THE EFFECT OF LOVASTATIN ON HYPERCHOLESTEROLEMIA IN EXPERIMENTAL CHRONIC RENAL FAILURE

by
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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master in Science.

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March, 1992
June 5, 1991

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Dear Raymonde:

Further to your letter of May the 15th, this letter will serve to give your student, Maria Christina Subang permission to reproduce tables and figures from the 1989 report of the Canadian Organ Replacement Register.

A suitable acknowledgement would read as follows:
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Le Registre Canadian Des Insuffisance Et Des Transplantations D'Organes, Hospital Medical Records Institute, Don Mills, Ontario, March 1991.

Yours sincerely,

S.S.A. Fenton, M.D., F.R.C.P.(C)
The effect of lovastatin on hypercholesterolemia in experimental chronic renal failure

Effect of lovastatin on hypercholesterolemia in chronic renal failure
my parents and
Mana Becca,
ABSTRACT

Hypercholesterolemia, a major risk factor for atherosclerosis, is present in many patients with chronic renal failure (CRF). The present study was carried out in order to determine the mechanisms which underlie this increase in serum cholesterol levels and to test the feasibility of using lovastatin, a HMG-CoA reductase inhibitor, in its treatment.

A mouse model of surgically-induced CRF was employed in the experiments. Five weeks after the onset of renal failure, the mice were characterized with regard to various biochemical and hematological parameters. At this time, treatment with lovastatin was initiated. The drug (50, 100, and 200 mg/kg BW/day) was incorporated in powdered diet and was given fresh daily for four weeks. Upon sacrifice, blood was collected for the estimation of blood urea nitrogen and serum lipids and livers were excised for the measurement of hepatic HMG-CoA reductase activity.

The mice exhibited the major manifestations of CRF - retention of nitrogenous wastes, elevated levels of alkaline phosphatase, suggesting the presence of bone disease, and severe anemia. CRF mice also had elevated serum total cholesterol levels with a concomitant, but not significantly correlated, increase in hepatic HMG-CoA reductase activity. Furthermore, their serum lipoprotein profiles were abnormal. Treatment with lovastatin resulted in a dose-dependent reduction in serum total cholesterol levels and correction of the serum lipoprotein profile. However, hepatic HMG-CoA reductase activity was unchanged.

These results indicate that the hypercholesterolemia observed in CRF mice is probably due to an increase in de novo synthesis of cholesterol in both the liver and extraneous tissues. Lovastatin may decrease serum total cholesterol levels in CRF mice by inhibiting peripheral, rather than hepatic, HMG-CoA reductase activity.
L’hypercholestérolémie, un important facteur de risque pour l’athérosclérose, se retrouve chez de nombreux malades souffrant d’insuffisance rénale chronique (IRC). La présente étude avait pour but de déterminer les mécanismes en jeu dans la hausse du taux sère de cholestérol et d’évaluer la faisabilité d’un traitement à la lovastatine, un inhibiteur de la HMG-CoA réductase.

Un modèle d’IRC induite par voie chirurgicale chez la souiss a été employé dans ces expériences. Cinq semaines après le début de l’insuffisance rénale, les souris ont été caractérisées en ce qui à trait à différents paramètres biochimiques et hématologiques. On a alors amorcé le traitement à la lovastatine. Le medicament (50, 100 et 200 mg/kg de poids corporel/jour) a été incorporé frais chaque jour dans la diète en poudre des souris pour une période de quatre semaines. Lors du sacrifice, on a recueilli le sang pour les titrages d’azote uréique et des lipides sérèques. On a également excisé les foies afin de mesurer l’activité de la HMG-CoA réductase hépatique.

On a retrouvé chez les souris les manifestations principales de l’IRC : rétention de déchets azotés, taux élevés de phosphatase alcaline semblant refléter une atteinte osseuse, et anémie grave. Les souris avec IRC présentaient également un taux sère élevé de cholestérol total ainsi qu’une hausse concomitante, mais sans corrélation importante, de l’activité de la HMG-CoA réductase hépatique. En outre le profil des lipoprotéines sérèques était anormal. Le traitement à la lovastatine a entraîné une réduction, proportionnelle à la dose administrée, du taux de cholestérol total et une correction du profil des lipoprotéines sérèques. Toutefois, l’activité de la HMG-CoA réductase hépatique est demeurée inchangée.

Ces résultats indiquent que l’hypercholestérolémie observée chez les souris atteintes d’IRC est probablement attribuable à une augmentation de la synthèse de novo du cholestérol aussi bien dans le foie que dans les tissus extra-hépatiques. Il est possible que la lovastatine réduise le taux sère de cholestérol total chez les souris atteintes d’IRC en inhibant l’activité de la HMG-CoA réductase périphérique, plutôt qu’hépatique.
ACKNOWLEDGEMENTS

I owe the success of this MSc project to my supervisors, Dr. Jean L. Stewart-Phillips and Dr. Raymonde F. Gagnon, who have encouraged and supported me throughout the program. I am also indebted to everyone in the two laboratories for their invaluable advice, technical help and, most of all, for simply being around.

My gratitude is extended to Dr. Emil Skamene and the McGill Centre for the Study of Host Resistance for the research studentship I held throughout the course of this study. My particular thanks go to Dr. Carolyne Pietrangelo, Administrative Assistant of the Centre, for her encouragement and support. I thank Dr. Peter Somerville for the opportunity to also work in the Division of Nephrology, Montreal General Hospital. The staff of the Department of Physiology, McGill University, especially Ms. Linda Tracey, have been very accommodating. I thank them for their help. I am grateful to Dr. Nigel Phillips and to Dr. Geoffrey Richards for their advice and to Dr. Peter Richardson for his patience in allowing me the time to finish this thesis.

I am indebted to Merck Frosst Canada for their generous support. I particularly thank Dr. Pierre Roland for approving the Grant-in-Aid which financed the project. Mr. Erskine Simons for a travel grant to the 9th International Symposium on Atherosclerosis and Mr. Alfred Alberts for providing pure lovastatin.

The following people deserve to be thanked for their technical assistance: Dr. Richard Sportsman and Ms. Judith Heisserman of Lilly Research Laboratories, Indianapolis, Indiana, Dr. Roger Illingworth and Dr. Anuradha Pappu of Oregon Health Sciences University, Portland, Oregon, Mr. Gerry Bibeault of the Division of Hematology and Dr. Andrew Weigensberg and Mr. Ron Kastelberger of the Department of Medical Biochemistry, Montreal General Hospital.

Lastly, I thank my family and my friends for their love, moral support and encouragement.
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HYPOTHESIS

The hypercholesterolemia associated with chronic renal failure can be treated with drugs of the HMG-CoA reductase inhibitor class.
INTRODUCTION

Chronic renal failure (CRF) is the result of a gradual process of glomerular and tubular functional impairment. Once a critical level of renal dysfunction has been reached, the progression to end-stage renal disease (ESRD) is usually unavoidable. Patients with ESRD suffer from uremia, a condition wherein substances normally removed by the kidney, such as nitrogenous compounds and acidic solutes, are retained in the body, causing major dysfunction of most physiological systems. These patients require renal replacement therapy by dialysis or kidney transplantation in order to survive.

Atherosclerosis and Chronic Renal Failure

The introduction of dialysis and renal transplantation has had a major impact on the management of patients with ESRD. However, these procedures have not prevented the occurrence of cardiovascular diseases, which are the major cause of morbidity and mortality of ESRD patients. Data from the 1989 Annual Report of the Canadian Organ Replacement Register [32] show that 45.6% of all deaths of ESRD patients can be attributed to cardiovascular diseases (Fig. 1). These figures have not changed significantly for over a decade (Fig. 2).

A wide spectrum of cardiovascular complications have been reported in ESRD [40, 122, 123, 124, 126]. These include pericarditis, left ventricular hypertrophy, heart failure, coronary artery disease and bacterial endocarditis [40]. Hypertension has been reported to precipitate left ventricular hypertrophy, heart failure, ischemia,
arrhythmias and myocardial fibrosis [124], while abnormalities of lipid metabolism have been implicated in the atherogenic process in ESRD patients.

In 1974, Lindner et al. [90] reported that the mortality rate of patients on long-term hemodialysis is similar to that of patients with Type II hyperlipoproteinemia and five times greater than that of hypertensive subjects. They postulated that atherosclerosis is accelerated in this patient population. Chest radiographs of dialysis patients of Schnyder et al. [127] revealed an increase in aortic calcification and a more rapid rate of calcification. Kudoh et al. [86] suggested that dialysis itself may promote atherogenesis.

The issue of whether "accelerated atherosclerosis" is a consequence of uremia per se or of its treatment has provoked considerable controversy. A historical review of atherosclerosis in CRF [105] revealed that atherosclerotic events were reported in untreated patients with CRF as long ago as 1827 when Bright [27] described "disease in the coats of the aorta" in 11 of his 100 patients. Nicholls [105] argued that atherogenesis may have occurred at an accelerated rate in CRF patients long before the advent of dialysis treatment. On the other hand, Ritz et al. [119] analyzed the concept of accelerated atherosclerosis in CRF and concluded that the available epidemiological evidence does not prove that atherogenesis is enhanced by uremia. However, the heterogeneity of the patient population makes it difficult to draw a conclusion from the data available in the literature.

It is undeniable that several risk factors for atherosclerosis are present in CRF subjects [62]. These include hypertension, hyperparathyroidism and lipid abnormalities. Indeed, the lactescence of sera from patients with kidney disease has been observed since the early 19th century. Furthermore, patients with CRF are
recommended to reduce their protein intake and therefore, in order to meet their daily caloric requirement, they must necessarily increase the amount of fat and carbohydrate in their diet. Hyperlipidemia, whether induced by uremia, diet or both, is thus undoubtedly operative as a risk factor for atherosclerosis in this patient population.

In order to understand the mechanisms underlying the changes in serum lipids exhibited by CRF patients and the ways in which such dyslipidemias might be treated, it is first necessary to review their synthesis, transport and metabolism.

**Cholesterol**

Cholesterol is a fat-like, essential alcohol that can be synthesized by the body or obtained from the diet [91]. It is an essential component of the plasma membrane and the myelin sheath, and a precursor of all other steroids, including bile acids, corticosteroids, sex hormones and vitamin D. About 93% of the body's cholesterol is found in cell membranes and only 7% is present in the circulation. When dietary cholesterol is not sufficient for the cellular sterol requirement, endogenous synthesis takes over and may contribute up to 70% of total body cholesterol.

Virtually every nucleated cell is capable of synthesizing cholesterol from acetyl-CoA. The biosynthetic pathway is a series of more than 20 reactions which occur mitochondrially. As illustrated in figure 3, it involves a) the formation of mevalonic acid from acetyl-CoA, b) the formation of isoprenoid units, c) the condensation of six isoprenoid units to form squalene and d) the conversion of squalene to lanosterol [97].

The rate-limiting step in cholesterol synthesis is the conversion of HMG-CoA to mevalonic acid which is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A.
(HMG-CoA) reductase (EC 1.1.1.34). This enzyme has a very short half-life (1-2 hours) and is inactivated by phosphorylation and cold temperatures [120]. Its activity is regulated by a number of factors including cholesterol and mevalonate levels, cAMP, Mg-ATP, insulin, glucagon, hydrocortisone and thyroid hormone [120].

Triglycerides

Triglycerides consist of three moles of fatty acids esterified to glycerol, the alcohol component of fats. The formation of triglyceride, which is illustrated in figure 4 [95], involves a) phosphorylation of free glycerol at the α-position, b) acylation with two moles of fatty acyl-SCoA, c) dephosphorylation of the compound and d) esterification with a third mole of fatty acyl-SCoA.

Lipoproteins

The transport of water-insoluble cholesterol and triglycerides through the circulation and into and out of cells is mediated by lipid:protein complexes called lipoproteins [40]. These spherical particles possess a hydrophobic core of triglycerides and cholesterol esters surrounded by a monolayer of free cholesterol, phospholipids and apoproteins (Fig. 5). The physical properties of lipoproteins are determined by their composition. They can be classified according to their density and electrophoretic mobility. The four major classes of lipoproteins - chylomicra, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) [41, 78, 129] are described in table 1, while the various interactions between themselves and different tissues are illustrated in figure 6.
a) Chylomicra

Triglycerides are transported to muscle fibres and adipose tissue by chylomicra and VLDL. Chylomicra are formed in the small intestine and consist of triglycerides from fatty acids derived from dietary fat, apoproteins, phospholipids, free cholesterol and cholesterol esters (Table 1). These particles are the source of triglycerides and cholesterol for the exogenous pathway of lipoprotein metabolism (Fig 6). They have a very short half-life in the circulation (less than 30 minutes), being hydrolyzed by endothelial-bound lipoprotein lipase as they pass through the capillaries of the muscle and adipose tissue. The enzyme is activated by apo-C-II bound to the chylomicra. The monoglyceride which results is then acted upon by monoacyl lipase which is free in the plasma and present in blood platelets and the recipient cell. The fatty acids released by the actions of these enzymes are taken up and reassembled into triglycerides for storage, while the cholesterol-rich chylomicron remnants are taken up by the liver via the apo-E receptor.

b) VLDL

VLDL are synthesized by hepatic parenchymal cells and are important in the transport of triglycerides from the liver to extrahepatic tissues where, like the chylomicra, they undergo hydrolysis, delivering free fatty acids to cells and forming cholesterol-rich VLDL remnants [96]. Some of these VLDL remnants, which are often referred to as intermediate density lipoproteins (IDL), return to the liver and are taken up by apo-B100:E receptors. VLDL remnants that escape clearance undergo further lipolysis by hepatic triglyceride lipase and form LDL.
c) LDL

The LDL carry the majority of the body's circulating cholesterol and deliver it to cells. The protein of LDL is almost exclusively apo-B100, which is recognized by LDL receptors. These receptors are found on cell membranes, in regions coated with clathrin [58]. As the LDL receptor also recognizes apo-E, it is also referred to as the apo-B100 E receptor. The catabolism of LDL involves a) the binding of apo-B100 to the receptor, b) the invagination of the coated pit regions which form endocytic vesicles, c) the fusion of these endocytic vesicles with lysosomes, d) the degradation of apo-B100 into amino acids and the return of the receptors to the cell surface and e) the hydrolysis of cholesterol esters of the LDL core to yield free cholesterol, which is released into the cytosol and used in various cell functions. The intracellular level of free cholesterol regulates the expression of LDL receptors on the cell surface.

d) HDL

High density lipoproteins, which are synthesized and secreted by the liver and the intestine, have been described as the "major surface remnant of the lipolytic process" or the "machinery for cholesterol esterification" [51]. They appear to have two roles - as a repository for apoproteins C and E, which are required in the metabolism of chylomicra and VLDL, and in reverse cholesterol transport. Production of HDL begins with the formation of apo-AI-phospholipid-free cholesterol complexes which act as substrates for the enzyme lecithin cholesterol acyltransferase (LCAT). This enzyme esterifies free cholesterol. The resulting cholesterol ester is hydrophobic and moves into the core of the particle, transforming its shape from a disc to a sphere. The HDL has a low molar ratio of free cholesterol to phospholipid making it
capable of receiving cholesterol from cell membranes or from other lipoproteins. The newly acquired free cholesterol is also esterified by LCAT and moves into the particle's core. Cholesterol esters in HDL can be transferred to LDL, VLDL and even to chylomicra in exchange for triglycerides [96]. This transfer is mediated by cholesterol ester transfer protein (CETP). The cholesterol esters will eventually be delivered back to the liver in a process called reverse cholesterol transport (Fig 7) [54, 96].

Dyslipidemias Associated with CRF

Hypertriglyceridemia is the most commonly observed lipid abnormality associated with CRF [10, 11, 12, 14, 34, 107]. However, it is not a consistent observation. Cattran et al [33] and Attman et al [5] reported that 60% and 71%, respectively, of their dialysis patients were hypertriglyceridemic while both Dieplinger et al [44] and Papadopoulos et al [109] observed hypertriglyceridemia in only 33% of their patients. On the other hand, Avashi et al [9] and Sharma et al [128] did not observe elevated triglyceride levels in any of their patients with CRF. Hypertriglyceridemia in uremic subjects has been attributed to increased triglycerides in any or all of the lipoprotein fractions [8, 11, 33, 34] which may result from their decreased removal or increased synthesis or both [11, 12]. Several studies have shown that defective triglyceride removal is caused by decreased lipoprotein lipase and/or hepatic triglyceride lipase activity [11, 12, 14, 36].

CRF also has effects on cholesterol levels (Table 2) and distribution. In a study by Chan et al [36], 31% of peritoneal dialysis and 10% of hemodialysis patients were shown to have elevated serum total cholesterol levels. Bagdade et al [14] reported
that serum LDL- and VLDL-cholesterol were both increased in hemodialysis patients. Norbeck et al [106] corroborated this observation on VLDL-cholesterol but not on LDL-cholesterol. Conversely, a low HDL-cholesterol level is one of the lipid abnormalities frequently reported in CRF patients [44, 117].

There have also been several studies which identified changes in cholesterol transfer induced by CRF [117]. The results of a study by Hsia et al [72] first revealed a defect in cholesterol transfer in hemodialysis patients. It was concluded that this defect resides in HDL as this fraction was found to contain less cholesterol than the HDL of control subjects. It was considered to be an "inferior donor for cholesterol transfer". Dieplinger et al [45] subsequently showed that in hemodialysis patients, esterification of cholesterol in HDL and the transfer of cholesterol esters from HDL to LDL and VLDL are reduced while the free cholesterol content of these lipoproteins is increased. Patients on continuous ambulatory peritoneal dialysis (CAPD), on the other hand, had normal cholesterol ester transfer rates [100].

The results of studies to determine the apoprotein content of serum lipoproteins from CRF patients are inconsistent (Table 2). Attman et al [7] found that, compared to normolipidemic control subjects, the serum apo-AI, apo-AII and apo-E levels of CRF patients before hemodialysis were reduced while the levels of apo-B and apo-CI were the same and the levels of apo-CII and apo-CIII were increased. On the other hand, Grutzmacher et al [67] did not observe a reduction in apo-AI in the serum of CRF patients not yet on dialysis, although they did find that apo-CII levels were increased and apo-B levels were the same. A study by Lacour et al [87] revealed that in both undialyzed and hemodialyzed ESRD patients, serum apo-A levels were decreased while a reduction in serum apo-B was only seen in hemodialyzed patients. The
elevation of apo-CIII levels observed by Attman et al [6] may explain the hypertriglyceridemia associated with CRF. Apo-CIII is an inhibitor of lipoprotein lipase and therefore increased levels of this apoprotein may lead to a decrease in triglyceride metabolism [67] and an increase in serum triglyceride levels.

**The Pathogenesis of Atherosclerosis**

The word atherosclerosis comes from the Greek "athero", meaning gruel, and "sclerosis", meaning hardening. It briefly describes the plaque formed in major arteries. Atherogenesis is a gradual process, with lesions progressing from fatty streaks to fibrous plaques to complicated lesions.

Fatty streaks, which have been observed even in children, are characterized by the presence of lipid-laden macrophages with small numbers of lipid-filled smooth muscle cells. These early lesions are found throughout the arterial tree, cause little obstruction of the vessels and present no symptoms.

Fibrous plaques are typically found in the abdominal aorta, and in the coronary and carotid arteries. They are characterized by a core of foam cells, necrotic debris and extracellular lipid, surrounded by layers of connective tissue matrix. These lesions may be subjected to calcification, thrombosis, ulceration or hemorrhage to produce complicated lesions.

Genesis of the atherosclerotic lesion has been attributed to the single influence of either plasma lipid infiltration or to endothelial injury. But more recently, a theory - the Unified Hypothesis [39] - has been proposed which links lipid imbibition with the disruption of endothelial integrity as the event which initiates the atherosclerotic process (Fig. 8).
Elevated levels of circulating LDL are associated with atherosclerosis [61, 121, 130, 135]. Indeed, Steinberg [130] contends that this dyslipidemia is sufficient to initiate atherogenesis in animals and humans deficient in LDL receptors. Furthermore, oxidized LDL, which is one of the modified forms of LDL present in serum, is very readily taken up by macrophages, the precursors of the foam cells present in early atherosclerotic lesions. Oxidized LDL has also been found to exert a cytotoxic effect on cultured endothelial cells. It is possible, therefore, that this modified lipoprotein acts \textit{in vivo} to damage the endothelial lining of blood vessels thus initiating the atherogenic process [121].

Damage to the vessel wall leads to the adhesion of platelets and monocytes [121]. Activated platelets release chemoattractants, like platelet-derived growth factor, which stimulate the migration and proliferation of smooth muscle cells. Proliferation of smooth muscle cells in the intima is accompanied by synthesis of connective tissue matrix and deposition of extra- and intracellular lipids.

The abnormal lipoprotein profile of uremic serum may provide clues to the mechanisms underlying the development of atherosclerosis in CRF. \(\beta\)-VLDL, which is a VLDL particle enriched with cholesterol esters and apo-E, has been identified in the sera of CRF patients [101, 106, 109]. This particle is a potential source of lipids for foam cell formation [83] and is also known to promote monocyte adhesion to the endothelium [130]. It is considered to be one of the most atherogenic of the lipoproteins [43].

Uremic serum contains several substances which may injure the endothelial lining of blood vessels. Among these are spermidine and spermine [93]. These polyamines are also known to promote cell growth which may possibly contribute to
the atherogenic process. Furthermore, spermidinepeptide, a spermidine-containing peptide, forms a stable complex with insulin and may inhibit the stimulatory effect of this hormone on lipoprotein lipase activity [93] resulting in decreased lipolysis of triglycerides and an elevation in serum triglyceride levels. This could lead to the development of hypertriglyceridemia in CRF.

Treatment of Hyperlipidemias Associated with CRF

In order to retard the progression of atherosclerosis in CRF patients and so to improve their quality of life, it is obviously essential to treat the dyslipidemias which they present. This may be achieved either by dietary manipulation, pharmacological means or both. To date, medication has not been universally adopted. Many nephrologists are reluctant to add more drugs to the long list already taken by their CRF patients, and there is the very real possibility that the effectiveness of the drug will be diminished or the side effects increased by the uremic state and/or dialysis procedure.

Carnitine, an ammonium compound, which is a cofactor for the transport of fatty acids into the mitochondria for fatty acid oxidation, has been used to reduce serum cholesterol levels in patients with CRF. Wanner et al [140] administered increasing doses (1, 5, and 15 mg/kg BW) of L-carnitine intravenously to CRF patients at the end of each hemodialysis session for a total period of nine months (each dose was administered three months). Not all patients responded to 1 mg/kg BW L-carnitine but in those who did, the drug reduced serum VLDL-cholesterol, -triglyceride and -phospholipid levels without affecting HDL and LDL. Higher doses (15 mg/kg BW) of L-carnitine led to an increase in serum triglycerides, particularly HDL- and VLDL-
triglycerides. This study corroborated the earlier findings of Chan et al [35] which demonstrated the "dual response" elicited by carnitine treatment. At lower dose ketogenic effects were observed while antiketogenic events occurred after administration of high doses of the compound. Treatment with this compound also produced severe problems in neuromuscular transmission, resulting in a condition similar to myasthenia gravis [35]. The unpredictability of individual responses to carnitine administration makes this compound unsuitable for the treatment of dyslipidemias in patients with CRF.

Fibrin acid derivatives, such as clofibrate, gemfibrozil and bezafibrate, have also been tested for their ability to lower serum lipids in CRF [37, 66, 141]. Fibrates stimulate lipoprotein lipase activity by increasing the apo CII/apo CIII ratio [142] and reduce VLDL production. Treatment with these agents thus leads to a significant decrease in serum triglyceride levels and a rise in HDL cholesterol levels. However, clofibrate has been reported to cause muscular pain and tenderness accompanied by an elevation in serum creatinine kinase [113]. It also increases plasma urea and creatinine levels. These side effects clearly make clofibrate unsuitable for patients with CRF. However, gemfibrozil, at a relatively lower dose has been effectively and safely administered to CAPD patients [37].

A new class of lipid-lowering drugs - the HMG-CoA reductase inhibitors - has recently become commercially available. The first of these, lovastatin (Mevacor®) decreases cholesterol synthesis and apo-B concentrations and increases LDL receptor activity. It also induces a decrease in plasma triglyceride and VLDL-cholesterol levels. Thus, it would appear to be a good candidate for treating the hypercholesterolemia and hypertriglyceridemia associated with CRF.
Lovastatin

a) Structure

Lovastatin, previously known as mevinolin, is a fungal metabolite which was first isolated from cultures of Monascus ruber [50] and Aspergillus terreus [2]. Chemically,Lovastatin is 1,2,6,7,8,8a-hexahydro-1H-dihydroxy-2,6-dimethyl-8-(2-methyl-1-oxobutoxy)-1-naphthaleneheptanoic acid δ-lactone. It is, in fact, an inactive lactone prodrug which is hydrolyzed to its active open hydroxy-acid form in the liver (Fig. 9). It belongs to the family of substituted hexahydroxynaphthalene lactones and is characterized by the presence of a 6-α-methyl group in the hexahydroxynaphthalene ring [3].

b) Mechanism of Action

Lovastatin reduces serum total cholesterol levels by two interrelated mechanisms. It partially inhibits HMG-CoA reductase, thus reducing endogenous synthesis of cholesterol [3], and it upregulates the expression of LDL-receptors on cell membranes. The hydroxy moiety of the hydroxy-acid form of lovastatin (Fig. 9) is structurally similar to the hemithioacetal intermediate in the HMG-CoA reductase reaction (Fig. 10) making the compound a transition state analogue inhibitor of the enzyme. The hydroxy moiety, which is the hydrolyzed δ-lactone moiety of the prodrug, is the active centre. Acetylation of the hydroxy group at either C3 or C5' of the hydroxy moiety reduces the inhibitory activity of the compound. Nakamura and Abeles [102] suggested that lovastatin and related compounds have the (R) configuration at C5' [15] of the hydroxy moiety and interact simultaneously with 2 separate regions of the enzyme's active site. 1) the part which binds the
hydroxymethylglutarate moiety of HMG-CoA and 2) the hydrophobic pocket which
binds CoA. The reduction in intracellular cholesterol levels induced by lovastatin
increases the number of LDL receptors expressed on the cell membranes. This in
turn increases the clearance and degradation of circulating LDL, IDL and VLDL.

c) Pharmacokinetic Properties

Ingested as an inactive lactone, lovastatin is absorbed in the small intestine.
The bulk of the absorbed compound is extracted in the liver where it is hydrolyzed to
the active open hydroxy-acid form [70]. It is believed that less than 5% of the oral
dose reaches the general circulation. Lovastatin and its major metabolites, 3-hydroxy
acid, a 6-hydroxy derivative and two, as yet, unidentified derivatives, have been found
to be highly bound to plasma proteins [85]. Radioisotope studies revealed that about
10% of the dose is excreted in the urine and 83% in the feces, which includes drug
excreted into the bile and unabsorbed drug [85]. In humans, the plasma concentration
of the drug reaches its peak 2 to 4 hours after administration and a steady state is
achieved within 2 to 3 days [70].

d) Clinical Studies

The findings of a number of selected clinical studies with lovastatin are
summarized in table 3. Studies involving patients with either familial or non-familial
hypercholesterolemia show that treatment with lovastatin (10-80 mg/day) for 4-18
weeks results in a significant reduction in plasma total and LDL-cholesterol levels and
an increase in HDL-cholesterol levels [63, 65, 69, 71, 74, 75, 76]. The study of Arad
et al [4] on patients with combined hyperlipidemia (hypercholesterolemia and
hypertriglyceridemia) demonstrated that lovastatin therapy lowers LDL-cholesterol and apo-B plasma concentrations by reducing the rate of entry of apo-B-containing plasma lipoproteins, either as VLDL or directly secreted LDL, into the circulation.

Bagdade et al [13] showed that treatment with lovastatin lowers plasma total and LDL-cholesterol and apo-B levels in patients with Type IIa hypercholesterolemia. However, neither the free cholesterol/lecithin ratio, a new index of cardiovascular risk, nor HDL- or VLDL-cholesterol were reduced. Cholesterol ester transfer remained unaffected after 2 months of treatment. Raveh et al [118] also observed reductions in serum LDL-cholesterol and apo-B levels following 12 weeks of lovastatin treatment in patients with familial or non-familial hypercholesterolemia. In addition, these workers were able to demonstrate an increase in LDL receptor activity in circulating monocytes isolated from their patients. Goldberg et al [57] showed that, although lovastatin therapy decreases mean plasma total and LDL-cholesterol levels in patients with hypercholesterolemia, it does not change whole body cholesterol synthesis. This finding suggests that a mechanism exists which compensates for the effects of the drug in order to maintain pretreatment levels of cholesterol production and tissue pool size.

Tobert et al [137] reported that in the course of a long-term (36 years) lovastatin study involving 744 patients, the following adverse effects were observed: asymptomatic transaminase elevations, myopathy, gastrointestinal symptoms, insomnia and skin rash. However, an overall assessment of the safety of the drug revealed that the incidence of adverse side effects is very small - less than 0.01% for myopathy or elevated serum levels of transaminases.
e) Clinical Studies in Patients with Renal Disease

Lovastatin has now been used in patients with nephrotic syndrome and in renal transplant recipients to lower serum total- and LDL-cholesterol levels. In a study by Kasiske et al [80], 13 patients with nephrotic syndrome were placed on a low-cholesterol diet, and given placebo for 4 weeks before the administration of lovastatin (20 mg twice a day) for 6 weeks. The treatment reduced serum total cholesterol and LDL-cholesterol levels by 27%, VLDL-cholesterol by 37%, triglycerides by 30% and apo-B by 29%. In this study, one patient was withdrawn after he developed diarrhea; the other 12 patients experienced no adverse effects.

The drug has also been used in renal transplant patients with hypercholesterolemia [81]. In a double-blind study involving 11 stable renal allograft recipients, who were first treated with diet, lovastatin (20 mg/day) was administered for 6 weeks followed by a crossover of lovastatin or placebo for another 6 weeks. Drug therapy led to a reduction in serum total- and LDL-cholesterol by 21% and 28%, respectively, without adverse side effects.

At the start of the current project there had been no studies involving the use of lovastatin in CRF patients. Late in 1991, the report of a study investigating the effects of lovastatin on hypercholesterolemia in hemodialyzed patients was published [142]. This study involved 40 patients who were hypercholesterolemic and moderately hypertriglyceridemic and who were receiving hemodialysis treatment three times a week. The study entailed a 4-week placebo period followed by administration of lovastatin or simvastatin, another HMG-CoA reductase inhibitor, in increasing dosages for a period of 3 months. Lovastatin treatment led to a lowering in serum total cholesterol of 24%, LDL-cholesterol of 30.8%, VLDL-cholesterol of 34.7% and apo-B
of 28.2%. One patient exhibited an increase in hepatic transaminases. No other side effects were observed.

One of the aims of the present study was to establish whether or not the hypercholesterolemia associated with CRF can be effectively and safely treated by a HMG-CoA reductase inhibitor. As clinical trials are complicated by the difficulty of patient selection - CRF patients present a wide spectrum of diseases and may be receiving any number of additional medications - it was decided to employ an original animal model of CRF.

The Mouse Model of CRF

A model of CRF has been developed in adult C57BL/6J mice [55, 56]. Renal failure is induced surgically by the electrocoagulation of the right kidney surface followed by contralateral nephrectomy two weeks later. Assessment done six weeks after the left nephrectomy reveals elevated levels of blood urea nitrogen, creatinine and glucose levels, hyperkalemia, acidosis, hyperphosphatemia, significant growth retardation, severe anemia and florid osteodystrophy. Serum cholesterol levels are also increased in CRF mice and histological examination by Stewart-Phillips et al. [132, 133] of the ascending aorta reveals the presence of foam cells in the aortic wall (Fig 11). These lesions are very similar to the fatty streaks found in humans. Thus, this model would appear to be eminently suitable for investigating the mechanisms underlying the hypercholesterolemia associated with CRF and for evaluating the possibility of using the HMG-CoA reductase inhibitor, lovastatin, for treating this complication.
Table 1. Composition of lipoproteins.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Source</th>
<th>Diameter (nm)</th>
<th>Density</th>
<th>Electrophoretic Behavior</th>
<th>Apoprotein Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicra</td>
<td>intestine</td>
<td>90-100</td>
<td>&lt;0.950</td>
<td>stays at origin</td>
<td>Apo A1, AII, B-48, CII, CIII, E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>intestine, liver</td>
<td>30-90</td>
<td>0.950 to 1.006</td>
<td>pre-β</td>
<td>Apo B-100, CI, CII, CIII, E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDL</td>
<td>VLDL</td>
<td>25-30</td>
<td>1.006 to 1.019</td>
<td>midway between pre-β and β</td>
<td>Apo B-100, E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>IDL</td>
<td>20-25</td>
<td>1.019 to 1.063</td>
<td>β</td>
<td>Apo B-100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>intestine, liver, other lipoproteins</td>
<td>7-20</td>
<td>1.063 to 1.210</td>
<td>α</td>
<td>Apo AI, AII, CI, CII, CIII, D, E</td>
</tr>
</tbody>
</table>
Table 2. Lipoprotein abnormalities observed in CRF subjects.

<table>
<thead>
<tr>
<th>Lipoprotein fraction</th>
<th>Lipid abnormality</th>
<th>Apoprotein abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>↑ Triglycerides</td>
<td>↑ Apo-CII</td>
</tr>
<tr>
<td></td>
<td>↑ Cholesterol</td>
<td>↑ Apo-CIII</td>
</tr>
<tr>
<td>LDL</td>
<td>↑ Triglycerides</td>
<td>↓ Apo-B</td>
</tr>
<tr>
<td>HDL</td>
<td>↑ Triglycerides</td>
<td>↓ Apo-AI</td>
</tr>
<tr>
<td></td>
<td>↓ Cholesterol</td>
<td>↓ Apo-AII</td>
</tr>
</tbody>
</table>
Table 3. Selected clinical studies with lovastatin.

<table>
<thead>
<tr>
<th>Ref</th>
<th>Treatment</th>
<th>Patient type</th>
<th>Lipoprotein changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>136</td>
<td>6.25-50 mg b.i.d., 4 wks</td>
<td>Normal</td>
<td>↓ (17-29%) TC, ↓ (23-45%) LDL-C</td>
</tr>
<tr>
<td>74</td>
<td>5-40 mg b.i.d., 4 wks</td>
<td>Hetero FH</td>
<td>↓ (13-33%) TC, ↓ (20-36%) LDL-C</td>
</tr>
<tr>
<td>76</td>
<td>40 mg/day, 6-9 wks</td>
<td>Hetero FH</td>
<td>↓ (21-30%) TC, ↓ (27-36%) LDL-C, ↑ (6%) HDL-C</td>
</tr>
<tr>
<td>69</td>
<td>5-40 mg b.i.d., 6 wks</td>
<td>FH</td>
<td>↓ (14-34%) TC, ↓ (17-39%) LDL-C, ↑ (6-18%) HDL-C</td>
</tr>
<tr>
<td>19</td>
<td>20-40 mg q.p.m., 8 wks</td>
<td>HC</td>
<td>↓ (35%) LDL-C, ↑ (21%) HDL-C</td>
</tr>
<tr>
<td>57</td>
<td>20 mg b.i.d., 15 mos</td>
<td>HC</td>
<td>↓ (19%) TC, ↓ (24%) LDL-C</td>
</tr>
<tr>
<td>139</td>
<td>20-80 mg/day, 6-14 wks</td>
<td>Type II HC</td>
<td>↓ (16-34%) TC, ↓ (18-42%) LDL-C, ↑ (6-13%) HDL-C</td>
</tr>
<tr>
<td>118</td>
<td>40-80 mg/day</td>
<td>FH</td>
<td>↓ (35%) LDL-C</td>
</tr>
<tr>
<td></td>
<td>Non-FH</td>
<td></td>
<td>↓ (47%) LDL-C</td>
</tr>
<tr>
<td>23</td>
<td>20-80 mg/day, 8 wks</td>
<td>HC</td>
<td>↑ (17-29%) TC, ↓ (24-40%) LDL-C, ↑ (7-10%) HDL-C, ↓ (10-19%) TG</td>
</tr>
</tbody>
</table>

Abbreviations:
FH = familial hypercholesterolemia
HC = hypercholesterolemia
TC = total cholesterol
LDL-C = LDL-cholesterol
HDL-C = HDL-cholesterol
TG = triglycerides
Figure 1. Causes of death in registered ESRD patients in Canada during the year 1989

Data from the 1989 Annual Report of the Canadian Organ Replacement Register, Hospital Medical Records Institute, Don Mills, Ontario, March 1991, p 64. Permission for reproduction granted by Dr S Fenton, Chairman of the Dialysis and Renal Transplant Sub-Committee of the CORR
Figure 2. Causes of death in registered ESRD patients in Canada between 1981 to 1989

Data from the 1989 Annual Report of the Canadian Organ Replacement Register, Hospital Medical Records Institute, Don Mills, Ontario, March 1991, p 65. Permission for reproduction granted by Dr S Fenton, Chairman of the Dialysis and Renal Transplant Sub-Committee of the CORR
Figure 4. Biosynthesis of triglycerides
Figure 5. The typical structure of a lipoprotein

A hydrophilic monolayer of phospholipids, apoproteins and cholesterol surrounds a hydrophobic core, which is comprised mainly of triglycerides and cholesterol esters.
INTESTINE
Dietary fat and cholesterol

Chylomicra

HDL

Apo C, E

Apo E receptor

LIVER
Apo B:E receptor

LDL

VLDL

HDL

Apo C, E

Apo C

VLDL remnants

Exogenous Pathway

Endogenous Pathway

Figure 6. Metabolism of lipoproteins by the exogenous and endogenous pathways

HDL = high density lipoprotein, LDL = low density lipoprotein; VLDL = very low density lipoprotein, LPL = lipoprotein lipase, HTGL = hepatic triglyceride lipase
**Figure 7.** Reverse cholesterol transport

PL = phospholipid, FC = free cholesterol, CE = cholesterol ester, TG = triglycerides, HDL = high density lipoprotein, LDL = low density lipoprotein, VLDL = very low density lipoprotein, HL = hepatic lipase, CETP = cholesterol ester transfer protein, LCAT = lecithin cholesterol acyltransferase
**Figure 8.** The Unified Theory of Atherogenesis which links the Lipid Infiltration Hypothesis with the Endothelial Injury Hypothesis.
Figure 9. Structure of lovastatin

Lovastatin is administered as a lactone prodrug and is hydrolyzed in the liver to the active hydroxy-acid form.
Figure 10. Mechanism of action of lovastatin

The active hydroxy-acid form of the drug inhibits HMG-CoA reductase by acting as an analogue of the half-reduced intermediate in the conversion of HMG-CoA to mevalonate.
Figure 11. A light photomicrograph of the ascending aorta of a CRF mouse stained with oil red O. Note the subendothelial accumulation of lipid-containing foam cells and cholesterol ester clefts.
AIMS OF THE STUDY

1. To further characterize a mouse model of CRF with particular attention to lipids;

2. To establish the effects of CRF on serum lipids in mice;

3. To determine the effects of CRF on the properties of hepatic HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis;

4. To investigate the possible effects of lovastatin on serum lipids in CRF mice;

5. To determine the mode of action of lovastatin in CRF mice;

6. To establish the feasibility of using the mouse model of CRF to screen HMG-CoA reductase inhibitors.
MATERIALS

Purina rat chow No. 5012 was purchased from J.E. Mundo Ltee, St. Leonard, Quebec, Canada. The commercial kits, thin layer chromatography (TLC) plates and the chemicals used in the HMG-CoA reductase assay were purchased from the Sigma Chemical Company, St. Louis, Mo, USA.

Escherichia coli alkaline phosphatase, obtained from the aforementioned company, came as a suspension in NH$_4$SO$_4$, which is inhibitory to glucose-6-phosphate dehydrogenase activity. Thus, the suspension was spun at 12,000 x g using a RC2-B Sorvall superspeed centrifuge with SM 24 rotor (Du Pont Canada, Inc., Mississauga, Ontario, Canada) for 45 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in a buffer containing 20 mM imidazole/chloride (pH 7.4) and 5 mM dithiothreitol.

Ecolume, the liquid scintillation counting solution, was purchased from ICN, Mississauga, Ontario, Canada. Unless otherwise stated all other chemicals were obtained from Fisher Scientific, Montreal, Quebec, Canada.

Frozen DL-3-[glutaryl-3-¹⁴C]-hydroxy-methylglutaryl coenzyme A, with a specific activity of 49.0 mCi/m mole, and RS-[5-³H(N)]-mevalonolactone, with a specific activity of 27.8 Ci/m mole, were obtained from New England Nuclear Research Products (Du Pont Canada, Inc., Markham, Ontario, Canada). The labeled HMG-CoA was thawed, aliquoted into 100 µl portions and stored at -20°C.
METHODS

1. Animals

a) Choice

Female mice of the C57BL/6J inbred strain were purchased at five weeks of age from Charles River Canada, Inc., St. Constant, Quebec, Canada. These animals were chosen for two reasons. 1) A reproducible, stable, severe CRF can be successfully induced by surgical procedures in young adult C57BL/6J mice [55, 56]. 2) This strain of mice is known to develop atherosclerosis in response to being fed a diet high in saturated fat and cholesterol [131, 132, 133, 134].

b) Maintenance

The mice were maintained under standard conditions of husbandry in the Animal Facilities of the Montreal General Hospital Research Institute. Groups of 5 animals were housed in shoe box plastic cages on woodshaving bedding. The mice had free access to untreated tap water and were fed ad libitum with Purina rat chow No. 5012 which contained 4% fat and 0.022% cholesterol. The cages were placed in a standing rack in a conventional animal room with a 12 h light (0600-1800 h), 12 h dark (1800-0600 h) cycle.

c) Induction of Renal Failure

Renal failure was induced surgically in the mice by electrocoagulation of the right kidney surface followed by left nephrectomy two weeks later. The right kidney was approached through a 2 cm long skin incision in the right flank. The kidney was
brought up to the skin with cotton applicators applied on both sides and freed by fine tissue dissection of the perirenal fat and adrenal gland. Using a foot-operated single point cauterizer (Hyfrecator, Model X-712, The Birtcher Corp., Los Angeles, California, USA) angled at 30 degrees, the entire surface of the kidney was electrocoagulated except for a 2 mm rim of intact tissue around the hilum (Fig. 12). After the electrocoagulation, the kidney was guided back into the renal fossa. Great care was taken to avoid damage to the ureter from either direct trauma or elongation/distortion during the kidney manipulations. The incision was closed with a running suture, using 5-0 silk for the deep tissue layers, and the skin was closed with stainless steel surgical autoclips. Two weeks later, a left nephrectomy was performed. The left kidney was exposed through the left flank as described above and the vascular pedicle was ligated using 6-0 silk. The kidney was then removed following vascular sectioning distal to the suture. The surgical wound was closed as above. Anesthesia was provided by diethyl ether in the vapour phase delivered continuously through a nose cone. The skin clips were removed 2 weeks after each procedure. Assessment of renal status was done 5 weeks after nephrectomy. To monitor the growth of the mice, body weight was measured weekly using a portable toploading balance (Model 1474, Sartorius GmbH, Germany).

d) Blood Collection and Processing

Blood was drawn from the retro-orbital venous plexus using a Pasteur pipette and transferred to 1.5 ml microcentrifuge tubes. It was allowed to stand at room temperature for at least 2 hours to allow clot formation and retraction with exudation of the serum. After centrifugation at 3,000 x g for 10 minutes at room temperature in
a micro-centrifuge (Model 235C, Fisher Scientific, Montreal, Que., Canada), the serum was separated and transferred to appropriately labeled microcentrifuge tubes. Serum was kept at 4°C for no more than 5 days, during which time all biochemical and electrophoretic procedures were performed.

For the measurement of plasma mevalonate, blood samples were drawn using heparin-coated Pasteur pipettes and transferred to heparinized 1.5 ml microcentrifuge tubes. The tubes were placed on ice in a cold room at 4°C for at least 2 hours. Plasma was separated by spinning the chilled blood at 3,000 x g for 15 minutes at 4°C. In order to remove substances which might interfere with the assay of mevalonate, the plasma samples were transferred to Centriflo™ cone assemblies (Amicon Division, W.R. Grace and Company, Danvers, MA, USA) fitted with ultrafiltration membranes with a 50,000 MW cut off. These were spun at 3,000 x g in a tabletop refrigerated centrifuge (Beckman GPR centrifuge, Beckman Instruments Inc., Palo Alto, CA, USA) for 45 minutes at 4°C. The plasma ultrafiltrates thus obtained were then transferred to 1.5 ml microcentrifuge tubes, labelled and stored at -20°C until they were assayed for mevalonate.

e) Urine Collection

A 17-hour, overnight, urine collection was performed using modified Gilman metabolic cages (Fig. 13). A single set-up included a plastic container provided with breathing perforations, a mesh wire floor placed on a plastic funnel and a 20 ml plastic tube into which the urine was collected. Each metabolic cage housed a single mouse. A slice of apple replaced the standard chow and water during the entire collection period. Contaminating feces and apple pieces were removed by centrifugation of the
urine at 1,000 x g for 30 minutes at 4°C. The volume of the urine was then measured before storage at -20°C prior to the assay of mevalonate.

2. Characterization of the CRF Mouse Model

a) Routine Biochemistry

Serum samples were tested at the Department of Medical Biochemistry of the Montreal General Hospital. A large series of biochemical tests was performed by autoanalyzer (Model 717, Boehringer-Manheim Hitachi, Japan) following standard procedures. This provided for a large range of biochemical tests as performed routinely on blood samples from patients, namely, urea, creatinine, calcium, phosphate, alkaline phosphatase, 5'-nucleotidase, glucose, albumin, iron, amylase, glutamic oxaloacetic transaminase (SGOT) and glutamic oxalpyruvic transaminase (SGPT).

b) Routine Hematology

Blood was drawn using a heparin-coated Pasteur pipette and was transferred to heparinized vacutainers and taken to the Hematology Department of the Montreal General Hospital. Routine hematological tests were performed using a Coulter Counter (Model STKS, Coulter Diagnostics Inc., Hialeah, Fla., USA).

c) Serum Insulin

Frozen serum samples were sent on dry ice to Lilly Research Laboratories, Indianapolis, Indiana for insulin determination by radio-immunoassay, using rat insulin as the standard. Results were analyzed using an in-house program, "RIASYS". This
program is based on a modification of the computer program developed by D. Robarb, Y. Feldman and M. Jaffe at the National Institutes of Health, Bethesda, USA

d) Plasma and Urine Mevalonate

The mevalonate content of plasma ultrafiltrates was determined using a modification of the method of Popjak et al [114]. Plasma ultrafiltrate or mevalonate standard (100 µl) was added to 50 µl of reaction mixture containing 1.0 M phosphate buffer (pH 7.5); 0.3 M MgCl₂; 1.0 mM (γ³²P) ATP, water and 10 units/ml mevalonate kinase. The reaction mixtures were incubated at room temperature for 2.5 hours. The following were then added in sequence: 25 µl conc. HCl, 20 µl of 5-phospho(¹⁴C)mevalonate and 25 µl of a solution containing 0.5 M ATP, 0.1 M ADP and 0.5 M KH₂PO₄. After thorough mixing, ethanol was added and the mixtures were chilled in ice for 30 minutes. The mixtures were then centrifuged at 500 x g for 10 minutes at 0°C. The supernatants were collected and added to 5.0 ml of 0.1 M triethylammonium carbonate buffer (pH 9.7) at room temperature. The diluted samples were then applied to an AG 1-X8 (carbonate) ion-exchange column (Bio-Rad Laboratories, Richmond, CA, USA) and eluted with a linear gradient of 0.1 M and 0.7 M triethylammonium carbonate buffers (pH 7.9). Fractions 5 to 9 (2.5 ml of eluate per fraction), which contained 5-phosphomevalonate, were collected and the radioactivity of each was measured in a gamma counter (Beckman Instruments Inc., Fullerton, CA, USA).

Urine mevalonate levels were also measured using the method described above. Mevalonate concentrations in urine samples were multiplied by their
corresponding urine volumes to obtain the overnight (17 hours) urinary mevalonate excretion.

3. Blood Urea Nitrogen

Renal function was assessed by measuring blood urea nitrogen (BUN) following Sigma Procedure No. 535. This procedure is based on the colorimetric method of Crocker [42] where diacetyl monoxime reacts with urea to produce a pink chromogen and hydroxylamine. Samples and standard (10 μl) were incubated for 10 minutes in a boiling water bath with a mixture of 3 parts BUN Acid Reagent (ferric chloride in phosphoric and sulfuric acids) and 2 parts BUN Color Reagent (diacetyl monoxime, 0.18% [w/v], and thiosemicarbazide). The tubes containing the samples and standards were placed in cold tap water for 5 minutes before absorbance was read against a reagent blank at 515 nm using a spectrophotometer (Bausch & Lomb Spectronic 1001, Milton Roy Company's Analytical Products Division, Fisher Scientific, Montreal, Que., Canada). The readings were completed within 20 minutes. A calibration curve (15-75 mg/dl) was constructed prior to each series of assays.

4. Serum Cholesterol

Serum total cholesterol levels were measured enzymatically following Sigma Procedure No. 352 which is based on a modification of the method of Allain et al [1]. This procedure involves the hydrolysis of cholesterol esters to cholesterol. Cholesterol is then oxidized and the hydrogen peroxide released reacts with 4-aminoantipyrine 0(4-AAP) and p-hydroxybenzenesulfonate (PHBS) to produce a quinonemine dye.
Samples and standard (10 μl) were pipetted into 1.5 ml plastic cuvettes and 1.0 ml of reagent (cholesterol oxidase, cholesterol esterase, peroxidase, 4-AAP, and PHBS in buffer) was added to each. After thorough mixing, the cuvettes were incubated for 5 minutes at 37°C. The absorbance of the quinonemine dye produced was read at 500 nm against a water blank in a spectrophotometer. All cuvettes were read within 30 minutes after the end of the incubation time.

5. Serum Triglycerides

Serum triglyceride levels were measured enzymatically following a modification of the method of McGowan et al [98] (Sigma Procedure No. 339). Following the same principle described above for the assay of cholesterol, triglycerides are hydrolyzed and oxidized. The hydrogen peroxide so produced reacts with 4-AAP and N-ethyl-N-(3-sulfopropyl)m-anisidine (ESPA) to produce a quinonemine dye.

Deionized water (blank), glycerol standard and samples (10 μl) were added to 1.5 ml plastic cuvettes containing 1.0 ml of GPO-Triglyceride Trinder Reagent (lipase, glycerol kinase, glycerol phosphate kinase, peroxidase, ATP, magnesium salt, Na ESPA and 4-AAP in buffer). The mixtures were then incubated at 37°C for 5 minutes. Absorbance was read against a water blank at 540 nm in a spectrophotometer.

6. Serum Lipoprotein Electrophoresis

Serum lipoproteins were separated by agarose gel electrophoresis. Serum samples (5 μl) were applied along one edge of the gels which were electrophoresed in barbital buffer (pH 8.6) for 30 minutes at 100V, 3A. The gels were then fixed for 5 minutes in a solution containing 95% ethanol:deionized water:glacial acetic acid.
(6:3:1). After fixation, the gels were completely dried before being stained with 0.07% (w/w in 95% ethanol) Sudan Black B stain. The gels were then destained three times with a solution of 95% ethanol and deionized water and finally washed with deionized water. Dried gels were scanned at 600 nm using a densitometer (Model CDS 200, Beckman Instruments Inc, Fullerton, CA, USA).

7. Hepatic HMG-CoA Reductase Activity

a) Preparation of the Liver

At the end of the experiment, after bleeding from the retro-orbital venous plexus under CO$_2$ narcosis as described earlier, the mice were sacrificed by cervical dislocation. Livers were collected for the measurement of HMG-CoA reductase activity. The hepatic vein was cut transversally and the liver was perfused with 0.9% saline through the portal vein to flush out residual blood. The gallbladder was ligated and removed after the perfusion to avoid possible contamination of the liver with bile. The liver was then excised, weighed and processed for isolation of microsomes.

b) Isolation of Microsomes

Hepatic total microsomes were isolated according to the method of Brown, Goldstein and Dietschy [30] which is outlined in figure 14. The liver tissue was placed in tubes with 25% (w/v) cold homogenization medium containing 0.3 M sucrose, 10 mM 2-mercaptoethanol, 10 mM EDTA and 50 mM sodium chloride, and homogenized to a thick soup. The homogenates were spun at 12,000 x g (Sorvall centrifuge with a SS 34 rotor) for 15 minutes at 4°C. The supernatants were then spun at 100,000 x g (Beckman ultracentrifuge with a SW 55 rotor, Beckman).
Instruments Inc., Fullerton, CA, USA) for 1 hour at 4°C. Pellets were collected, transferred to properly labelled 1.0 ml cryotubes and stored in liquid nitrogen.

c) HMG-CoA Reductase Assay

The activity of HMG-CoA reductase in liver microsomal preparations was determined using the method of Brown, Goldstein and Dietschy [30] as outlined in figure 15. Microsomal pellets were resuspended in Buffer A containing 20 mMimidazole/chloride (pH 7.4) and 5 mM dithiothreitol. Resuspended microsomal samples (25 µl) were incubated with 25 µl of buffer A and 10 units of Escherichia coli alkaline phosphatase for 1 hour at 37°C to activate the enzyme. Buffer B (100 µl), which contained 0.2 M potassium phosphate (pH 7.4), 40 mM glucose-6-phosphate, 0.7 unit glucose-6-phosphate dehydrogenase, 20 mM EDTA, 10 mM dithiothreitol and 5 mM NADPH, was added to the assay mixture. The reaction was initiated by adding 5 µl of DL-(3-14C)-HMG-CoA and the mixtures were incubated at 37°C for 30 minutes. Addition of 25 µl of 6 M HCl stopped the reaction and lactonized the newly formed product. The standard used in the assay was (3H)-mevalonate which was added to the reaction mixture before lactonization of the newly formed product with HCl. Addition of buffer A to the reaction mixture in place of the microsomal preparation served as the blank. The protein was pelleted down by spinning the sample at 3,000 x g (Baxter Megafuge, Heraeus Sepatech GmbH, Germany, with 2101 rotor) for 10 minutes at room temperature.

Newly formed 14C-mevalonolactone was separated from 14C-HMG-CoA by thin layer chromatography (TLC), using plates coated with a 250 µm layer of polyester silica gel. Samples (10 µl) of the reaction mixtures and standards, (14C)-HMG-CoA
and R-mevalonolactone, were spotted along the edge of the TLC plates, which had been heat-activated at 160°C for 1 hour. The plates were run in one direction in a 1:1 benzene:acetone mixture. The spots were visualized by exposing the plates to iodine vapour and rinsing. The iodine was blown off and each spot was scraped into a vial containing 10 ml Ecolume. Radioactivity was counted in a beta counter (Beckman LS 6000IC, Beckman Instruments Inc., Fullerton, CA, USA).

Hepatic HMG-CoA reductase activity was calculated using the formula:

\[
\text{cpm of } ^{14}\text{C-mevalonolactone} \times \frac{\text{spec act. of } ^{14}\text{C-HMG-CoA}}{\text{cpm/0.1 } \mu\text{Ci of } ^{14}\text{C-HMG-CoA}}
\]

and expressed in pmol/min/mg microsomal protein.

d) Protein Assay

Microsomal protein was measured following Sigma Procedure No. 5656 which is based on Peterson's modification of the micro-Lowry method [112]. As the microsomal pellets had been resuspended in buffer containing dithiothreitol and this chemical interferes with the protein assay, proteins were removed from the buffer by precipitation. Protein samples were diluted to 1 ml with distilled water before the addition of sodium deoxycholate (0.1 ml of a 1.5 mg/ml solution in water). The mixtures were left at room temperature for 10 minutes before the addition of 0.1 ml of trichloroacetic acid. After spinning at 3,000 x g for 10 minutes, the supernatants were discarded and blotted out. The pelleted proteins were each redissolved in 1 ml Modified Lowry Reagent and transferred to appropriately labeled test tubes. The microcentrifuge tubes were rinsed with 1 ml distilled water and the rinsings were added to their respective test tubes. The mixtures were allowed to stand for 20
minutes at room temperature before adding 0.5 ml Folin and Ciocalteu's Phenol Reagent Working Solution to each tube. After allowing the mixtures to stand at room temperature for a further 30 minutes, the absorbances of the samples and standards were read against a water blank at 750 nm in a spectrophotometer. A calibration curve (50-400 μg/ml bovine serum albumin) was constructed for each assay.

e) Kinetic Properties of HMG-CoA Reductase

The Michaelis-Menten constant (Km) and maximum velocity (Vmax) for the enzymes prepared from normal and CRF mice were compared. A number of reaction mixtures containing increasing concentrations (10 to 160 μM) of the substrate, 14C-HMG-CoA, were prepared. The assay was carried out as described above. The results were used to construct a Lineweaver-Burk plot from which the values of Km and Vmax were calculated.

f) Effect of Alkaline Phosphatase in vitro on Enzyme Activity

In the standard HMG-CoA reductase assay described above, alkaline phosphatase was used to activate the enzyme. To establish the native state of activation of HMG-CoA reductase from the livers of normal and CRF mice, enzyme activity was determined in the presence and in the absence of alkaline phosphatase in the reaction mixture.

g) Effect of Lovastatin in vitro on Enzyme Activity

Lovastatin is a competitive inhibitor of HMG-CoA reductase. This drug was used to confirm the presence of the enzyme in the microsomal preparations from
normal and CRF mice. Varying concentrations (0.01 to 1.00 nM) of the sodium salt form of lovastatin was incubated with the microsomal samples during the activation of the enzyme with alkaline phosphatase.

8. Conditions for Sacrifice

A diurnal variation in HMG-CoA reductase activity, which parallels cholesterol synthesis, has been described in various animal species. In order to establish the times of optimal hepatic enzyme activity and serum cholesterol levels in normal and CRF mice, animals were sacrificed at 3 different time points 1200, 1800 and 2400 h.

To determine if fasting affects hepatic HMG-CoA reductase activity and/or serum cholesterol levels in C57BL/6J mice, food was withdrawn from groups of normal and CRF animals 12 hours before sacrifice.

At the end of both of the above experiments, mice were bled under CO₂ narcosis for serum cholesterol and BUN determinations, and then sacrificed by cervical dislocation. Their livers were excised and microsomes immediately isolated and frozen in liquid nitrogen for the subsequent determination of HMG-CoA reductase activity.

9. Treatment of Hypercholesterolemia in CRF with Lovastatin

a) Drug Preparation and Administration

Lovastatin, being highly water-insoluble, was well mixed with rat chow powder at a concentration of 0.02% (50 mg/kg BW), 0.04% (100 mg/kg BW) or 0.08% (200 mg/kg BW). Each mouse was estimated to have a daily consumption of 5 grams of
chow. The diet was prepared fresh daily and was given to the mice in special metal containers placed directly into the cages.

b) Experimental Protocol

To study the efficacy of lovastatin in treating the lipid abnormalities of CRF, the drug was given to mice starting at 5 weeks after the onset of renal failure. The drug was also administered to a group of age-matched, normal mice. Before starting the drug treatment, both experimental and control mice were bled to assess renal status and to determine serum cholesterol levels. At the end of the treatment period, the mice were bled under CO₂ narcosis for various biochemical tests and then sacrificed by cervical dislocation. Livers were removed to measure HMG-CoA reductase activity.

In most experiments, the following protocol was employed:

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Arrival of mice in animal facilities</td>
</tr>
<tr>
<td>0-1</td>
<td>Acclimatization</td>
</tr>
<tr>
<td>3</td>
<td>Electrocoagulation of right kidney surface</td>
</tr>
<tr>
<td>5</td>
<td>Left nephrectomy</td>
</tr>
<tr>
<td>10</td>
<td>Blood collection to assess renal status</td>
</tr>
<tr>
<td>10-14</td>
<td>Treatment with lovastatin</td>
</tr>
<tr>
<td>14</td>
<td>Sacrifice and evaluation</td>
</tr>
</tbody>
</table>
c) Assessment

Based on the results of the experiments to determine the optimal conditions for sacrifice, mice were killed at midnight in the non-fasting state. Thus, the treated mice had access to lovastatin up to the moment of sacrifice.

10. Data Analysis

The results were analyzed and compared using Student’s t-test for grouped data and one-way ANOVA using the computer statistical program, Stat View II (Abacus, Concepts Inc.).
Figure 12. A photograph of an anesthetized mouse immediately after the electrocoagulation by cautery of the exposed surface of the right kidney. Note the minimal blood loss associated with this method of controlled thermal injury.
Figure 13. Schematic representation of the modified Gilman's metabolic cage used for overnight urine collection from mice.
ISOLATION OF LIVER MICROSONES

Collection of the liver

Homogenization of liver slices

Centrifugation of homogenates at 12,000 x g for 15 min at 4°C

Pellet - discard  Supernatant - centrifugation at 100,000 x g for 1 hr at 4°C

Pellet - store in liquid nitrogen  Supernatant - discard

Figure 14. The important steps in the isolation of liver microsomes for the measurement of HMG-CoA reductase activity
HMG-CoA REDUCTASE ASSAY

Isolation of liver microsomes

Initiation of the reaction by the addition of \((^{14}C)\)-HMG-CoA

Termination of the reaction and lactonization of the product by HCl

Centrifugation at \(3,000 \times g\) for 10 min at room temperature

Pellet - discard

Supernatant - thin layer chromatography

Counting of radioactive mevalonolactone

**Figure 15.** The important steps in the determination of HMG-CoA reductase activity
RESULTS

Characterization of the Mouse Model

The results of the biochemical tests performed by autoanalyzer on the sera of normal and CRF mice are presented in Table 4. Mice with CRF had a 4 to 5-fold increase in both serum urea and creatinine levels, indicating that the total number of functioning nephrons remaining was approximately 20-25% of the normal.

The levels of serum calcium and alkaline phosphatase were significantly elevated in CRF mice. Serum alkaline phosphatase levels, but not calcium, were correlated with serum urea levels (Fig 16). Surprisingly, serum phosphate levels remained unchanged in CRF mice.

The serum albumin and total protein levels of CRF mice were not significantly different from those of normal mice (Table 4). These findings suggest that the renal failure is not associated with significant proteinuria.

There was an increase in serum amylase levels in CRF mice which may be due to a decrease in the filtered load resulting from renal damage, while the serum levels of the liver enzymes SGPT, SGOT and 5'-nucleotidase (Table 4) were not significantly different from those of normal animals.

The reduction in renal function was accompanied by anemia as indicated by a fall in hemoglobin concentration and hematocrit (Table 5). In addition, serum iron levels and total iron-binding capacity (TIBC) were not altered. These results were not unexpected as the anemia associated with CRF is not due primarily to iron deficiency but rather to a relative deficiency in erythropoietin production. Other hematological indices were not modified in CRF mice.
The serum insulin levels of normal (0.793 ± 0.387 ng/ml) and CRF (0.669 ± 0.439 ng/ml) mice were not significantly different. Correspondingly, serum glucose levels in CRF mice (8.919 ± 1.095 mmol/l) did not differ from those of normal animals (9.457 ± 1.293 mmol/l). These results suggest that glucose intolerance, which is often observed in CRF patients, is absent in CRF mice.

Plasma mevalonate, which may reflect total body cholesterol synthesis in subjects with normal renal function, was significantly increased in CRF mice as shown in table 6. Conversely, the urinary mevalonate excretion by CRF mice was found to be significantly decreased (Table 6). These changes are probably the result of impaired renal function.

Blood urea nitrogen, which is twice the concentration of urea, was also found to be significantly higher in CRF mice. The results from a representative experiment are shown in figure 17. In subsequent experiments BUN was performed routinely to monitor renal function in mice.

Serum total cholesterol levels were significantly increased in CRF mice (Fig 18). Furthermore, there was a significant positive correlation between BUN and cholesterol levels (Fig. 19). The hepatic HMG-CoA reductase activity was also evaluated in this group of mice and was found to be significantly higher than in normal mice (Fig. 20). However, there was no correlation between the rise in enzyme activity and the increase in either serum cholesterol or BUN levels.

Serum triglyceride levels varied widely in both CRF and normal mice and, although the mean serum triglyceride level in CRF mice (1.106 ± 0.351 mmol/l) was higher than that in normal mice (0.974 ± 0.355 mmol/l), the difference was not statistically significant.
Separation of lipoproteins by agarose gel electrophoresis revealed the presence of \(\beta\)-VLDL and an increase in HDL in the sera of CRF mice (Fig. 21). In addition, the electrophoretic mobility of HDL of CRF mice was faster than that of normal mice.

**Evaluation of Hepatic HMG-CoA Reductase**

The kinetic parameters of the enzyme isolated from the livers of CRF and normal mice were compared. There were no significant differences between the Km of the enzyme prepared from CRF mice (12.904 ± 0.120 \(\mu\)M) and that prepared from normal mice (12.265 ± 0.898 \(\mu\)M). Similarly, the Vmax of the enzyme prepared from CRF mice (9.960 ± 1.456 nmol/min) did not differ significantly from that of normal mice (8.730 ± 1.117 nmol/min) (Fig. 22).

Preincubation of either enzyme preparation with varying concentrations of lovastatin resulted in a dose-dependent, competitive inhibition of the conversion of \(^{14}\)C-HMG-CoA to \(^{14}\)C-mevalonate (Fig. 23). The inhibition constant (Ki) for lovastatin determined using HMG-CoA reductase prepared from CRF mice was not significantly different from that calculated for lovastatin using hepatic HMG-CoA reductase prepared from normal mice.

These results showed that HMG-CoA reductase from CRF mice is identical to that from normal mice and can be inhibited by lovastatin *in vitro*.

HMG-CoA reductase exists in 2 forms - phosphorylated (inactive) and dephosphorylated (active). In these experiments an activation step whereby the microsomal preparations were preincubated with alkaline phosphatase was always included in any assay of the enzyme. However, as serum alkaline phosphatase levels are elevated in CRF mice (Table 4), it was considered prudent to investigate the
native activation state of liver HMG-CoA reductase in CRF mice. Pre-incubation of the microsomal preparation with alkaline phosphatase did not increase the conversion of HMG-CoA to mevalonate (1.897 ± 0.476 vs. 1.914 ± 0.612 nmol/min/mg protein). On the other hand, alkaline phosphatase slightly increased the activity (1.412 ± 0.392 to 1.638 ± 0.336 nmol/min/mg protein) of HMG-CoA reductase isolated from the livers of normal mice. The activity of the enzyme in either state was higher in CRF mice than in normal mice.

**Conditions for Sacrifice of Experimental Animals**

In order to establish the optimum conditions for sacrifice, normal and CRF mice were killed at different time points, in a fasting or non-fasting state, and their serum total cholesterol levels and liver HMG-CoA reductase activities were measured.

Figure 24a shows that although serum total cholesterol levels were always significantly higher in CRF mice than in normal mice, there were no diurnal variations within the groups. Mice sacrificed at 1200 h exhibited the same cholesterol levels as animals sacrificed at 1800 h or at 2400 h.

On the other hand, a diurnal variation in HMG-CoA reductase activity was observed in both normal and CRF mice (Fig. 24b). Peak activity occurred at midnight, while activity was lowest at noon. No significant difference in HMG-CoA reductase activity was observed between normal and CRF mice at noontime, but enzyme activity was increased more than 100% in CRF mice compared with normals at the other time points.

As shown in figure 25a, there was no significant difference in serum total cholesterol levels between mice sacrificed in a non-fasting state and mice sacrificed
after an overnight fast. Serum total cholesterol levels in CRF mice were significantly higher compared to those of normal animals. Figure 25b shows that the HMG-CoA reductase activity of normal mice sacrificed in a non-fasting state was significantly higher than that of normal animals sacrificed after an overnight fast. This difference in enzyme activity between the non-fasting and fasting state, however, was not seen in CRF mice. Surprisingly, in this experiment there was no significant difference in enzyme activity between CRF and normal mice killed in a non-fasting state.

Based on these results, it was decided to sacrifice the animals in a non-fasting state and at midnight in all future experiments.

Treatment with Lovastatin

The possibility of using lovastatin to prevent the rise in serum total cholesterol levels induced by CRF was first investigated. Lovastatin (50 mg/kg BW/day) was administered to mice for 5 weeks from the onset of renal failure (the day following left nephrectomy). Figures 26a and 26b show that lovastatin was not effective in preventing the rise in either serum total cholesterol levels or HMG-CoA reductase activity which are associated with CRF. It was decided to investigate whether or not lovastatin can reduce serum cholesterol levels once they have been increased by CRF. Therefore, in the next experiments, the drug was administered at a dose of 50 mg/kg BW/day for 2 weeks starting 5 weeks after the onset of renal failure. The results show that, although serum total cholesterol levels were reduced in CRF mice treated with lovastatin, the changes were not significant (Fig. 27a). Furthermore, the activity of HMG-CoA reductase activity in CRF was unaltered by 50 mg/kg BW lovastatin administered for two weeks (Fig. 27b). In normal mice, however, serum
total cholesterol levels remained unaltered while enzyme activity increased, though not significantly.

As there was a trend towards a reduction in serum total cholesterol levels in CRF mice with 50 mg/kg BW lovastatin treatment, it was decided to extend the time of administration to 4 weeks and to increase the dosage to 100 and 200 mg/kg BW.

From figures 28a and 28b, it can be seen that CRF induced a significant increase in serum total cholesterol levels in mice which was accompanied by an increase in hepatic HMG-CoA reductase activity. Again, treatment with 50 mg/kg BW/day lovastatin did not appear to have a significant effect on either serum total cholesterol levels or HMG-CoA reductase activity in CRF mice. In normal mice, 50 mg/kg BW/day lovastatin significantly increased hepatic HMG-CoA reductase activity without affecting serum total cholesterol levels. Lovastatin at 100 and 200 mg/kg BW/day produced significant dose-related decreases in serum total cholesterol levels in CRF mice. Indeed, at 200 mg/kg BW/day, lovastatin lowered cholesterol levels to normal levels in these animals (Fig. 28a). The changes in serum total cholesterol levels, however, were not reflected in similar changes in enzyme activity. At 100 mg/kg BW, lovastatin appeared to reduce HMG-CoA reductase activity, but this change was not statistically significant. At 200 mg/kg BW/day, lovastatin had no effect on enzyme activity in CRF mice. Similarly, in normal mice, this high dose had no significant effect on enzyme activity but significantly decreased serum total cholesterol levels.

Serum triglyceride levels were also investigated in this experiment. Lovastatin at 100 and 200 mg/kg BW/day also resulted in a dose-related reduction in triglyceride levels (Fig. 29). However, these changes were not significant.
Agarose gel electrophoresis of the serum lipoproteins of the mice receiving 100 and 200 mg/kg BW/day lovastatin revealed that the drug altered the lipoprotein profile of CRF mice (Fig. 30). In fact, treatment with 200 mg/kg BW/day lovastatin resulted in a profile similar to that of normal mice.

It is conceivable that any effects lovastatin might have on HMG-CoA reductase activity, may have been masked by liver damage induced by such relatively high doses of the drug. Therefore, it was decided to measure serum transaminases as indicators of possible hepatic dysfunction. Lovastatin did not induce an increase in levels of serum transaminases in treated mice (Table 7), indicating there was no liver damage even after 4 weeks of administration of the highest dose.
Table 4. Comparison of the serum biochemical profiles of normal and CRF mice 5 weeks after the onset of renal failure.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal (n=14)</th>
<th>CRF (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mmol/l)</td>
<td>7.836 ± 1.137</td>
<td>35.294 ± 6.568 *</td>
</tr>
<tr>
<td>Creatinine (mmol/l)</td>
<td>12.286 ± 1.267</td>
<td>54.688 ± 15.452 *</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>2.347 ± 0.120</td>
<td>2.893 ± 0.229 *</td>
</tr>
<tr>
<td>Phosphate (mmol/l)</td>
<td>2.777 ± 0.307</td>
<td>2.994 ± 0.485</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>89.000 ± 8.840</td>
<td>217.875 ± 51.305 *</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>28.846 ± 1.676</td>
<td>28.688 ± 1.662</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>52.643 ± 2.590</td>
<td>53.187 ± 3.816</td>
</tr>
<tr>
<td>Amylase (U/l)</td>
<td>4349.429 ± 783.615</td>
<td>6742.062 ± 409.892 *</td>
</tr>
<tr>
<td>SGOT (U/l)</td>
<td>52.929 ± 10.986</td>
<td>54.375 ± 12.082</td>
</tr>
<tr>
<td>SGPT (U/l)</td>
<td>17.520 ± 5.841</td>
<td>18.230 ± 6.455</td>
</tr>
<tr>
<td>5'-Nucleotidase (U/l)</td>
<td>27.731 ± 4.373</td>
<td>27.320 ± 3.258</td>
</tr>
</tbody>
</table>

Abbreviations:
SGOT= serum glutamic oxaloacetic transaminase;
SGPT= serum glutamic oxalpyruvic transaminase.

Data represent mean ± SD
* = significant difference between animal groups (p<0.05)
Table 5. Hematological features of normal and CRF mice sacrificed 5 weeks after the onset of renal failure.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>CRF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=14</td>
<td>n=16</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>7.836 ± 1.137</td>
<td>35.294 ± 6.565 *</td>
</tr>
<tr>
<td>Creatinine (mmol/l)</td>
<td>12.286 ± 1.267</td>
<td>54.688 ± 15.452 *</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12.250 ± 2.201</td>
<td>8.231 ± 2.178 *</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.314 ± 0.085</td>
<td>0.220 ± 0.062</td>
</tr>
<tr>
<td>MCV (μm³)</td>
<td>44.700 ± 0.355</td>
<td>44.719 ± 1.996</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>19.785 ± 1.421</td>
<td>16.875 ± 1.184</td>
</tr>
<tr>
<td>Platelets (x 1000/mm³)</td>
<td>868.923 ± 234.196</td>
<td>928.188 ± 185.000</td>
</tr>
<tr>
<td>MPV (μm³)</td>
<td>5.235 ± 0.276</td>
<td>5.450 ± 0.470</td>
</tr>
<tr>
<td>Iron (μmol/l)</td>
<td>14.692 ± 4.800</td>
<td>17.000 ± 3.645</td>
</tr>
<tr>
<td>TIBC (μmol/l)</td>
<td>61.231 ± 5.231</td>
<td>70.033 ± 6.595</td>
</tr>
</tbody>
</table>

Abbreviations:
MCV= mean corpuscular volume; MCH= mean corpuscular hemoglobin; MPV= mean platelet volume; TIBC= total iron binding capacity.

Data represent mean ± SD.
*= significant difference between animal groups (p<0.05).
Table 6. Plasma and urine mevalonate in normal and CRF mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal (n=7)</th>
<th>CRF (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood urea nitrogen (mmol/l)</td>
<td>5.852 ± 0.983</td>
<td>34.153 ± 4.455 *</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/l)</td>
<td>2.977 ± 0.287</td>
<td>5.963 ± 0.653 *</td>
</tr>
<tr>
<td>Plasma mevalonate (pmol/l)</td>
<td>292.305 ± 28.951</td>
<td>504.659 ± 86.841 *</td>
</tr>
<tr>
<td>Urine mevalonate (nmol/17 h)</td>
<td>134.758 ± 34.029</td>
<td>3.982 ± 2.687 *</td>
</tr>
</tbody>
</table>

Data represent mean ± SD.
*= significant difference between animal groups (p<0.001).
Table 7. Serum transaminase levels in normal and CRF mice treated with lovastatin

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>BUN (mmol/l)</th>
<th>SGOT (U/l)</th>
<th>SGPT (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>12.920 ± 1.316</td>
<td>67.000 ± 10.382</td>
<td>20.700 ± 3.632</td>
</tr>
<tr>
<td>Normal + lov 100</td>
<td>10</td>
<td>13.580 ± 1.944</td>
<td>56.400 ± 11.057</td>
<td>14.800 ± 6.546</td>
</tr>
<tr>
<td>Normal + lov 200</td>
<td>10</td>
<td>12.670 ± 1.083</td>
<td>65.300 ± 8.982</td>
<td>19.000 ± 1.333</td>
</tr>
<tr>
<td>CRF</td>
<td>10</td>
<td>34.800 ± 6.883</td>
<td>58.200 ± 23.145</td>
<td>11.600 ± 2.881</td>
</tr>
<tr>
<td>CRF + lov 100</td>
<td>7</td>
<td>37.700 ± 5.522</td>
<td>73.000 ± 13.275</td>
<td>12.600 ± 1.578</td>
</tr>
<tr>
<td>CRF + lov 200</td>
<td>5</td>
<td>34.180 ± 3.176</td>
<td>82.714 ± 10.714</td>
<td>15.714 ± 2.430</td>
</tr>
</tbody>
</table>

Abbreviations:
BUN=blood urea nitrogen;
SGOT= serum glutamic oxaloacetic transaminase;
SGPT= serum glutamic oxalopruvic transaminase;
lov 100= 100 mg/kg BW/day lovastatin treatment;
lov 200= 200 mg/kg BW/day lovastatin treatment.

Data represent mean ± SD.
Figure 16. Correlation between serum urea and alkaline phosphatase levels in CRF mice (n=18)
Figure 17. Blood urea nitrogen levels in normal (n=7) and CRF (n=9) mice from a representative experiment.

Data are the mean ± SD  № = significant difference between animal groups (p<0.05)
Figure 18. Serum total cholesterol levels in normal (n=7) and CRF (n=9) mice from a representative experiment

Data are the mean ± SD  # = significant difference between animal groups (p<0.05)
Figure 19. Correlation between blood urea nitrogen and serum total cholesterol levels in CRF mice (n=16)

$r=0.8723$
$p<0.0001$
Figure 20. Hepatic HMG-CoA reductase activity in normal (n=14) and CRF (n=16) mice from a representative experiment.

Data are the mean ± SD. # = significant difference between animal groups (p<0.05).
Figure 21. Electrophoretic profile of sera from CRF (lanes 1-3) and normal (lanes 4-6) mice. HDL=high density lipoprotein, LDL=low density lipoprotein, VLDL=very low density lipoprotein.
Figure 22. Lineweaver-Burk analysis of the kinetics of hepatic HMG-CoA reductase activity isolated from normal and CRF mice.

Each data point represents the mean value from 3 assays using different microsomal preparations.
Figure 23. Dose-dependent inhibition of hepatic HMG-CoA reductase activity from normal and CRF mice by the sodium salt form of lovastatin

Each data point represents the mean value from 3 assays using different microsomal preparations.
Figure 24a. The diurnal variation in serum total cholesterol levels in non-fasting mice.

Data represent mean ± SD  # = significant difference between animal groups (p<0.05)  
n=4-6  Mean BUN levels in normal mice ranged from 7 220 to 8 275 mmol/l and in CRF mice from 31 840 to 37 500 mmol/l.
Figure 24b. The diurnal variation in hepatic HMG-CoA reductase activity in non-fasting mice.

Data represent mean ± SD # = significant difference between animal groups (p<0.05) and * = significant difference within the CRF group (p<0.05)

n and BUN levels as per legend to Figure 24a.
Figure 25a. The effect of fasting on serum total cholesterol levels in mice sacrificed at midnight.

Data represent mean ± SD. * = significant difference between animal groups (p<0.05). 
n=10 for each group. Mean BUN levels in normal mice ranged from 8.244 to 8.345 mmol/l and in CRF mice from 32.130 to 33.861 mmol/l.
Figure 25b. The effect of fasting on hepatic HMG-CoA reductase activity in mice sacrificed at midnight.

Data represent mean ± SD. # = significant difference between animal groups (p<0.05) and * = significant difference within the normal group (p<0.05). n and BUN levels as per legend to Figure 25a.
Figure 26a. The effect of lovastatin on serum total cholesterol levels in CRF mice

Treatment was for 5 weeks starting on the day following left nephrectomy (time of onset of renal failure). Data represent mean ± SD. # = significant difference between animal groups (p<0.05) and * = significant difference within the normal group (p<0.05). n=10 for each group. Mean BUN levels in normal mice ranged from 10781 to 13495 mmol/l and in CRF mice from 24347 to 28132 mmol/l.
Figure 26b. The effect of lovastatin on hepatic HMG-CoA reductase activity in CRF mice.

Treatment was for 5 weeks starting on the day following left nephrectomy (time of onset of renal failure). Data represent mean ± SD. * = significant difference within each group (p<0.05).

n and BUN levels as per legend to Figure 26a.
Figure 27a. The effect of short-term administration of a low dose of lovastatin on serum total cholesterol levels in CRF mice

Treatment was for 2 weeks starting 5 weeks post-nephrectomy (time of onset of renal failure). Data represent mean ± SD. # = significant difference between animal groups (p<0.05)

n=10 for each group. Mean BUN levels in normal mice ranged from 6.74 to 7.959 mmol/l and in CRF mice from 21.991 to 24.4972 mmol/l
Figure 27b. The effect of short-term administration of a low dose of lovastatin on hepatic HMG-CoA reductase activity in CRF mice.

Treatment was for 2 weeks starting 5 weeks post-nephrectomy (time of onset of renal failure). Data represent mean ± SD. * = significant difference between animal groups (p<0.05) n and BUN levels as per legend to Figure 27a.
Figure 28a. The effect of lovastatin treatment on serum total cholesterol levels in CRF mice

Mice were treated daily with the drug for four weeks starting 5 weeks after the onset of renal failure. Data represent mean ± SD. # = significant difference between animal groups (p<0.05) and * = significant difference within each group (p<0.05).

n=10 for all groups, except for CRF treated with 100 mg/kg BW/day lovastatin where n=7 and CRF treated with 200 mg/kg BW/day lovastatin where n=5. Mean BUN levels in normal mice ranged from 12.550 to 13.580 mmol/l and in CRF mice from 32.705 to 37.700 mmol/l.
Figure 28b. The effect of lovastatin treatment on hepatic HMG-CoA reductase activity in CRF mice

Mice were treated daily with the drug for four weeks starting 5 weeks after the onset of renal failure. Data represent mean ± SD. * = significant difference between animal groups (p<0.05)  * = significant difference within the normal group (p<0.05) n and BUN levels as per legend to Figure 28a
Figure 29. The effect of lovastatin treatment on serum triglyceride levels in CRF mice.

Mice were treated daily with the drug for four weeks starting 5 weeks after the onset of renal failure. Data represent mean ± SD. $n=10$ for all groups, except for CRF treated with 100 mg/kg BW/day lovastatin where $n=7$ and CRF treated with 200 mg/kg BW/day lovastatin where $n=5$. Mean BUN levels in normal mice ranged from 12.550 to 13.580 mmol/l and in CRF mice from 32.705 to 37.700 mmol/l.
Figure 30. Electrophoretic profile of sera from normal (lanes 1 & 2) and CRF mice untreated (lanes 3 & 4) and treated with 200 mg/kg BW lovastatin (lanes 5 & 6) and 100 mg/kg BW lovastatin (lanes 7 & 8). HDL=high density lipoprotein LDL=low density lipoprotein VLDL=very low density lipoprotein
SUMMARY OF RESULTS

1. CRF does not alter the kinetics of mouse hepatic HMG-CoA reductase.

2. Inhibition of the hepatic HMG-CoA reductase by lovastatin *in vitro* is not affected by CRF.

3. CRF does not affect the observed diurnal rhythm in hepatic HMG-CoA reductase activity.

4. CRF increases serum total cholesterol levels and hepatic HMG-CoA reductase activity in mice.

5. CRF decreases urinary mevalonate excretion which is reflected in higher plasma concentrations.

6. Lovastatin does not prevent the rise in serum total cholesterol levels nor the increase in hepatic HMG-CoA reductase activity induced by CRF in mice.

7. Lovastatin negates the increase in serum total cholesterol levels induced by CRF in mice without significantly affecting hepatic HMG-CoA reductase activity.
DISCUSSION

The mouse model used in this investigation exhibited the major manifestations of chronic renal failure - metabolic abnormalities with retention of nitrogenous compounds, severe anemia and possible bone disease. These findings confirmed some of the previous observations on this animal model made by Gagnon and Gallimore [56].

The degree of renal failure was assessed by serum urea and creatinine levels. In CRF mice these parameters were elevated, indicating a reduction in glomerular filtration rate (GFR). GFR, an index of renal function, is usually measured by the clearance of substances which are neither reabsorbed nor secreted by the renal tubule. In the steady state, levels of serum urea and creatinine are commonly used to monitor renal function, with the knowledge that these indices do not always provide exact measurements of GFR. Both substances are freely filtered at the glomerulus but their excretion in the final urine is also dependent on tubular processing. Substantial tubular reabsorption of urea occurs during volume contraction, thus underestimating GFR. On the other hand, creatinine secretion by the tubule is increased during renal failure, leading to an overestimation of GFR.

Serum alkaline phosphatase levels were found to be significantly increased in CRF mice. As the levels of alkaline phosphatase and serum urea were significantly correlated, it would appear that the rise in this enzyme level is a function of renal failure. The rise in serum alkaline phosphatase levels could be a result of increased release of the enzyme from the bone, rather than the liver, as the levels of serum...
5'-nucleotidase, SGOT and SGPT, which provide a measure of liver damage, did not differ significantly between normal and CRF mice.

The results of the present experiments confirmed the report by Gagnon and Gallimore [56] that CRF in mice is associated not only with elevated serum alkaline phosphatase levels, but also with increased serum calcium levels. These findings suggested the presence of renal osteodystrophy, which was later confirmed by bone histology demonstrating extensive changes compatible with ostertis fibrosa. The observations in CRF mice differ in some respects from those reported in CRF patients with significant bone disease. Although these patients exhibit elevated serum levels of alkaline phosphatase, they usually present with hypocalcemia, not hypercalcemia, and hyperphosphatemia.

Another interesting observation in this study was that of elevated serum levels of amylase in CRF mice, which may have been caused by decreased filtered load at the glomerulus. Several compounds, including hormones, by-products of protein metabolism, sulphates and unidentified anions, are known to be retained in the blood of CRF subjects due to a fall in GFR. This is the first demonstration of elevated serum amylase levels in CRF mice.

Anemia, as indicated by a reduced hemoglobin concentration and low hematocrit, was also observed in CRF mice. As serum iron levels and iron binding capacity were not reduced in these animals, iron deficiency was safely ruled out as the cause of anemia. In humans, anemia is also a major manifestation of CRF. Here it is ascribed primarily to an insufficient production of red blood cells by the bone marrow, resulting from decreased erythropoietin production by the diseased kidney.
Another reason for inadequate erythropoiesis in CRF is a reduced responsiveness of the bone marrow to erythropoietin.

A number of endocrine abnormalities are observed in patients with CRF, the best-known being glucose intolerance resulting mainly from peripheral insulin resistance [53, 116, 145]. However, in CRF mice, neither basal serum glucose nor insulin levels were found to be significantly different from those of normal mice. Different results might have been obtained had the mice been challenged with a glucose load prior to testing or if proinsulin, rather than insulin, had been measured.

In this study, mevalonate, the product of the reaction catalyzed by HMG-CoA reductase, which is normally metabolized by the kidney, was found to be markedly affected by renal failure. Mice with CRF exhibited significantly increased plasma mevalonate levels and significantly decreased urinary mevalonate excretion compared to normal animals. The increased levels of mevalonate in the plasma of CRF mice are likely to result from reduced GFR. Similar findings were reported in the rat by Kopito et al. [84] who observed a 4-fold rise in plasma mevalonate levels following bilateral nephrectomy.

Abnormalities in the lipid profile and the enzymes involved in the metabolism of lipids were also observed in this mouse model of CRF. These included elevated serum total cholesterol levels and HMG-CoA reductase activities and the presence of β-VLDL in the serum, as detected by agarose gel electrophoresis. These changes are similar to those seen in the human situation where over 30% of patients with CRF are reported to exhibit elevated cholesterol levels and triglyceride-rich β-VLDL appears in the serum [101, 106]. The presence of β-VLDL in the sera of CRF patients is manifested as hypertriglyceridemia. However, despite showing similar increases in
In the present experiments, mice with CRF exhibited only a slight increase in serum triglyceride levels. This suggests that other lipoproteins, like HDL, may be losing triglycerides to β-VLDL, becoming enriched with cholesterol instead. HDL in CRF mice were observed to have a faster electrophoretic mobility which may result from cholesterol-enrichment.

The present study confirmed that there is a strong correlation between serum total cholesterol and BUN levels in CRF mice as previously reported by Stewart-Phillips et al. [132, 133]. This suggests that there are factors present in uremic serum which either accelerate the synthesis of cholesterol or inhibit its cellular uptake. As previously mentioned, one substance which is retained in the circulation of CRF mice is mevalonate. Mevalonate is believed to regulate HMG-CoA reductase through one of its derivatives, which exerts a negative feedback action on the synthesis of the enzyme (Fig. 31) [59, 111, 115]. However, mevalonate may also exert a stimulatory effect on the activity of HMG-CoA reductase by providing it with substrates through the shunt pathway of mevalonate metabolism (Fig. 32). The overall effect of mevalonate on serum total cholesterol levels probably depends on the balance between these two processes.

Plasma mevalonate which is extracted by the kidney has three fates. About 70% is synthesized to squalene and sterols [99, 144], 20% is excreted unchanged in the urine [31] and the rest is metabolized via the shunt pathway [73, 77, 88]. In this pathway, carbon 1 of mevalonate is lost as CO₂, carbons 2, 3 and 6 are utilized in the formation of acetoacetate and carbons 4 and 5 become the carbons of acetyl-CoA [88]. Acetyl-CoA and acetoacetate are both precursors of HMG-CoA, the substrate for HMG-CoA reductase.
Feinhold et al. [52] found that 5/6 nephrectomy in rats resulted in a 50% reduction in shunt pathway activity and a concomitant increase in sterol formation from circulating mevalonate in the liver and carcass. The reduction in shunt pathway in the kidney correlated with serum creatinine and BUN levels. The kidney, however, accounts for only 1% of shunt activity in the rat [144]. Weinstock et al. [143] reported that in humans the liver is the major site of the shunt pathway of total body mevalonate metabolism. Plasma mevalonate makes a further contribution to mevalonate shunting in the organ [144].

Although information regarding mevalonate shunting is not available for the mouse, it is reasonable to suppose that the pathway does exist in this species. Thus, in mice with elevated levels of plasma mevalonate may upregulate the shunt pathway in the liver, thereby increasing the availability of the substrate precursors acetoacetate and acetyl-CoA, and consequently HMG-CoA, for the synthesis of cholesterol. Increased levels of its substrate might be expected to increase the activity of HMG-CoA reductase. Indeed, in the present experiments there was a significant increase in hepatic HMG-CoA reductase activity in CRF mice. However, this increase did not correlate with the rise in serum total cholesterol levels which was also observed. The absence of such a correlation indicates that the elevation in serum total cholesterol levels was not entirely due to an increase in its de novo synthesis in the liver. It is possible that increased peripheral synthesis of cholesterol from the increased circulating mevalonate present in renal failure, as previously observed in rats [84], also occurred. Decreased cellular cholesterol uptake by the liver, resulting from inhibition of CETP activity as described in CRF patients.
is another possible mechanism contributing to the rise in serum total cholesterol levels in mice with CRF.

It is also possible that the increased serum total cholesterol levels observed in CRF mice did not result from increased availability of substrate but from structural, functional or activational changes in HMG-CoA reductase induced by the uremic state. However, the enzyme isolated from the livers of CRF mice was found to have identical kinetic properties and the same capacity to be inhibited by lovastatin in vitro as the enzyme from normal mice, thus indicating that CRF did not alter either the structural or functional properties of hepatic HMG-CoA reductase.

Before the enzyme can convert HMG-CoA to mevalonate, it must be dephosphorylated by phosphatases. In the liver, only 10-25% of the total enzyme is reported to be activated at any given time [30] and so, in the assay procedure employed in the present study [30], alkaline phosphatase is used to activate HMG-CoA reductase present in liver microsomal preparations. However, as serum alkaline phosphatase levels were found to be increased in CRF mice, it is possible that hepatic HMG-CoA reductase from these animals was already in an activated state. To test this hypothesis, reaction mixtures containing no exogenous alkaline phosphatase were first assayed for HMG-CoA reductase activity. The addition of commercially available alkaline phosphatase to the reaction mixtures had no significant stimulatory effect on the activity of the enzyme, indicating that it was indeed extracted from liver microsomes in the activated state. However, a similar result was obtained using microsomal enzyme isolated from the livers of normal mice, indicating that it, too, was already activated. As normal mice do not have increased levels of serum alkaline
phosphatase, activation of the enzyme probably resulted from the procedure employed to isolate the liver microsomes.

The inactivation of HMG-CoA reductase, on the other hand, may be controlled by one of three kinase systems: a) the cyclic cascade system consisting of HMG-CoA reductase, reductase kinase and reductase kinase kinase [16], b) calcium-activated and phospholipid-dependent protein kinase [17] and c) calcium- and calmodulin-dependent protein kinase [18]. It is interesting to note that two of the kinases involved in the regulation of HMG-CoA reductase are dependent on calcium, which was observed to be significantly elevated in CRF sera. However, despite the possible enhancement of inactivation of the enzyme by these kinases, hepatic HMG-CoA reductase activity was found to be increased in CRF mice.

The activation by dephosphorylation of the enzyme and its degradation by kinases may be considered to provide short-term control. There is also a long-term regulation of HMG-CoA reductase activity through negative feedback inhibition (Fig. 31). This involves two mediators: cholesterol, which is normally obtained from LDL, and a nonsterol by-product of mevalonate metabolism. The exact mechanisms by which these substances regulate the enzyme are still unclear. It has been reported that sterols partially inhibit the synthesis of HMG-CoA reductase mRNA (transcription) [29, 92] and the nonsterol derivative of mevalonate suppresses the translation of the mRNA to the reductase protein [103]. However, Ness and colleagues [104] reported that dietary cholesterol does not affect transcription of the HMG-CoA reductase gene.

Edwards et al [49], on the other hand, were able to show that mevalonate not only decreased enzyme synthesis but it also increased its degradation. Furthermore, in cultured cells, inhibition of mevalonate production by lovastatin has been shown to
induce a 200-fold increase in the translation of the enzyme protein [58]. This increase in enzyme synthesis was abolished by adding mevalonate to the cell cultures.

The diurnal rhythm of hepatic HMG-CoA reductase activity, which was previously observed in normal mice by Kandutsch and Saucier [79] and Berndt et al [21], was also observed in CRF mice in the present experiments. However, whereas Berndt et al reported that peak activity occurred between 1300 h and 1800 h, here peak hepatic HMG-CoA reductase activity was observed at midnight and lowest activity was seen at noon in both normal and CRF mice. It should be noted that Berndt et al did not measure enzyme activity between 1800 and 0830 h. If they had done so, perhaps they would have observed the same peak and nadir as have been reported here. The increase in HMG-CoA reductase activity appears to be a direct result of increased enzyme protein synthesis [21, 79] as prior administration of protein synthesis inhibitors prevents the observed peak. This finding indicates that newly synthesized enzyme is necessary to maintain its rhythm of activity.

Rodwell et al [120] suggested that certain hormones have an important influence on the diurnal variation in HMG-CoA reductase activity. Insulin and thyroid hormone have been implicated in the enhancement of enzyme activity and are proposed to act on the rising phase of the rhythm while glucagon and hydrocortisone appear to suppress enzyme activity [120]. Probably many factors interact to control the rise and fall in enzyme activity.

The effect of fasting on hepatic HMG-CoA reductase activity was also studied in the present experiments and was found to decrease enzyme activity in normal mice but not in CRF animals. This result supports the finding of Berndt et al [21] that fasting in normal mice led to a reduction in HMG-CoA reductase activity but is in
disagreement with the finding of Kandutsch and Saucier [79] that fasting had no effect on enzyme activity. Why fasting would decrease HMG-CoA reductase activity in normal mice is open to speculation. Indeed, one might expect an increase in enzyme activity as increased endogenous synthesis of cholesterol compensates for a decrease in the exogenous supply of this lipid.

Lovastatin, at a dose of 50 mg/kg BW, did not prevent the rise in HMG-CoA reductase activity induced by CRF in mice nor did it have a significant cholesterol-lowering effect in these animals. However, at doses of 100 and 200 mg/kg BW, lovastatin produced a dose-dependent reduction in serum total cholesterol levels, without hepatic enzyme inhibition, in CRF mice. On the other hand, in normal mice, 50 mg/kg BW lovastatin significantly increased hepatic HMG-CoA reductase activity while higher doses had no significant effects.

These results are in keeping with the findings that lovastatin, which is expected to inhibit the conversion of HMG-CoA to mevalonate has, in fact, different actions in different animal species. The effect of this drug on HMG-CoA reductase activity and cholesterol levels in rats is the opposite of that seen in humans and dogs [2]. Similarly, in mice, Kita et al. [82] demonstrated that the inhibition of mevalonate production from HMG-CoA by lovastatin led to an increase in hepatic HMG-CoA reductase activity even when the animals were fed a high cholesterol diet. The increase induced by the drug could be prevented only by the concomitant administration of mevalonate. Yamauchi et al. [146] studied rat and human liver microsomes and demonstrated that the species difference in response to lovastatin administration may lie in differences in the membrane fluidity of their microsomes. The rat was found to have a more rigid microsomal membrane than humans which
might render it less permeable to lovastatin. A similar property of mouse microsomal membranes may explain the high lovastatin dosage needed to have a lipid-lowering effect in this species in the present study but does not explain why HMG-CoA reductase activity was not apparently affected in CRF mice.

The results of this study demonstrate the complexity of the effects of lovastatin on HMG-CoA reductase in CRF mice. The fact that no inhibition of the hepatic enzyme by the drug was observed suggests that a reduction in the level of cholesterol or some intermediate in the cholesterol synthetic pathway upregulates HMG-CoA. As discussed above, the long term regulation of the enzyme is mediated by cholesterol together with a second, unknown derivative of mevalonate (Fig. 31). Therefore, when levels of these substances are reduced due to the inhibition of HMG-CoA reductase by lovastatin, there may be a compensatory upregulation of the enzyme. This theory is supported by the finding of Kita et al. [82] that lovastatin increases hepatic HMG-CoA activity in mice and that this increase could be prevented by mevalonate.

In the present experiments with CRF mice, in which plasma mevalonate levels were significantly increased, hepatic HMG-CoA reductase levels were found to be unchanged. This suggests that the rebound upregulation of the enzyme resulting from lovastatin therapy may have been countered by downregulation by mevalonate.

The availability of lovastatin to the different organs of the body following oral administration has been studied extensively and should also be considered in any discussion of the mechanisms underlying the lipid-lowering effect of lovastatin in CRF mice. Lovastatin is administered as a hepatoselective, lipophilic prodrug [110]. Once inside the liver it is reversibly transformed into its active open hydroxy-acid form which is able to competitively inhibit HMG-CoA reductase.
During its first pass through the liver, 95% of lovastatin is extracted and 5% remains in the circulation where it may be hydrolyzed by esterases to its active form [138]. The majority of the absorbed drug is eventually excreted in the bile [48]. However, a significant fraction enters the bloodstream as active hydrophilic metabolites [108]. Circulating lovastatin metabolites are normally excreted by the kidneys [85] but renal failure may lead to their eventual accumulation in extrahepatic tissues where they could produce a reduction in HMG-CoA reductase activity sufficient to be reflected as a reduction in serum total cholesterol levels. Indeed, cases of peripheral lipid-lowering effects of the drug have recently been reported [28, 89, 125].

In the current experiments, relatively large doses of lovastatin were employed which may have produced high levels of circulating active metabolites. As lovastatin did not appear to inhibit hepatic HMG-CoA reductase activity, it may have reduced serum total cholesterol levels in CRF mice through the inhibition of peripheral cholesterol synthesis by its circulating active metabolites.

In summary, hypercholesterolemia in CRF mice was found to be accompanied by an increase in hepatic HMG-CoA reductase activity. However, the increase in enzyme activity was not correlated with the rise in serum total cholesterol levels, suggesting that hypercholesterolemia in CRF mice is only partly due to an increase in de novo cholesterol synthesis in the liver. CRF did not appear to alter either the kinetic properties of the enzyme or its diurnal rhythm. Lovastatin was effective in reducing serum total cholesterol levels associated with CRF. However, the results of this investigation suggest that such a reduction in cholesterol levels did not result directly from inhibition of hepatic HMG-CoA reductase activity. Although lovastatin may indeed have inhibited the hepatic enzyme, other factors which stimulate its
activity may have countered the effect of the drug. Inhibition of cholesterol synthesis in extrahepatic tissues by circulating active lovastatin metabolites is possible and may be responsible for the decrease in serum total cholesterol levels which result from lovastatin treatment in CRF mice.

A paper was recently published by Wanner et al [142] which describes the effect of lovastatin therapy on hypercholesterolemia in hemodialysis patients. The results of the present experiments using the mouse model are in agreement with the findings of this study where lovastatin (20-40 mg/day) was shown to promote a reduction in serum total, LDL- and VLDL-cholesterol levels. One subject developed a mild elevation in liver transaminases suggesting liver damage. The mice, on the other hand, showed no significant elevations in transaminase levels, indicating that liver damage did not occur even with relatively high doses of lovastatin. It should be emphasized that Wanner's study was published two years after this M.Sc. research project commenced.

During the course of the project, several difficulties were encountered. For example, the severity of renal failure was unpredictable as it depended on the degree of electrocoagulation applied to the right kidney. If limited electrocoagulation of the kidney was performed, ample renal parenchyma would be left, resulting in only mild renal failure (as assessed by BUN values). On the other hand, if the electrocoagulation was excessive, not enough renal parenchyma would remain after the left nephrectomy to support life, resulting in large animal losses. It was crucial to induce renal failure of sufficient severity to produce significant serum lipid changes without compromising the survival of the animals.
A further major difficulty was encountered in the *in vivo* studies with lovastatin. In order to keep conditions similar to those employed for the administration oflovastatin to humans, it was decided to give the drug orally every day. In a pilot experiment which involved administration of lovastatin by gavage, ethanol was used to dissolve the drug before the addition of isotonic saline. This method had to be discontinued as ethanol itself decreased serum total cholesterol levels. Therefore, it was decided to incorporate lovastatin into powdered standard rodent diet and to give it fresh daily. However, incorporating the drug into the diet did not ensure that each mouse received the required dosage. It was very difficult to evenly distribute the small amount oflovastatin employed into the diet.

Despite the difficulties encountered, the aims of the research project were effectively met:

1. Further characterization of the mouse model of chronic renal failure was achieved.
2. The effects of CRF on serum lipids in mice were identified. Five weeks after the onset of CRF, serum total cholesterol levels were found to be significantly increased, while serum triglyceride levels were moderately elevated. β-VLDL appeared in the serum.
3. The properties of hepatic HMG-CoA reductase were studied and found to be unaffected by CRF.
4. The effects of lovastatin on serum lipids in CRF mice were established. The drug does not prevent the rise in serum total cholesterol levels induced by CRF but it is effective in reversing the hypercholesterolemia once it has been established. Lovastatin also lowers serum triglyceride levels in CRF mice.
5 Although the exact way in which lovastatin lowers serum lipid levels in CRF mice was not determined, the results suggest that it involves mechanisms other than the direct inhibition of hepatic HMG-CoA reductase.

6 In view of the results obtained, the feasibility of employing this mouse model of CRF to screen HMG-CoA reductase inhibitors for possible use in treating the hypercholesterolemia associated with CRF was established.
Figure 31. The long-term regulation of HMG-CoA reductase

The solid line represents the known pathway of negative feedback on the enzyme by cholesterol. The broken lines represent three possible mechanisms of feedback inhibition of HMG-CoA reductase mediated by non-sterol derivatives of mevalonate.
Figure 32. The shunt pathway of mevalonate metabolism
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LIST OF PUBLICATIONS

A. PAPERS

Submitted:

1. Subang MC, Stewart-Phillips JL and Gagnon RF
   Effect of lovastatin on hypercholesterolemia in chronic renal failure (CRF)
   (Accepted)

In Preparation:

2. Subang MC, Stewart-Phillips JL and Gagnon RF
   Lovastatin reduces cholesterol levels in experimental chronic renal failure (CRF)
   ASAIO Transactions: The Proceedings of Selected Presentations at The 38th Annual Meeting of the American Society for Artificial Internal Organs, Nashville, Tennessee, USA, May 7-9, 1992

B. ABSTRACTS

1. Subang MC, Stewart-Phillips JL and Gagnon RF
   Effet de la lovastatine durant l'insuffisance rénale chronique expérimentale
   (Poster Presentation)

2. Subang MC, Stewart-Phillips JL and Gagnon RF
   Effect of lovastatin on experimental chronic renal failure-associated atherosclerosis.
   9th International Symposium on Atherosclerosis, Chicago, Illinois, USA, October 6-11, 1991
   (Poster Presentation)

3. Subang MC, Stewart-Phillips JL and Gagnon RF
   An experimental study of the possible mechanisms underlying the hyperlipidemia of chronic renal failure
   24th Annual Meeting of the American Society of Nephrology, Baltimore, Maryland, USA, November 17-20, 1991
   (Not Presented)
4 Subang MC, Stewart-Phillips JL and Gagnon RF
Effect of lovastatin on the dyslipidemia of chronic renal failure (CRF).
12th Annual Conference on Peritoneal Dialysis, Seattle, Washington, USA,
February 19-21, 1992
(Poster Presentation)

5 Subang MC, Stewart-Phillips JL and Gagnon RF
Lovastatin reduces cholesterol levels in experimental chronic renal failure
(CRF)
38th Annual Meeting of the American Society for Artificial Internal Organs,
Nashville, Tennessee, USA, May 7-9, 1992.
(Oral Presentation)

6 Subang MC, Stewart-Phillips JL and Gagnon RF
The effect of Lovastatin on the hypercholesterolemia associated
with chronic renal failure
61st Annual Meeting of the Royal College of Physicians and Surgeons of
Canada, Ottawa, Ontario, Canada, September 11-14, 1992
(Submitted)

7 Subang MC, Stewart-Phillips JL, Pappu A and Gagnon RF
Hypercholesterolemia in experimental chronic renal failure
Vth Congress of the International Society for Peritoneal Dialysis, Thessaloniki,
Greece, October 1-4, 1992
(Submitted)

8 Subang MC, Stewart-Phillips JL, Pappu A and Gagnon RF.
Role possible du mévalonate dans la génèse de l'hypercholestérolémie
durant l'insuffisance rénale chronique expérimentale
34th Annual Meeting of the Club de Recherches Cliniques du Québec,
October 8-10, 1992, Montebello, P.Q., Canada
(Submitted)