Hypercholesteremia and bacterial infections in rabbits
by Rosalyn Woodland White

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology
Montana State University

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In additional studies with mice, injection of cholesterol into neonatal mice apparently produced no ill effects, but addition of cholesterol, particularly with lard, for a long period of time to the diet of young and adult mice resulted in the development of lung tumors in some of the mice.

It was concluded that the addition of cholesterol and fat to the diets of rabbits and mice may result in an impairment of the immune mechanism which renders rabbits susceptible to their normal flora and mice susceptible to the development of tumors.

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Approved:

William G. Walter
Head, Major Department

Berenice G. Bayliss
Chairman, Examining Committee

K Goering
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

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ABSTRACT

Hypercholesteremia can be induced in laboratory animals by feeding high-fat diets or diets supplemented with cholesterol with or without fat. These hypercholesteremic animals become subject to a variety of lesions according to species including atherosclerosis, anemia, xanthomatosis, and cancer.

In this study, rabbits made hypercholesteremic by the addition of 1% cholesterol and 4% corn oil to their feed either died or incurred infections leading to sacrifice. Cultures of material from lesions and organs of these animals at death yielded only bacteria considered part of the normal flora of the rabbit.

In additional studies with mice, injection of cholesterol into neonatal mice apparently produced no ill effects, but addition of cholesterol, particularily with lard, for a long period of time to the diet of young and adult mice resulted in the development of lung tumors in some of the mice.

It was concluded that the addition of cholesterol and fat to the diets of rabbits and mice may result in an impairment of the immune mechanism which renders rabbits susceptible to their normal flora and mice susceptible to the development of tumors.

INTRODUCTION

Cholesterol is a white, waxy solid which is insoluble in water but soluble in fats and fat solvents. It is found in the tissues of all vertebrates and is a normal constituent of the human body, being derived from the diet or from synthesis by the tissues. Most tissues tested in vitro, including liver, adrenal cortex, arterial wall, etc., can synthesize cholesterol from acetate. When present in the diet, cholesterol is absorbed from the intestinal lumen into the intestinal lacteals, thence to the blood by way of the thoracic duct. In the process of adsorption the major portion is esterified with fatty acids and appears in the lymphatic chylomicra as cholesterol esters. Cholesterol is also found in the blood plasma where roughly two-thirds is esterified with fatty acids. The liver appears as the main source of plasma cholesterol and the chief agent for its disposal. Approximately 80% of the cholesterol metabolized in the body is transformed by the liver to bile acids. Cholesterol also appears to be the precursor of the adrenal cortical steroids and the sex hormones (White, Handler, and Smith, 1964).

The involvement of cholesterol in the etiology of atherosclerosis was speculated following the report of Windaus in 1910 (cited by Moore and Williams, 1964) who ascertained that human atheromatous aortas contained six times as much free cholesterol and about twenty times as much esterified cholesterol as normal aortas.

Atherosclerosis is a disease characterized by thickening of areas of the arteries due to localized accumulations of lipids. The earliest lesions are the subintimal fatty streaks seen in the thoracic aorta of

children, which either retrogress or grow larger, forming plaques. The plaques (atheromas), which are rich in cholesterol and other lipids, may involve any artery, reducing the lumen. The centers of the plaques may become necrotic, leading to rupture and extrusion of the grumous material into the blood stream. A thrombus then forms over the plaque. Microscopically, the intimal lesions consist of varying proportions of fat droplets foam cells, fibrous proliferation and necrotic debris containing cholesterol crystals. Calcification may occur. Atherosclerosis is of great clincial importance because of its predilection for coronary and cerebral arteries and is the most common cause of coronary artery disease (Lyght, 1961).

In 1908 and 1909, Saltykow and Ignatowski respectively, (cited by Clarkson, 1963) found that diets of milk, meat and eggs were capable of producing atherosclerosis in the rabbit. In 1913, Anitschow (cited by Moore and Williams, 1964) reported that the inclusion of pure cholesterol in the diet of rabbits resulted in elevated levels of cholesterol in the blood and atheromatous degeneration of the aorta.

Since that time the induction of hypercholesteremia (higher then normal levels of cholesterol in the blood) in various laboratory animals and its relation to the production of atherosclerosis has been the subject of numerous investigations. A complete compilation of these studies is beyond the scope of this paper but an attempt has been made to select some of the studies which deal with the more direct effects of the administration of cholesterol to laboratory animals, particularly the rabbit, and to review these in some detail.

Clarkson (1963) in a review of spontaneous and induced atherosclerosis found that the laboratory animals used included rabbits, chickens, turkeys, pigeons, swine, dogs, baboons, chimpanzees, Rhesus monkeys, Cebus monkeys, squirrel monkeys, rats, hamsters, guinea pigs, and the Mongolian gerbil. The most commonly used of these was the rabbit.

Hypercholesteremia and atherosclerosis can be produced in the rabbit by feeding butter (Funch, Krogh and Dam, 1960), hydrogenated arachis (peanut) oil (Funch, Kristensen, and Dam, 1962), hydrogenated coconut oil (Lambert et al., 1958), a high starch diet (Moore and Williams, 1964), and cholesterol with or without added fat (Kritchensky et al., 1961). Of these, cholesterol has undoubtedly been the most effective. According to Whereat (1967) no other comparable atherogenic agent has been described. That the effects are due to pure cholesterol rather than any impurities present in the commercial product was shown by Schwenk, Stevens and Altschul (1959) by producing a greater degree of atherosclerosis in rabbits with pure cholesterol than with commercial cholesterol. The impurities removed from the cholesterol had no atherogenic effect when added to the diet without cholesterol.

A comprehensive study of the effects of feeding rabbits a diet supplemented with 1 g of cholesterol a day was presented by Prior, Kurtz, and Ziegler (1961). Thirty-one New Zealand white rabbits were used in the study. A few were kept on Purina rabbit chow for controls, the others were fed the chow supplemented by dissolving purified

cholesterol in ether, mixing it with the pellets, and allowing the ether to evaporate. Serum for cholesterol determinations was obtained at the beginning and at intervals throughout the study. Some of the rabbits were killed at the end of each month, the last of the animals being sacrificed at the end of five months. Autopsies were carried out on each animal and histological studies made on selected tissues.

Normal serum cholesterol levels ranged from 20 to 110 mg/100 ml initially but at the end of one month on the cholesterol diet values up to 1120 mg/100 ml were obtained. At this time only microscopic lesions were found in the aorta. These lesions consisted of collections of foam cells resting on the internal elastic membrane beneath an intact endothelial cell surface. The coronary arteries showed deposition of lipid in the subendothelial coat but, in contradistinction to man, only the intramyocardial branches were affected while the larger epicardial arteries were uninvolved. The liver and kidneys showed no changes at this time but the adrenal glands were enlarged and grossly yellow in color. Isolated lipid-containing histiocytes were present in the spleen, bone marrow and lymph nodes.

After two months of cholesterol feeding, blood cholesterol levels averaged 1800 mg/100 ml. At this time, the lipoid was increasingly prominent in the lymph nodes, bone marrow and spleen. The pulmonary arteries and veins showed severe luminal narrowing by masses of sub-intimal lipoid histiocytes. Gross lesions of a raised, yellow nature were noted along the aortic intima. Microscopically there was intimal

thickening produced by both intra- and extracellular lipoid, and signs of early "degenerative" changes in the media. The stroma of the ovaries was infiltrated with lipoid as was the cortex of the adrenal glands with some evidence of cellular necrosis and inflamation of the cortical cells.

After three months of cholesterol administration, serum cholesterol averaged 3000 mg/100 ml. The changes seen at two months were present but exaggerated. The myocardial vessels were transformed into a lipoid mass with extreme luminal narrowing. The aortic intima showed evidence of surface fibrosis, and the extension of the fatty process into the medial coat was apparent. At this time, lipoid deposition became prominent in the hind paws, liver and stomach. Scattered lipoid-containing histiocytes were seen throughout the lower part of the mucosa of the stomach.

At the end of four months, serum cholesterol levels had reached 4000 mg/100 ml and the animals showed a consistent weight gain since the beginning of the experiment. At this time splenic parenchymatous tissue was virtually replaced by lipoid-containing histiocytes. The gastrointestinal tract showed mucosal lipoid deposition which was most severe in the stomach. Lipoid deposition was apparent in the kidneys. Striated muscle showed replacement by lipoid histiocytes. The fatty material in the intima of the aorta had undergone extension into the medial coat. Gross lipid deposition could be seen in the irises of the eyes.

By the end of the fifth month, the remaining animals were showing a consistent weight loss and were sacrificed. Serum cholesterol levels

had dropped to an average of 2000 mg/100 ml. There was little variation from the changes at four months except that the kidneys showed a replacement of cytoplasm of the proximal convoluted tubles by lipoid and the tongue showed slight sub-epidermal lipoid deposition.

Prior, Kurtz and Ziegler (1961) concluded that the widespread distribution of cholesterol in rabbits fed cholesterol for a short time resembled a "lipid storage disease" and wondered if the hypercholesteremic rabbits showing a weight loss were not also suffering from functional failure of organs showing evidences of necrosis at that time, i.e., adrenal cortex, liver, kidney, reticulo-endothelial system, etc.

Wang, Strauss and Adlersberg (1957) reported on a study of experimental xanthomatosis (a generalized eruption of xanthomas which are localized accumulations of cells containing lipid, mainly cholesterol) in the rabbit. One hundred and twenty male rabbits were fed cholesterol-treated Purina chow for periods varying from two weeks to one year. Animals were killed at intervals and organs and tissues taken at autopsy were subjected to extensive pathological studies. Four untreated animals served as controls. After two to three months of cholesterol feeding, 2.5% of the rabbits exhibited cutaneous xanthomata. After six months, 21% of the animals, and after nine months 50%, and after twelve months 100% showed skin xanthomata. All plasma lipids increased progressively during the first three months of the experiment to 6.5 to 7.5 times normal, then decreased moderately after five or six months and became stabilized. Plasma total cholesterol levels for these periods showed an

increase from 50 to 1280 mg/100 ml, a decrease to 1098 mg/100 ml, then stabilized around 787 mg/100 ml. No strict correlation was found between the degree of hypercholesteremia and the onset of skin xanthomatosis. These appeared first on the paws as small, reddish-yellow nodules 1-3 mm in diameter. The skin of the soles became thickened and cracked. After six months, small elevated tophi, 1-3 mm in diameter, were seen scattered over the ears. Previous injury to the ear, e.g., sites of puncture for bleeding, usually predisposed the skin to development of these nodules. In some instances, the skin on the back and neck showed loss of hair and appeared reddish and thickened (hyperkeratosis). In many cases, ulceration of the xanthomata and secondary infection, with abscess formation, occurred. Occasionally, large abscesses were noted over the limbs, one abscess reaching the size of an orange. These abscesses contained a cholesterol-rich, whitish, cheesy material, cultures of which yielded no bacterial growth.

Gross atherosclerotic yellowish-white plaques could be seen in the ascending part and the arch of the aorta, especially around the orifices of branching arteries at the end of one month of cholesterol feeding. These became larger and more numerous and confluent with continued cholesterol feeding. After four to six months the plaques of the aorta and pulmonary artery were so extensive that relatively normal intima was seen only in small areas.

Roentgenological studies were carried out serially on some of the animals. Considerable thinning of the cortex of the long bones was

noticed after three to five months on the cholesterol diet. Pathological fractures occurred in three animals. The deposition of foam cells in the synovial membrane, periosteum, and bone marrow was noted long before the development of any roentgenological changes in the bones and joints. Isolated foam cells and clusters of them were seen in the bone marrow of the long bones and of the ribs, especially in animals with active hemopoiesis. In the adult animal with inactive bone marrow of the long bones, large foam cells and cholesterol crystals were frequently observed in the bone lacunae. Cartilages, tendons and ligaments, which are characterized by highly differentiated cells and poor blood supply, exhibited no infiltration of foam cells. In bones with fractures, the deposition of foam cells and cholesterol crystals was more abundant than in intact bones and the deposition of cholesterol was particularly heavy at the site of the fracture.

Pinter and Baily (1961) made a study on anemia in rabbits fed a cholesterol-containing diet, an effect they had observed in previous experiments. One group of ten rabbits received 1 g of cholesterol, and 2.7 g of hydrogenated vegetable oil per 100 g of Purina rabbit chow. Controls consisted of a second group of five animals which received the chow and oil without cholesterol, and a third group of five which received the chow only. Red blood cell, white cell, differential and reticulocyte counts, and hemoglobin and hematocrit measurements were made for each animal weekly. There were no differences between the two control groups during the entire experiment and all measurements obtained for them were

in accordance with normal values. The cholesterol-fed animals, however, starting three to four weeks after the beginning of the test, showed anisocytosis, anisochromia, erythrocytes with punctate basophilia, and nucleated red cells of different maturity on stained slides of peripheral blood. High reticulocyte counts and decreasing hematocrit and hemoglobin values were found. After about twelve weeks, the morphological picture showed some improvement and became stabilized.

Three of the rabbits fed cholesterol and two of the rabbits fed chow only died during the experiment. Causes of death were not determined. Autopsy revealed no pathology in the control animals but the cholesterol-fed rabbits showed signs of jaundice, fatty livers, enlarged spleens, atheromatous-like lesions in the thoracic aortas, and atrophic gastritis in the stomach wall. Microscopic examination of the bone marrows showed extremely high activities with 70 to 80% of the cell populations belonging to the erythrocyte series. Red blood cells were tagged with Cr^{51}. The average apparent half-life of normal cells in normal environment was 12 days, while that of cells from the cholesterol-fed animals was 1.7 days. These values were obtained whether the cells were reinjected into the donor animals or transfused into a littermate on the same diet. Results from cross-transfusion experiments (cells from cholesterol-fed animals into animals on the control diet and vice versa) indicated that the anemia developed as a consequence of the production of red cells with decreased resistance. The authors' conclusion was that the total picture supported a diagnosis of hemolytic anemia in

the cholesterol-fed animals and that cholesterol brings about an alteration in the function of the erythropoetic tissue.

Silver, McMillan and Silver (1964) made a further study of anemia in cholesterol-fed rabbits which confirmed that the anemia produced was of the hemolytic type. A marked polymorphonuclear leukocytosis developed in the latter part of each animals's anemia. Platelet counts were usually two to three times normal values. Autopsies showed marked bone-marrow hyperplasia. The spleens weighed three to ten times normal and some showed an abundance of hemosiderin. Extramedullary hemopoiesis, usually consisting mainly of myeloid elements, was found frequently in sinusoids of the inner half of the adrenal cortex and was also present in lymph nodes of one anemic animal and the liver of another. Splenectomy was performed on some of the anemic animals but an ameliorating effect was only temporary. Addition of olive oil to the cholesterol diet potentiated the anemia. The animals most susceptible to anemia were those showing the earliest, highest and most prolonged hypercholesteremia, the least weight gain and the most severe liver disease.

Grice, Beare and Balazs (1963) also reported anemia in rabbits on diets containing cholesterol, cholesterol and lard, and cholesterol, olive oil and tallow. Animals on the diets supplemented with cholesterol and fats were more anemic than those on diets supplemented with cholesterol only, and the stomachs of the latter showed only a mottling or roughening of the mucosa while the cholesterol-fat-fed rabbits showed gastric lesions which appeared as "cauliflower-like" growths on the mucosa of

the stomach. When the rabbits were taken off the supplemented diets the lesions showed slow regression only after the anemia had disappeared.

Okey (1944) found the guinea pig quite susceptible to the effect of cholesterol. Cholesterol feeding resulted in increases in free as well as esterified cholesterol in the liver and blood, gross enlargement of the spleen, hyperplasia of the bone marrow, anemia, and sometimes formation of gallstones. No cholesterol deposits or sclerotic plaques, however, were observed in the heart or blood vessels. Four to six months were required for removal of cholesterol ester deposits from the liver after cessation of cholesterol feeding. A few of the guinea pigs which developed abscesses of the mammary gland during this experiment had markedly lower liver and blood cholesterol contents. Okey concluded in her report that phagocytic cells played a considerable part in removing cholesterol esters from the liver and that the rapid disappearance of cholesterol deposits in animals infected with pus-forming organisms suggested that leukocytes also function in cholesterol mobilization.

Ostwald and Shannon (1964) reported similar results for 44 guinea pigs fed a diet supplemented with 1% cholesterol. Thirty survived the experimental period of 50-60 days. (Death of 9 experimental and 5 control animals was attributed to failure to eat.) The cholesterol-fed guinea pigs developed hemolytic anemia and grossly enlarged spleens and livers. Livers showed fat infiltration and cell destruction, spleens were spongy, gall bladders extended and lungs were occasionally hemorrhagic. The cholesterol esters of the liver were increased about 30-fold

and the free cholesterol 2-to 5-fold, with a large decrease in the ratio of phospholipid to cholesterol. The percentage of triglycerides in the liver was doubled. The total cholesterol content of the plasma increased from 80 mg to 400 mg/100 ml. The total erythrocyte lipid content as calculated from the sum of cholesterol and phospholipids was 900 mg/100 ml in contrast to 400 mg/100 ml for the control group.

Yamanaka, Winchell and Ostwald (1967) reported that the mean survival time of red blood cells (RBC) from normal guinea pigs was 59-64 days while that of RBC from guinea pigs with anemia induced by cholesterol feeding was 5-20 days. A marked reduction of the mean survival time of normal RBC when transfused into an anemic recipient suggested the presence of an extracorpuscular, hemolytic factor in the anemic guinea pig. The mean survival time of RBC from an anemic animal transfused into a normal recipient remained well below normal indicating an intracorpuscular defect of these cells. The authors suggested that both the intracorpuscular defect and the extracorpuscular factor are the result of an abnormal lipid composition of the RBC membrane secondary to abnormal proportions of plasma lipids.

The dog is quite resistant to the induction of atherosclerosis but in 1946 Steiner and Kendall (cited by Clarkson, 1963) produced atheromatous lesions in the dog by feeding 10 g of cholesterol and 1.2 g of thiouracil (to depress the thyroid) along with a diet high in fat for 52 to 60 weeks.

Stepenson, Younger and Scott (1962) achieved a more rapid production of arteriosclerotic lesions by first giving dogs a thyroidectomy by administration of radioactive iodine, I^{131}, then after a two-week lapse putting them on a high-fat, high-cholesterol diet. Serum cholesterol levels rose above 1000 mg/100 ml. Animals whose average serum cholesterol level reached 1923 mg/100 ml showed weight loss and a profound anemia. Seventeen out of 20 animals autopsied showed gross lesions of coronary arteriosclerosis, in some animals approaching total occlusions of the coronary arterial system.

Okey, Gillum, and Yokela (1934) found that rats on a high cholesterol intake developed fatty livers which contained at least 20 times the normal percentage of cholesterol but otherwise the rats appeared no worse for their diet. In another study, to determine the effect on rats of cholesterol feeding from weaning to old age, Okey (1941) again found an extraordinary capacity of the rat to remain in apparent good health for long periods of time with cholesterol feeding. Grossly enlarged and fatty livers were present throughout the life span but histological examination of these livers showed fatty infiltration rather than degeneration of functioning tissue. However, 4 out of 6 cholesterol-fed animals living more than 700 days developed tumors of considerable size while only 3 of 15 control animals had tumors.

Another observation from her study was that cholesterol disappeared with rapidity from the liver of animals which developed infections or became ill. Four male rats with weight losses of about 50 g and with

lung abscesses had total liver cholesterol values averaging 1.6% against an average of 8.7% for this group. Two females with uterine hemorrhages a week before sacrifice had cholesterol values of 0.8% and 1.6%. In comparison, a healthy rat changed from a cholesterol-rich to a cholesterol-poor diet took from 6 to 9 weeks to clear its cholesterol-ester stores. Likewise, the pus sac in the lung of one rat (Okey, 1944) contained 149 mg of total cholesterol, 5/6 of which was esterified, and had only 781 mg of total cholesterol left in the liver. Two littermates on the same diet had 1351 and 1768 mg of liver cholesterol. (No information on serum cholesterol levels were given in these experiments by Okey.)

Thomas, Hartroft and O'Neal (1959) produced hypercholesteremia in rats by using a diet of 40% butter or lard, 0.3% propylthiouracil, 2% sodium cholate and 5% cholesterol. In 6 separate experiments using 775 rats fed 38 modifications of this diet they produced serum cholesterol levels ranging from 717 to 3315 mg/100 ml and arterial thrombosis with resultant myocardial and renal infarction.

Later, O'Neal, Still and Hartroft (1961) produced hypercholesteremia and gross atherosclerosis in rats without the use of an anti-thyroid drug by feeding a diet high in saturated fat (40% butter) with 5% cholesterol and 2% sodium cholate. Average serum total cholesterol values obtained were 1110 mg/100 ml. Enlarged and extremely fatty livers and spleens were found at necropsy. The spleens contained large numbers of fat-filled macrophages. Similar macrophages were abundant in lymph glands and in alveolar walls and air spaces of the lungs. Eleven animals

surviving 9 months had gross atherosclerosis of adbominal and thoracic portions of the aorta.

Priest and Normann (1962) induced hyperlipemia, hypercholesteremia and anemia in rats by feeding diets containing 40% butter, 5% cholesterol, 20% casein and 2 or 3% sodium cholate. The two rats showing the highest serum cholesterol levels were the most anemic. In anemic animals a pronounced erythrocytic hyperplasia was present in the bone marrow and the anemia was also associated with an increase in extramedullary hemopoiesis in the spleen. Hemosiderin was deposited in large quantities in tublar epithelium of the renal cortex.

Wohl and Mersky (1964) also reported anemia in rats fed a diet containing 40% beef fat, 5% cholesterol, 0.3% thiouracil and 2% cholic acid, and in rats fed a diet without the beef fat but containing the other three ingredients. Anemia developed at 50-70 days in the rats fed the experimental diet containing fat and at 90-100 days in the rats on the diet without the fat. Survival of Cr^{51}-labelled RBC from the anemic rats was shortened when injected into control animals and also when injected into other rats on the experimental diets. RBC from control animals survived the normal length of time when injected into the anemic rats or other controls. The authors interpreted this as indicative of a defect in the RBC rather than an effect of the environment. In addition to the anemia they also reported weight loss, alopecia, dermatitis and diarrhea in the cholesterol-fat-fed rats.

Mice, like rats, were for a long time considered resistant to the induction of atherosclerosis. Clarkson (1963) was unable to find in the literature any information on pathological changes in the cardiovascular system of mice.

Brull and Keil in 1957 and Ball, Williams and Collum in 1963 (both cited by Wang, Chang, and Kuan, 1965) obtained high serum cholesterol and mycardial necrosis by dietary means; atherosclerotic lesions were not observed. Wang et al. (1965) induced atherosclerosis in mice by using diets supplemented with lard, cholesterol and sodium cholate. Mice fed diets supplemented with cholesterol and sodium cholate but without lard had increased serum cholesterol levels but no aortic lesions were observed histiologically.

Heiger (1959, 1962) reported the induction of sarcomas in mice by subcutaneous injections of cholesterol dissolved in olive oil. Olive oil injection alone had very low sarcoma-inducing potency. Three injections of 0.2 ml of a 9% suspension of pure or commercial cholesterol given subcutaneously gave an incidence of 5% sarcomas at the site of the injection in one strain of mice and of 40% in a different strain. Incidence of sarcomas could sometimes be increased either by enlarging the dose of cholesterol, by giving the first injection when the mice were only one day old, by crowding the mice into one large cage rather than housing them 5 to a cage, or in one or two instances by substituting lard for the olive oil. Different strains of mice and even different batches of the same pure strain showed gross differences of susceptibility to carcinogenesis by cholesterol.

Szepsenwol (1966) produced mammary cancer and lung adenocarcinomas in mice by feeding diets supplemented with cholesterol and with cholesterol plus lard. In one group of mice on the diet supplemented with cholesterol and lard, 48.8% developed malignancies which were mainly lung adenocarcinomas and mammary cancer (50% of the breeding females developed the latter). A second group on the same diet had an incidence of 68.1% malignancies with 65.5% of the females developing mammary cancer. On the diet supplemented with cholesterol only, 58 mice (81.6%) developed malignancies and 54 of these were lung adenocarcinomas. Mammary gland cancer appeared in only 3 of this group or an incidence of 8.6%.

Microscopic examination revealed changes in the liver, ovaries and pituitary gland of mice on the diet supplemented only with cholesterol; these changes were not seen in mice on the control diet or mice on the diet supplemented with cholesterol and lard. On the basis of these results the author concluded that the difference in reactivity to a fat-enriched diet between the lungs and connective tissue on one hand and the mammary glands and the skin on the other hand is due to a difference in these two groups of organs or tissues, the former being affected by steroids while the other is affected by lipids which act, not directly, but through the ovaries.

It seems, therefore, that inducing hypercholesteremia in laboratory animals by feeding cholesterol and/or fat can lead to a variety of lesions. Data in some publications have included the number of animals dying during the experiments and in the reports of Funch et al. (1960),

Funch et al. (1962), and Moore and Williams (1964) the causes of death. The latter concluded that the causes of death were in no way connected with the experimental diets. Except for the statement by Wang et al. (1957) that the large abscesses they found on the limbs of their cholesterol-fed rabbits were sterile, no bacteriological studies were mentioned in the experiments reviewed or in numerous others not cited. Consequently, the purpose of this study was to look for correlations between atherosclerosis, hypercholesteremia, and bacterial infections in rabbits with a view to finding some etiological relationship. In the course of the study the experiments were extended to mice.

MATERIALS AND METHODS

A. Experimental Animals

Sixteen rabbits of mixed breed, five males and eleven females, approximately nine weeks old, were purchased locally. On delivery each animal was injected subcutaneously with one ml of a solution containing streptomycin 20 mg/ml and penicillin 300 units/ml as a precaution against bringing infection into the laboratory. The rabbits were housed in pairs in metal cages except two which were caged individually. They were designated by letters A through P and identification recorded by sex, color, markings and cage.

All mice used were Swiss Manor white mice obtained from the stock colony of the Botany and Microbiology Department at Montana State University. Mice were caged individually except that litters were left with their mothers for six weeks before separation.

B. Administration of Cholesterol

Cholesterol was administered to the test rabbits (Experiments #1 and #2) in their diet by supplementing commercial rabbit pellets (Purina) with 1% (by weight) cholesterol U.S.P. from Nutritional Biochemicals Corporation (NBC) and 4% (by weight) Mazola corn oil (grocery grade). The cholesterol was dissolved in heated corn oil which was then poured over the pellets and the mixture stirred until the pellets were uniformly coated. The diet for the control rabbits was the commercial pellets only. After several months, some alfalfa hay (purchased locally) was fed daily to both test and control rabbits. Feed and water were

furnished ad libitum.

Cholesterol was administered to neonatal mice (Experiments #3 and #4) by subcutaneous injections into the dorsal neck region. In experiment #3 the cholesterol U.S.P. (NBC) was dissolved in corn oil (Mazola) containing 10% Tween 80, polyoxyethylene sorbitan monooleate, (Fisher Scientific Co.) for better solvation. Test injections were 0.05 ml of the oil solution containing 0.50 mg of cholesterol. Control injections were 0.05 ml of the Tween 80-corn oil solution without cholesterol. In experiment #4 the cholesterol was dissolved in olive oil (pharmaceutical grade). Test injections were 0.05 ml of olive oil containing either 0.5, 1.0, 1.5, or 2.0 mg of cholesterol. Control injections were 0.05 ml of olive oil only. Solutions for both experiments were prepared in vaccine bottles, autoclaved at 15# and 121 C for 15 minutes, and injected aseptically. The mother mice and litters were fed Purina Laboratory Chow and water ad libitum.

Cholesterol was administered to young and adult mice (Experiment #5) in their feed. In the first part of the study three diets were used: #1 -- laboratory chow plus 1% cholesterol and 4% corn oil (prepared as described for the rabbits); #2 -- laboratory chow plus 4% corn oil only; #3 -- laboratory chow only. Because extension to studies with germ-free mice was contemplated, Purina Laboratory Chow (Special Formula) 5010C was used and all diets were autoclaved for thirty minutes at 15# and 121 C to conform to germ-free techniques (Braaten, 1966, and Reed, 1967). After 25 weeks, because no deviations from the normal controls

were being obtained, procedures were changed. Instead of the Special Formula 5010C, Pur Pac Purina Mouse Breeder Chow was used because it had a rough, porous structure which adsorbed more of the cholesterol-oil mixture. Diets were not autoclaved and five compositions were used as follows:

Diet	Chow	Cholesterol	Corn oil	Lard (Armour's)	Sucrose (grocery grade)
I	500 g	25 g	40 g	----	----
II	500	25	----	40 g	----
III	500	25	----	40	100 g*
IV	500	----	----	40	----
V	500	----	----	----	----

(* The sucrose was dissolved in 100 ml of water and boiled to make a sticky syrup which was then poured over the fat-coated pellets and stirred throughly to get an even distribution.)

The amounts of the ingredients were later changed to 400 g for the chow, 40 g for the corn oil and lard, and 200 g for the sucrose.

C. Procedures for Obtaining Blood and Weights.

Rabbits were restrained in a rabbit box and weighed. One ear was shaved, sponged with 70% alcohol and lightly coated with vaseline. The ear was warmed with a heat lamp to dilate the blood vessels, then a small cut was made in the marginal vein with a razor blade and the blood allowed to drip into a test tube.

Rapid clotting made it difficult to obtain blood from the mice by clipping their tails. This may have been due to the fat in their diets since Gresham and Howard (1963) reported that a butter-cholesterol diet increased the coagulability of the blood of rats. To facilitate bleeding each mouse was injected intraperitoneally with 0.4 ml of saline containing 2.4 mg of 100 units/mg Heparin-Ammonium (NBC), then restrained in a well-perforated plastic bottle with its tail extending through a hole in the lid and weighed. After warming the tail with a heat lamp the tip was cut off and the blood collected in a test tube.

All serums were stored at -5 C until used.

D. Serum Cholesterol Determinations.

Serum total cholesterol determinations were done by the Abel et al. (1952) method which utilizes saponification with alcoholic KOH, extraction of cholesterol in N-hexane and color development by the Leiberman-Burchard reagent (acetic anhydride-sulfuric-glacial acetic acids). The method was adapted to use of 0.25 ml of serum and results were read in a Klett-Summerson photoelectric colorimeter. Cholesterol standards were prepared from Cholesterol S.C.W. (NBC). Other chemicals used were reagent grade.

E. Bacteriological Procedures.

Bacteriological studies were done on material from rabbits if they were found very soon after death and on rabbits sacrificed (by chloroform inhalation) due to infection.

The aorta, brain, internal organs and abscesses when present were removed with sterile instruments and placed in sterile petri dishes. Organs were examined macroscopically and their appearance recorded. Surfaces on organs and abscesses were seared with a large iron kept red-hot over a Bunsen burner, then incisions made with a sterile scalpel. A flamed loop was inserted into an incision and twisted to pick up fragments of tissue. Inoculum obtained in this manner was streaked on blood agar plates and inoculated into brain heart infusion broth, and thioglycolate broth. Plates and broths were incubated aerobically at 37 C and examined for growth each day for several days. In one case (Rabbit M), blood agar plates and cooked meat media were inoculated and incubated anaerobically in a Brewer jar at 37 C. Positive cultures were subcultured on blood agar and TSY (trypticase-soy-yeast) agar plates for isolation. The media were prepared from the dehydrated products of Difco and Baltimore Biological Laboratories (BBL). Identifications were based on classifications in Bergey's Manual (Breed, Murray, and Smith, 1957).

F. Histological Procedures.

Rabbit aortas were preserved by immediate freezing, then later were fixed in 10% formalin made with physiological saline and buffered by saturation with calcium carbonate. After fixation the aortas were dyed by placing them in a solution of Sudan IV, 0.5% w/v in ethanol/acetone/water 35:35:30 v/v/v (Moore, 1967) which was then agitated over

a magnetic stirrer for four hours after which the aortas were washed in running water for two hours (adapted from WHO Chronicle, 1965).

Mice were sacrificed by chloroform inhalation; organs were then removed and preserved in 10% formalin for further studies which have not yet been done.

RESULTS

A. Studies with Rabbits.

1. Weights and serum total cholesterol levels of rabbits on a diet supplemented with cholesterol and corn oil and of control rabbits.

Experiment #1. Eight rabbits, four males and four females, were placed on the diet supplemented with cholesterol and corn oil, and one male and seven females on the control diet. All rabbits gained weight during the first four and one-half months. Individual and mean weights are shown in Table I. The control animals showed a slightly greater mean increase than the test animals for this period. Weights at death (of those obtained) show a slight loss for three of the rabbits (A, C, J) on the test diet and for one of the controls (O).

After a month the eight rabbits on the cholesterol-oil-supplemented diet were hypercholesteremic. Their mean serum total cholesterol was 2260 mg/100 ml at 33 days while that of controls was 100 mg/100 ml. At 137 days the mean for the test animals was 2490 mg/100 ml and for controls 73 mg/100 ml. Cholesterol levels for each animal at these times, the mean for each group, and the values obtained at the death of some of the animals are shown in Table II. The levels at death were decreased for three and increased for one of the test rabbits and increased for the two controls.

The higher mean cholesterol values for the control rabbits at 33 days than at 137 days as well as the slower rate of increase in the cholesterol values for the test animals for the period from 33 to 137

Table I

Experiment #1

Individual and mean weights and survival times of rabbits on a diet supplemented with cholesterol and corn oil and of control rabbits.

Test rabbits	Sex	Weights in Kg at 0 days	33 days	136 days	death	Days of survival
A	male	1.8	2.2	2.7	2.6	283
B	"	1.9	2.2	3.4	3.7	247
C	"	2.2	2.6	3.2	2.8	183
D	"	1.7	2.2	3.2	3.3	226
G	female	1.9	2.4	3.0	---*	178
H	"	1.9	2.2	2.7	2.7	279
I	"	2.0	2.3	2.7	---*	273
J	"	2.2	2.5	3.1	2.9	268
Mean		1.9	2.3	3.0		
Control rabbits						
E	male	2.0	2.5	3.6	---	---**
F	female	1.8	2.4	2.9	---	---**
K	"	2.0	2.3	3.1	---	---**
L	"	1.9	2.4	3.9	---	---**
M	"	1.6	2.1	2.4	2.8	225
N	"	2.0	2.6	3.9	---	---**
O	"	1.8	2.3	3.3	3.1	191
P	"	2.1	2.4	3.1	---	---**
Mean		1.9	2.4	3.3		

* Weights not obtained
** Still alive at end of experiment

Table II

Experiment #1

Individual and mean serum total cholesterol levels of rabbits on a diet supplemented with cholesterol and corn oil and of control rabbits.

Test rabbits	Sex	Total serum cholesterol mg/100 ml at 33 days	137 days	death	Days of survival
A	male	2793	2056	2423 S	283
B	"	720	3715	---* D	247
C	"	2835	2497	1999 S	183
D	"	2604	2808	630 S	226
G	female	2159	1631	656 S	178
H	"	2108	3016	---* D	279
I	"	2281	2238	---* S	273
J	"	2583	1955	---* D	268
Mean		2260	2490		
Control rabbits					
E	male	95	35	---**	---**
F	female	134	66	---**	---**
K	"	113	78	---**	---**
L	"	113	74	---**	---**
M	"	88	118	195 S	225
N	"	94	78	---**	---**
O	"	92	84	200 S	191
P	"	70	46	---**	---**
Mean		100	73		

* Values not obtained
** Still alive at end of experiment
S Sacrificed
D Found dead

days could have been due to heat stress. Serum at 33 days was obtained in August and at 137 days in December and the animals were kept in a room with windows to the south and no cooling equipment. Sodeman and Logue (1960) found higher mean cholesterol levels for both control and cholesterol-fed rabbits maintained at environmental temperatures of 26-30 C than at 4-7 C. The increased levels at death obtained for the two control rabbits, however, is not easily explained since they were sacrificed during cold weather. Also, considered individually the variations in cholesterol levels in the test rabbits are not consistent with heat stress. Addition of alfalfa hay to the diet in the latter months may have made some differences (Cookson, Altschul, and Fedoroff, 1967 reported a lowering of serum cholesterol levels when alfalfa was added to the cholesterol diets) but changes should have been consistent. The lowered cholesterol values at death for three of the test animals could have been due to anorexia caused by infections since two of them suffered from abscess and the other from a middle ear infection. However, the other test rabbit had a respiratory illness and showed increased levels of cholesterol and only one of the two control rabbits had an infection but both showed increased cholesterol levels at death. Results do not disagree with reports in the literature, however, as Mann and Stare (1954) found spontaneous fluctuations of serum cholesterol in the cholesterol-fed rabbits and Gaman et al. (1963) reported a variation in serum cholesterol levels in different strains of rabbits in response to cholesterol feeding. Also, serum cholesterol values for

normal rabbits as reported in the literature show a wide range, for example: Prior et al. (1961) -- 30 mg/100 ml; Funch et al. (1960) -- 50 to 75 mg/100 ml; Seifter et al. (1953) -- 52 to 101 mg/100 ml; and Sokoloff et al. (1966) -- 105 mg/100 ml. Variations could be due not only to different strains but also to different methods of cholesterol determinations (Ham, 1962).

Because of the wide range of variables -- known and unknown -- which influence serum cholesterol levels, individual responses remain unexplained. The significant conclusion to be drawn from the values shown in Table II is that the serum total cholesterol levels of the cholesterol-oil-fed rabbits were considerably higher than that of the control rabbits.

Experiment #2. The six control rabbits surviving the first experiment were used. The one male and two of the females were left on the control diet. The other three females were placed on the test diet.

One of the two test animals when sacrificed showed a weight loss, the other did not. Serum cholesterol levels per 100 ml for the two test animals at the time of sacrifice were 2709 mg and 2200 mg while that of the control rabbit at the time it was sacrificed was 113 mg. These results are shown in Table III and are consistent with the results of the first experiment.

Table III

Experiment #2

Individual weights and serum total cholesterol levels in rabbits on a diet supplemented with cholesterol and corn oil and of control rabbits.

Test rabbits	Sex	Weight in Kg		Days survival	Serum total cholesterol at death in mg/100 ml
		Initial	At death		
F	female	2.9	---*	67	D	---*
N	"	3.9	2.9	164	S	2709
P	"	3.1	3.3	87	S	2200
Control rabbits						
E	male	3.6	---	---**	-	---
K	female	3.9	---*	165	S	133
L	"	3.1	---	---**	-	---

* Values not obtained
** Still alive at end of experiment
D Found dead
S Sacrificed