned anxiety stress, and found that the plasma cholesterol level was not significantly affected.

Let us consider very briefly some particular aspects of stress.

a. Cold

Like most of the reports from studies on stress in general, the findings from studies on cold stress in relation to serum cholesterol level are somewhat conflicting. It was shown in 1953 by Mann and White that animmediate

decrease of plasma total cholesterol occurred in rats when exposed to moderate cold temperature. However, Kuhl et al. (1955) found a significant increase in the serum cholesterol of 55 male subjects upon immersion in cold water.

More recently still Sellers and You (1956) reported an increase in the plasma cholesterol of rats exposed to a temperature of 1-3°C for 12 months.

b. Tissue injury

Tissue injury is another form of stress which might influence the serum cholesterol level. It has been shown by Mann et al. (1946), that the serum lipids, including total and free cholesterol, decrease after surgical operations. Later in 1952, Kyle et al. reported that in 9 out of 10 cases subjected to the operative stress of surgery there was a decrease in plasma cholesterol. They also observed reduction in the serum cholesterol level upon administering ACTH. They interpreted the response as being caused by the direct action of the adrenal cortical secretion, either by inducing a temporary impairement of the hepatic function, or more specifically, by suppressing the cholesterol synthesis.

c. Mental and emotional stress in relation to serum cholesterol level.

A study made by Grundy and Griffin (1959) on college students indicated that during examination periods the

serum cholesterol levels increased 23 to 27 per cent over control periods of relative relaxation. Another study made in 1958 by Friedman et al. on the effect of occupational stress on the serum cholesterol level of accountants, revealed that the highest serum cholesterol level for each individual occurred during severe occupational stress periods while the lowest levels were at periods of minimal stress. Wertlake et al. (1958) found a statistically significant increase by 11 mg per cent in the serum cholesterol of 44 students during examination periods as compared to the level during free periods.

Studies done on rats subjected to a stressful environmental situation and maintained on a high fat, high cholesterol diet for 10 months, showed greater degree of hypercholesteremia and hyperlipemia and more atherosclerosis compared with the control group put on a normal diet (Uhley and Friedman, 1959).

d. Starvation

Starvation has been widely used by a number of investigators as a form of stress in relation to the serum cholesterol level.

Such studies have been performed by different investigators under various conditions and on different animals. From the reports given, it is seen that there exist specific differences in the response of serum cholesterol level to starvation (Deuel, 1955).

It is known that during starvation, the major source of calories for the body are the fat depots. The fat from there is carried to the blood stream, liver and then to the extrahepatic tissues for oxidation.

There are differences in the experimental data of the effect of fasting on the composition of blood lipids. Earlier it was mentioned that studies on dogs indicated no change in serum lipids upon fasting. However, Terroine, (1914) subjected 7 dogs to a period of 22-35 days partial starvation and observed changes in the total lipids from +45 to -54 per cent of prefasting level. In 1916 Greene and Summers subjected puppies to starvation and found an increase of 200% in their blood lipids. On the other hand, upon subjecting adult dogs to fasting for long periods of time, they could obtain no significant rise.

Other studies on starvation in relation to blood lipids, including cholesterol, are those done by Underhill and Baumann (1916) who found first a decrease in blood lipids during the beginning of the starvation period, and then an increase towards normal, as starvation progressed.

Later, in 1940, Entenman et al. after subjecting dogs to 30 days partial starvation or to prolonged periods of undernutrition, reported no lipemia at all. However, when there was much loss in body weight, they observed a reduction in blood cholesterol, parallel to that in the fatty acids and phospholipids.

Kartin et al. (1944) reported that fasting did not affect the blood lipids in dogs, but that in man and monkey it resulted in an increase.

Starvation studies in relation to blood lipids were also made on rats. Sure et al. (1933) noted a drop in both fatty acids and phospholipids in the blood of fasted rats, but no change in the cholesterol. Kohn (1950) reported that in rats, there exist differences in the response of blood cholesterol between the different strains when subjected to one week of fasting. He interpreted this on a genetic basis involving a number of genes. However, hypophysectomy caused the same increase in the blood cholesterol level of different strains. In mice, as was known by Mac Lachlan in 1944, there was an increase in the total blood lipids upon fasting despite differences in strains. However, Hodge et al. (1947) showed that in fasting mice, lipemia occurred only during the first part of the fasting period and then decreased on the fifth day.

Studies made on rabbits indicated an increase in the blood cholesterol. (Ellis and Gardner, 1912; Shope, 1927). According to Shope (1927) fasting in the cat, guinea pig, swine and man resulted in a decrease of blood cholesterol level.

In 1953, Mann and White reported that inanition in dogs caused hypocholesteremia, resulting in a disproportionate reduction of the cholesterol ester fraction. At the same time they observed parallel changes in both the size and the cholesterol content of the adrenal glands. Their experiments on rats also showed a reduction. They further found that the administration of ACTH to normal dogs would have the same effect on plasma cholesterol as inanition, while cortisone was without effect.

As to the behaviour of plasma cholesterol of man during inanition, the general tendency is towards hyperlipemia. Early in 1932, Fahrig and Wacker reported a rise in all blood lipids of man during starvation. Later, in 1944 Kartin et al. came to the same conclusion, and reported a significant rise in blood cholesterol and blood phospholipids starting on the second day of complete starvation.

In 1932 Deuel and Gulick showed that the rise in total blood lipids of women was sharper than of man during starvation.

In line with the effect of inanition on blood cholesterol, it should be mentioned that a carbohydrate deficiency results in an increase in the serum cholesterol values, as seen in epileptic children (McQuarrie, 1933; Tolstoi, 1929).

F. Importance of blood cholesterol in atherosclerosis

Atherosclerosis is a very serious pathological condition occurring in the intimal and subintimal layers of arteries, specially the coronary arteries. Early in 1910, Windaus demonstrated that these depositions were lipids, fibrous tissues and cholesterol. Recently, this condition has become more common, and its seriousness has attracted the attention of many investigators. Lipids and liporoteins, particularly cholesterol, seem to play an important role in the occurrence of this disease. This was first suggested by Morrison et al. in 1948 as a result of their clinical and experimental observations. The general tendency has been the occurrence of atherosclerosis in persons with high serum cholesterol level, while in case of patients with hypochol esteremia the incidence of atherosclerosis has been very low (Wilens, 1947).

Some of the factors considered to be related to serum cholesterol level and their impact on atherosclerosis are hypothyroidism, adrenal cotex and corticosteroids, sex hormones, hypertension, occupational stress, and diet (Cook, 1958). Atherosclerosis has been induced in experimental animals, specially the rabbit, by feeding cholesterol. This is accompanied with a rise in the plasma cholesterol level as well (Anitschkow, 1933; Davidson, 1951; and Mann, 1956).

A good systematic study on rabbits is that of Wang et al. (1954) in which rabbits were fed cholesterol with a fat free diet. This resulted in a rise of plasma cholesterol, 30 times the normal level after 3 months of feeding. The first appearance of atheromatous plaques started one month after feeding, and continued increasing. This condition resembled the pathological condition found in human vascular disease.

There seems to be a general agreement that experimental atherosclerosis depends on the plasma cholesterol level and on the duration of hypercholesteremia. However, as indicated by many investigators, other factors could be involved in the development of atherosclerosis (Duff and McMillan, 1951; Kellner et al., 1951; Oppenheim and Bruger, 1952; Seifter et al., 1953; Wang et al., 1955a; Aldersberg et al., 1957).

VI. Tissue Cholesterol

Cholesterol is the major sterol present in animal tissues. The source of tissue cholesterol is (1) from biosynthesis, as occurring in the liver, intestine, adrenal glands, gonads and (2) absorption from the intestine.

Plasma cholesterol originates mostly from liver biosynthesis. There is a continuous interchange of plasma cholesterol with that of liver, and less so with that of spleen, intestine, adrenals, muscle, adipose tissue, skin, and arterial walls (Cook, 1958). However, its amount varies from tissue to tissue and from species to species. Cholesterol is a constituent of all body cells. Tissues and organs can be grouped into three categories as to their cholesterol content. Those with a high cholesterol level, containing more than 1 per cent of their fresh weight. The adrenal glands (rich in the ester form), the nervous tissue (rich in the free form), and the outer layer of skin belong to this class. The second class is medium in cholesterol content. This includes the liver, the kidneys, the lungs, glandular and adipose tissues, and the dermis of the skin. These have about 0.2 - 0.4 gm cholesterol per 100 gm fresh weight. The third category is that of low cholesterol content and involves the muscular and the supportive tissues, such as the bone and cartilage (Cook, 1958). It was estimated by Parker (1948) that in a normal man weighing 70 kg 0.2 per cent of his body weight is represented by the total cholesterol content, i.e., an amount of about 140 gm. Tables showing the amount of cholesterol in the different body tissues of different

animal species are presented by Cook (1958) in his review.

B. Effect of starvation on the content of body cholesterol

Fasting, as shown by Tomkins and Chaikoff (1952) in their in vitro experiment on rat liver slices, caused a reduction in the liver biosynthesis of cholesterol. The rate of biosynthesis decreased to 10 per cent of the normal value after 24 hours of fasting, and still more after 72 hours.

In vivo experiments showed also a decrease in the rate of synthesis, although it was less than those performed in vitro (Van Bruggen et al., 1952). More recently, Gould et al. (1959) have shown, by using radioisotope techniques, that the rate of incorporation of labelled acetate into cholesterol by liver homogenates of fasted rats was much less than that of normal control rats.

Whitney and Roberts (1955) suggested that the prefasting kind of diet plays a role in the influence of fasting upon hepatic cholesterol biosynthesis. A prefasting high fat diet seemed to counteract the effect of fasting on the rate of biosynthesis.

Chapter One

Effect of various forms of starvation on the serum cholesterol level of rats

1. Background

Data on the effects of complete starvation on serum cholesterol are scaree and what is found in the early literature seems to be somewhat conflicting. Lennox et al(1926) studied the effect of fasting on human subjects and found conflicting results for blood cholesterol. In one of the patients, the blood cholesterol level was very low at the beginning of starvation, then it increased gradually during the 11-day period of starvation. However, the patient died after this period. On the other hand, in two other subjects, the same investigators found much lower cholesterol levels during fasting compared to the prefasting levels as well as the levels after refeeding.

Also conflicting results have been reported to occur in laboratory animals. Bloor (1914) found that the blood lipids of dogs subjected to fasting increased during the first four or five days of fasting in three dogs, while it remained constant in three others.

In 1916, Greene and Summers found that the blood lipids increased in fasting puppies while it remained constant in fasting dogs. Terroine (1914) subjected dogs to a prolonged period of fasting and obtained a decrease in their blood lipids while Rothshild (1915) got an increase in four rabbits subjected to fasting for two to nine days.

Shope in 1927 using rabbits, cats, guinea pigs, swine and human subjects noted an increase in serum cholesterol during fasting. This was followed by an immediate drop upon the intake of food at the termination of the fasting and then by a rise sometime after the intake. Presumably the latter is due to the utilization of the ingested food.

Entenman et al. (1940)could find no striking changes in the levels of total cholesterol in dogs subjected to 30 days of acute starvation.

Kartin in 1944, reported a significant rise in the plasma cholesterol of human subjects fasted for 3 to 6 days.

The accumulated data obtained from a comparative analysis of the work done on mammals indicate that in most of this class of animals, (dogs and some species of rats being exceptions), short periods of fasting seem to induce hyperlipemia (Gillman et al., 1959). These data also indicate that the rise in the circulating lipids is mainly attributable to a rise in cholesterol.

In this study, the animal species used for the study of the effect of starvation on cholesterol level is the albino rat raised in the Biology Department of the American University of Beirut.

In an early study done by Sure et al (1933) on albino rats, it was observed that complete fasting with liberal supply of fresh distilled water caused no demonstrable change in the blood cholesterol content. Later in 1945, Levin,

studying the effect of stressful conditions, e.g. starvation on the adrenal and serum cholesterol content in albino rats, found a decrease in both levels.

In this study, interest is focused on the effect of starvation of varying degrees on the total serum cholesterol of adult male albino rats of a given age group.

II. Review of some various methods for the determination of serum cholesterol

From the time Salkowski (1872) discovered that cholesterol could develop a color with sulfuric acid, various quantitative methods have been developed (Liebermann, 1885; Zlatkis et al.,1953; Zak et al., 1954; MacIntyre and Ralston, 1954; King and Wootton, 1956). All these methods and various modifications of them have been shown to produce consistent results among themselves. These procedures vary depending on whether cholesterol is extracted from the serum with various solvents or whether it is determined directly on the serum.

Free cholesterol forms an insoluble precipitate with the alkaloid digitonin (Windaus, 1909; 1910). This property has been used by Schoenheimer and Sperry (1934) to develop a microgravimetric determination. This method and various modifications of it (Burn, 1939; Sperry and Webb, 1950) are the standard methods for the determination of cholesterol.

The digitonin precipitation can be used in conjunction with a colorimetric method or an oxidative method for

the estimation of ester and free cholesterol (Kirk et al., 1934; Folch et al., 1940).

III. Method used in this work and the reasons for choosing it

Among the several methods known for serum cholesterol determination, the one used throughout this study is the MacIntyre's method, as described by King and Wootton (1956). However, it was modified slightly by using half the quantities mentioned. This is the method that was developed by Zlatkis et al. (1953) as mentioned previously, and it depends on the production of a purple red color when an acetic acid solution of cholesterol is treated with $FeCl_3$ and H_2SO_4. The reasons for which this method was chosen in this study are the following:

1. All reagents needed are easy to get and prepare, and are not very expensive.
2. Stock solutions can be prepared and stored in the refrigerator.
3. It is not a time consuming method, and it saves a lot of trouble in that there is no need to extract the cholesterol and precipitate the proteins, as is done in the Liebermann-Burchard method of King (1951).
4. The color developed is stable, giving constant readings over three hours.
5. Reproducibility of the method is another advantage. MacIntyre (1954), ran ten determinations on the same sample of human serum and got them all in the range of 235-240 mg per 100 ml. Also, as MacIntyre and Ralston (1954)

mention in their article, when the results of this method were compared with those obtained by other methods, the analysis of variance showed no significant difference between them. The standard deviation they got was ± 12 mg per 100 ml. Furthermore, determinations on the same samples of chicken sera were done in the Biochemistry Laboratory and by myself independently. The results showed very good agreement.

IV. Materials and procedure

0.05 ml serum samples are measured with a 0.1 ml blow pipette and placed in test tubes. Duplicate samples are used whenever available. To each sample, 3 ml of the stock aldehyde-free glacial acetic acid are added. This solution is then treated with 2 ml of a $FeCl_3$ - H_2SO_4 color reagent added to the side of the tube, thus forming a separate layer at the bottom. The contents of the tube are mixed to allow uniform heat distribution. Upon mixing, a yellow to brown color develops which finally turns purplish. Cooling to room temperature is necessary before attempting to read the optical density. The cholesterol concentration is calculated in terms of mg of cholesterol per 100 ml of serum according to the formula:

$$Cu = \frac{O.Du}{O.Ds} \times Cs$$

where Cu and Cs are the concentrations of the unknown and the standard, and O.D.s are their respective optical densities.

The measured standard solution consists of a mixture of 0.2 ml standard stock solution (containing 0.2 mg per ml), 0.05 ml distilled water, 2.75 ml acetic acid and 2 ml $FeCl_3$ - H_2SO_4 color reagent. The color developed is a light purple.

All readings are made against a blank solution consisting of 0.05 ml distilled water, 3 ml acetic acid, and 2 ml. color reagent.

V. Experiments performed

A. Effect of complete starvation carried to death on the total serum cholesterol level

(1) Experimental

This part of the experiment consisted in subjecting a group of seven month-old rats to complete starvation until they died, and studying the changes that occurred in the level of total serum cholesterol.

Studies were first made on a group of six 7 month old male rats. (Rats No. 81, 83, 84, 103, 105 and 106). Their normal serum cholesterol level was determined three times, then all food was removed. The animals were allowed to drink ad libitum. Serum cholesterol determinations were performed at intervals of two to three days. The weight of the rat was recorded before fasting and at the time of death. The same kind of study was later performed on two more rats, 42, and 43, starved until death, and used for tissue cholesterol determination. (see chapter II). For these two rats the body weight was recorded every time blood was drawn.

The procedure for drawing blood and obtaining serum for cholesterol determination was as follows:

The blood samples were obtained from the rat's tail as described by Farris and Griffith (1949). The rat was wrapped in a piece of cloth with only its tail protruding. The tail was dry-shaved and then dipped into warm water for one to two minutes with slight massage, to cause vasodilation. Blood was drawn from the vein with a 2 ml-Syringe and a 25-gauge needle and collected in small 1-ml tubes, allowed to clot, and centrifuged after 15-20 minutes. The serum was pipetted out and kept frozen until used.

In case it was difficult to obtain blood with the procedure described as after a long period of starvation when the blood flow becomes very slow, the tail bleeding method described by Farris and Griffith (1949) was used. In this method the tip of the tail was cut, allowing the blood to flow directly into the tubes.

(2) Results and discussion

The results of this part of the work are shown in Tables I and II. Table I shows the changes that have occurred in the body weight and in the serum cholesterol level of each of the 6 rats subjected to complete starvation until they died.

From these results we see that the body weight undergoes upon starvation a great reduction: the mean weight having dropped from 380 $\pm$ 8 to 205 $\pm$ 7 when the rats died. The

reduction appears to be quite uniform in all the animals.(1)

The serum cholesterol of all rats has increased on the second day after starvation and this continued until about the seventh day, after which the level tended to go down towards normal. Such a rise does not seem to agree with the results of Levin (1945) who found a decrease in the serum cholesterol of rats subjected to starvation.

Also it does not seem to agree with Sure's findings (1933) who, upon completely starving rats, could find no striking change.

Table II shows the effect of complete starvation on body weight and the serum cholesterol level in two one year-old rats, No. 42 and 43, which upon dying were used for tissue cholesterol determination.

As can be seen, the serum cholesterol level has increased after starvation and reached its peak at about the seventh day of starvation, after which it started decreasing.

B. Effect of short-term starvation on the serum cholesterol level

(1) Experimental

In this experiment two rats (No. 68 and 80) were starved for seven days, after which they were sacrificed and used in the tissue cholesterol determination.

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(1) Unfortunately the data is not complete as this was the first experiment performed and the procedure was not then completely defined.

(2) Results and discussion

The results are indicated in Table III. Here too there is a rise in serum cholesterol levels. However, in case of rat No. 68 there is a decline on the third day; at sacrificing the level was back to the prefasting value.

C. Effect of 7 day starvation followed by refeeding on the serum cholesterol level

(1) Experimental

In this experiment two groups of rats were studied. The first consisted of 5 eight month old male rats, starved for six days, then refed for 81 days. Serum cholesterol was determined daily during starvation and the body weight recorded. After termination of fasting, the serum cholesterol determinations were done at weekly intervals, during the refeeding period. The results for this group are presented in Table IV.

The second group of rats consisted of 4 nine month old male rats which also were starved for 5 days then refed for 92 days. Daily cholesterol determinations were done during starvation, and weekly determinations were done during the refeeding period for 11 weeks. After the 92 days of refeeding the animals were sacrificed and their tissue cholesterol content determined.

Table V includes the data on this group.

(2) Results and discussion

From what is seen in Tables IV and V there is definitely a rise in the serum cholesterol level immediately after starvation. This is maintained in most of the animals for the first 5 to 7 days, after which there is a decline towards the prefasting level.

However, for almost every rat, one can notice the presence of a period at which the cholesterol value has reached a peak. This is usually seen at around the 3rd day of starvation.

During the refeeding period, it is seen from the Tables that serum cholesterol suddenly rises on the second day of refeeding and then starts approaching the prefasting level, and by the seventh week, as seen in Table IV, this level is reached.

In the second group, serum cholesterol determination after refeeding was done for 7 weeks, and on the 7th week the tendency towards the prefasting level is shown very clearly.

In this group blood was drawn on the 92nd day of refeeding for serum cholesterol determination, and the rats were subsequently sacrificed for tissue cholesterol determination. The results at various intervals in both experiments are summarized in Table VI.

VI Conclusion

From the results, it is seen that in all these rats of the group, the serum cholesterol had stabilized at a normal level before the beginning of starvation.

We could probably say, by looking at both Tables (IV and V), as well as at the Tables discussed before (I,II and III) that starvation has caused in most cases an immediate rise of total serum cholesterol, reaching a maximum level at about the third day of starvation and then starting to decrease gradually. In some cases it was maintained high until the seventh or eighth day after which an approach towards the normal prestarving level was seen. In case refeeding was done after a 5-6 day starvation period, the serum cholesterol dropped and from what is seen, it could be suggested that it takes the rats 7-8 weeks of refeeding to have their serum cholesterol back to normal.

If we consider all the possible metabolic pathways of cholesterol and try to see how each one is behaving in case of starvation, we might be able to limit ourselves down to some possible explanations for the behaviour of serum cholesterol during starvation. Figure (1) represents the various metabolic pathways of serum cholesterol.

The main two sources of blood cholesterol are dietary or exogenous cholesterol and hepatic or endogenous cholesterol. Friedman et al (1951) have shown in their partial hepatectomy experiment on rats, that the liver is a main source of plasma

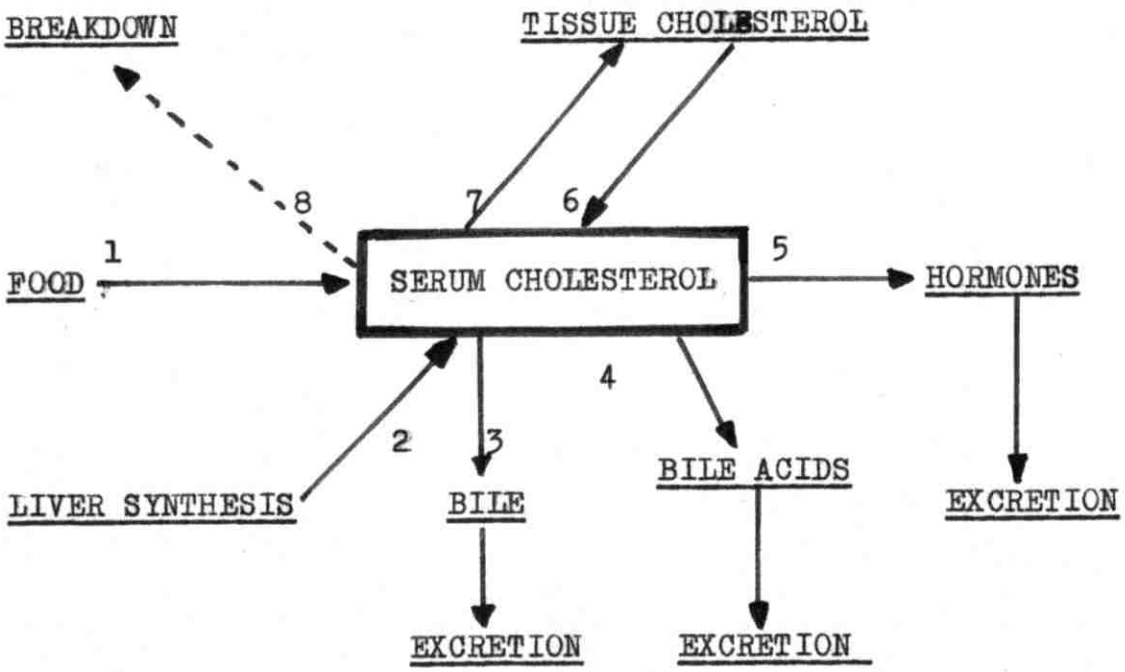

Figure I Diagram showing the metabolic pathways of serum cholesterol in the animal body.

cholesterol. Gould, (1951a), Harper et al.(1953), and later Eckles et al. (1955) came to the same conclusion, using the radioisotope technique on dogs. Upon hepatectomy, there was no indication of newly synthesized cholesterol molecules in the plasma. If we now consider each one of the pathways separately under the condition of starvation, we see that the food pathway (1) is blocked since the animals are receiving no food at all, and therefore there is no possibility of having any exogenous cholesterol. Pathway (2) refers to the hepatic synthesis. From earlier experiments done both in vivo and in vitro, it was found that there is a decreased hepatic biosynthesis of cholesterol in fasted rats. This was shown by Tomkins and Chaikoff (1952) who studied the synthesis in fasted rat liver slices. They could detect a reduction in the hepatic synthesis rate to about only 10% of the rate of synthesis in control animals, after 24 hours of starvation. The value decreased much more after 72 hours of starvation.

Van Bruggen et al. (1952) came to the same conclusion that the hepatic cholesterol synthesis decreases upon starvation, after their experiments on rats.

Therefore, the possibility that the liver as being the contributor to the increased serum cholesterol level could be eliminated.

In pathway (3) cholesterol is excreted directly in bile. However, the amount excreted in rats is only about 1.8 mg daily on the first day of bile fistula and less on the following days as determined by Friedman et al. (1950). Eriksson's findings (1956; 1957) confirmed the above. Consequently one can say that this pathway is of little quantitative significance.

As to pathway (4) dealing with the excretion of cholesterol in the form of bile acids, what happens exactly to this pathway during starvation is not known, but what we might be able to say is that excretion is being reduced greatly as a result of the absence of food ingestion, and therefore it could be that we get neither a loss nor a formation of new bile acids because of a feedback mechanism through which bile acids inhibit the breakdown of cholesterol into bile acids. Thus a block in pathway (4) could be an explanation for this initial rise. As to pathway (5) this is considered to be a minor one during which only about 5 per cent of the serum cholesterol is converted into steroid hormones. Whether this pathway is blocked or not during starvation should not really be of a great quantitative importance in the serum cholesterol level.

Pathways (6) and (7) deal with cholesterol deposition and mobilization in the tissues. Not much is known about these routes in starvation.

A possibility is an increase in the rate of mobilization of cholesterol from the tissues, thus accounting for a rise in serum cholesterol level.

As seen in Tables I-VI, after about 5 to 6 days there is a tendency towards a decrease and the serum cholesterol level approaches the normal value. Obviously this decrease could not be explained by pathways (3), (4) or (5) as (3) and (5) are of minor importance and from a quantitative consideration, (4) should be inhibited by the accumulation of bile acids.

Pathway (8) has never been shown to be of great significance when radioactive isotope was administered to normal rats (Chaikoff et al., 1952a). However, whether starvation could cause the production or release of a new enzymatic pathway is a question which has not been yet answered.

VII Summary

Some investigators have found an increase of serum cholesterol upon starvation, while others have found a decrease. In this work, done on albino rats, the following was found:

1. An initial increase for the first few days reaching the peak at about the third or fourth day.

2. This was followed by a gradual decrease until the level approached normal.

3. In most cases, on the second day after refeeding, there was a sudden rise in serum cholesterol, and then it approached normal after about two weeks.

TABLE I

The effect of total starvation on body weight and serum cholesterol level

Days Starved	Body weight in gms and serum cholesteron in mgs per 100 ml											
	Rat 81		Rat 83		Rat 84		Rat 103		Rat 105		Rat 106	
	wt.	chol.	wt.	chol.	wt.	Chol.	wt.	chol.	wt.	chol.	wt.	chol.
0	350							90		94		90
0		80				79						
0	350	73	365	72	393	82	405	88	390	74	375	87
1				139		135		137		141		163
2				138				130				148
3		141								132		
6		145		151	224	110-		142		160		128
7		125	197	-				110		153		142
8		80						120		108		112
9	200	-						134				87
10								130	222	-	188	-
12							199	-				

(-) Indicates day of death

Some results in this table are missing as this was the first experiment and the plan was not completely set

TABLE II

The effect of starvation till death on the weight and the serum cholesterol. (Rats used for tissue cholesterol. See Chapter Two)

Days Starved	Body Weight in gm and mg cholesterol per 100 ml serum			
	Rat 42		Rat 43	
	Weight	Choles.	Weight	Cholesterol
0	502	97	462	85
0	500	98	458	85
0	484	96	433	87
2	422	116	387	116
3	405	135	370	105
5	388	122	350	122
8	347	118	310	104
10	318	122	278	109
12	301	120	264	-
13	290	123		
14	274	120		
15	265	-		

- Indicates day of death

TABLE III

The level of serum cholesterol upon starvation for seven days. (Rats were sacrificed after the 7 day period for tissue cholesterol determination (See Chapter Two)

Days Starved	Rat 68		Rat 80	
	Wt. gm	mg Chol. per 100 ml serum	wt. gm	mg. Chol. per 100 ml serum
0	450	87	418	96
1	412	101		
2		110	392	104
3	384	76		
4			363	119
5	356	72		
6				
7	338	80	330	

TABLE IV

The effect of 7-day starvation on and refeeding on the body weight and serum cholesterol level. (I)

	Date	Rat 31 wt.gm.	Rat 31 Chol.*	Rat 107 wt.gm	Rat 107 Chol.*	Rat 111 wt.gm.	Rat 111 chol.*	Rat 112 wt.gm	Rat 112 Chol.*	Rat 115 wt.gm	Rat 115 Chol*
Pre-fasting	Nov. 13	308	82.8	375	103	338	94	389	106	439	120
	Nov 14	286	89.5	358	96.1	328	101	379	109	420	116
	Nov 16	302	83.3	357	92.2	340	83.4	379	107	431	122
	Nov 29	310	92.6	360	94.4	343	96.8	389	106	442	122
	Dec 4	315	95.2	363	98.5	345	92.8	397	104	447	115
Starvation	Dec 5	280	130	328	117	306	125	336	129	414	122
	Dec 6	263	136	309	120	291	124	346	131	393	120
	Dec 7	250	132	281	123	295	146	333	120	379	134
	Dec 8	240	124	260	109	289	112	325	115	370	117
	Dec 9	227	112	258	96.8	272	110	315	91.2	357	114
	Dec 10	212	-**	241	-**	256	-**	305	-**	340	-**
Refeeding	Dec 11	241	88.8	250	110	290	93.6	335	103	340	119
	Dec 18	277	132	339	137	350	136	388	118	416	143
	Dec 26	281	127	342	106	361	125	402	119	396	135
	Jan 2	297	98.8	357	110	338	143	421	122	415	122
	Jan 8	311	108	366	93.6	379	112	441	105	434	111
	Jan 15	320	109	373	104	384	116	444	109	462	123
	Jan 22	281	147	379	110	396	119	461	111	475	129
	Jan 31	341	98	402	80	410	81.7	489	80	496	96
	Feb 5	343	97.9	388	88	402	102	492	100	490	101
	Feb 12	348	94.3	366	89	391	98.7	495	107	501	106
	Feb 19	353	107	402	89.6	378	99.4	507	88	490	96.8

* Cholesterol in mg per 100 ml. serum

** No determination was done on this day

TABLE V

The effect of 7-day starvation and refeeding on body weight and serum cholesterol level.(II) Rats were sacrificed on April 12th for tissue cholesterol studies -(See Chapter Two)

Date		Rat 34		Rat 36		Rat 37		Rat 39	
		wt.gm.	Chol.*	wt.gm.	Chol.*	wt.gm.	chol.*	wt.gm.	Chol.*
Dec 20	Pre-fasting	425	111	390	100	391	101	395	102
Dec 27		427	112	411	96	388	102	388	102
Dec 30		428	114	401	98	397	104	390	104
Jan 3		440	115	-	-	409	103	395	105
Jan 4	Starvation	393	117	357	108	357	134	353	100
Jan 5		374	144	339	128	342	144	323	136
Jan 6		357	159	327	142	329	142	318	128
Jan 7		337	149	306	142	310	144	302	131
Jan 8		320	-	290	131	300	136	290	132
Jan 9		299	146	284	127	284	127	268	130
Jan 10	Refeeding	337	122	310	102	328	108	304	105
Jan 17		391	152	388	133	377	141	362	142
Jan 24		415	120	402	116	402	120	363	121
Jan 31		426	114	426	118	421	110	404	115
Feb 7		429	104	429	98	420	100	416	100
Feb 20		444	120	438	119	427	114	435	117
Feb 28		441	116	422	101	425	108	432	109
Apr 12		436	90	470	74	437	74	475	80

* Cholesterol in mg. per 100 ml. serum

TABLE VI

The changes in weight and serum cholesterol upon starvation and refeeding - Mean values and standard deviation of the mean

	Experiment I* (Table V)		Experiment II * (Table VI)	
	Body Weight gms	Choles. mg/100 ml	Body Weight gms	Choles. mg/100 ml.
Initial	373 ± 23	101 ± 3.9	404 ± 8.3	105 ± 3.3
3 days starvation	307 ± 16	131 ± 4.6	333 ± 8.4	143 ± 6.3
6 days starvation	271 ± 23	105 ± 4.6	279 ± 9.0	133 ± 4.6
	(5 days starvation)			
1 week refeeding	354 ± 24	133 ± 4.2	380 ± 6.6	142 ± 3.9
4 weeks refeeding	386 ± 24	106 ± 3.2	423 ± 3.3	114 ± 1.7
7 weeks refeeding	426 ± 26	87 ± 4.0	430 ± 4.2	109 ± 3.1
10 weeks refeeding	426 ± 31	96 ± 3.4		

* Experiment I included 5 rats; experiment II included 4 rats

CHAPTER TWO

STUDIES ON THE DISTRIBUTION OF CHOLESTEROL IN THE RAT'S VISCERA AND CARCASS, AND THE EFFECT OF STARVATION ON THESE

I. Brief literature review and purpose of the experiment

As it was stated previously in the "Historical Review", cholestefol is present in various tissues of the body in different concentrations. There are also species variations. Certain factors seem to affect the concentration of cholesterol in the tissues, among which are age, sex and diet (Cook, 1958).

Studies have been done on the equilibration of cholesterol between blood and tissues. Gould, in 1952, studied the passage of cholesterol from the blood into the extrahepatic tissues in dogs after having given them a blood transfusion containing labelled cholesterol in the normal lipoprotein form. His findings showed that the liver and spleen are the first organs to equilibrate with the blood, because he found that the cholesterol of these organs had a specific activity equal to half that of the plasma after few hours of introduction of labelled cholesterol. He also found a slower rate of equilibration for the kidney, lung, heart, intestine and diaphragm. In these organs, it took one to two days to have the equilibration accomplished. Still slower were the adrenal glands, the aorta, and the skin.

In rats, studies done in 1953 by Chevalier on the distribution of cholesterol in the tissues, have shown that upon feeding cholesterol-C^{14} to rats, it takes 9 days for the intestine, liver and serum to reach a high specific activity. The lung, heart, adrenals, fat depots, and spleen had between 60 to 80 per cent of the serum value, while the kidney, skin, and muscle had about only 30 per cent. The brain showed no equilibration at all, since no specific activity could be detected in it.

Among all tissues, the liver has been always shown to be the major site for cholesterol metabolism, including biosynthesis, catabolism and conversion.

The purpose of this investigation was to study the cholesterol distribution in the normal rat's tissues, divided in all cases into viscera and carcass, and to see how starvation, applied for different periods of time, affects the cholesterol contents, taking into account each time the behaviour of the serum cholesterol level.

Since the body weight of the rat is decreasing to almost its half upon starvation, then the concentration of tissue cholesterol, if cholesterol remained intact during starvation, would come out to be about double the amount we have in the normal non starved rats.

II. Method for isolating and determining body cholesterol

The method used in this experiment for isolating the body cholesterol is a modification of the

Sperry and Schoenheimer method developed in 1935. Determination of the cholesterol after extraction was done by the MacIntyre's method described previously.

Principle of the Method

The isolation procedure used for cholesterol is based on the principle of saponification and separation of the non-saponifiable fraction which would include the sterols.

The principle consists in hydrolyzing the material with an alcoholic potassium hydroxide solution, thus forming soaps of the fatty acids. The steroids, including cholesterol would remain ether soluble when saponification is complete. The non-saponifiable fraction is extracted with diethyl ether.

III. Procedure for cholesterol extraction from the rat's tissues

Living rats were killed by a head stroke, cut, and blood drained as much as possible(1). The latter was deemed necessary, because it has been reported (Hansard, 1956) that the amount of blood left in the organs after death varied greatly, thus affecting the tissue cholesterol concentration

(1) The amount of blood drained was in all cases very small, not exceeding 5 to 10 ml. which would amount to about 10 mgs. cholesterol.

In case the rats had died from starvation, they were taken directly through the extraction procedure. The rat was opened, all its viscera removed, weighed, and placed in a Waring Blender with an equal volume or more of 7% KOH in 95% ethanol, and homogenized well until it formed a uniform suspension. The whole carcass with the skin was weighed and subjected to the same process.

After homoginization, the total volume of the homogenate was measured and kept in closed bottles. From the total homogenates small aliquot samples were taken into test tubes and diluted with 7% alcoholic KOH in such a way as to have about 20 fold the total dilution of the original tissue. Saponification was allowed to take place for 2 to 3 hours by placing tubes into a hot water bath and refluxing the contents, using small reflux condensers which fitted into the tubes.

After saponification, the tubes were removed, opened and placed in a hot water bath under an electric fan to allow maximum evaporation of the alcohol. The residue in the tubes is a mixture of both saponifiable and non-saponifiable matter. To this, about 1 ml. of distilled water was added, followed by 5 ml. of diethyl ether and the mixture was shaken. The upper ether layer was pipetted out into a large test tube. The extraction was repeated twice, each time using about 5 ml.

ether to ensure complete extraction of cholesterol. The combined ether extracts were then evaporated to dryness. Then the tubes were closed with sheet paraffin and kept at room temperature until used.

When determination was to be performed, the dry residue was dissolved in 5 ml. glacial acetic acid, and the same method described previously for serum cholesterol determination was followed.

The determination was made on aliquots of 0.5 ml. of the acetic acid solution.

The amount of cholesterol in the tissue was calculated as follows:

$$Cu = \frac{ODu}{ODs} \times Cs\ (0.04\ mg) \times \frac{5ml}{0.5ml} \times \frac{\text{Total Volume of Homogenate}}{\text{No. of ml. of Homogenate aliquot}}$$

= mg Cholesterol per Total Tissue Weight

Where Cu and Cs refer to the concentration of the unknown and the standard respectively. The O.D.u. and O.D.s. refer to the optical densities of unknown and standard.

Viscera and carcass cholesterol determinations were performed on normal and experimental rats. The latter group involved 4 sub-groups classified on the basis of the duration of starvation.

The sub-groups were:

1. Animals starved until death. These consisted of rats No. 42, 43, 59 and 62.

2. Animals (No. 64 and 61) starved for 2-3 days, then sacrificed. (A period of 2-3 days of starvation was found to be the critical period during which the serum cholesterol level was raised and reached a peak (Please refer to Chapter One).

3. Animals (No. 68 and 80) starved for 7 days and then sacrificed. (This period was chosen so that these rats would act as a second control for the rats in group 4.

4. Animals that had been starved previously for 7 days and then refed. This group consisted of rats No. 34, 36, 37 and 39. When these were sacrificed, they had been on ad libitum diet for a period of 92 days after the termination of the fasting period.

IV. Results and discussion

The results of this experiment are represented in Tables VII, VIII, IX and X.

Table VII shows the effect of starvation on the body weight, serum cholesterol and total body cholesterol. On analysing the data it is clearly seen that, with the exception of one animal, which died after only 4 days of starvation, the total body cholesterol is remarkably lower in starved animals compared to that of the animals kept on ad libitum diet. On the other hand, the serum cholesterol level preceeding death is higher compared to the prefasting level. Rats No. 68 and 80 starved for 7 days and then sacrificed

also show a low total body cholesterol while at the same time their serum cholesterol is elevated.

Table VIII shows the effect of starvation on the weight of the tissues, the concentration of cholesterol in them and their cholesterol content. It is seen that upon starvation, the cholesterol level of both the viscera and the carcass are low compared with the animals given ad libitum diet, despite the higher level in serum cholesterol compared with the normally fed animals (Table VII). It is interesting to note that in 4 animals dying of starvation, the level of cholesterol in the viscera as well as the relative distribution of it between viscera and carcass is extremely low.

Table IX shows the body weight, serum cholesterol and total body cholesterol of animals which were fasted, refed and then sacrificed. Unfortunately, serum cholesterol levels were not available at the time of sacrifice due to an unanticipated mishap. However, as can be seen on comparison with the data in Table V, serum cholesterol has stabilized after the 3rd week of refeeding and remained constant for 5 weeks.

These data show that while serum cholesterol is back to or near normal level, the total tissue cholesterol is remarkably low when compared with animals not subjected to fasting at all (Table VII), even though they had free access to food for a period of 92 days after the termination of fasting.

The distribution of cholesterol in viscera and carcass of these refed rats is presented in Table X. It is seen from the comparison of these with the unstarved rats presented in Table VIII, that the viscera cholesterol to carcass cholesterol ratio is lower in the fasted and refed animals compared with the normally fed ones.

V. Conclusion

From the above studies done on the effect of starvation on cholesterol distribution in the tissues, starvation seems to cause a decrease in the total body cholesterol content. This decrease is reflected in both viscera and carcass. On the other hand, starvation seems to increase the serum cholesterol concentration specially for the first few days after the initiation of starvation. In refed animals where serum cholesterol tends to reestablish itself back to normal in about 3 weeks of refeeding, tissue cholesterol level is still markedly lower in the animals of this experimental group compared with those which were not denied food at all, despite their being on ad libitum diet for a period of 92 days after the termination of fasting. This is even more striking when the concentration of cholesterol in the tissue rather than the total amount is considered.

In distinction to the results presented for the investigation in Chapter One, where serum cholesterol was studied and where each rat acted as its own control,

individual variations among the animals studied in this investigation contribute somewhat to the lack of closer uniformity in results. For example, it is seen that a comparison of the total body cholesterol of rats No. 50 and 56, both subjected to exactly the same laboratory conditions, indicate a tremendous variation.

However, in spite of this variation, the tissue cholesterol in the starved rats, specially in those starved to death, is much lower than the normal range thus reflecting a real substantial reduction due to starvation. It would be desirable to perform the experiment on a larger number of rats, preferably littermates, raised under more constant conditions of temperature, humidity, etc.....

Also, it would be desirable to run the starvation experiment using radioactive cholesterol as an internal control. A preliminary attempt at doing the latter by preparing biologically labelled cholesterol and injecting it to a rat was undertaken. The results showed that the total body cholesterol had dropped to 37% of its original value in animals starved to death.

One question which may arise is: What is the fate of the cholesterol which presumably is being utilized by the starving animal? From the discussion in Chapter One, it is clear that its contribution to the synthesis of steroid hormones could not be an explanation, because of the relatively small magnitude of the catabolic pathway involved in this.

It is unlikely that it could have gone into bile acids because of the feedback mechanism whereby bile acids inhibit their own synthesis from cholesterol. These points were confirmed by the results of the preliminary radioactive experiment. The hypothetical pathway through which cholesterol is broken into CO_2 (pathway (8), Chapter One, Fig. 1) does not seem to be operating in a normal rat. However, it is not unlikely that such a pathway could exist and acquire importance during starvation. Future experiments will have to be done to bear evidence upon this point.

VI. Summary

1. There was a decrease in tissue cholesterol upon starvation which was more pronounced in extreme cases where the total body cholesterol reached below half its initial value.
2. The concentration of cholesterol in both the viscera and the carcass decreased in starvation. The concentration in the viscera decreased very sharply shortly before the rats died.
3. Upon refeeding, although the serum cholesterol went back to the prefasting level, the tissue cholesterol did not, even after 92 days of refeeding.
4. A preliminary experiment with radioactive cholesterol confirmed the marked decrease of total tissue cholesterol upon starvation.

Table VII

The effect of starvation on body weight and tissue cholesterol

Rat No.	Days starved	Rat wt. gms		Serum cholesterol mg/100 ml		Total body cholesterol mg
		Initial	Final	Initial	Final	
50	0	482		89		1730
52	0	421		91		1499
54	0	416		75		**
56	0	455		84		890
64	2	524	472	92	103	989
61	3	550	315	86	89	612
59	4 death	366	278	95	*	1273
68	7	450	338	85	72	637
80	7	418	330	96	119	926
43	10 death	451	264	86	109	305
62	12 "	437	275	78	*	465
42	14 "	495	265	97	120	352

* The serum cholesterol level was not determined because the rat died from starvation before its blood was collected

** The total body cholesterol could not be determined in this rat because the carcass homogenate was lost

Table VIII

The effect of starvation on the weight and distribution of cholesterol in the tissues

Rat No.	Days starved	Viscera			Carcass			percentage distribution of cholesterol	
		Wt. gms	Conc. chol. mg/100 gm	Total chol. mgs.	wt. gms	Conc. chol. mg/100 gm	Total chol. mgs	Viscera	Carcass
50	0	88	535	470	372	349	1260	27	73
52	0	86	451	386	336	298	1113	26	74
54	0	75	168	126	390	*	*		
56	0	88	170	150	326	227	740	17	83
64	2	81	404	323	364	180	666	33	67
61	3	80	193	155	371	125	457	24	76
59	4+	49	185	90	224	190	1183	7	93
68	7	54	302	160	272	173	477	25	75
80	7	66	392	259	303	220	668	28	72
43	10+	32	111	35	241	112	270	11	89
62	12+	29	124	36	252	170	428	8	92
42	14+	32	98	31	235	137	321	8	92

* Carcass sample lost

\+ Died as a result of. starvation

Table IX

The effect of starvation and refeeding on body weight and serum and tissue cholesterol

Rat No.	Rat weight in gms *			Serum choles. mgs/ 100 mls *;+			total body cholesterol mgs
	Pre-fasting	End of fasting	7 week refeeding	Pre-fasting	end of fasting	7 weeks re-feeding	
34	440	299	442	115	146	116	517
36	401	284	422	98	127	101	602
37	409	284	425	103	127	108	582
39	395	268	432	105	130	109	803

* For details see Table V

+ The last reliable serum determination is given - The one at 92 days refeeding is not reliable.

Table X

The effect of starvation and refeeding on the weight and distribution of cholesterol in tissues

Rat No.	Viscera			Carcass			Percentage distribution of cholesterol	
	Wt. gm.	Conc. chol. mg/100 gm	total chol. mgs.	wt. gm.	Conc. chol. mg/100 gm	total chol. mg.	Viscera	carcass
34	77	125	96	327	129	421	18	82
36	99	165	163	330	133	439	27	73
37	85	140	119	318	145	462	21	79
39	84	213	180	337	185	623	22	78

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