Pathogenesis of Cholesterol-Induced Glomerulosclerosis in Guinea Pigs

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Abstract

The role of cholesterol-rich diet and of high protein supplement on the development of a glomerular lesion was studied in male guinea pigs. The possible pathogenesis of lipid-induced glomerulosclerosis was investigated. Four experiments were carried out. Four groups of guinea pigs were used in experiment I: CONT group was kept on normal guinea pig chow for 70 days; HC group was kept on 2% cholesterol diet for 70 days; HP group was kept on 50% casein diet for 70 days, and HCHP group received 2% cholesterol diet for 30 days and 2% cholesterol/50% casein diet for another 40 days. In experiment II two groups were used: CONT group and acetyl phenylhydrazine (APH)-treated group in which haemolytic anaemia was induced. In the third experiment the same dietary regimens as described in experiment I were used. In experiment IV three groups, namely CONT, HC, and HCHP, were employed. The animals in experiment IV were sacrificed after 5, 10, and 30 days. The first experiment explored the role of high cholesterol - and high cholesterol/high protein diet in the development of glomerulosclerosis. The other three experiments were designed to learn about the possible mechanism of lipid-induced glomerulosclerosis. Lipid analyses of plasma, erythrocytes and kidney tissue as well as complete blood count, erythrocyte osmotic fragility and blood cell morphology studies were performed. Kidney histology, histochemistry, immunohistochemistry, electron microscopy, morphometry, and renal and liver function tests were also carried out. *De novo* cholesterol synthesis was assessed by measuring HMG COA reductase activity and incorporation of tritiated water into cholesterol in the kidneys.

Cholesterol-fed animals showed decreased weight gain, increased cholesterol concentration in plasma, erythrocytes, and kidney tissue. Haemolytic anaemia was documented after 70 days on this dietary regimen. Glomerular proliferation lesion was first noted at day 30 and progressed by day 70. Moderate proteinuria and haematuria were observed at day 70. Addition of protein to the high cholesterol diet led to a further decrease in weight gain. It also increased the mortality rate to 40% by day 70. The glomerular lesion, proteinuria and haematuria, and possibly haemolysis were more marked in the HCHP group. No causal relationship was found between liver function, immune complexes, haemolysis and glomerulosclerosis. Serum phosphate levels did not differ among the groups. The lipid found in the kidney of both HC and HCHP groups was mostly of plasma origin, since the kidney cholesterol de novo synthesis was suppressed in these two groups compared to the CONT group. There was a concommitant increase in the lipid content of kidney tissue and the mesangial expansion (MA/GTA) at day 30. No significant increase in the intraglomerular monocyte/macrophage was found at day 30 in the HCHP group compared to the HC group. However, a significant correlation (r=0.678, p 0.001) was found between the number of these cells and MA/GTA ratio among the four experimental groups at day 70.

These data indicate that lipid deposits in kidney tissue may induce a glomerulosclerotic lesion in the absence of monocytes. However, these cells likely augment the proliferation of mesangial cells. We postulate that high protein diet could worsen the lipid-induced glomerular lesion by increasing delivery of abnormal lipoproteins to the kidney which could trigger mesangial cellular proliferation directly and indirectly by a macrophage-mediated process.

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This thesis is dedicated

To The Glorious IRAQ

To The Strong People

AFAF

KUTAIBA

OSAMA

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Glossary of Abbreviations

LCAT	Lecithin:cholesterol acyltransferase
CONT	Control
HC	High cholesterol
HCHP	High cholesterol/high protein
APH	Acetylphenylhydrazine
TC	Total cholesterol
FC	Free cholesterol
CE	Cholesteryl ester
TPL	Total phospholipids
TG	Triglycerides
HDL-C	Cholesterol of high density lipoprotein
VLDL	Very low density lipoprotein
LDL	Low density lipoprotein
EM	Erythrocyte membrane
HCC	Hypochromic cell
FER	Fractional esterification rate
MER	Molar esterification rate
Ccr	Creatinine clearance
BUN	Blood urea nitrogen
Cr	Serum creatinine
HMG-COA reductase	Hydroxymethylgluteryl coenzyme A reductase
LLM	Lipid-laden monocyte
MA/GTA	Mesangial area/glomerular tuft area ratio
NSE-positive cells	Non-specific esterase positive cells

INTRODUCTION

I Preface:

This investigation was initially designed to test a hypothesis that a high protein dietary supplement is necessary to augment the renal lesion and to induce deterioration of renal function in patients with familial lecithin: cholesterol acyltransferase (LCAT) deficiency. This hypothesis formulated on the basis of clinical observations in such patients (1). Typical microscopic findings in this disorder include accumulation of lipid particles in different parts of the glomeruli and fusion of foot processes. However, similar findings were observed in a biopsy of one patient without proteinuria (3). Flatmark et al. (1977) reported the same renal lesion in a normal kidney several months after its transplantation into a patient with familial LCAT deficiency, though the renal function was normal (2). Frohlich and Mcleod (1) pointed out that LCAT-deficient patients on high protein diets, e.g. the Scandinavian patients, had a much higher frequency (90%) of renal complications than the Japanese patients on lower protein diets (43%). In addition, the Scandinavians developed uraemia and 60% of them ultimately died of the disease, whereas none of the Japanese were uraemic. (1) A higher ratio of BUN to serum creatinine was demonstrated in those who died of their kidney disease than the survivors (1). Regarding the fact that urea clearances were initially normal in the first group, the high BUN level was largely determined by the dietary protein intake. These data suggested that the patients with serious renal involvement were on substantially higher protein diets than the others.

The cholesterol-fed guinea pig was chosen as an experimental model, since it has similar lipoprotein abnormalities, haematological alterations and renal lesions to those described in LCAT-deficient patients (see

"Introduction" - Section V). However, although lipoprotein abnormalities and haemolytic anaemia were documented in this model, no marked reduction in LCAT activity was demonstrated in our study. Thus a new set of hypotheses arose:

1- Plasma lipid deposition in glomeruli results in significant structural change. Intraglomerular monocyte infiltration may contribute to the structural change.

2- High protein intake augments these changes and may play an important role in the development of functional impairment.

A study that tests the above hypotheses could help us understand the mechanism of renal manifestations found in some dyslipidaemias, among them LCAT deficiency. Perhaps, more importantly, it may explain the role of plasma lipids in the pathogenesis of glomerulosclerosis. In this chapter, the literature on renal lesions in hyperlipidaemias and effect of high protein diet on renal disease will be reviewed. In addition, the cholesterol-fed guinea pig model and its relationship to human familial LCAT deficiency will be considered. Finally, the objectives of this study will be stated.

II Plasma Lipids in Renal Disease

The role of lipid deposits in renal glomeruli in the pathogenesis of renal disease is unclear. Most studies of the abnormalities (and their possible effects on renal function) have been confined to patients with the nephrotic syndrome. However, hyperlipidaemia in the nephrotic syndrome is a secondary one (4) which might be attributed to a renal lesion. In this syndrome there is an increase in levels of plasma total cholesterol, triglycerides, and phospholipids (5-7) and variable patterns of lipoproteins (6,7). It has been speculated that the loss of the activator of lipoprotein lipase might lead to persistence of hyperlipaemia due to the disturbance of triglyceride-rich lipoprotein metabolism (8). Alternatively, hyperlipidaemia might be due to increased synthesis of lipoproteins by the liver (9). Despite that hyperlipidaemia is secondary, Moorhead et al. (1982) suggested a potential role for plasma lipids in chronic renal disease (10). They postulated that the excessively filtered lipoproteins may accumulate in the mesangium and lead to proliferation of mesangial cells and deposition of matrix tissue. In addition to nephrotic syndrome, glomerular lipid deposits have been reported in Fabry's disease (11), lecithin: cholesterol acyl-transferase (LCAT) deficiency (12-15), cholestatic liver disease (16), and arteriohepatic dysplasia (Alagille's syndrome) (17). The syndrome of partial lipo-dystrophy in humans has also been described with glomerular changes and renal failure (9). Edward (1981) noted a significant inverse correlation between fasting serum triglycerides and mean glomerular filtration rate in patients with analgesic nephropathy (18). Diabetic glomerulosclerosis has also been attributed to fat emboli in the glomerular capillaries (19).

Experimental evidence suggests that lipid may have an important role in the development of some glomerular lesions. Long-term treatment of rats with aminonucleoside and adriamycin resulted in lipid deposition in the mesangium followed by the development of focal and segmental hyalinosis and sclerosis (20). A similar finding was reported earlier by Silva et al. (1979): amino nucleoside-induced focal glomerulosclerosis was enhanced by a lipid-rich diet while lowering of serum lipids by halofenate protected rats against this effect (21). Kelley and Izui have demonstrated that an enriched lipid diet (51.7% fat) resulted in increased lipid accumulation in glomeruli and accelerated glomerular injury in NZBXW mice with lupus nephritis (22). Guinea pigs kept on a 1% cholesterol rich diet have developed glomerulosclerosis (23) (see "Introduction" - Section V). Furthermore, it has been reported that at physiological pH and ionic strength, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) can bind with polyanionic glycosaminoglycans (24). This binding may alter the permeability of the glomerular barrier by changing the net charge state of the membrane which normally governs the permeation of the particles with a surface charge (24). In concert with this report, an increase in basement membrane lipid has been noted (25).

In short, a growing body of evidence has been presented which strongly suggests a role for hyperlipidaemia in the development of renal lesions in humans and in experimental animals. Hence, we tried to study the pathogenesis of glomerulosclerosis accompanying hyperlipidaemia, using the cholesterol-fed guinea pig model.

III Dietary Protein and Renal Disease:

Examination of the effect of high protein supplementation on lipid-induced glomerular lesion is one of the objectives of this study. This section reviews some of the literature dealing with the effect of protein-rich diet on renal disease.

Renal structural abnormalities in the experimental animals fed high protein diet were reported early in this century (26-28). Klahr et al. (1983) suggested high dietary protein as a risk factor in the progression of chronic renal disease (29). These authors reviewed the evidence of deleterious effect of dietary protein on the course of experimental nephritis. All partially nephrectomized rats (which developed uraemia) were kept on 51% protein-supplemented diet died within 11 days after the surgery. On the other hand, another group of rats subjected to the same surgery but kept on a low-protein dietary regimen lived for 30 days after renal ablation (30).

Both renal blood flow and glomerular filtration rate were increased in animals kept on the protein rich diet (31). Brenner (1983) postulated that the syndrome of progressive azotaemia, proteinuria and glomerulosclerosis is related to high protein intake (32). He suggested that this dietary supplement to experimental animals with partial nephrectomy leads to sustained elevation in glomerular capillary pressure and flow and ultimately to renal failure. In rats, renal mass was found to be increased after long-term feeding of protein (33). Conversely, feeding a low protein diet to rats with remnant kidneys has largely prevented the striking increase in glomerular plasma flow and capillary pressures. Furthermore, the accompanying proteinuria and structural alterations were found to be less

severe (29, 33). Pennell et al. (1975) found decreased glomerular filtration rate (GFR) values in weaning rats fed a low protein diet. Such protein restriction also reduced the kidney mass (34).

Protein-restriction in rats accounted for a fall in glomerular capillary plasma flow rate and a reduction in glomerular cross sectional area (35). The increase in GFR in a remnant kidney was associated with remarkable changes in glomerular structure. There were epithelial cell adhesions to Bowman's capsule, detachment of some of the epithelial cells from the underlying basement membrane, and a prominant increase in mesangial cells and matrix (33). Others described an increase in glomerular mass, vacuolization of glomerular epithelial cells and effacement of foot processes (36). In humans, restriction in dietary protein may have a similar effect on renal function to that shown in experimental animals. Pullman et al. reported a decrease in GFR and renal plasma flow in low-protein diet-consuming healthy individuals (37). Normal subjects also reduced their GFR on feeding a caloric-deficient diet (38). Klahr and Alleyne (1981) found decreased values for GFR and renal plasma flow in ten adults with protein malnutrition; both values returned to normal level after protein repletion (39).

All these findings in both experimental animals and humans support the notion that high dietary protein has a deleterious effect on kidney disease.

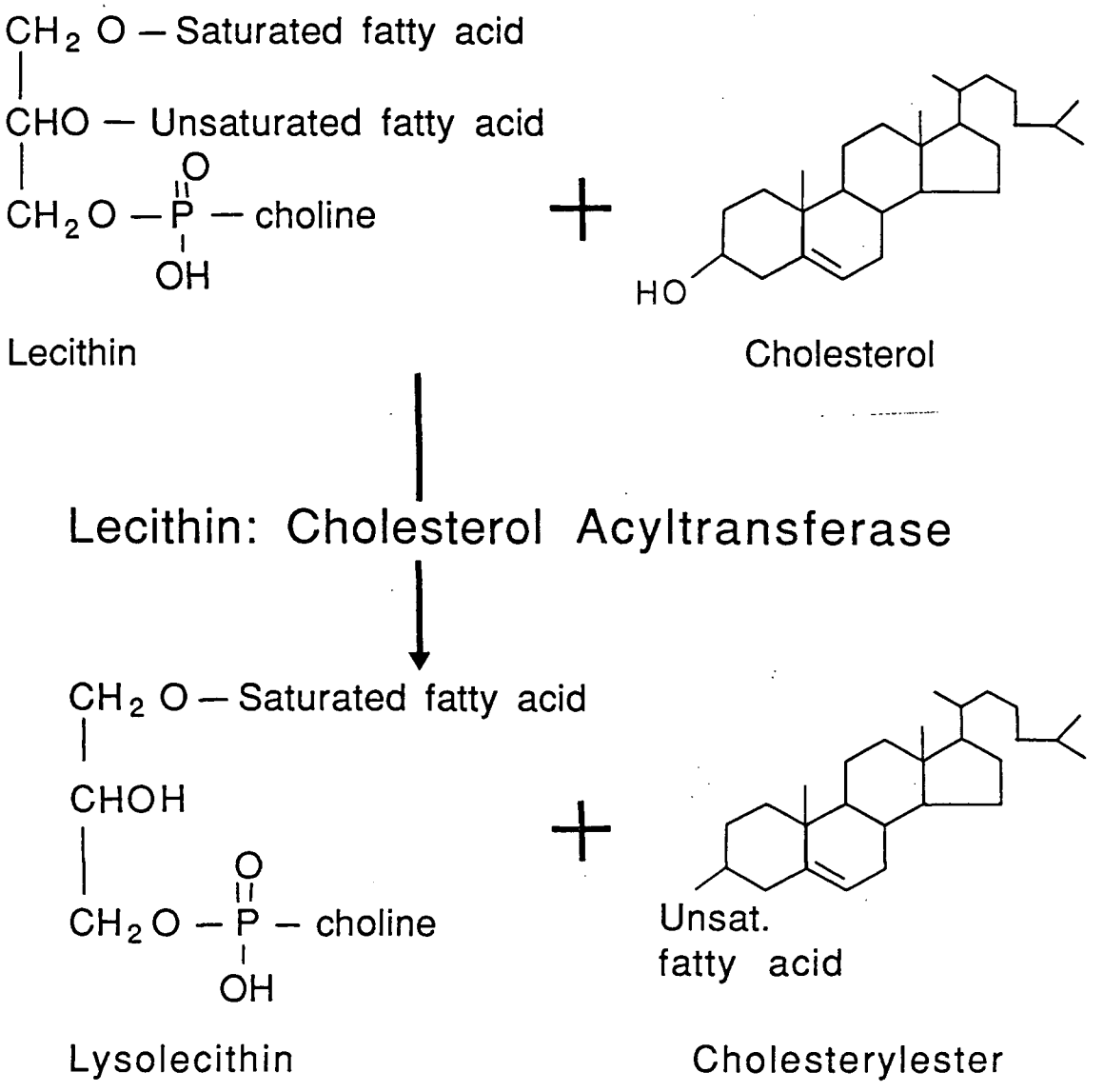
IV Familial Lecithin: Cholesterol Acyltransferase Deficiency:

The data obtained in the present study which contribute to an understanding of lipid-induced glomerulosclerosis are pertinent to the glomerular lesion described in familial LCAT deficiency. The following review provides a background for this disease and highlights its major features; besides, it facilitates introducing a comparison between familial LCAT deficiency and the cholesterol-fed guinea pig in the next section.

Lecithin: Cholesterol acyltransferase (EC 2.3.1.43) catalyzes in plasma the transfer of a fatty acyl group from lecithin to cholesterol (40) (Figure 1). The enzyme is synthesized and secreted by the liver (41, 42) and activated mainly by apoprotein AI (43) and to a lesser degree by apoproteins CI (44), A IV, E_2, and E_3 (45). The major portion of LCAT circulates in plasma with HDL components (46) particularly with the smaller subfraction of HDL (47).

Familial LCAT deficiency was first described in 1968 in 3 sisters of a Norwegian family (48). Since then 43 cases have been detected including six patients currently being investigated (3) (Table 1). The disease has an autosomal recessive mode of inheritance (12). The LCAT gene is located between the middle and terminal points of the long arm of chromosome 16 (12). Mclean et al. (1986) isolated the human LCAT cDNA clones (49). The typical features of the clinical presentation of familial LCAT deficiency are corneal opacities, haemolytic anaemia and various degrees of renal involvement ranging from an increase in albumin and B_2 microglobulin excretion to uraemia (3) (Table 2).

Fig - 1



Principal lipid reactants in the LCAT reaction

All LCAT deficient patients have a marked decrease in LCAT activity (Table 3 shows the methodology of LCAT activity assessment), relatively high concentration of plasma unesterified cholesterol (UC) and phosphatidyl choline (PC), and all have low concentrations of cholesteryl ester (CE) and lysolecithin (50). The plasma triglycerides (TG) are also increased in some patients (50). All plasma lipoprotein classes in LCAT deficient patients are abnormal. Upon filtration through 2% agarose gel, different subfractions of VLDL have been recovered: Large particles excluded from the gel with more lipid relative to protein content and more UC and PC relative to TG (51); smaller VLDL particle had unchanged ratio of CE to TG or UC. Upon electrophoresis of plasma on agarose gel, VLDL demonstrates slow pre-beta migration (51). Low-density lipoprotein particles of different size and lipid composition are also recovered from plasma of LCAT deficient patients (50, 52). Generally, abnormal LDL subfractions have large amounts of FC and PC compared to protein, however, more protein and less lipids are found in the intermediate-sized subfraction (50).

Although the HDL protein in LCAT deficient patients has been reported to be about one-third normal, the total concentration of HDL UC was in the normal range due to the high proportion of UC in the HDL subfractions (53). Upon filtration through Sephadex G200, the HDL of LCAT deficient patient has been subfractionated into: large, normal and small-sized components (50). The large HDL subfraction forms stacked discs (54) and contains apoprotein E (55).

In short, the plasma lipid and lipoprotein abnormalities might directly or indirectly be implicated in the development of renal disease in familial LCAT deficiency syndrome. Employing an experimental animal model

which has a substantial number of these abnormalities could help investigating the pathogenesis of renal disease in hyperlipidemic patients.

M + LCAT deficient patients have moderate target-cell haemolytic anaemia with reduced compensatory erythropoiesis (56), and normochromic erythrocytes. Several investigators (57-59) have reported an increase in erythrocyte free cholesterol, PC and a decrease in phosphatidylethanolamine. Moreover, the erythrocyte lipid abnormalities can be reverted when patients' erythrocytes are incubated in normal plasma (12). Different features of these abnormalities have been reported in cholesterol fed guinea pigs (see next section). Those patients have sea-blue histocytes in spleen and bone marrow (60). It has been found that the lamellar-arranged membranes detected inside these cells consisted of PC and UC (13, 61) which might be due to phagocytosis of abnormal lipoproteins by reticuloendothelial cells (62). However, no foam calls have been reported in the blood circulation of LCAT deficient patients. On the other hand, foam cells have been described in the renal lesion (12). Thus, the interrelationships between plasma lipid alterations, the lipid-laden phagocytes and renal lesion are considered, too, in this study. Also, the possible role of cholesterol-rich erythrocytes in the development of a renal lesion was investigated in our study.

Renal disease is the most serious outcome of familial LCAT deficiency syndrome. Moderate proteinuria has been reported early in life in most patients (12, 62). Beside protein, urine of those patients contains erythrocytes and hyaline casts; albumin, alpha-1, and alpha-2 globulins are detected on urine electrophoresis (12, 62). Nevertheless, renal function in LCAT-deficient patients indicated by serum creatinine and urea concentration in addition to the creatinine, inulin, and PAH clearance is normal before

the end-stage disease (62). Grossly, the kidneys of LCAT deficient patients are pale and slightly enlarged while microscopically, the glomeruli show expanded mesangial areas and Bowman's capsule is thickened (12). The glomerular tuft capillaries usually have thickened walls and lumens containing amorphous materials (12, 62). Ultrastructural studies of the lumen material have shown that the capillary lumens were partly filled with a membraneous meshwork (13, 14). Frequent loss of capillary endothelial cells and fusion of epithelial foot processes are frequently noted (12). Some of these findings have been described earlier in cholesterol-fed guinea pigs (see next section). In addition, the subendothelial lipid deposits and the presence of foam cells in the tunica media of renal arterioles of LCAT deficient patients (12) have been reported in cholesterol-fed guinea pigs (23). An additional feature of the renal manifestations in familial LCAT deficiency is the increase in the lipid content in kidney tissue (63). Implication of large LDL as causally related to renal injury in familial LCAT deficiency is still equivocal (64, 1). However, the presence of plasma lipid and lipoprotein abnormalities besides the lipid deposition in kidney tissue may implicate a causal relationship with renal lesion. The mechanism of such a relation could be studied in an animal model which exhibits a similar plasma lipid and kidney structural alteration. Hence, we used cholesterol-fed guinea pigs for this purpose (see next section).

Table 1: Patients with Familial LCAT Deficiency

FAMILY	PATIENT	SEX	DOB	ETHNIC ORIGIN	ANAEMIA	RENAL INVOLVEMENT
I	1	F	1936	Norwegian	+	+
	2	F	1934	Norwegian	+	+
	3	F	1946	Norwegian	+	+
II	4	F	1921	Swedish	+	+
	5	M	1935	Swedish	+	+
III	6	F	1926	Norwegian	+	+
	7	M	1932	Norwegian	+	+
IV	8	M	1918	Norwegian	+	+
	9	F	1914	Norwegian	+	-
V	10	M	1942	Italian	+	+
	11	M	1944	Italian	+	+
VI	12	M	1955	Indian	-	-
VII	13	M	1945	Eng-Cdn	+	?
	14	M	1940	Eng-Cdn	+	+
VIII	15	F	1934	French	+	+
	16	F	1937	French	+	?
IX	17	M	1959	Ital-Dutch	+	+
	18	F	1954	Ital-Dutch	+	-
X	19	F	1946	Japanese	+	MIN
	20	M	1948	Japanese	+	+
	21	M	1950	Japanese	+	+
XI	22	M	1950	Eng-Irish	+	+
	23	F	1955	Eng-Irish	+	-

Table 1 Cont'd

FAMILY	PATIENT	SEX	DOB	ETHNIC ORIGIN	ANAEMIA	RENAL INVOLVEMENT
XII	24	F	1932	Irish	+	+
	25	F	1925	Irish	-	-
	26	M	1932	Irish	+	+
	27	F	1931	Irish	+	-
XIII	28	F	1954	Norwegian	+	-
XIV	29	F	1949	Germ-Irish	+	MIN
	30	M	1958	Germ-Irish	-	+
	31	M	1955	Germ-Irish	-	+
XV	32	M	1933	English	MIN*	MIN
XVI	33	M	1965	Italian	-	+
XVII	34	F	1937	Japanese	+	-
	35	M	1933	Japanese	+	-
XVIII	36	M	1934	Japanese	+	+
	37	F	1924	Japanese	+	-

* Min - Minimum

Table 2: Frequency of Clinical Findings in Two Groups of Patients with Familial LCAT Deficiency

	Scandinavian (10 patients)	All Other (27 patients)	Number Affected
Corneal opacities	100%	100%	37
Anaemia	100%	82%	32
Proteinuria	80%	74%	22
Uraemia	70%	14%	10
Death	60%	4%	7

Table 3

Methodology of LCAT Activity Assessment

Method	Substrate for LCAT Reaction	Remarks
I. Glomset and Wright	Pooled human plasma (heat denatured substrate)	-Requires long incubation time -Low reproducibility
II. Stokke and Norum	Patient's own plasma (endogenous substrate)	-Time consuming -Influenced by the quality of substrate
III. Enzyme Immunoassay	---	-Quantitative measurement of the enzyme mass -Low sensitivity may be due to complete Ag-Ab reaction (antigenic determinants of the enzyme are obscured by HDL/LCAT complex)
IV. Common-Substrate Method	Apo AI-containing liposome (exogenous substrate)	-Preferred method -Overcomes the limitations imposed by the variability of the endogenous substrate -Rapid, reproducible, and sensitive -Correlates well with enzyme mass measurement

V-Cholesterol-fed Guinea Pig:

Historical Background

The investigation of the biological effect of a cholesterol-rich diet on guinea pigs goes back to 1939 when Okey and Greaves (64) studied the metabolic activities of cholesterol. They chose guinea pig over rat because the first, like human, has a gallbladder and requires ascorbic acid in its diet. In that study they reported a severe anaemia with splenic enlargement and fatty liver in guinea pigs fed a 1% cholesterol-rich diet. Severe anaemia appeared after five weeks of the experiment and the enormous splenomegaly was evident after 7-9 weeks. In 1944 Okey demonstrated that haemolytic anaemia and splenic hyperplasia were correlated with the excess esterified cholesterol in liver and free cholesterol in plasma (65). Also, they showed that phagocytic cells play a role in removing cholesteryl ester from the liver. Most of these data were later confirmed and studied in detail by Ostwald and Shanon (1964), as discussed below (66). In 1967 French et al. reported a glomerulosclerotic lesion in cholesterol-fed guinea pigs (23).

(i) Plasma Lipids and Lipoprotein Abnormalities:

Guinea pigs kept on a 1% cholesterol diet for 10-14 weeks have an increased level of both UC and CE in plasma (66, 67); UC was increased 11-fold while EC showed a 2.5-fold increase. Total phospholipid and phosphatidylcholine (PC) concentrations in plasma were also elevated; the plasma CE had a decreased proportion of linoleic acid (66).

The most striking abnormalities in cholesterol-fed guinea pig which resemble those in familial LCAT deficiency have been seen in the plasma lipoproteins (Table 4) (68, 69). On agarose gel electrophoresis, ß-or slow

pre-β VLDL was demonstrated. Compared to the protein content, VLDL of guinea pigs fed a 1% cholesterol diet has increased UC (50). The concentration of UC and phospholipid was also increased in the LDL of the same animals. The subfractions of LDL which have been described in LCAT deficient patients after filtration through 2% agarose gel have the same characteristics as those in the cholesterol-fed guinea pig. Glomset and Norum (1973) pointed out that besides the similarity in size distribution of the LDL subfractions to those in patients of familial LCAT deficiency, the large and intermediate LDL particles unlike the smaller ones were rich in UC and phospholipid (50). Sardet et al. (1972) demonstrated LDL heterogeneity on electron microscopy (69). Large and intermediate molecular weight subfractions appeared as flat discs of 80-100 nm in diameter while the small subfraction was indistinguishable from LDL of the control animals.

The HDL of cholesterol-fed guinea pigs has been shown to be rich in UC and phospholipid and large enough to be excluded from Sephadex G200 (68). They formed stacked discs upon electron microscopy (69).

Finally, plasma of cholesterol-fed guinea pigs contains the abnormal lipoprotein LP-X. This particle has the composition of the abnormal intermediate-sized LDL reportedly similar to the LP-X described by Seidel et al. (1969) (70). Upon electron microscopy they are disc-shaped with a major axis of 40-60 nm (71, 72).

(ii) Haematologic Abnormalities:

Haemolytic anaemia has been found consistently after 7-10 weeks in guinea pigs fed 1% cholesterol-rich diet (66, 73, 74). The half-life of erythrocyte is decreased (66) and the fall in the erythrocyte count is accompanied by an increase in the erythrocyte mean corpuscular volume (MCV).

Sardet et al. (1972)demonstrated a 10-35% increase in reticulocytes in anaemic cholesterol-fed guinea pigs (75). Numerous studies have shown that cholesterol content of erythrocyte is increased in this animal model (67, 68, 73, 75). In an in vitro study, cholesterol-loaded RBC from cholesterol-fed guinea pig lost its excess cholesterol when incubated with normal guinea pig's plasma; in the same way, unesterified cholesterol mostly transferred from a hypercholesterolaemic plasma to normal RBC (75) (also, see "Discussion"). Erythrocyte cholesterol has been shown to increase within 1-3 days after supplementation of the guinea pig diet with 1% cholesterol, simultaneously with the increase in plasma cholesterol content (76).

Sardet et al. (1972) suggested that UC transfers from the abnormal HDL to erythrocyte membrane in guinea pigs fed a 1% cholesterol-rich diet (69). They found that worsening of haemolytic anaemia was concomittant with the appearance of new species of HDL and abnormal LDL particles. Cholesterol-rich erythrocytes could be a possible contributor to the lipid-induced glomerulosclerosis; hence, we investigated this possibility in a separate experiment.

In conclusion, cholesterol-fed guinea pig has striking similarities in plasma lipid and lipoprotein abnormalities to those in the familial LCAT deficiency syndrome. On the other hand, although this animal model presents haemolytic anaemia after feeding cholesterol-rich diet, it has some differences in the haematological findings: first, haemolytic anaemia is severe in the animal model (23, 66, 69); second, the erythrocytes of cholesterol-fed guinea pigs are mainly spur cells (69), while in LCAT deficient patient they are mainly of target cell type (as described in a previous section). (Also see next section for further conclusions.)

(iii) Renal Findings:

The renal findings in cholesterol-fed guinea pigs are similar to those in human LCAT deficiency (see section IV). French et al. (1967) demonstrated the following in the kidneys of anaemic guinea pigs on 1% cholesterol-rich diet: (1), a focal or diffuse thickening of the glomerular stalk with an increase in the number of stalk cell and the connective tissue elements; (2), hyaline material in glomeruli together with Sudan IV positive droplets; (3), fat and hyaline in the media and the subintima of the renal arterioles (23). Epithelial cells of the proximal convoluted tubules loaded with haemosiderin were noted in another study (77). This finding indicates the massive filtration of haemoglobin due to cholesterol-induced haemolytic anaemia.

There are several differences in the renal lesions between LCAT-deficient patients and cholesterol-fed guinea pigs. Extramedullary haemato- poiesis and erythrophagocytosis were detected in the affected glomeruli of the guinea pigs (23) but not in LCAT deficient patients. Mesangial hypercellularity was demonstrated in the animal model, while in familial LCAT deficiency the glomeruli are normocellular despite the increase in mesangial matrix (15). As well, in LCAT deficiency the lumens of the glomerular capillaries have a mottled structure with a meshwork of membranes and particles, a finding which has not been described in cholesterol-fed guinea pigs, which show intraluminal lipid-laden macrophages (78). Unlike in LCAT deficiency, no glomerular basement membrane thickening was demonstrated in cholesterol-fed guinea pigs.

Since the cholesterol-fed guinea pig is intended, in our study, to be a model for the investigation of the pathogenesis of the renal lesion in hyperlipidaemic diseases, we review here the features of renal lesions in two of these diseases.

In Fabry's disease (11) the kidney lesion is characterized by the foamy vacuolation of the cells of the glomerular tuft, (endothelial, mesangial, and epithelial). The foamy cells contain Oil Red O-positive material which is clearly recognizable by electron microscopy. Furthermore, irregular thickening of the glomerular basement membrane and subendothelial lipid deposition are described, as well. In hepatic disease, Hovig et al. (1978) reported several cases of hyperlipidaemia with renal involvement (79). Kidney biopsy revealed widening of mesangial region, irregular thickening of basement membranes, and occasional pronounced glomerulosclerotic alterations. Again, lipid deposition in different parts of the glomeruli were detected by electron microscopy. In Fabry's disease, no mesangial proliferation was reported, while in cases of hepatic disease moderate glomerulo- sclerosis was present.

In the hyperlipidemic diseases described above including familial LCAT deficiency and in the cholesterol-fed guinea pig model, a consistent relation is found between hyperlipidaemia, lipid deposition in renal tissue, and the glomerular lesion. This observation constitutes the basis for the most current hypothesis to explain the glomerular lesion in hyperlipidaemic diseases (10). Moreover, since the cholesterol-fed guinea pig has most of the histopathological features of the renal lesion presented in human hyperlipidaemic disease, it may serve as a suitable model to study the mechanism of development of the renal pathology reported in these diseases.

Table 4

Similarities in plasma lipoproteins between familial LCAT deficiency and cholesterol-fed guinea pig

1. Very low density lipoprotein:
   a-Increase UC
   b-Slow pre-beta mobility
2. Low density lipoprotein:
   a-Increase UC
   b-Yield of three subfractions upon 2% agarose gel filtration
   c-Large and intermediate molecular weight subfractions of (b) are rich in UC and phospholipids
   d-Upon electron microscopy, large and intermediate molecular weight subfractions are flat discs
   e-On analytical centrifugation, presence of fast floating components of S_f7 to 12 and S_f 12
3. High density lipoprotein*: (large HDL)
   a-Excluded from sephadex G200
   b-Increased UC and phospholipid
   c-Yield stacked discs upon electron microscopy
4. Presence of LP-X

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*HDL is virtually absent in normal guinea pig

VI Objectives of This Study:

This investigation aimed to:

A. Study the effect of cholesterol feeding on the kidney in guinea pigs, and to elucidate the mechanism underlying renal alterations. The role of the following in the development of renal lesion was studied:

1- Lipid deposition *per se* and kidney cholesterol *de novo* synthesis
2- Haemolysis
3- Liver changes
4- Immune complex mediation
5- Monocyte/macrophage mediation

B. Study the role of high protein intake in augmenting the renal changes. Again, the above-suggested mechanisms were examined.

MATERIALS AND METHODS

I. Animals, diets and materials

Animals and diets

Hartley male guinea pigs were supplied by the animal unit at the University of British Columbia; their initial weights ranged from 330 and 370 gm. Normal guinea pig chow based on Reid and Brigg's formula and modified high protein diet (4% corn oil, 50% protein (casein), 37.5% carbohydrate, 8.5% vitamins and minerals, plus 7 gms. fiber/100gm diet) (Table 5) were supplied by ICN (Nutritional Biochemical, Cleveland, Ohio). To prepare the 2% cholesterol-enriched diet and 2% cholesterol-enriched high protein diet, ether-dissolved cholesterol was added to both normal and modified high protein diets.

Table 5

[Composition of the experimental diets]

Type of diet	Cholesterol gm/100gm diet	Fat	Protein (casein)	Carbohydrate (dextrose)	Vitamins & minerals	Fibres gm/100gm diet
Normal Guinea Pig Chow	-	4% (8)	18.5% (20)	69% (72)	8.5%	7
Cholesterol-Rich Diet	2	4% (8)	18.5% (20)	69% (72)	8.5%	7
Protein-rich Diet	-	4% (8)	50% (52)	37.5% (40)	8.5%	7
Cholesterol/ Protein-Rich Diet	2	4% (8)	50% (52)	37.5% (40)	8.5%	7

The numbers between brackets represent the percentage of total calories.

Materials

1. For composing an artificial substrate for the measurement of LCAT activity; egg yolk phosphatidylcholine was purchased from Sigma (type III-E 5mg/ml in absolute ethanol). Free cholesterol was Sigma CH-S (1mg/ml in absolute ethanol). Apoprotein AI was purified by PBE 94 chromatofocussing by Roger Mcleod - Shaughnessy Hospital. Bovine serum albumin (BSA) was Sigma and was essentially fatty acid free.
2. Reagents and quality control materials used in the determination of plasma total and free cholesterol and triglycerides were supplied by Abbott and Eastman Kodak Ektachem.
3. Plasma lipoprotein electrophoresis was done on universal electrophoresis film agarose which was supplied by Corning Universal.
4. The radioactive substances used were purchased from New England Nuclear (Boston, Massachusetts, USA).
5. The purity of cholesterol used in preparing different dietary regimens was equivalent to USP specification.
6. All reagents and quality control materials used in the analyses of the liver function tests were supplied by (Eastman Kodak EKTA Chem).
7. All other reagents used in this study were of analytical grade.
8. Plasma ultracentrifugation for lipoproteins isolation was done on (Beckman L8-70). (Details of preparation for ultracentrifugation is described in Section III).
9. Acetylphenylhydrazine was purchased from BDH (British Drug House).

II Experimental Design

All the animals were fed normal guinea pig chow for one week as an adaptation period, and kept in couples per (60 x 45 x 45cm) cage. Throughout the experimental period the animals had free access to food and water. Water was supplemented with ascorbic acid (1gm/L) to meet the animal's daily requirement. Blood and urine samples were obtained and weights were recorded at the beginning of each experiment and at different experimental periods (see details about blood and urine collection in Section III).

A. Experiment I

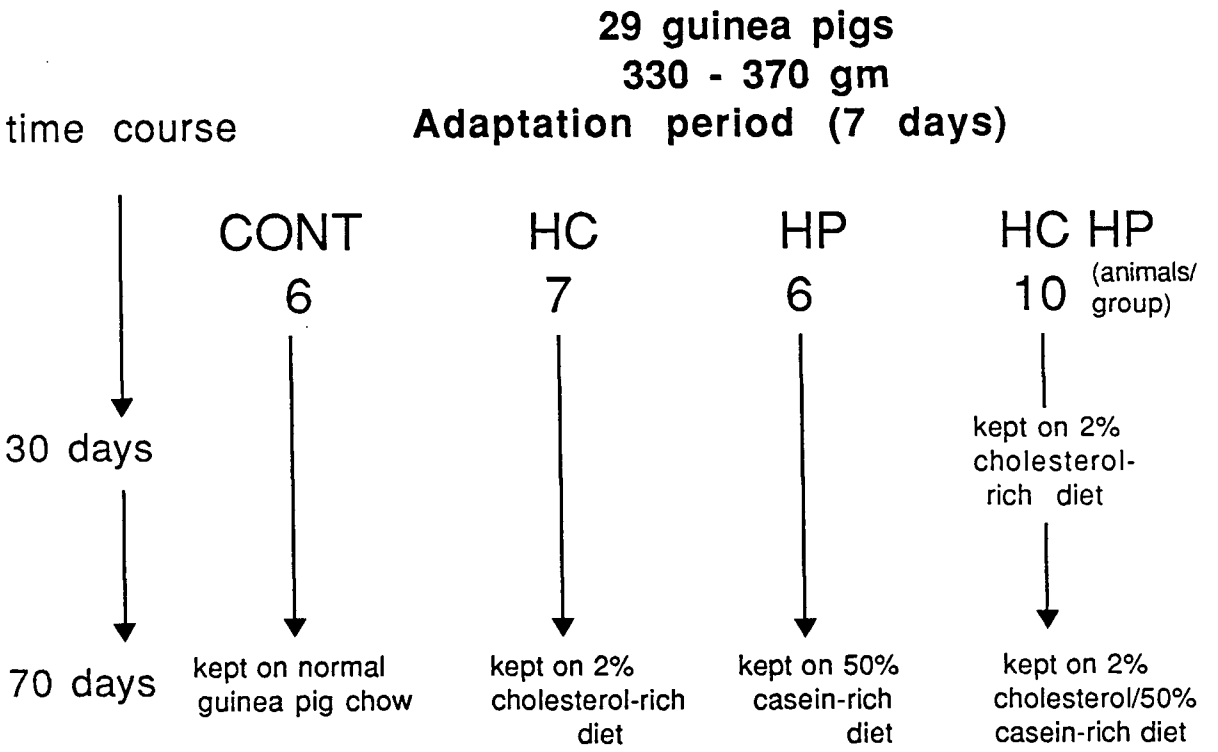
This experiment was done to study the effect of cholesterol-rich diet in inducing glomerulosclerosis in guinea pigs and to determine the effect of a cholesterol/protein-rich diet in aggravating this lesion.

Twenty-nine male guinea pigs were used. The animals were randomly allocated to four groups:

Control group (CONT) was kept on normal guinea pig chow for seventy days; high cholesterol diet (HC) group received 2% cholesterol-rich diet for seventy days; high protein (HP) diet group was kept on 50% casein-enriched diet for seventy days, and high-cholesterol high-protein diet (HCHP) group started on 2% cholesterol-rich diet for 30 days then was shifted to a cholesterol/ protein-rich diet (2% cholesterol/50% casein-rich diet) for another 40 days (Figure 2).

Fig - 2

Flow chart of the experimental design of experiment - I



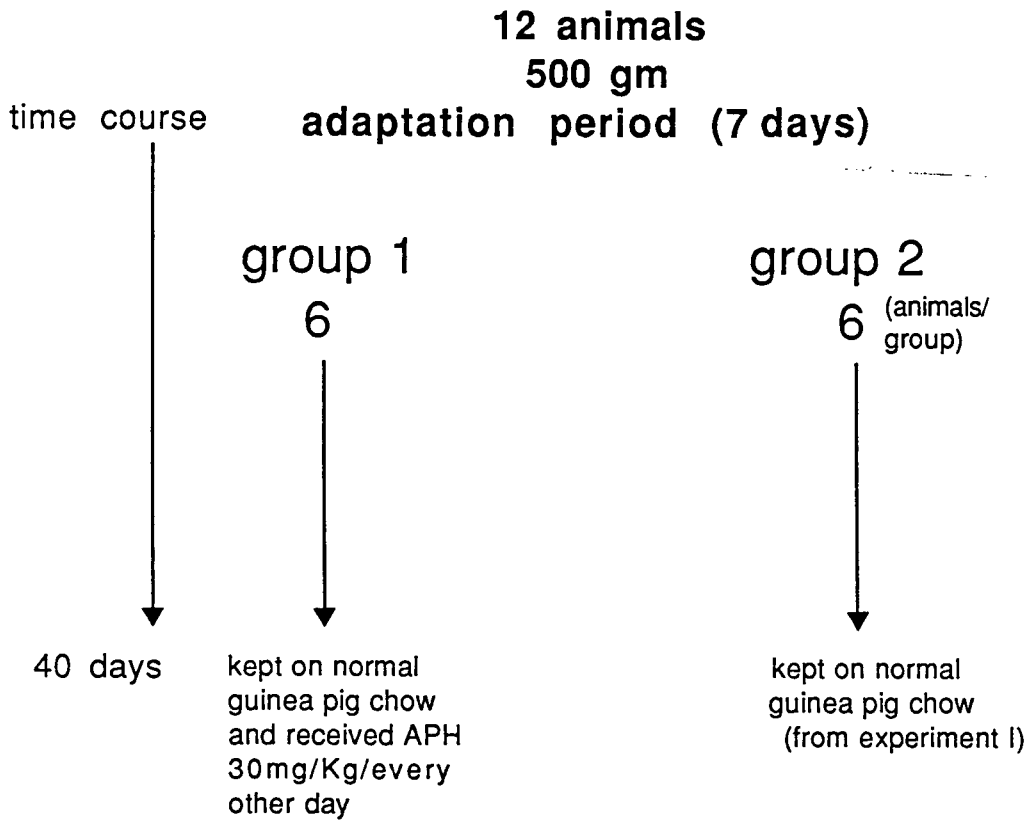
CONT = Control group
HC = High cholesterol group
HP = High protein group
HC HP = High Cholesterol/High Protein Group

B. Experiment II

This experiment was conducted to examine the effect of haemolytic anaemia in inducing a glomerulosclerotic lesion in guinea pigs. Six male guinea pigs were used with a starting weight of 500 gm. They were injected with acetyl phenylhydrazine (APH) (30mg/kg body weight/every other day). The experiment continued for forty days and the results were compared to those of the control group in Experiment I. Animals in this experiment were kept on normal guinea pig chow. Blood samples were obtained at day 0 and day 40, in addition to random sampling along the experimental periods. Urine samples were collected at day 40 (Figure 3).

Fig - 3

Flow chart of the experimental design of the experiment - II



APH = Acetylphenyl hydrazine

C. Experiment III

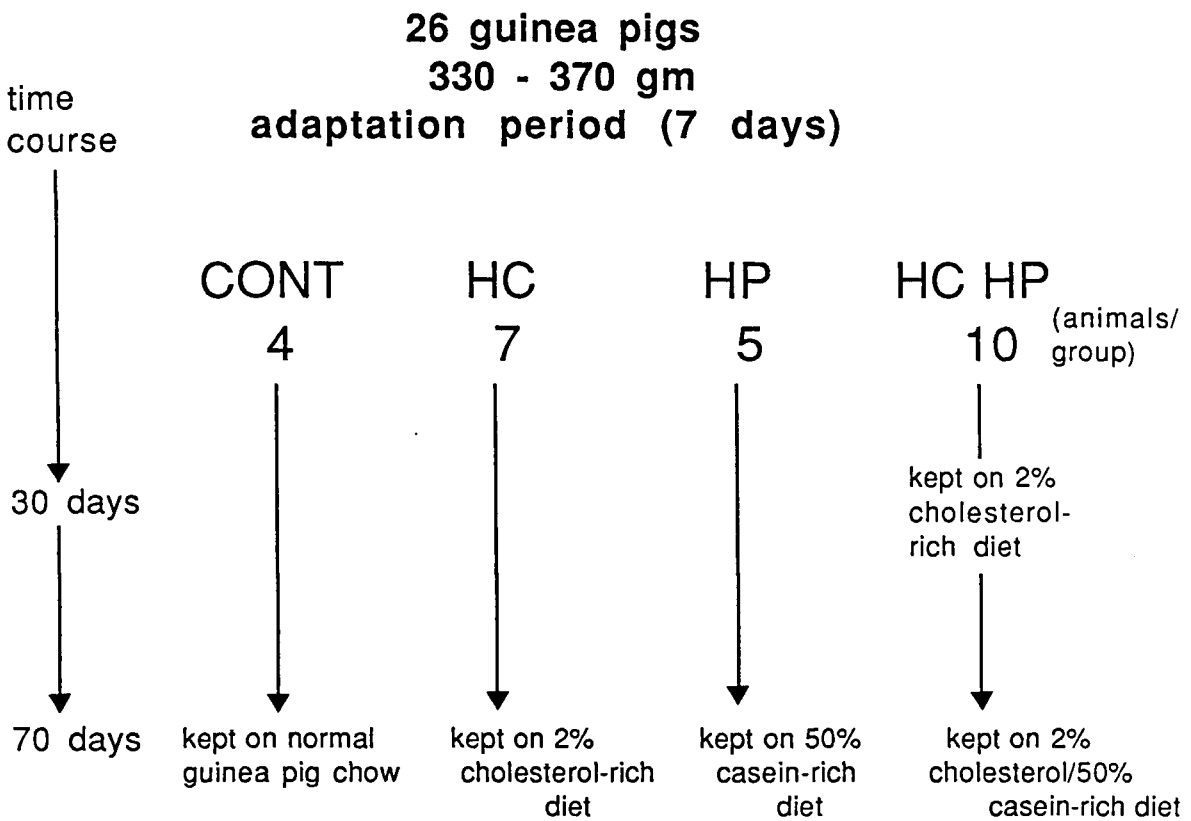
This experiment aimed to study the possible pathogenesis of cholesterol-induced glomerulosclerosis and the role of the high-protein supplement in aggravating such a lesion (see "Objectives" in "Introduction", too).

Twenty-six male guinea pigs were used. They were assigned randomly to the following groups: HCHP group had ten animals; HC group had seven animals; HP group had five animals and CONT group had four animals. All the groups ran through a similar time course and received the same diets described in Experiment I (Figure 4).

In this experiment, sixteen additional animals were used in the assessment assay of HMG-COA reductase activity. Five animals served as the HC group, five animals as the HCHP group, three animals as the HP group and three animals as the CONT group. To measure cholesterol de novo synthesis by another method, namely, measuring incorporation of tritiated water into cholesterol, another ten animals were used in the following groups: HC group (three animals), HCHP group (three animals), HP group (two animals), and CONT group (two animals) (Figure 5).

Fig - 4

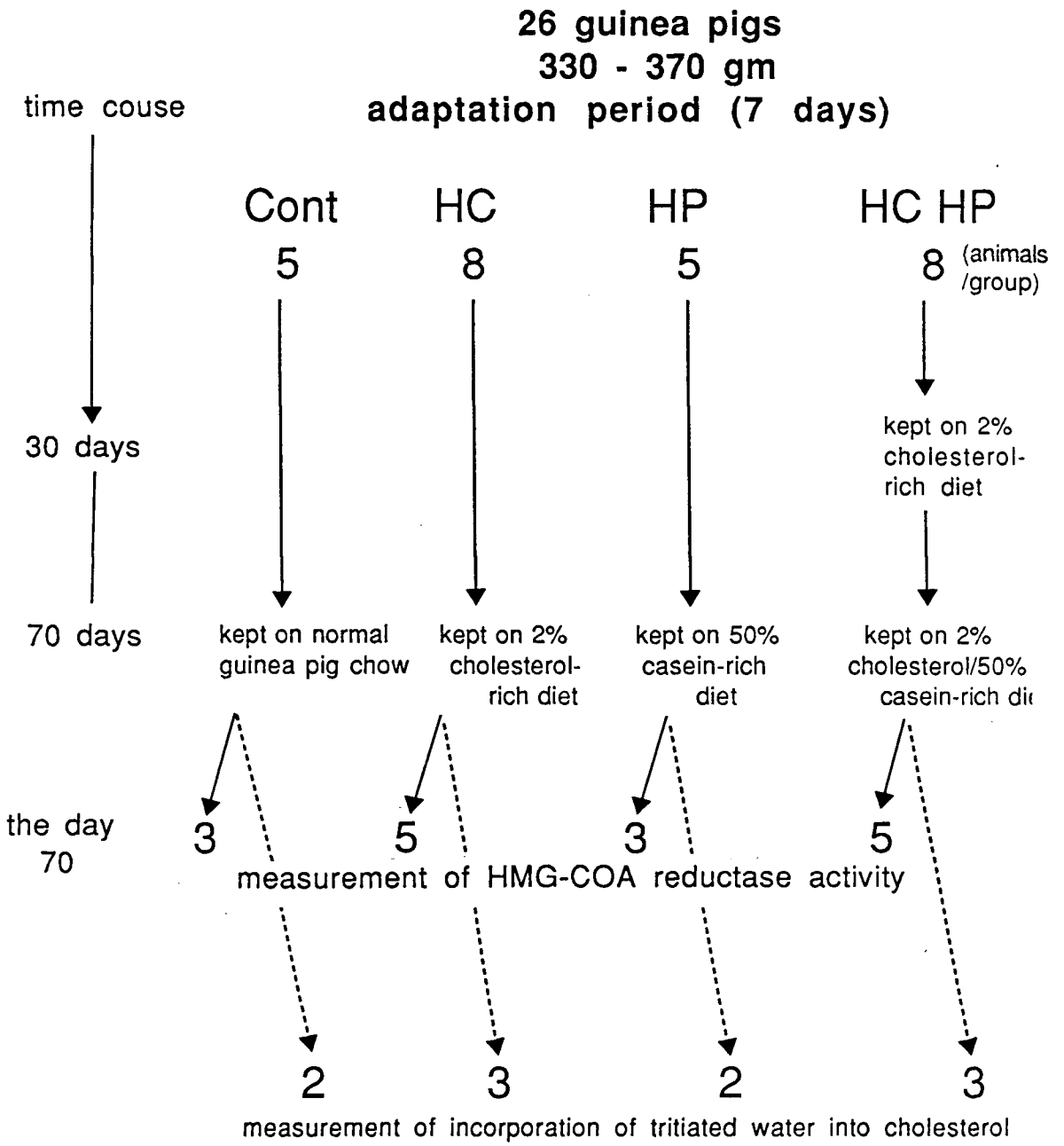
Flow chart of the experimental design of experiment - III



CONT = Control group
HC = High cholesterol group
HP = High protein group
HC HP = High Cholesterol/High Protein Group

Fig - 5

Flow chart of the experimental design of the assessment assays of cholesterol *de novo* synthesis

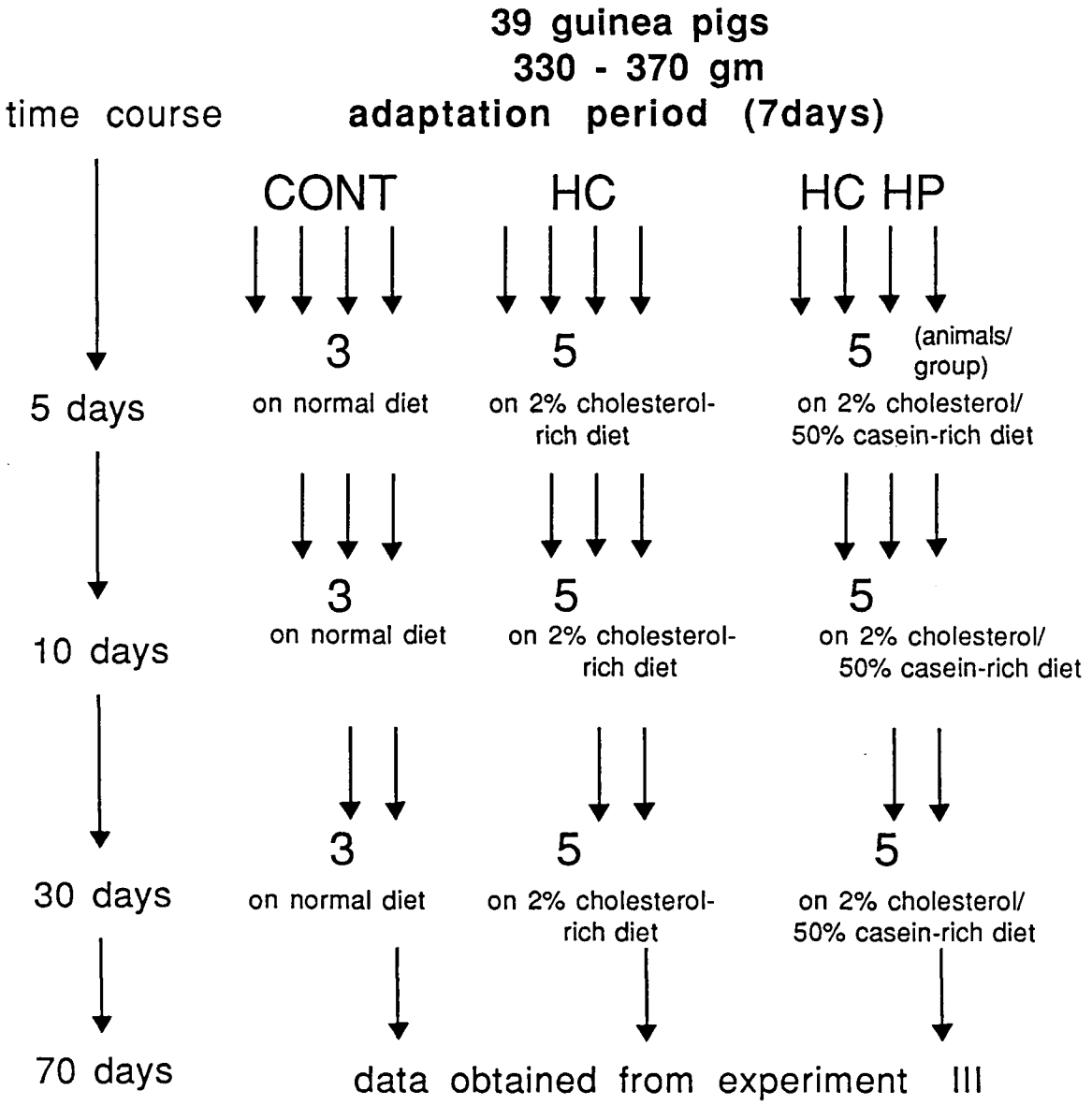


D. Experiment IV

This experiment was performed to sequentially follow up the changes in plasma and kidney tissue lipids versus the kidney histopathological alterations in an effort to elucidate the pathogenetic role of plasma lipid and lipoproteins in inducing glomeruloclerosis. A total of thirty-nine male guinea pigs were used. Groups of animals were sacrificed at day 5, 10, and 30 (Figure 6). In the result analysis, the data for day 70 were derived from the Experiment III. The following dietary regimens were instituted: five animals on the cholesterol-rich diet, five animals on the cholesterol/protein-rich diet, and three animals on the normal control diet.

Fig - 6

Flow chart of the experimental design of experiment - IV



III Methods

A. Blood and Urine Collections:

Blood was collected into EDTA containing tubes from ether anaesthetized animals from a 1mm long incision in the lateral metatarsal vein. After separation of plasma by centrifugation at 1000xg for 15 minutes, whole RBC and plasma lipid extractions were performed.

24-hour urine collections were done at day 70 in all of the animals in Experiment III. The animals spent one day in metabolic cages as an adaptation period, then two separate 24-hour urine specimens were obtained.

B. Erythrocyte lipid extraction:

The solvent system used to extract erythrocyte lipid was chloroform: isopropanol (2:1 v/v) with solvent-to-plasma sample ratio (10:1v:v). Samples were re-extracted to increase lipid recovery, and all extractions were performed at 0°C in an ice bath (80). The lipid extracts were dried under a nitrogen stream and reconstituted with 200ul of chloroform:methanol (2:1 v/v). Butylated hydroxy toluene (BHT) 0.05% (w/v) was added to the solvents to prevent lipid peroxidation.

C. Plasma:

(i) Enzyme assay and lipid Profile:

1. Lecithin:cholesterol acyltransferase activity using endogenous substrate:

Plasma LCAT activity was measured according to Stokke and Norum (1971) (81). Plasma sample (0.25ml) was incubated with 50ul of dithionitrobenzoic acid 4.2 mM (DTNB) for 30 minutes at 37°C in a shaking water bath. ^{3}H-cholesterol/50% bovine serum albumin emulsion (75ul) was added and the

mixture was incubated for 4 hours at 37°C water bath. After this incubation period, 50ul of 2-mercaptoethanol was added and a 150ul aliquot of the mixture was removed to represent activity at time 0 (t=0). After one hour incubation another 150ul aliquot was removed into a separate tube to represent activity after one hour (t=1). Each aliquot was mixed with 2ml of chloroform:methanol (2:1 v/v) to allow lipid extraction. The extract was recovered from the chloroform layer after mixing with saline and centrifugation. The lipid extract was dried down under a nitrogen stream at 60°C; it was reconstituted by chloroform and streaked onto 10 x 20cm silica gel G plastic plates. Separation of free cholesterol and cholesteryl ester was obtained by using petroleum ether:ether:acetic acid solvent (70:10:1 v/v/v) and the spots were visualized by iodine vapour. Free cholesterol and cholesteryl ester spots were mixed with 5mls Omniflour in toluene and liquid scintillation counting was done in ^{3}H window (A15) for 5 minutes. Fractional esterification rate (FER) was calculated as follows:

$$FER = \frac{CPM - EC}{CPM - EC + CPM\text{-}FC} \times \frac{100}{1\ hr}$$

where

CPM = Count per minute

EC = Esterified cholesterol

FC = Free cholesterol

To calculate molar esterification rate (MER):

MER = FER/hr x FC (nmol)

= nmol FC/hr/ml plasma

2. Lecithin:cholesterol acyltransferase activity - using artificial (exogenous) ethanolosome substrate:

Liposomes were prepared by mixing 0.26mls egg yolk phosphatidylcholine (PC) (5mg/ml in absolute ethanol), 0.15mls free cholesterol (FC) (1mg/ml in absolute ethanol), and 12ul ^{3}H-FC (1mCi/ml), giving a molar ratio of PC:FC of 4:1. The mixture was dried down and taken up in 125ul of absolute ethanol and injected rapidly into 10mls of 10mM Tris-HCl pH 7.4/5mM EDTA/0.15M NaCl. The final volume of 2.5ml was obtained by concentration with an Amicon Ym-30 membrane.

Thirty microlitres of liposomes were pre-incubated at 37°c for 30 minutes with apo AI (20ul of 0.5mg/ml) and Tris-HCL buffer. 50ul bovine serum albumin (BSA), and 10ul of 2-mercaptoethanol were added and the reaction was started by adding 15ul of plasma. After 30 minutes incubation, the reaction was stopped by adding 4mls chloroform:methanol (2:1). The extracted lipid was dried under nitrogen, and 20ul of FC/esterified cholesterol (EC) were added together with 70ul chloroform and the mixture was streaked on silica gel TLC plates. FC and EC spots were separated and radioactivity was determined.

Calculation of the activity:

MER = FER x 6.21 (nmol FC esterified/hr/ml).

3. Total and Free Cholesterol.

Cholesterol was measured enzymatically (82) using an "ABBOTT (ABA-100) Bichromatic Analyzer". The principle of this assay is converting cholesterol to cholest-4-ene-3-one and H_2O_2 by cholesterol oxidase; H_2O_2 reacts with 4-aminoantipyrine in the presence of phenol and hydrogen peroxidase to produce quinoneimine dye that absorbs at 500nm.

4. Phospholipids.

Plasma phospholipids were determined by one dimensional thin layer chromatography (TLC) with subsequent assay of the phosphorus content of individual resolved phospholipids according to the Bartlett method (83). An aliquot of 200ul of a chloroform:methanol (2:1 v/v) reconstituted lipid extract was applied to pre-coated silica gel F-254 (0.2mm thickness, Brinkman) which was activated by heating at 110°C for 30 minutes. The solvent mixture contained chloroform:methanol:ammonia (14:6:1 v/v/v). Visualization of phospholipid spots was obtained by iodine vapor; aminophospholipids were visualized after ninhydrin spraying. Individual phospholipids were hydrolyzed by 70% perchloric acid at 230°C for 1/2 hr; after cooling down and mixing with water and centrifuging the mixture, an aliquot from the supernatant was reacted with 5% ammonium molybdate and ANS (1-amino-2-napthol-4-sulfonic acid) in a boiling water bath for 7 minutes. The cooled samples were read at 830nm. Plasma total phospholipids were estimated according to Anderson and Davis (1982) (also, see kidney lipid analysis for more detail).

5. HDL-Cholesterol (HDL-C).

Plasma HDL-C was measured after heparin-manganese precipitation of the lipoproteins containing apo-B. A mixture of manganese chloride 1.0mol/L and heparin 4000u/mL was used to precipitate chylomicrons, VLOL, and LDL. The HDL-C found in the supernatant was determined as described above (82).

6. Triglycerides.

Triglycerides were determined enzymatically by "ABBOTT (ABA-100) Biochromatic Analyzer" based on the method of Bucolo and David (1973) (84). The triglycerides are hydrolyzed to glycerol and fatty acids by lipase. The produced glycerol is phosphorylated by ATP which is catalyzed by glycerol kinase producing ADP. ADP is rephosphorylated by phosphoenol pyruvate to produce pyruvate. In the presence of NADH + H^+ and lactate dehydrogenase, pyruvate is converted to lactate. The decrease in absorbance at 340nm is proportional to the concentration of glycerol.

(ii) Lipoprotein studies.

1. Lipoprotein isolation.

Lipoproteins were isolated according to their hydrated densities. The lipoprotein with hydrated density 1.006 was isolated by putting one volume of plasma in a centrifuge tube and the level was marked. One half volume of d=1.006g/ml saline was layered over the sample and centrifuged in a 75 Ti rotor for 18 hr at 40,000 rpm, 15°C. The upper two mls were removed by a tube slicing technique and placed in a clean tube, whereas the infranatant under the intermediate clear zone proceeded for further lipoprotein

isolation. To isolate the lipoprotein fraction at the hydrated density range of d=1.006 - 1.063, 0.5 volume of 1.182g/ml saline solution was mixed with 1 volume of the infranatant recovered from the first isolation. The tube was sealed and centrifuged under the same conditions for 20 hrs. The upper two mls were kept as the lipoprotein fraction with hydrated density of d=1.006-1.063. After removing the middle clear zone, one volume of the infranatant was mixed with 0.5 volume of d=1.478g/ml saline solution. The sealed centrifuge tubes were spun at 40,000 rpm, 15°C for 24 hrs. The top two mls contained the lipoprotein fraction with hydrated density of (d=1.063-1.210). The isolated lipoprotein fractions were dialysed prior to SDS-polyacrylamide gel electrophoresis.

1. Electrophoresis.

One ul plasma was applied to 1% agarose gel (1% agarose/barbital buffer, pH 8.6). After 35 minutes electrophoresis gels were dried and overlayered with fat stain. The stained gels were dried under hot air.

2. SDS Polyacrylamide gel electrophoresis.

Acrylamide 30% (73gm acrylamide and 2gm Bis, made up into 250ml) was used after filtration. To prepare gel, 10mls acrylamide 30% was added to 7.5ml lower buffer (1.5M Tris-HCl pH 8.8/0.4% SDS) and 12.6ml water. To the mixture 10ul TEMED and 150uL ammonium persulfate were added. To the casted gel, stacking gel [3ml acrylamide 30%, 5ml upper buffer (0.5M Tris-HCl pH 6.8/0.4% SDS), 12mL d H_2O, 20uL TEMED, and 60 uL ammonium persulfate] was added. After insertion of an appropriate comb, the stacking gel was allowed to polymerize for one hour. One volume of sample buffer pH 6.8 (20gm

glycerol, 4.6gm SDS, 0.76gm Tris base/in 50mls d H_2O; plus 1 part mercaptoethanol and 1/2 part 0.5% bromophenol blue) was added to 9 parts (2 x concentration) of the sample buffer. This mixture was used in the electrophoresis after mixing 1 volume sample buffer (dilute) with 1 volume dialyzed sample and heating in a boiling water bath for 3-5 minutes and cooling down to room temperature. Electrophoresis was carried out at a constant 200 volts with tapwater cooling of the electrophoresis chamber. The gel was stained with Coomassie blue R250 overnight at room temperature. Destaining of the background was achieved by using destaining solution (100mls acetic acid, 450mls methanol, and 450 mls dH_2O). Low molecular weight standards were included in the separation.

D. Erythrocyte Analyses:

1. Complete blood count:

It was performed using an ELT-8 (Ortho instruments) automatic counter.

2. Erythrocyte osmotic fragility:

An aliquot of packed RBC was used to measure osmotic fragility as described by Godin *et al*. (1978) (58). Erythrocytes were incubated in different concentrations of NaCl, and the degree of hemolysis was monitored by spectrophotometry at 540nm.

3. Erythrocyte morphology:

Peripheral blood films were stained with:

* Wright-Giemsa stain (Giemsa stain 5ml/L mixed with Wright stain 3g.L in absolute methanol).
* Oil-Red O stain:

  Blood films were fixed by exposure to formalin fume. A saturated solution of Oil Red O (0.25-0.5%) in isopropyl alcohol was mixed with water 6:4 (v/v) and the slides were stained in a closed container for 10-15 minutes.
* Nonspecific esterase (NSE) stain:

Monocytes in peripheral blood and macrophages in renal tissue were detected by the alpha-naphthyl acetate for NSE stain based on the method described by Yam et al. (1971 (85). The non-specific esterase of these cells librates alpha-napthol from the substrate alpha-napthyl acetate. The alpha-napthol is coupled to the dye hexazonium pararosaniline to form dark red granules in the cytoplasm.

Estimation of vacuolated monocytes in the peripheral blood:

The NSE-positive white blood cells are mainly monocytes (85); neutrophils are slightly positively stained. Differentiation between the two cell types was achieved by applying other morphological criteria. One hundred monocytes were counted in each blood film and the percentage of the vacuolated monocytes was derived. Scanning the periphery of each smear by a zig-zag pattern was the method applied to all samples.

4. Lipid analyses were carried out as mentioned previously (see section III (i)).

E. Kidney:

(i) Renal Function Tests.

1. Serum creatinine:

Serum creatinine was determined on EKTACHEM automatic analyzer. In this method, serum creatinine is hydrolyzed by creatinine iminohydrolase into N-methylhydantoin and ammonia. The produced ammonia reacted with bromophenol blue (ammonia indicator) and produced a blue dye. The reflection was measured at 600nm.

2. Blood urea nitrogen (BUN):

BUN was measured by EKTACHEM automatic analyzer. The principle is based on releasing ammonia from urea by the action of urease; the rest of the measurement followed the same reaction described under serum creatinine.

3. Creatinine clearance:

Serum and urine creatinine were measured by EKTACHEM automatic analyzer as described above. Creatinine clearance was obtained by applying the equation:

$$\text{Creatinine clearance (mL/min)} = \frac{\text{urinary creat. conc. x volume of urine/24hrs.}}{\text{serum creat conc}}$$

4. Twenty-four hour urinary protein:

Urinary protein was measured by ABBOTT ABA-100 automatic analyzer (86). Coomassie brilliant blue G250 was used in the presence of phosphoric acid and 95% ethanol. The diluted dye reagent is bound to the protein in urine samples and absorbance is measured spectrophotometrically at 595nm.

5. Routine urinalysis (chemical and microscopic):

A semiquantitative assessment of white blood cells, bilirubin, and erythrocyte in urine was achieved by using a dipstick method.

(ii) Renal histology.

1. Blocks of $1cm^3$ size were taken from kidneys and fixed in Karnovsky's fixative (2 mls of 70% gluteraldehyde, 14mls of 38-40% formalin, and 126mls phosphate buffer of pH 7.2). Thin sections of 3 microns were obtained from paraffin blocks and stained with different stains.

a. Routine histological stain (hematoxylin and eosin stain).

b. Perls Prussian blue stain:

Iron containing pigments are soluble in acids, and insoluble in alkalis and fat solvents. The specimens were exposed to a fresh solution of equal parts of 2% aqueous potassium ferrocyanide and 2% hydrochloric acid for 30 minutes. After a wash with distilled water, a counter stain (1% neutral red) was applied for 10-15 seconds. The ferric-iron-containing pigments appeared blue.

c. Periodic acid-Schiff (PAS) and PASM stains were used to elucidate the connective tissue elements (collagenous and elastic fibres), (for PASM, 1-micron thick sections were used after embedding of tissues in polyglycol methacrylate).

d. Von Kossa stain for calcium.

2. Renal frozen sections.

a. Oil-Red O Stain (as described above).

b. Immunohistofluorescent study:

Fluorescein conjugated goat anti-guinea pig IgG (heavy and light chain specific) (Cappell, Cockranvile, PA) was used with an antibody protein concentration of 4.2mg//ml. Frozen sections were examined, after incubation, for immunofluorescent complexes.

c. Non specific estrase (NSE) stain (as described above).

3. Electron microscopy:

Blocks of $1mm^3$ were taken from kidney and fixed in ice cold 3% glutaraldyehyde in 0.1M sodium phosphate, pH 7.3 (Sorensen's solution). After washing in 0.1M phosphate buffer for approximately 30 minutes, the tissue was post fixed in 1% osmium tetroxide in 0.1M phosphate buffer for one hour at 4°C. After washing in phosphate buffer, the tissues were dehydrated in a graded ethanol series and embedded in an epon-araldite (Polyscience In. Co., PA). Ultrathin sections were cut from appropriate blocks with a diamond knife. The sections were stained with uranyl acetate followed by lead citrate and examined by a Zeiss EM 109 electron microscope.

4. Morphometric study:

Glomerular mesangial expansion was measured by examining histological sections of kidney stained by PASM stain at high magnification (1000x) using an eyepiece net micrometer (20 divisions/cm). The relative mesangial area (mesangial area/glomerular tuft area) was determined by a standard point-counting method (87). Mesangial cells were also determined.

5. Lipid analysis - extraction and measurement:

Kidney tissues were homogenized and lipid was extracted with

chloroform:methanol 2:1 (v/v). The extract was dried down under nitrogen and reconstituted with chloroform to be streaked on the TLC plates. The TLC solution solvent system used was petroleum ether:ethyl ether:acetic acid (85:15:3, v/v/). The phospholipid spots were visualized under ultraviolet light, while the spots of the different lipids were visualized by brief exposure to iodine vapour. Phospholipids were eluted by chloroform:methanol: acetic acid:water (25:15:4:2, v/v/v/v) then by methanol alone followed by methanol:acetic acid:water (95:1:5, v/v/v) (88). Free cholesterol, cholesteryl esters and triglycerides were eluted from the silica powder by a mixture of chloroform:methanol (89).

a. Tissue free and esterified cholesterol.

Cholesterol was determined by a modified Liebermann-Burchard reaction. This reaction involves strong acid medium-sulfuric acid, and glacial acetic acid. Ferric chloride is added to yield tetraenylic cation which absorbs at 560nm.

b. Phospholipids.

Phospholipids were measured according to Anderson and Davis (1982) (90). Phospholipids were digested with concentrated sulphuric acid at 155°C. To accomplish oxidation of the organic compounds hydrogen peroxide was used subsequently. Colorimetric reaction was achieved when a mixture of 10.1nmol/L ammonium molybdate and 0.28mol/L ascorbic acid (1:1, v/v) was added; the absorbance was read at 797nm.

c. Triglycerides.

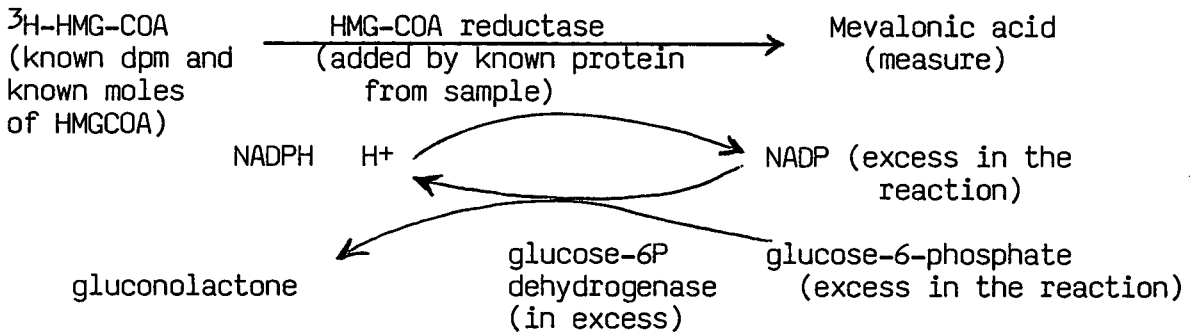
Triglycerides in renal tissue extract were determined according to Fossati and Lorenzo (1982) (91). Triglyceride was converted by lipase into glycerol and free fatty acids. By virtue of glycerol kinase and in the presence of ATP, glycerol-1-phosphate was formed. The latter is oxidized by glycerol phosphate oxidase in the presence of O_2 to produce H_2O_2 which gives a quinoneimine dye after reacting with 4-amino antipyrine. The produced dye absorbed at 515nm.

6. Assessment of tissue protein was done by Lowry et al. method (92). The value of protein served in expressing the lipid values in kidney tisse; the unit was ug/mg protein.

(iii) Estimation of cholesterol de novo synthesis in renal tissue:

1. Measurement of hydroxy methyl glutaryl-COA (HMG-COA) reductase activity.

Measurement of HMG-COA reductase activity was performed according to Goodwin and Marglis (93). Kidneys were homogenized and the microsomal fraction was prepared as follows: The homogenate (1 part tissue:5 parts of 225mM sucrose/25mM Tris HCl buffer pH 7.8) was spun at 2500 rpm for 10 minutes at 4°C. The supernatant was spun again at 8000 rpm for 20 minutes at 4°C. A third spin of the supernatant was performed at 25000 rpm (105000g) for 60 minutes at 4°C. The pellet was resuspended in 1ml (100mM sucrose/50mM KCl/40mM KH_2PO_4/30mM EDTA buffer, pH 7.2). Fresh 20mM dithiothreitol (30.86mg/10ml) was added to the resuspension before storing at -20°C. The assay is conducted according to the following principle:



The reaction was stopped at every 15 sec. by adding 12N HCl, and incubated for 30 minutes at 37°C. Sodium sulfite was added to raise the pH of the reaction mixture to 6.5 which enhanced the retention of HMG COA in the aqueous phase. The produced mevalonate was extracted by toluene. Scintillation fluid was added to 10ml of the extract and radioactivity was counted.

2. Estimation of incorporation of tritiated water into cholesterol:

Guinea pigs were injected intravenously with 200mCi/kg tritiated water between 9:00 and 11:00 a.m. (94). One hour later, the animals were sacrificed and blood samples were taken for the measurement of the specific activity. Kidneys were removed; thin slices were prepared and washed with cold isotonic saline. The tissues were weighed and homogenized in 0.9% NaCl/3mM EDTA. Tissue lipids were extracted with chloroform:methanol (2:1, v/v). After adding saline, the mixture was centrifuged at 2500 rpm for 5 min. and the bottom phase was recovered for further lipid separation. Free cholesterol and cholesteryl esters were separated by TLC chromatography (as described above). Radioactivity detected in the cholesteryl esters was negligible. Thus, the tritiated water was incorporated into the free cholesterol fraction only. The calculations were performed according to Turley et al. (95).

F. Liver Function Tests

1. Serum total protein and albumin:

All analyses were done on an EKTACHEM automatic analyzer by the chemistry unit in Shaughnessy Hospital. The method for determining total protein was based on the biuret reaction (96) in which a violet-colored complex is generated when protein is treated with cupric ion (Cu^{2+}) in an alkaline medium. The density of the resulting complex is related to the concentration of total protein in the sample, and a spectrophorometric measurement can be achieved at 540nM.

Serum albumin was measured using the same automatic analyzer. The method was based on the binding of bromcresol green dye to albumin, resulting in a substantial shift in the wavelength of light absorbed by the free dye. The density of the albumin-bound dye was related to the concentration of albumin in the sample. It was measured spectrophotometrically at 630nm.

2. Blood urea nitrogen. (see above).

3. Serum aspartate transaminase (SGOT or AST).

In this enzymatic assay, the amino group of L-aspartate is transferred to alpha-keto glutarate in the presence of sodium pyridoxal-5-phosphate to produce glutamate and oxalo-acetate. Oxalo-acetate is converted to malate by malate dehydrogenase in the presence of NADH which is oxidized at 37°C to NAD^{+}. The oxidation of NADH is monitored by reflectance spectrometry at 340nm.

4. Serum bilirubin.

Determination of both conjugated and unconjugated bilirubin was performed simultaneously on EKTACHEM autoanalyzer. The analysis is based on a modification of the classical diazo reaction (97). A dual-wave length reflectance spectrophotometric method was used. Dyphylline and surfactant are used to dissociate unconjugated bilirubin from albumin. Unconjugated and conjugated bilirubin reacts with the diazonium salt [4-(N-carboxymethyl-sulfamyl)-benzene diazonium hexafluorophosphate] to produce azobilirubin which has an absorbance maximum around 520nM. Unconjugated and conjugated bilirubin bound to a cationic mordant (polymeric quaternary amine). As a result of this interaction, the absorbance peaks of the bilirubin fractions were shifted about 10nms, and the molar extinction coefficients were significantly increased.

G - Serum phosphate was done on EKTACHEM automatic analyzer.

IV Statistical Analyses.

All data were assessed by Student's t-test, both paired and unpaired. Accordingly, this statistical test examined the difference between-group means at each period and between-period means in each group. Correlations were determined by multiple linear regression analysis.

RESULTS

I General Observations

In both experiment I and experiment III, the animals kept on cholesterol-rich and cholesterol/protein-rich diets showed smaller weight gain at day 70. Compared to the CONT group (422 $\pm$ 52.6)gms and HP group (363.3 $\pm$ 24.9) gms, HC and HCHP groups gained (235 $\pm$ 29.3)gms and (105 $\pm$ 39.9) gms, respectively. A significant reduction ($p < 0.01$) was found in HCHP group compared to HC group (Fig. 7), but no change found between CONT and HP groups.

As shown in (Table 6), the differences in weight gain were first noticed at day 30. Both HC and HCHP groups had lower weight gain compared to the CONT group ($p < 0.05$) and ($p < 0.01$), respectively. These results suggested that keeping guinea pigs on cholesterol-rich or cholesterol/protein-rich diet does not affect the animals' ability to gain weight during the first two weeks of the experiment. Addition of protein supplement to the cholesterol-rich diet affected weight gain more severely than cholesterol-rich diet alone ($p < 0.02$). Animals made anaemic by acetylphenylhydrazine also showed a significant reduction in weight gain comparable to that found in the HCHP group. They gained 171.7 $\pm$ 55 gm compared to 370.1 $\pm$ 50 gm in the CONT group.

Mortality rate in experiment III was almost the same as in experiment I. At day 70 of both experiments the HCHP group had 40% mortality compared to almost 10% mortality in the HC group. There were no deaths among the control animals or in those kept on any of the diets for 30 days.

As depicted in Fig. 8, the liver was enlarged and yellowish in the cholesterol-fed guinea pigs at day 70 compared to the animals of the CONT and HP groups. Table 7 summarizes the organ weight changes in the individual groups. The animals of the HC and HCHP groups had significant enlargement

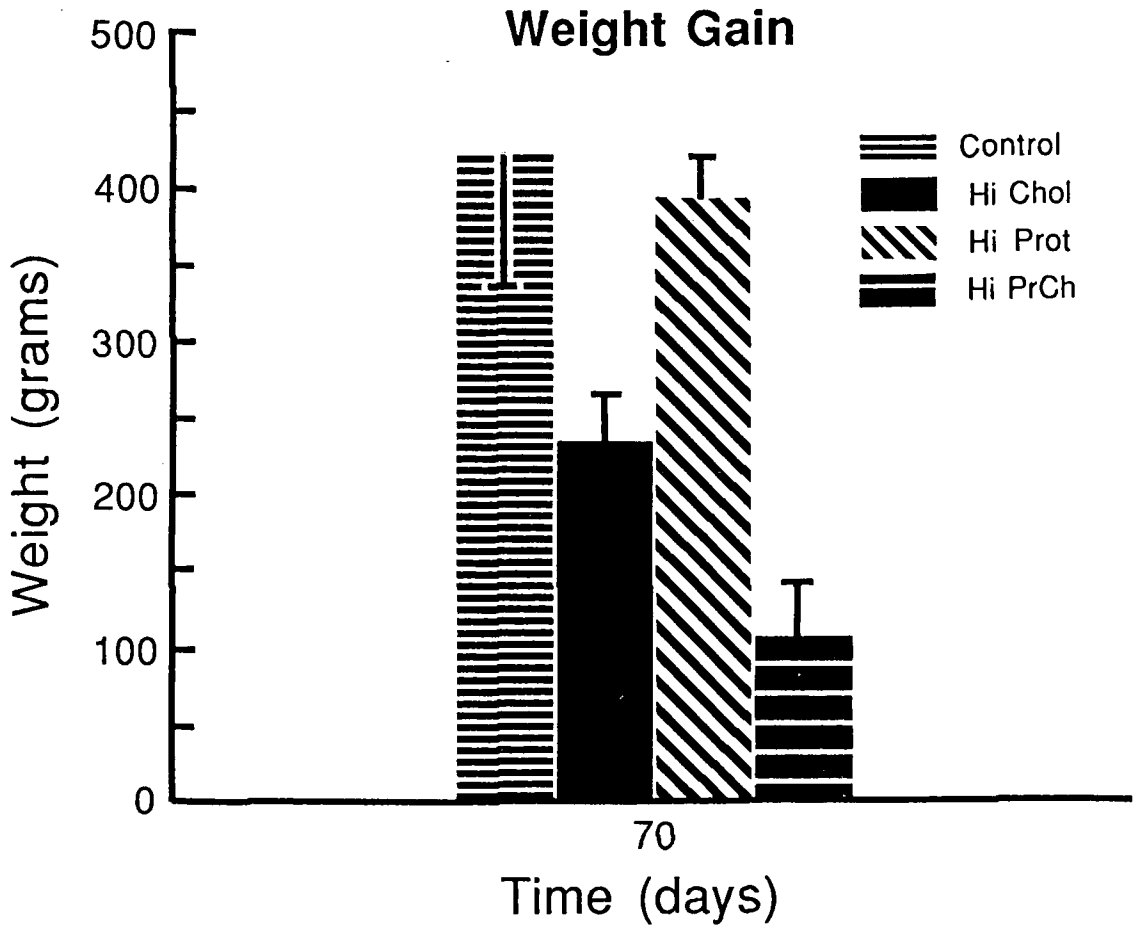
Table 6

[Weight Gain (gm). Mean ± SD]

Group	Day 5	Day 10	Day 30
	(n = 5)	(n = 5)	(n = 5)
CONT	60 ± 81	136.7 ± 16.9	353.3 ± 49.9
HC	64 ± 8	116 ± 10.2	236 ± 45.9[a]
HCHP	58 ± 7.5	112 ± 16	132 ± 32.5[b]

n = Number of animals/group
CONT = Control
HC = High cholesterol
HCHP = High cholesterol/protein
a = Significantly different from CONT ($p < 0.05$)
b = Significantly different from CONT ($p < 0.01$)

Fig - 7


Weight Gain
500
400
300
200
100
0
Weight (grams)
70
Time (days)
Control
Hi Chol
Hi Prot
Hi PrCh

in liver size compared to the control groups ($p < 0.01$). The spleen was enormously enlarged in these two groups (Fig. 8). Almost ten-fold enlargement was found in the HC and HCHP groups compared to the normal-sized spleen in the CONT and HP groups. Table 7 also shows the significant increase in weight of the kidney in the HP group compared to other groups ($p < 0.05$). There was no change in the HC or HCHP groups. As shown in Table 7, the only significant change was present in spleens of the HC and HCHP groups at day 30; there was a significant increase ($p < 0.05$) in weight compared to the CONT group. There was a significant increase in the weight of the spleen and the liver from day 30 to day 70 in the HC and HCHP groups ($p < 0.01$) (Table 7).

Figure 8:

A severely enlarged spleen from the HCHP group compared to a normal-sized spleen from the CONT group (upper left corner). The right side of the figure shows enlarged yellowish pale liver from the same animal compared to a normal liver. (All specimens were obtained at day 70.)

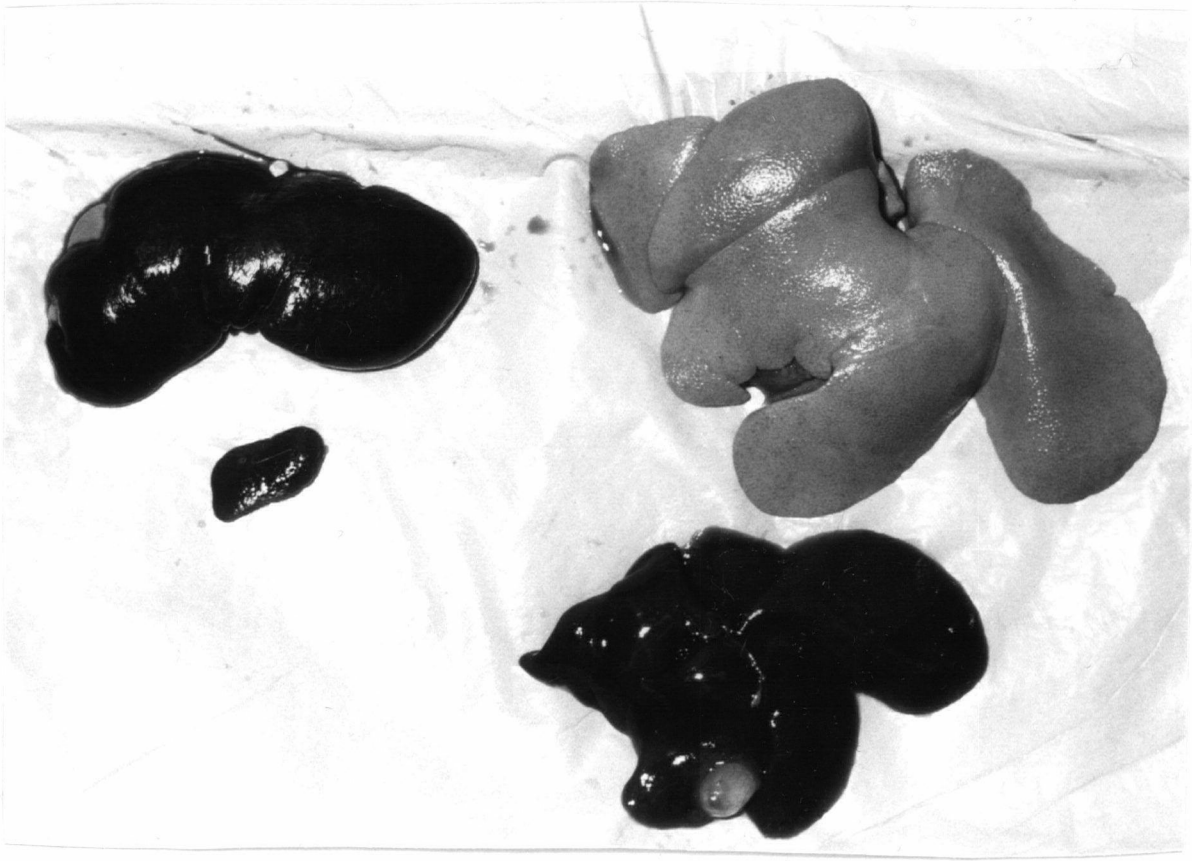


Table 7

[Organ weight (gm). Mean ± SD]

Group	Organ	Day 5	Day 10	Day 30	*Day 70	
		(n = 5)	(n = 5)	(n = 5)		
	Kidney	2.1 ± .1	2.2 ± .2	2.6 ± 3	2.1 ± .6	
CONT	Liver	24.3 ± 1.7	23.7 ± 1.2	34.1 ± 7.2	22.3 ± 2.6	n = 4
	Spleen	.8 ± .1	.8 ± .1	.74 ± .1	.8 + .1	
	Kidney	2.3 ± .1	2.1 ± .1	2.3 ± .3	2.3 ± .4	
HC	Liver	25.9 ± .7	25.8 ± 1.9	40.2 ± 4.2[b]	59.5 ± 4.5[b]	n = 6
	Spleen	.8 ± .1	.8 ± .1	1.2 ± .4[c]	9.5 ± 7.3[d]	
	Kidney	2.1 ± .1	2.1 ± .1	2.7 ± .5	2.3 ± .3	
HCHP	Liver	25.9 ± .7	25.6 ± 1.0	35.6 ± 8.2[b]	46.8 ± 9.3[b]	n = 6
	Spleen	.8 ± .1	1.0 ± .2	2.0 ± 1.8[c]	9.5 ± 6.6[d]	
	Kidney				3.3 ± .3[a]	
HP	Liver				21 ± .9	n = 5
	Spleen				.8 ± .1	

n = Number of animals/group

* = Data from experiment III

a = Significantly different than other groups ($p < 0.05$)

b = Significantly different than the control groups ($p < 0.01$)

c = Significantly higher than the control groups ($p < 0.05$)

d = Significantly higher than the control groups ($p < 0.01$)

II Plasma Lipid and Lipoprotein Changes

In experiment I, plasma total cholesterol (TC) was significantly increased at day 30 in both HC and HCHP groups compared to the CONT and HP groups ($p < 0.001$) (Figure 9). As shown in Figure 10, the increase in plasma TC in the HC and HCHP groups was already significant at day 5. There was only a numerical, but not a statistical, difference in plasma TC between HC and HCHP groups (Figures 9 and 10). The changes in plasma TC after 5, 10, 30 and 70 days of the feeding are shown in Figure 10. Only the increase between days 10 and 30 was not statistically significant.

The increase in plasma free cholesterol (FC) generally paralleled that in TC (experiment I) except for a significant increase in the HCHP group over the HC group ($p < 0.01$) at day 70 (Figure 11). As shown in Table 8, the percentage of plasma free cholesterol (FC%) was significantly higher at both day 30 and day 70 in the HC and HCHP than in the CONT and HP ($p < 0.05$). Plasma triglycerides were only measured in experiment I. There was no significant difference between the groups (Table 8). Plasma TC was slightly elevated in the CONT and HP groups at day 70 compared to day 0. As shown in Table 9, plasma total phospholipids (TPL) were significantly increased in the HC and HCHP groups at the day 70 of the experiment relative to the CONT group ($p < 0.001$). Once again the highest values were found in the HCHP group versus HC group ($p < 0.01$). This increase in plasma TPL in both groups was first noted at day 30 ($p < 0.02$) compared to the CONT group (Table 9). Plasma cholesterol of HDL (HDL-C) as presented in Table 9 was unchanged at days 5 and 10 in all groups of the experiments. At day 30, HDL-C increased significantly in the HC and HCHP groups compared to the CONT group. This

Fig - 9

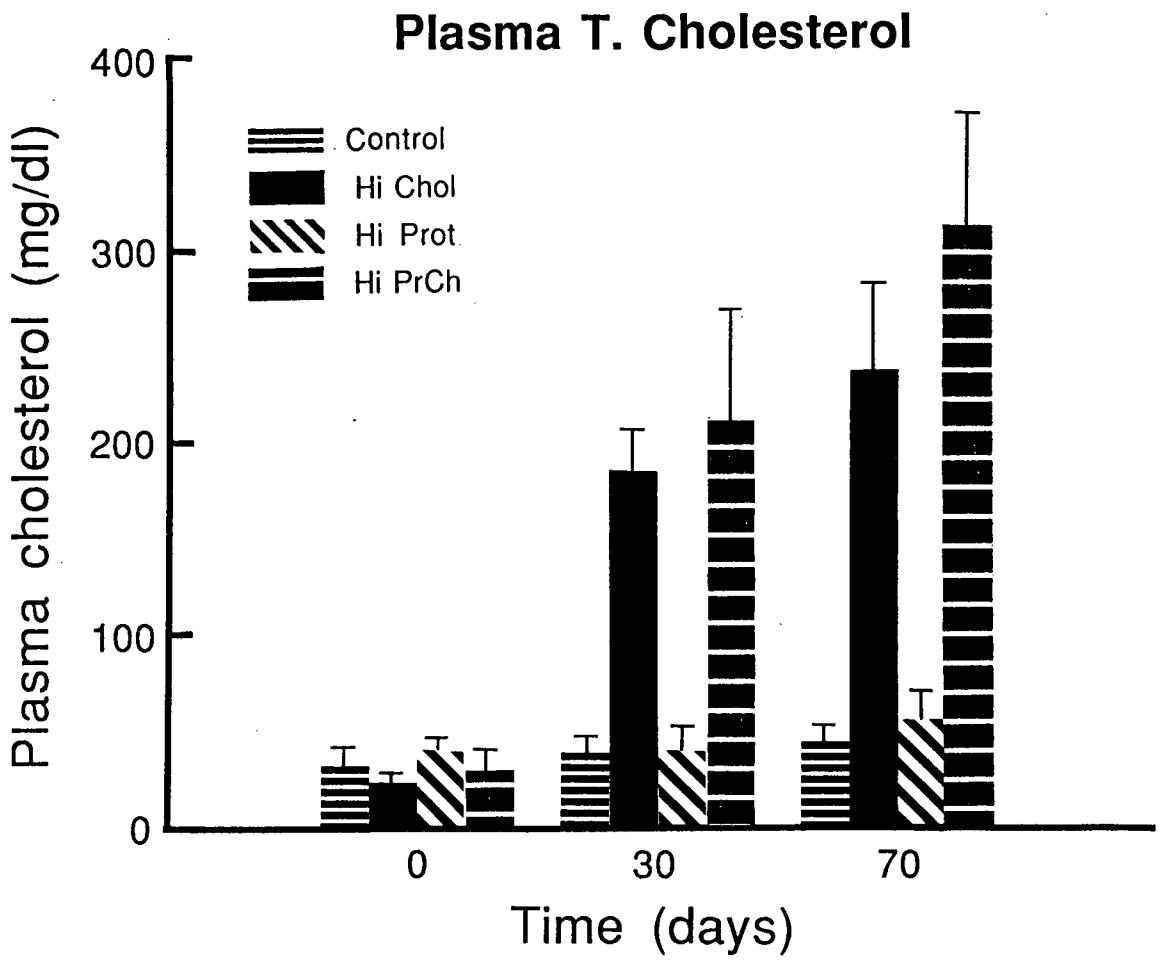

Plasma T. Cholesterol
Control
Hi Chol
Hi Prot
Hi PrCh
Plasma cholesterol (mg/dl)
400
300
200
100
0
0
30
70
Time (days)

Fig - 10

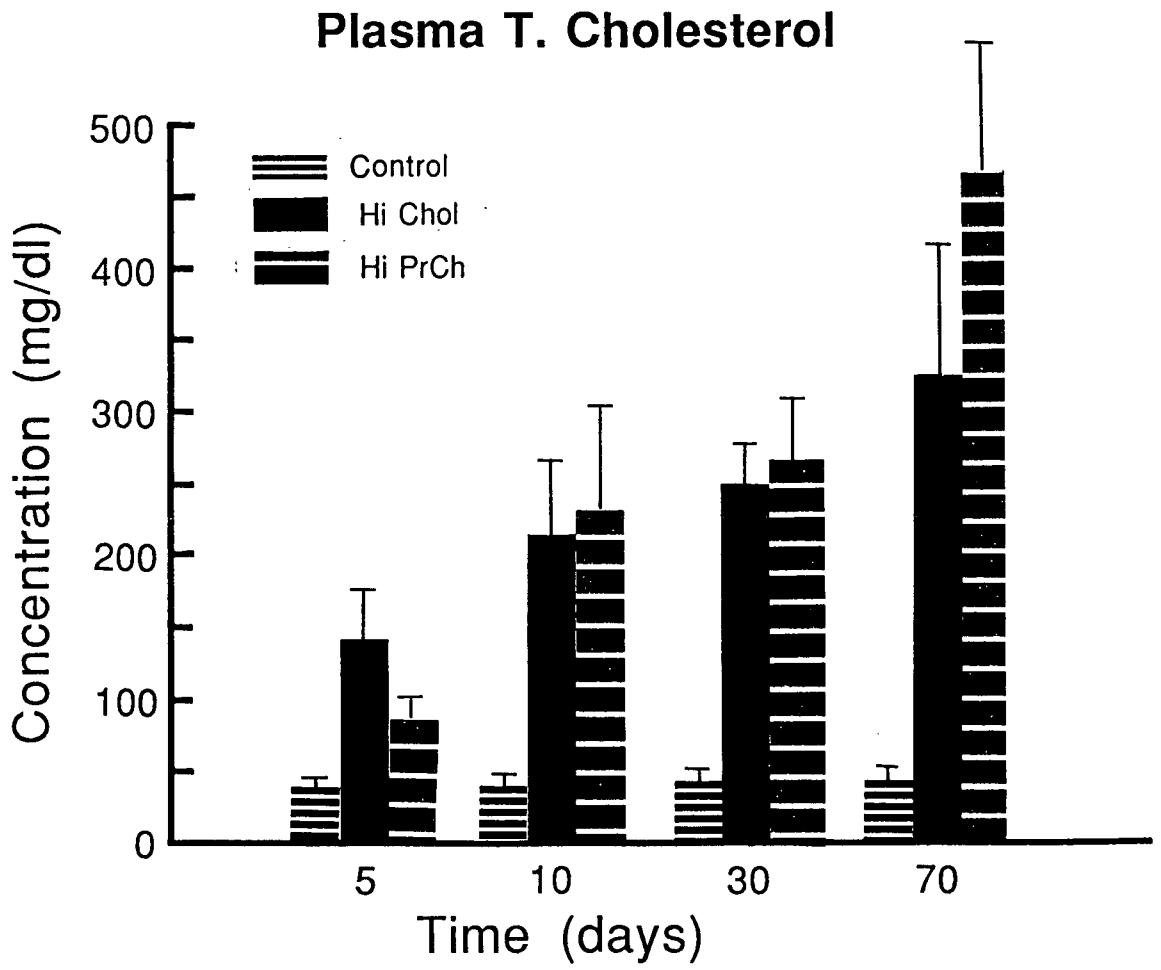

Plasma T. Cholesterol
Control
Hi Chol
Hi PrCh
Concentration (mg/dl)
500
400
300
200
100
0
5
10
30
70
Time (days)

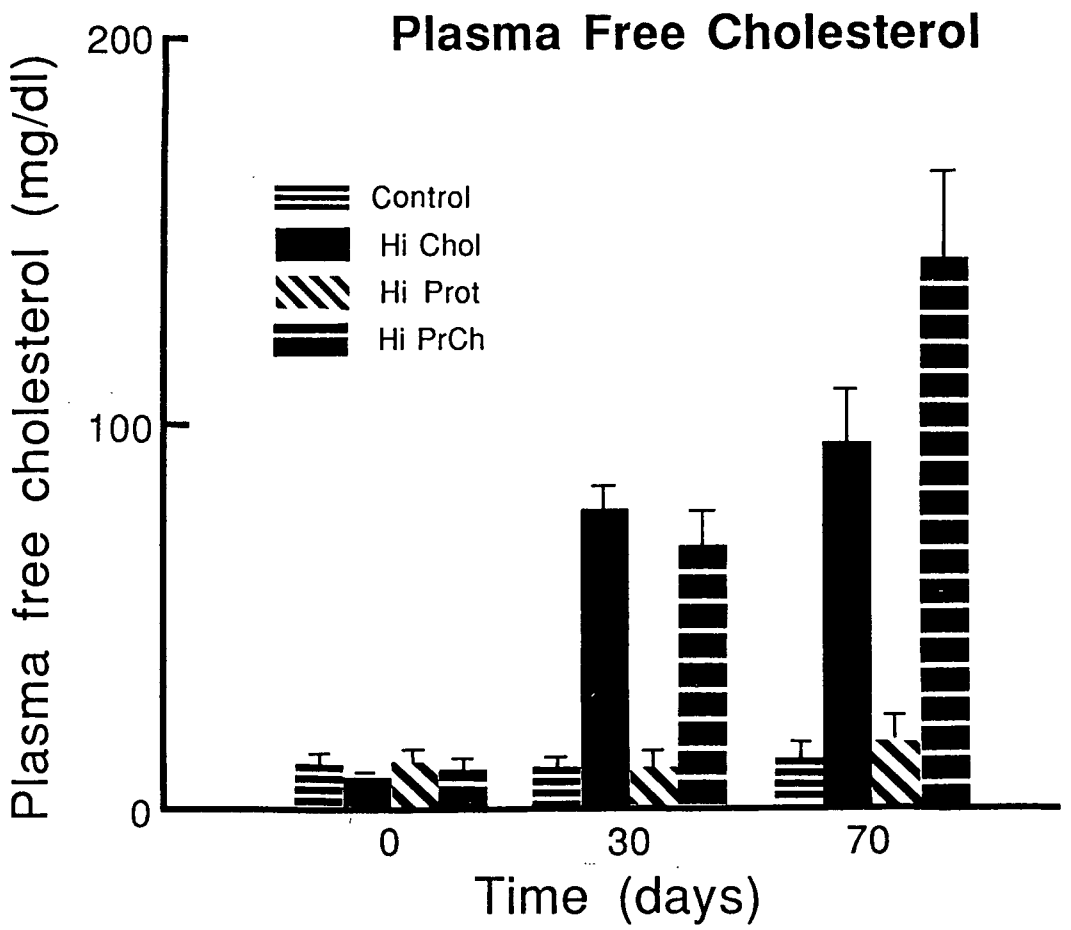
Table 8

[Plasma Lipid Profile. Mean ± SD]

Group*		Day 0	Day 30	Day 70
CONT	%FC	32.9 ± 4.7	27.2 ± 4.4	29.8 ± 5.3
**n=6	TG(mg/dL)	73.3 ± 31.8	72.3 ± 23.5	65.3 ± 14.6
HP	%FC	28.4 ± 8.2	27.6 ± 5.6	30.8 ± 11.8
n=6	TG	73.8 ± 30.1	82.2 ± 22.6	70.5 ± 11.8
HC	%FC	30.2 ± 3.9	40.0 ± 3.3[a]	40.9 ± 5.5[a]
n=6	TG	62.3 ± 23.3	68.4 ± 23.4	61.3 ± 9.2
HCHP	%FC	31.0 ± 9.7	33.7 ± 8.5[a]	46.3 ± 12.0[a]
n=6	TG	69.2 ± 29.4	71.8 ± 28.7	60.3 ± 29

*CONT = Control
HP = High Protein
HC = High Cholesterol FC = % Free Cholesterol
HCHP = High Cholesterol & High Protein TG = Triglycerides
**n = Number of animals/group
a = Significantly higher than the control groups ($p < 0.05$)

Fig - 11


Plasma Free Cholesterol
Plasma free cholesterol (mg/dl)
200
100
0
Control
Hi Chol
Hi Prot
Hi PrCh
0
30
70
Time (days)

increase was even more pronounced by day 70 in both HC and HCHP groups. No significant change was detected between the HC and HCHP groups. No visible band was detected at the alpha region on agarose gel electrophoresis at day 30 which looked indistinguishable from the CONT group (Figure 12). The alpha band appeared at day 70 in both HC and HCHP groups (Figure 13). Other abnormalities in electrophoretic profile of plasma lipoproteins were as follows: the prebeta band in the HC and HCHP group disappeared from the region where it runs in the CONT group (Figure 13). Only one beta band appeared on the electrophoresis gel of plasma from HC and HCHP groups at day 30 (Figure 12) while the CONT group shows both beta and prebeta bands. At day 70 there was an additional alpha-migrating band in the HC and HCHP groups (Figure 13). Also, a slow broad beta band was found in these two groups at day 70 compared to the control beta band. The slow beta could be possibly a combination of slow prebeta and beta bands (Figure 14). The slowness of the prebeta band was monitored in experiment IV, where there was a gradual decrease in the anodal movement of the prebeta band which ultimately blended with beta band (Figure 14). The earliest change in the pattern of electrophoretic mobility was noticed at day 10 in the HC and HCHP groups and this was limited to the slowness of the prebeta band (Figure 14).

The apoprotein profile of the lipoproteins isolated by preparative ultracentrifugation in the hydrated density ranges $<$ 1.006 gm/ml and 1.063-1.21 gm/ml showed no salient differences in patterns of the HC and HCHP groups at day 70. The lipoprotein species isolated in the hydrated density range of 1.063-1.21 gm/ml from these two groups showed a doublet protein band with an apparent molecular weight of $>$ 31000 (Figure 15). This band was not observed in the CONT group. This band(s) may represent apoprotein E.

Table 9

[Plasma Lipid Profile. Mean ± SD]

Group n		Day 5	Day 10	Day 30	*Day 70
CONT**					
n=12	HDL-C	6.5 ± 1.6	6.5 ± 1.6	6.5 ± 1.6	6.5 ± 1.6
	TPL	63.2 ± 11.2	63.2 ± 11.2	63.2 ± 11.2	63.2 ± 11.2
HC					
	HDL-c	6 ± 1	9.8 ± 1.6	18.8 ± 9.4	79.8 ± 20.1[e]
	TPL	65 ± 19	71 ± 21	133 ± 34.4	314.5 ± 50.3[e]
HCHP					
	HDL-C	8.5 ± 3.4	11.4 ± 5.8	19.4 ± 6.7	68.4 ± 23.9[e]
	TPL	68 ± 15	75 ± 18	145.2 ± 7.8	475.2 ± 79.4[e]

n = Number of animals (5)/group/5, 10, and 30 day periods.
The number of animals at day 70 was: HP(5), HC(6), HCHP(6)

* = Data from experiment III

HDL-C = Cholesterol of HDL (mg/dL)

TPL = Total plasma phospholipids (mg/dL)

e = Significantly different than CONT ($p < 0.001$)

** = The data of days 5, 10, 30 and 70 are combined due to the absence of significant changes among the control animals.

Figure 12:

An electrophoretogram on agarose gel of the CONT, HC, and HCHP groups at day 30. The CONT group reveals beta and prebeta bands while both HC and HCHP groups have only one darkly stained band runs in the beta region.

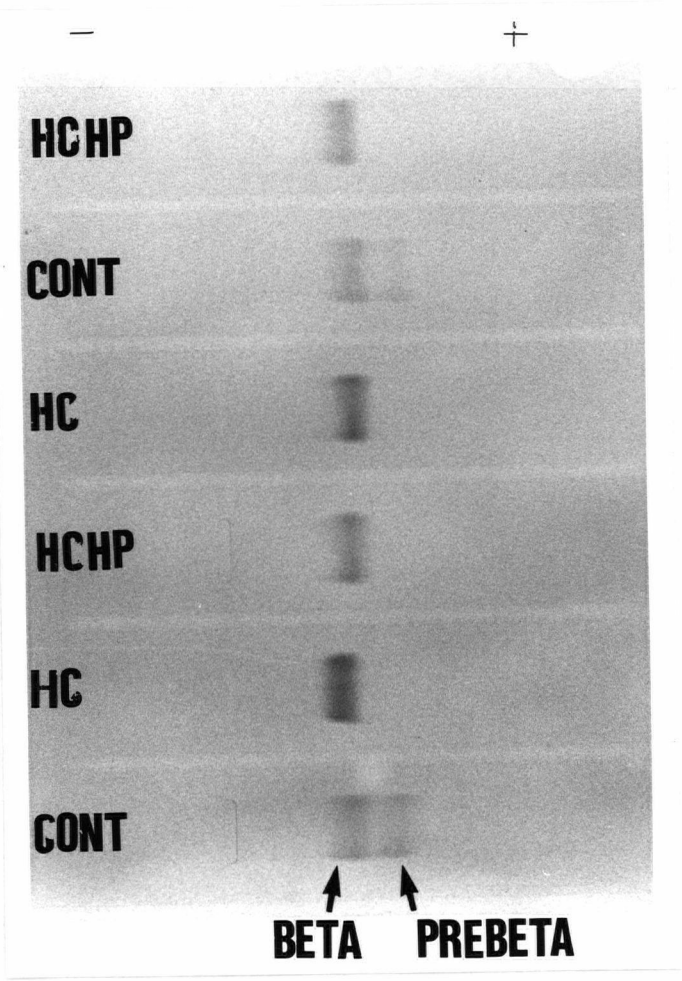

—
+
HCHP
CONT
HC
HCHP
HC
CONT
BETA
PREBETA

Figure 13:

An electrophoretic profile on agarose gel of the HC, CONT, and HCHP groups at day 70. Both HC and HCHP groups reveal a clear alpha band. Prebeta band in these two groups are absent, instead, there is a darkly stained beta band which migrates slower than the beta band of the CONT group. Both beta and prebeta bands are clear in the CONT group, while there is no alpha band.

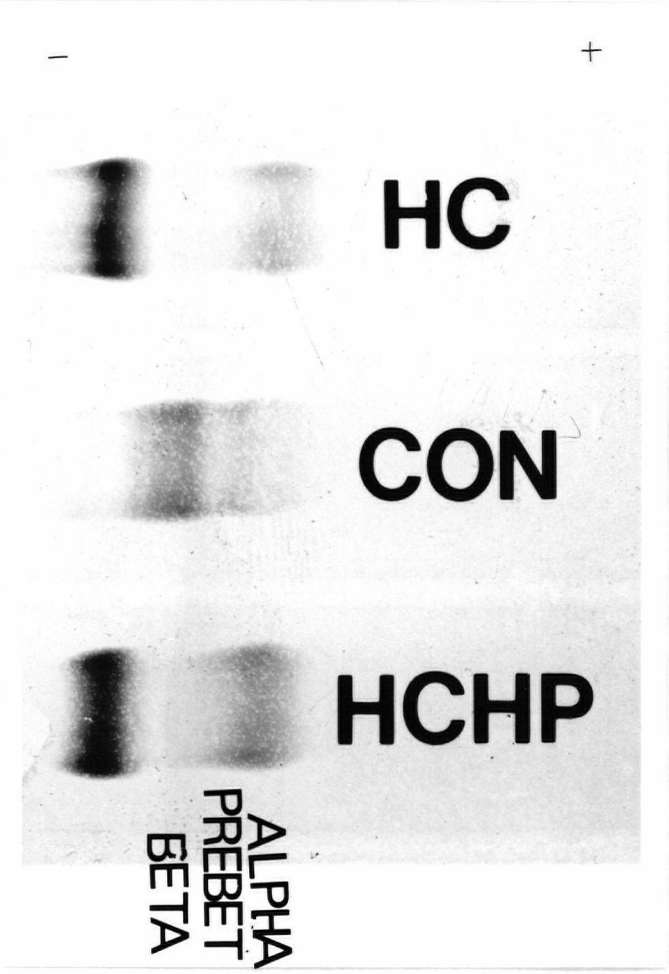
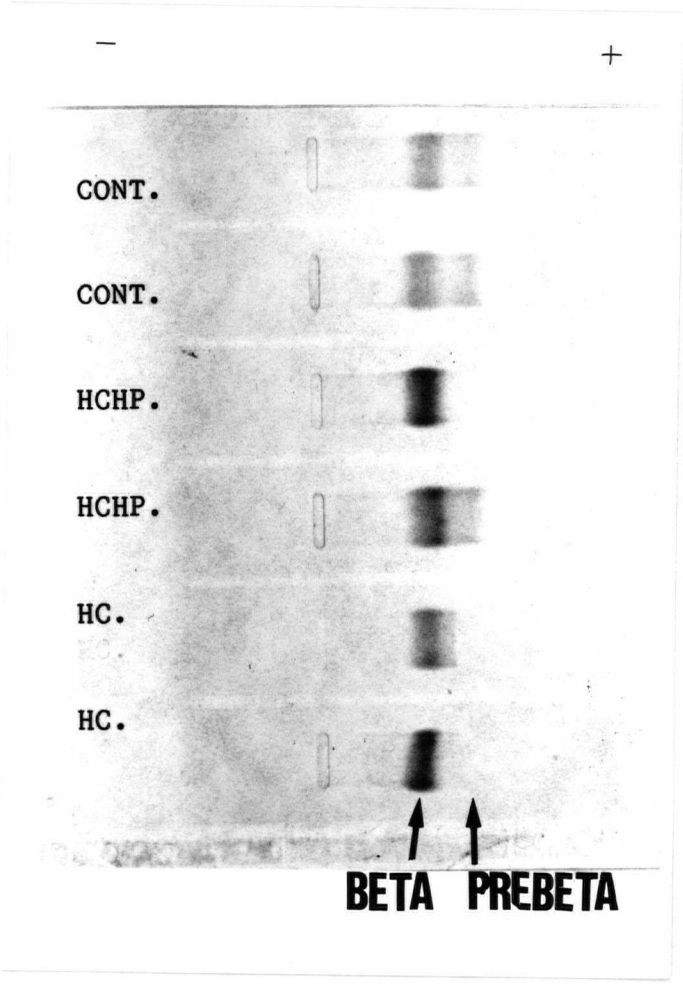

−
+
HC
CON
HCHP
ALPHA
PREBET
BETA

Figure 14:

Electrophoretic profile of the CONT, HCHP, and HC groups at day 10 on an agarose gel. Compared to the CONT group which shows beta and prebeta bands, different stages of blending of the slow prebeta band into the beta band can be seen in the HC and HCHP groups. The complete blending is presented as a single dark thick band.


−
+
CONT.
CONT.
HCHP.
HCHP.
HC.
HC.
BETA
PREBETA

III. Plasma LCAT Activity

Cholesterol feeding alone or with high protein supplement lowered plasma LCAT activity expressed by either FER or MER. However, the magnitude of the change differed with two different subtrates used. As shown in Table 10, with an endogenous substrate, FER was remarkably decreased at both day 30 and 70 in the HC and HCHP groups compared to the CONT and HP groups ($p < 0.01$). However, there was no significant change in MER in any of the four groups at the different experimental periods. On the other hand, using an exogenous substrate, both FER and MER showed a significant moderate decrease ($p < 0.05$), at day 70 in the HC and HCHP groups relative to the CONT group (Figure 16). As shown in Table 10, the CONT and HP groups had a significant decrease in FER ($p < 0.05$), at day 70 compared to day 0.

Figure 15:

Two lipoprotein fractions separated by preparative ultracentrifugation and resolved in 10% SDS-polyacrylamide. The HCHP, HC and CONT groups are presented in the two fractions. The same pattern of protein distribution is present in the two fractions in the HC and HCHP groups. In the fraction with hydrated density of d = 1.063 - 1.21 gm/ml, both HC and HCHP groups have double bands with an apparent molecular weight > 31000.

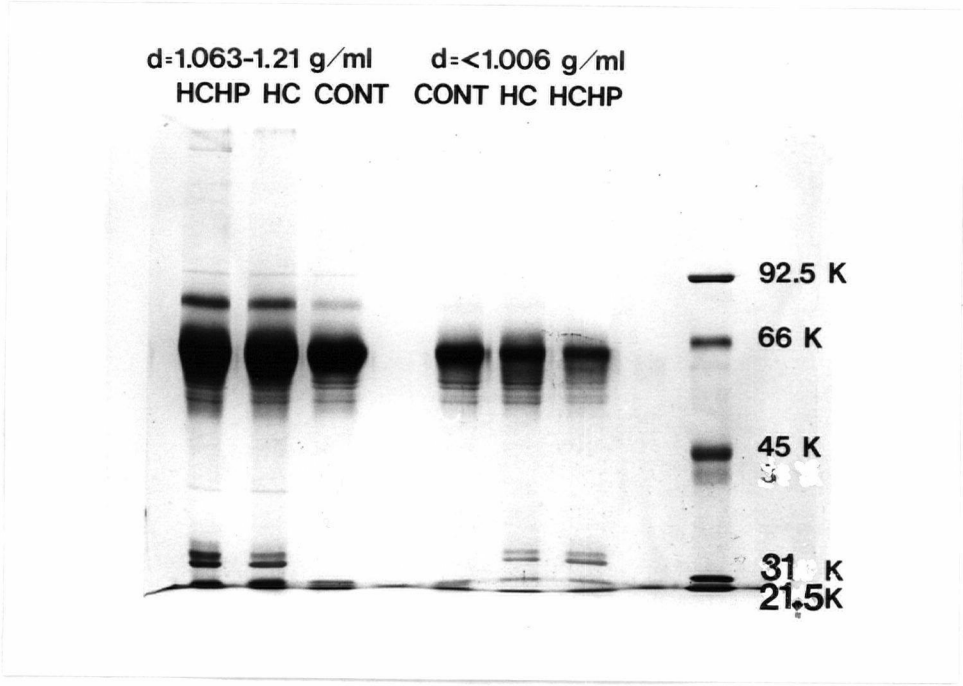

d=1.063-1.21 g/ml
d=<1.006 g/ml
HCHP HC CONT
CONT HC HCHP
92.5 K
66 K
45 K
31 K
21.5K

Fig - 16

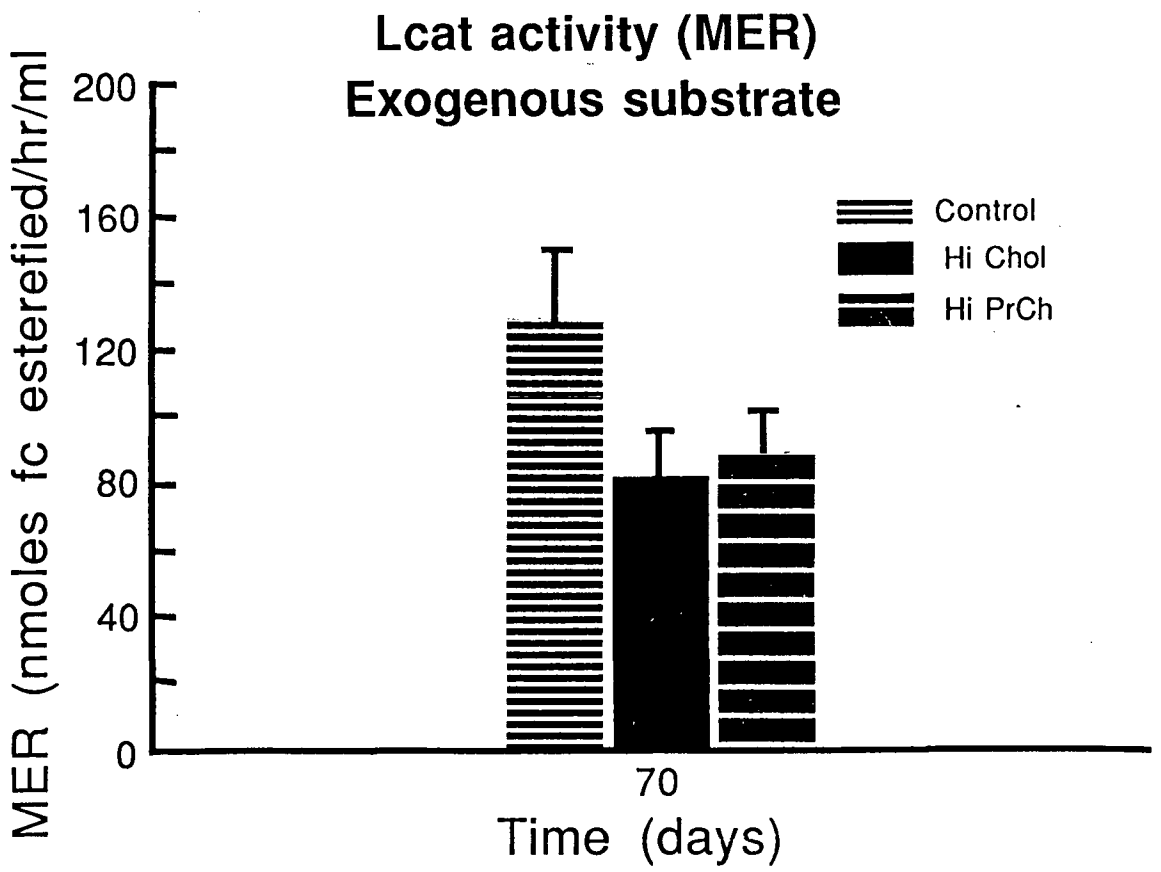

Lcat activity (MER)
Exogenous substrate
MER (nmoles fc esterefied/hr/ml
200
160
120
80
40
0
Control
Hi Chol
Hi PrCh
70
Time (days)

Table 10

[LCAT Activity Using Endogenous Substrate. Mean ± SD]

Group		Day 0	Day 30	Day 70
CONT n=6	FER[1]	10.5 ± 1.1	9.5 ± 2.2	7.7 ± 1.0[a]
	MER[2]	31.4 ± 6.4	27.6 ± 5.7	28 ± 8.3
HP n=6	FER	10.7 ± 3.4	8.6 ± 1.5	6.8 ± 0.8[a]
	MER	33.8 ± 13.8	25.3 ± 6.2	31.9 ± 12.0
HC n=6	FER	13.1 ± 2.5	1.0 ± 0.3[b]	1.2 ± 0.2
	MER	24.9 ± 4.9	21.6 ± 9.9	20.3 ± 2.1
HCHP n=6	FER	11.4 ± 1.5	1.0 ± 0.3[b]	1.4 ± 0.3
	MER	28.3 ± 5.9	20.6 ± 6.0	27.4 ± 14.2

(1) FER = Fractional Esterification Rate (%/hr)

(2) MER = Molar Esterification Rate [nmole (FC)/hr/ml plasma].
a = Significantly different than the control groups ($p < 0.01$)
(CONT at day 70 is compared to day 0)
b = Significantly less than the level at day 0 ($p < 0.05$)

IV Haematological Changes

1. Complete Blood Count:

Anaemia developed by day 70 in the HC and HCHP groups (Table 11). There were significant decreases in erythrocyte count, haemoglobin concentration, and haematocrit at day 70 in the HC and HCHP groups compared to the CONT and HP groups ($p < 0.001$). There was no sign of reduction in any of these parameters in any of these groups at day 30. Compared to day 0, the HP group at day 70 showed a significant increase in haematocrit ($p < 0.01$). In experiment II after treatment with acetylphenylhydrazine) there was a significant decrease at day 40 in the erythrocyte count, haemoglobin concentration ($p < 0.01$) and haematocrit ($p < 0.001$) compared to the values at day 0 (Table 12). Also, a remarkable increase in the mean corpuscular volume of erythrocyte ($p < 0.001$) was found at day 40. This finding was in accordance with the extensive reticulocytosis detected in the treated group. Mean corpuscular haemoglobin was also significantly increased at day 40 in these animals.

2. Erythrocyte Morphology and Fragility:

Numerous spiked-surface cells (echinocytes) were seen in the blood films of the HC and HCHP groups at day 30 (Figure 17). These echinocytes had a smaller size than normal cells. Poikilocytosis, anisocytosis, and reticulocytosis were not evident at that time. Abnormalities in size and shape of erythrocyte were more obvious at day 70. A population of target cells (an erythrocyte with a dark centre surrounded by a haemoglobin-poor zone) was detected in the HC and HCHP groups (Figure 18). These two animal

Table 11

[Complete Blood Count (CBC) and Erythrocyte Osmotic Fragility. Mean ± SD]

Group		Day 0	Day 30	Day 70
	RBC[1] count	5.27 ± 0.19	5.37 ± 0.33	5.43 ± 0.23
	Hb[2]	13.17 ± 0.54	13.7 ± 0.7	14.42 ± 1.1
CONT	HTC[3]%	41.4 ± 1.7	43.4 ± 1.9	44.4 ± 2.6
n=6	MCV[4]	79.2 ± 2.7	80.7 ± 3.4	81.7 ± 2.9
	O.F[5]	62 ± 4	61 ± 2	63 ± 3
	RBC count	5.03 ± 0.35	5.55 ± 0.11	5.85 ± 0.33
	Hb	12.48 ± 1.08	14.5 ± 0.84	15.6 ± 0.85
HP	HTC%	40.4 ± 3.2	45.4 ± 1.5	48.2 ± 3.6
n=6	MCV	80.8 ± 2.9	82.0 ± 1.6	82.8 ± 1.8
	O.F	58 ± 7	62 ± 5	66 ± 2
	RBC count	4.96 ± 0.27	5.39 ± 0.29	3.79 ± 0.81[e]
	Hb	13.12 ± 1.01	13.4 ± 0.78	7.9 ± 2.14[e]
HC	HCT%	40.9 ± 1.8	42.2 ± 2.4	30.0 ± 4.3[e]
n=6	MCV	82.7 ± 1.5	78.1 ± 0.8	84.2 ± 3.8
	O.F	64 ± 1	48 ± 4[e]	56 ± 5[b]
	RBC count	4.99 ± 0.45	5.61 ± 0.45	3.90 ± 0.96[e]
	Hb	12.33 ± 0.83	14.22 ± 1.11	9.45 ± 2.7[e]
HCHP	HCT%	40.8 ± 2.0	43.9 ± 4.0	27.8 ± 4.9[e]
n=6	MCV	80.0 ± 2.4	78.2 ± 2.9	82.0 ± 2.5
	O.F	62 ± 4	52 ± 4[b]	57 ± 4

(1) RBC count = RBC x 10^6/ml blood

(2) Hb = Haemoglobin (gm/dL blood)

(3) HCT% = Percentage of haematocrit (packed cell volume)

(4) MCV = Mean Corpuscular Volume (femto liter)

(5) O.F = Osmotic Fragility x 10^{-3}

(NaCl molar solution producing 50% haemolysis)

b = Significantly less than the values at day 0 ($p < 0.01$)

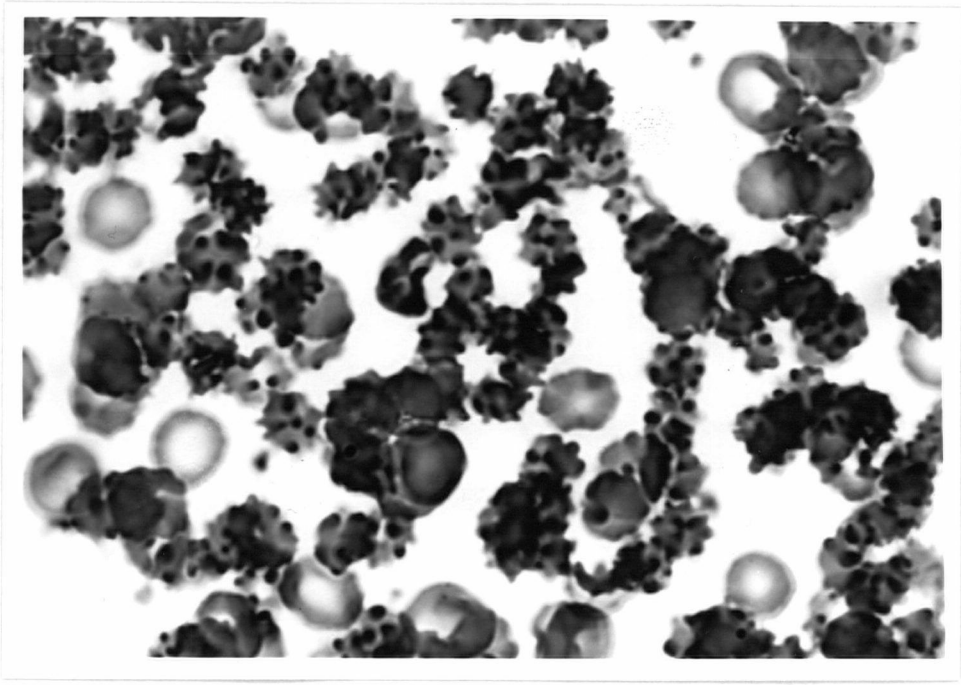
e = Significantly different than the control groups ($p < 0.001$)

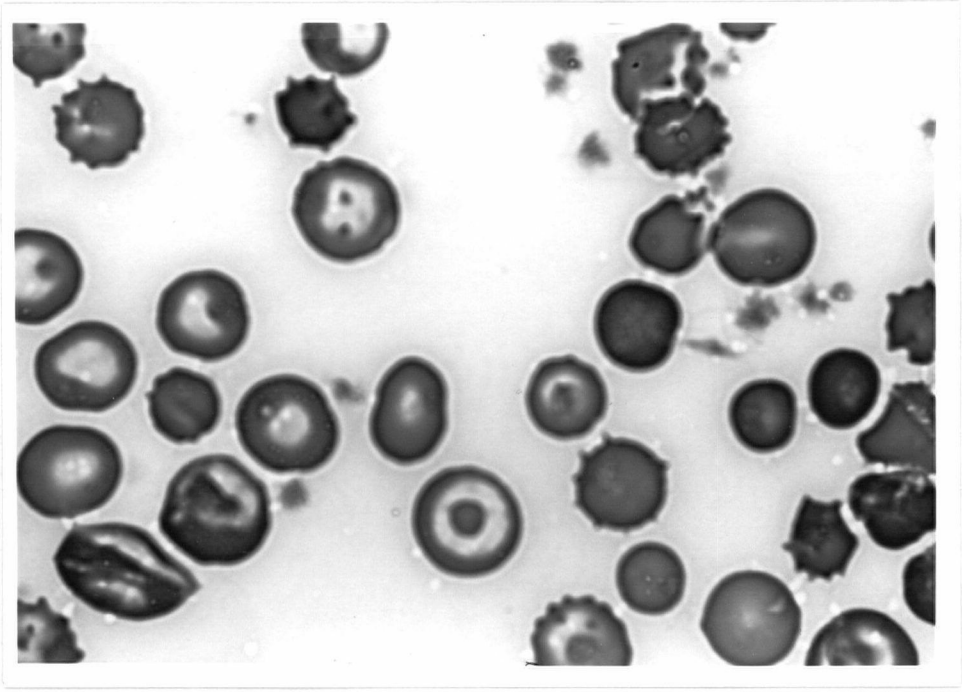
Figure 17:

A peripheral blood smear from an animal kept on a cholesterol-rich diet at day 30. A large number of echinocytes (spiked-surface erythrocytes) are present. (Wright-Giemsa Stain x 1000)

Figure 18:

A peripheral blood smear from a cholesterol-fed guinea pig revealing a target cell, few echinocytes, and stomatocyte. (Wright-Giemsa stain x 1000).





groups had another abnormal erythrocyte form, a hypochromic cell (HCC) (a bigger-than-normal erythrocyte with a remnant amount of haemoglobin located at the periphery of the cell). Morphologically, the HCHP group had more HCC than HC group (Figure 19). The number of echinocytes decreased by day 70 in both groups. Reticulocytosis was also evident at day 70 in the HC and HCHP groups, although its magnitude did not correspond to the degree of anaemia in these animals. However, reticulocytosis was more marked in the HCHP group than in the HC group.

As shown in Table 11, erythrocyte osmotic fragility was significantly decreased at days 30 and 70 in the HC group ($p < 0.001$, < 0.01, respectively) compared to the value at day 0. The animals of the HCHP group had a significant decrease only at day 30 ($p < 0.01$) but not at day 70.

3. Erythrocyte Lipid Profile:

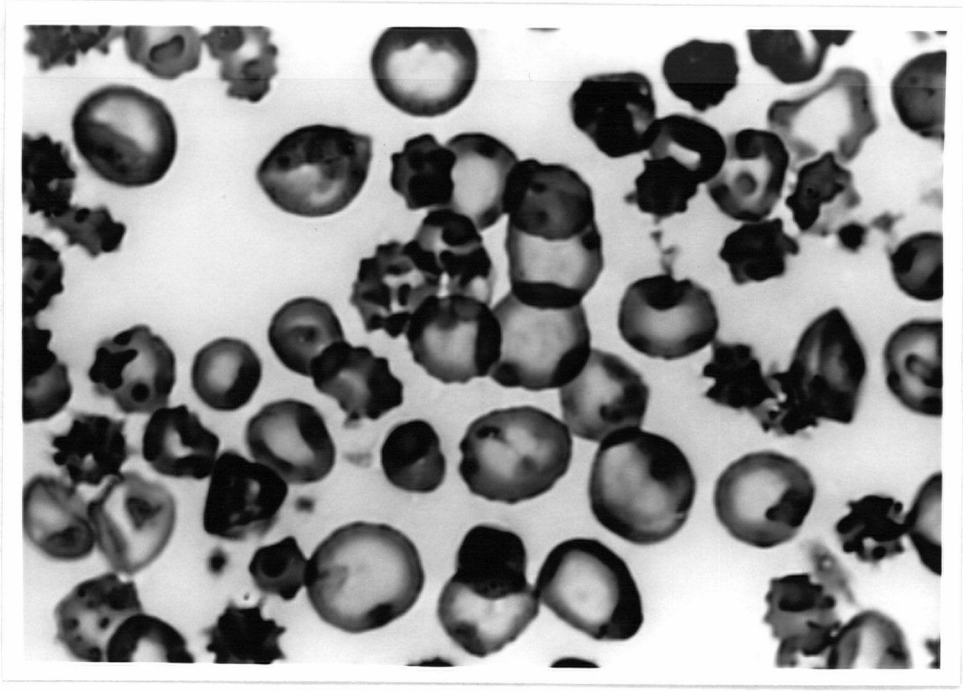
Table 13 summarizes the observed changes in total cholesterol and phospholipids in erythrocytes. A significant increase in cholesterol was found at days 30 and 70 in the HC group compared to the CONT group and to the day 0 level ($p < 0.05$). While the HCHP group showed the same increase at day 30, there was no statistically significant change at day 70 compared to the CONT group. A negative insignificant correlation was found between the increase in erythrocyte cholesterol and the decrease in osmotic fragility among the experiment groups ($r = -0.69$, $p > 0.1$). It appeared that increases in erythrocyte TC and sp/pc were accompanied by the appearance of large numbers of echinocytes in the peripheral blood, whereas the appearance of HCC was noticed when there was no significant change in TC and sp/pc.

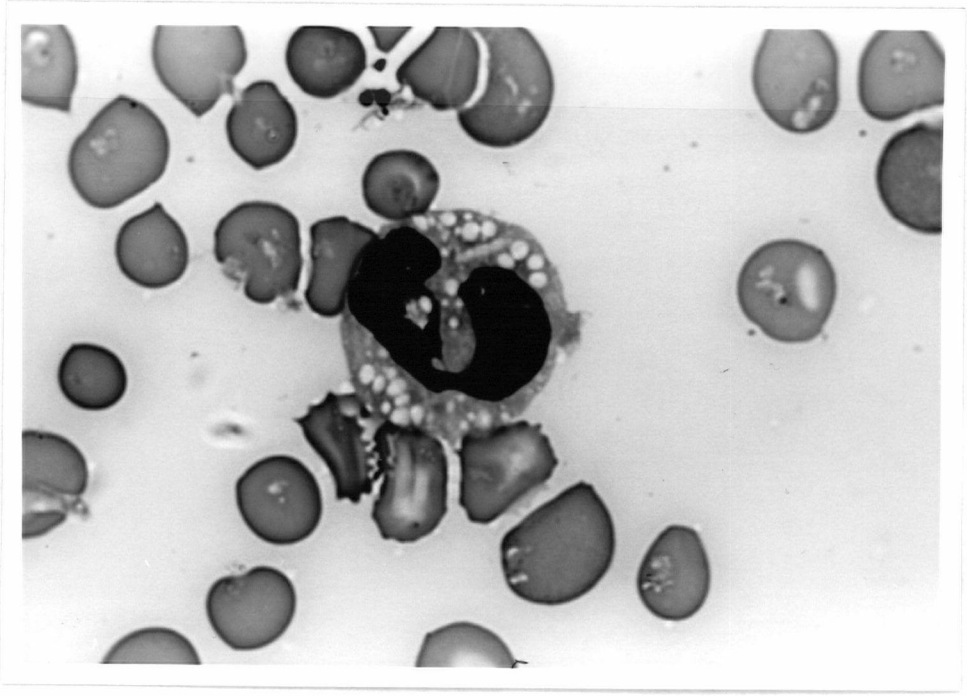
Figure 19:

A peripheral blood smear from the HCHP group at day 70. The echinocytes are diminished in number, while the predominant erythrocyte form is a hypochromic cell (HCC) (larger-than-normal erythrocyte with a fine rim of haemoglobin and a big pale center with a corrugated boundary). (Wright-Giemsa stain x 1000).

Figure 20:

A peripheral blood smear from the HCHP group at day 70 showing a vacuolated monocyte. (Wright-Giemsa x 1000).





4. Characterization of the Vacuolated White Blood Cells:

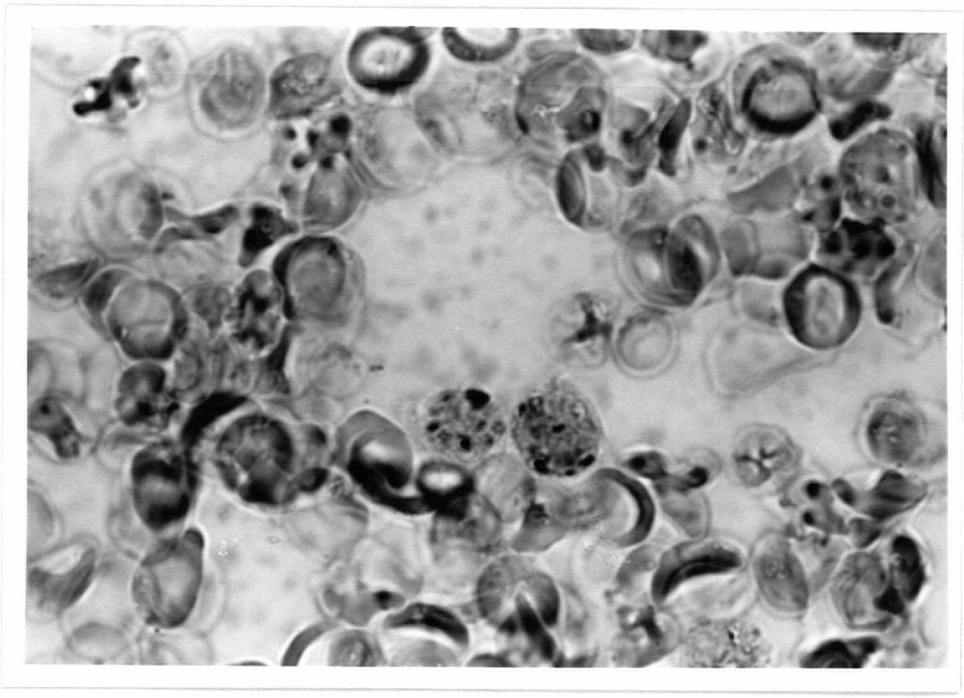
Peripheral blood films from the HC and HCHP groups at day 70 stained with Wright-Giemsa stain showed vacuolated white blood cells (WBC) with indented nuclei; unlike lymphocytes, these cells have less compact nuclear chromatin, and the nuclei do not occupy the major part of cytoplasm, which seems agranular. Such cell was considered vacuolated when showing more than three cytoplasmic vacuoles (Figure 20). To identify the nature of the vacuole, blood films were fixed with formalin fumes and stained with Oil Red-O (ORO). As illustrated in Figure 21, the vacuoles stained positively with ORO. Further identification of these cells was undertaken using nonspecific esterase stain (NSE). As shown in Figure 22, the cytoplasm of these cells contained reddish brown different-sized particles. Neutrophils, usually, show a mild positive reaction, but the extensiveness of the reaction and the morphological characteristics (as described above) suggest strongly that these cells are monocytes laden with lipid particles. These cells were not seen in peripheral film of the HC group at days 5, 10 or 30. However, at day 70 these cells with a 14.6% of peripheral blood monocytes were found in the HC group. On the other hand, in the HCHP group, lipid-laden monocytes (LLM) appeared with a 0.4% of peripheral blood monocytes at day 30, whereas at day 70 they showed a larger population of 31.2% of the monocytes. A positive correlation was found between the percentage of LLM among blood monocytes and the concentration of plasma TC at day 70 ($r = 0.70$, $p < 0.05$).

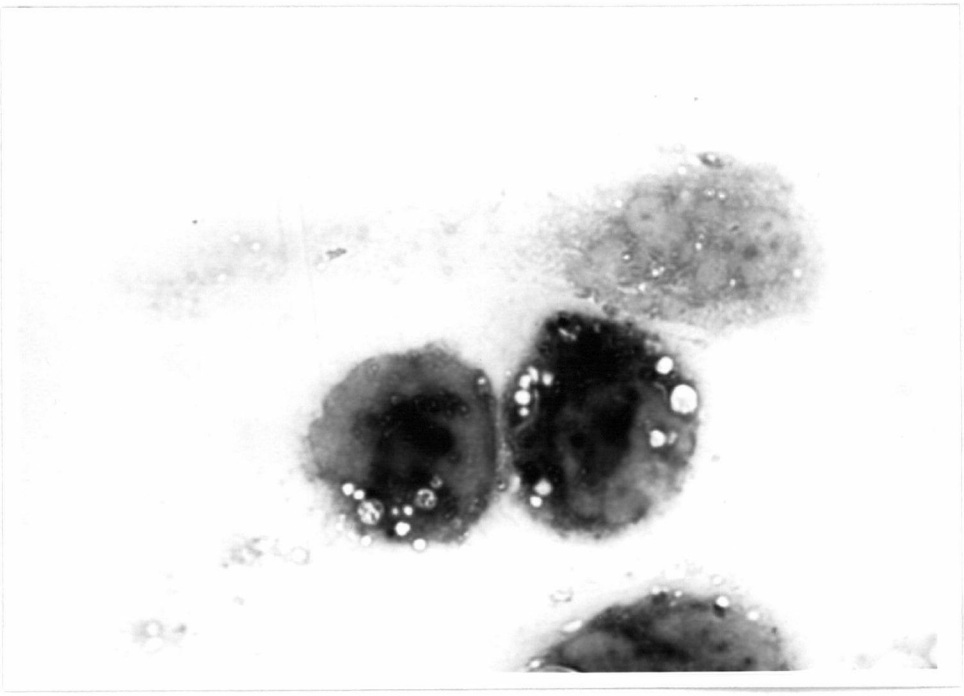
Figure 21:

A peripheral blood smear from a cholesterol-fed animal at day 70 depicting two monocytes in the center of the field with intracytoplasmic Oil-Red O positive droplets (ORO-Stain x 1000).

Figure 22:

A peripheral blood smear from a cholesterol-fed animal at day 70. Two vacuolated NSE-positive monocytes are shown (NSE-stain x 1000).





(AcetylphenylHydrazine (APH) - Induced Anaemia Experiment)

Table 12

[Complete Blood Count. Mean ± SD]

Experimental Period	RBCx 10^6	Hb(g/dL)	HTC%	MCV(fL[1])	MCH(Pg[2])
Day 0	4.9 ± 0.1	12.8 ± 0.3	40.7 ± 1.1	82.7 ± 1.2	25.9 ± 0.6
Day 40	3.9 ± 0.2[b]	11.7 ± 0.3[b]	35.9 ± 0.9[e]	95.3 ± 1.2[e]	30.7 ± 0.9

(1) MCV (fL) = Mean Corpuscular Volume (femtoliter)

(2) MCH (Pg) = Mean Corpuscular haemolglobin (Picogram)

n = 6 (number of the animals)

b = Significantly different than the values at day 0 ($p < 0.01$)
e = Significantly different than the values at day 0 ($p < 0.001$)

Experiment I

Table 13

[Lipid Profile of Erythrocyte. Mean ± SD]

Groups		Day 0	Day 30	Day 70
CONT n=6	TC[1]	0.94 ± 0.9	0.87 ± 0.17	0.94 ± 0.2
	SP/PC[2]	0.35 ± 0.03	0.35 ± 0.03	0.43 ± 0.11
HP n=6	TC	1.11 ± 0.17	1.22 ± 0.21	1.29 ± 0.26
	SP/PC	0.26 ± 0.1	0.41 ± 0.09	0.39 ± 0.12
HC n=6	TC	1.04 ± 0.11	1.42 ± 0.05[a]	1.77 ± 0.5[a]
	SP/PC	0.20 ± 0.10	0.57 ± 0.11[a]	0.61 ± 0.14[a]
HCHP n=5	TC	0.93 ± 0.21	1.61 ± 0.37[a]	1.39 ± 0.32
	SP/PC	0.33 ± 0.01	0.55 ± 0.03[a]	0.38 ± 0.04

(1) TC = Total Cholesterol (mg/mL packed RBC)

(2) SP/PC = Spingomyelin/Phosphatidylcholine ratio

a = Significantly different than the control groups ($p < 0.05$)

V Changes in Liver Function:

Liver function was assessed by measuring serum direct and indirect bilirubin, SGOT(AST), total protein, and albumin at different experimental periods (see "Methods"). The earliest change was detected in AST, namely, a significant increase ($p < 0.05$) at day 5 of the experiment in the HCHP group compared to the CONT group (Table 14). At day 10 both HC and HCHP groups showed a significant elevation in AST ($p < 0.01$) compared to the CONT group. Such an increase with a higher level of significance was also present at day 30 and 70 in the HC and HCHP groups. Interestingly, the HP group also showed a significant increase in AST at day 70 ($p < 0.02$) compared to the CONT group. However, the CONT group showed a gradual increase in AST throughout the experimental periods; the first significant increase, compared to the day 0 level, was noticed at day 30 ($p < 0.01$).

The AST changes were the same in the HCHP and HC groups. Indirect bilirubin increased significantly in the HCHP group at day 70 compared to the HC group ($p < 0.002$). Both groups also had an elevated direct bilirubin at day 70 compared to the CONT group. However, there was no significant change between the HC and HCHP groups. Except for the day 70, no significant differences between the HC and HCHP groups were detected throughout the experimental periods. The increase in indirect bilirubin (in the HCHP group) and direct bilirubin (in the HC and HCHP groups) appeared to be correlated with the severity of haemolysis in both groups (see "Discussion" for further analysis of the data).

Table 14 also shows the serum total protein and albumin levels at different periods. No change in albumin was found between the groups at

Table 14

[Liver Function Tests at Different Periods. Mean ± SD]

Days		Group	Ind.bili	D.bili	SGOT(AST)	TP	Alb.
	n=13	CONT**	.08 ± .02	.09 ± .02	100.4 ± 49	4.6 ± 0.3	2.3 ± .1
5	n=5	HC	.07 ± .01	.08 ± .02	86.5 ± 37.9	4.7 ± .19	2.3 ± .07
	n=5	HCHP	.09 ± .02	.09 ± .03	117.2 ± 74.6[a]	4.7 ± .2	2.4 ± .2
10	n=5	HC	.08 ± .03	.09 ± .01	225 ± 51[b]	4.8 ± .2[b]	2.3 ± .1
	n=5	HCHP	.09 ± .02	.09 ± .02	181 ± 44.8[b]	5.1 ± .3[b]	2.4 ± .1
30	n=5	HC	.09 ± .01	.09 ± .02	366.6 ± 105[b]	4.82 ± .2[b]	2.4 ± .1
	n=5	HCHP	.09 ± .02	.09 ± .03	316 ± 83.4[b]	5.03 ± .2[b]	2.5 ± .14
70*	n=6	HC	.12 ± .05	.3 ± .02[b]	510 ± 30[b]	5.4 ± .2[b]	2.6 ± .1
	n=6	HCHP	.5 ± .3[f]	.7 ± .5[b]	510 ± 10[b]	5.3 ± .2[b]	2.4 ± .1
	n=5	HP	.09 ± .02	.3 ± .2	209 ± 15	5.4 ± .2	2.6 ± .1

n = Number of animals/group

* = Data from experiment III

SGOT(AST) = Aspartate transaminase (IU/L)

Ind. bili = Indirect bilirubin (mg/dL)

D. bili = Direct bilirubin (mg/dL)

TP = Total protein (gm/ L)

Alb = Albumin (gm/ L)

a = Significantly different than CONT ($p < 0.05$)

b = Significantly different than CONT ($p < 0.01$)

f = Significantly different than HC at day 70 ($p < 0.002$)

** = Data of days 5,10,30 and 70 are combined due to the absence of significant difference

all periods, whereas total protein in the HC and HCHP groups was significantly increased ($p < 0.01$) at days 10, 30 and 70 compared to the CONT group.

Taken together, although hepatic injury was evident by the increase in AST particularly in the HCHP and HC groups, hepatic failure was unlikely, as indicated by the normal level of albumin and normal conjugation response for the increase in indirect bilirubin in the HCHP group.

VI Renal Function

Changes in blood urea nitrogen (BUN), serum creatinine (Cr), 24-hour urinary protein (UPr), creatinine clearance (Ccr) and haematuria are shown in Table 15.

1. Urinary Findings:

Twenty-four-hour urinary protein significantly increased at day 70 in the HC and HCHP groups ($p < 0.001$) compared to the CONT and HP groups (Figure 23). A markedly elevated UPr was found in the HCHP group at day 70 compared to the HC group ($p < 0.001$). Despite the tendency to show higher UPr than CONT group, the difference was not statistically significant in the HP group. Twenty-four-hour urinary protein was not measured before day 70 because the semiquantitative data obtained in the preliminary experiments showed absence of significant proteinuria.

No significant change could be detected in Ccr at day 70 between the four groups, except for a tendency toward an increase in the HP group . This was not the case with haematuria as an indicator for glomerular basement membrane damage. Semiquantitatively, HC group had at day 70 (1+) level of erythrocyte in urine compared to the (-) level in the CONT and HP groups, while the HCHP group showed haematuria of (4+) level. This finding was positively correlated ($r = 0.73$, $p < 0.01$) with the mesangial area/glomerular area ratio. Again, no haematuria was detected in the preliminary study (up to day 30).

Fig - 23

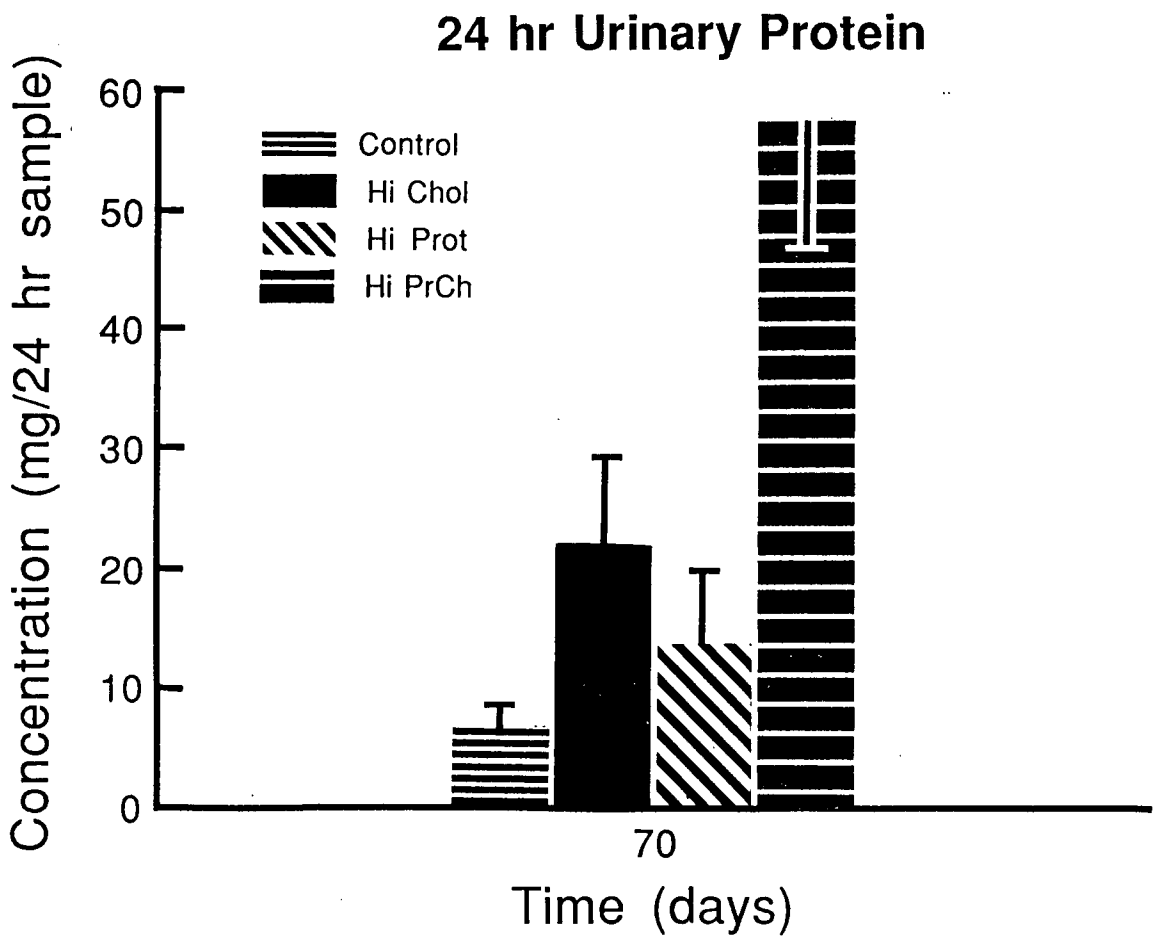

24 hr Urinary Protein
Concentration (mg/24 hr sample)
60
50
40
30
20
10
0
Control
Hi Chol
Hi Prot
Hi PrCh
70
Time (days)

Table 15

[Kidney Function Tests at Day 70. Mean ± SD]

Group	Ccr[1]	U.Prot.[2]	BUN[3]	Cr[4]	RBC/urine[5]
CONT (n=4)	8.3 ± 0.4	6.4 ± 2.3	18 ± 2.8	0.3 ± 0.05	(-)
HP (n=5)	10.2 ± 4.9	13.7 ± 6.3	38.7 ± 4.5[e]	0.27 ± 0.09	(-)
HC (n=6)	9.4 ± 2.3	22.1 ± 7.2[e]	36.6 ± 2.7[g]	0.24 ± 0.05	(+)
HCHP (n=6)	8.2 ± 3.7	57.9 ± 10.3[e]	64 ± 27[e]	0.25 ± 0.1	(4+)

(1) Ccr = Creatinine Clearance (mL/min)

(2) U.Prot. = Twenty four-hour urinary protein (mg/24hr)

(3) BUN = Blood Urea Nitrogen (mg/dL)

(4) Cr = Serum creatinine (mg/dL)

(5) RBC/Urine = Number of erythrocytes in 24-hr urine sample (high-power field)

n = Number of animals/group
e = Significantly different than other groups ($p < 0.001$)
g = Significantly different than CONT ($p < 0.02$)

2. Serum Findings:

As shown in Tables 15 and 16, BUN was increased in the HCHP group at days 5, 10, 30 and 70. This increase was significantly different from that in HC and CONT groups ($p < 0.001$), while in the HC group the increase was significant ($p < 0.02$) only at day 70. Both HC and HCHP groups showed a significant increase in BUN relative to the CONT group at day 70. However, this was less than in the HCHP group ($p < 0.001$). Serum creatinine (Tables 15 and 16) did not show a significant change in any of the groups throughout the different experimental periods. The HP, HC, and CONT groups showed a significant increase in Cr at days 30 and 70 compared to that at days 0 and 10 ($p < 0.01$), whereas there was no change in this parameter in the HCHP group.

Serum phosphate: No significant change was found between the different experimental groups.

VII Renal Tissue Alterations

1. Histopathology, histochemistry, histoimmunofluorescence, and electron microscopy:

Histological sections of kidneys from the CONT group revealed normal glomeruli and renal tubules (Figure 24). The glomeruli had patent capillary lumina with a modest amount of mesangial matrix and cellularity. The HP group had a similar normal kidney structure except for enlarged glomeruli. At day 70, the histopathological changes were variable in the HC and HCHP groups. The increase in mesangial matrix and cellularity varied from glomerulus to glomerulus. Glomerular hypercellularity (Figure 25) in the HC and HCHP groups was constituted by an increased number of mesangial and

Table 16

[Blood Urea Nitrogen and Serum Creatinine. Mean ± SD]

Group		Day 5	Day 10	Day 30
CONT** (n=9)	BUN	16.7 ± 2.6	16.7 ± 2.6	16.7 + 2.6
	Cr	0.2 ± .07	0.2 ± .07	0.2 ± .07
HC (n=5)	BUN	15.8 ± 1.6	15.7 ± 1.2	20.8 ± 1.3
	Cr	.3 ± .07	.15 ± .02	.3 ± .07[b]
HCHP (n=5)	BUN	28.2 ± 7.6[e]	32.5 ± 5.8[e]	40.3 ± 3.6[e]
	Cr	.18 ± .04	.13 ± .05	.2 ± .07

n = Number of animals/group

BUN = Blood Urea Nitrogen (mg/dL)

Cr = Serum Creatinine (mg/dL)

b = Significantly different than days 5 and 10 ($p < 0.01$)

e = Significantly different than other groups ($p < 0.001$)

** = Data of days 5,10,30 and 70 were combined due to the absence of significant changes among the animals of the control group

intracapillary cells - mostly mononuclears with few neutrophils. Intracytoplasmic vacuoles were noted in some intracapillary and mesangial cells; few vacuoles were extracellularly distributed. The intracapillary vacuolated cells were mononuclear leukocytes and endothelial cells. No changes were noted in the interstitium, tubules or small arteries or arterioles except for an occasional intracytoplasmic vacuole in arteriolar endothelium. Mild mesangial expansion was detected in both HC and HCHP groups at day 30, but there were no changes either at day 10 or 5. Occasional extramedullary haematopoiesis was found in the glomeruli of the HCHP and HC group at day 70 (Figure 26).

In the animals injected with APH, there were no salient glomerular histological alterations. However, their renal tubular epithelial cells were loaded with Perl's Prussian blue (PPb) positive particles (Figure 27).

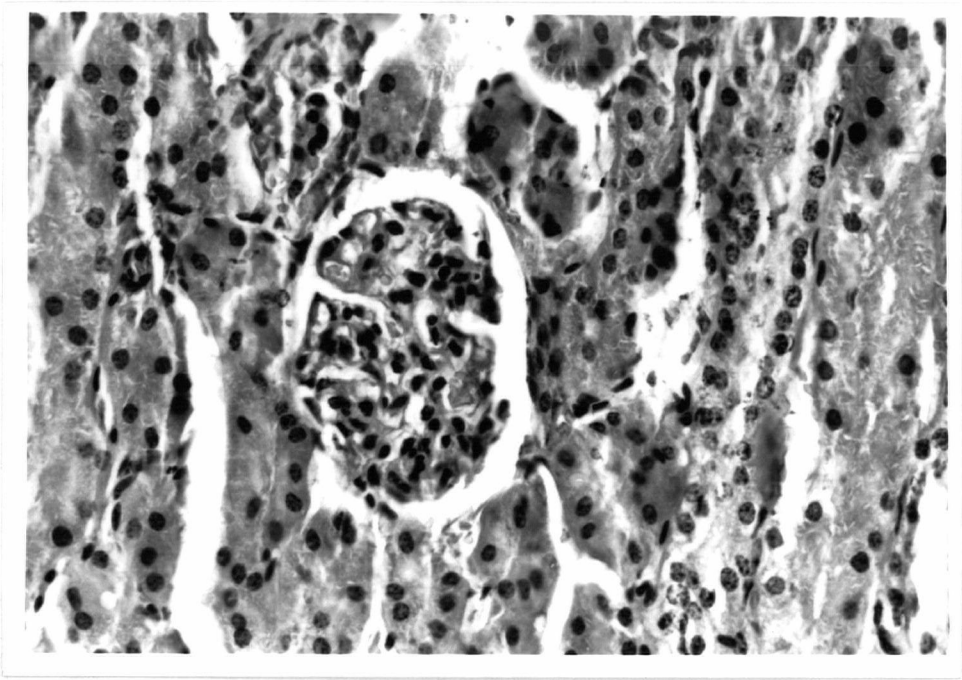
Oil-red O (ORO) staining was diffusely positive in glomeruli and focally present in tubules and arterioles of the HC and HCHP groups at day 70 (Figure 28 and 29). A positive ORO reaction, although variable was detected predominantly in the glomeruli and some tubules at day 30 in both HC and HCHP groups. Also some animals of these two groups showed mild staining for ORO at day 5 and 10. Frozen histological sections from the CONT group were negative for this stain. Proximal tubular epithelial cells of the CONT group positively showed a strong diffuse cytoplasmic reaction for NSE stain. However, most of the glomeruli in the CONT group had no NSE-positive cells. On the contrary, many glomeruli in the HC and HCHP groups at day 70 contained moderate numbers of NSE-positive cells (Figure 30). Some of these cells were vacuolated. There was no significant

Figure 24:

A kidney section from the CONT group at day 70. A normal glomerulus is depicted. (H&E x 250).

Figure 25:

A kidney section from the HCHP group at day 70. The glomerulus is enlarged and hypercellular (H&E x 250).



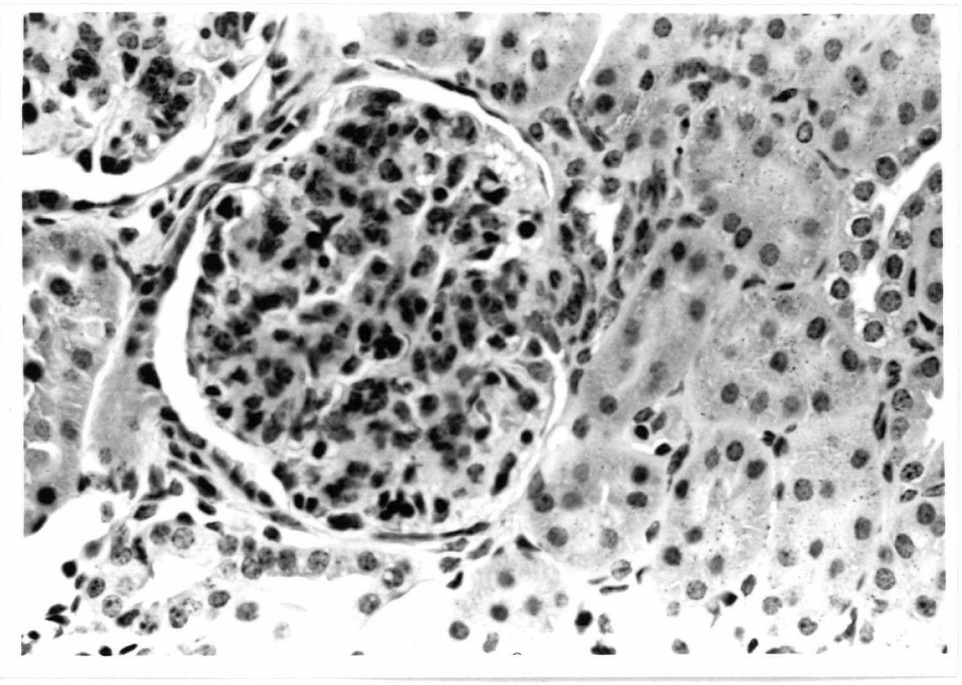
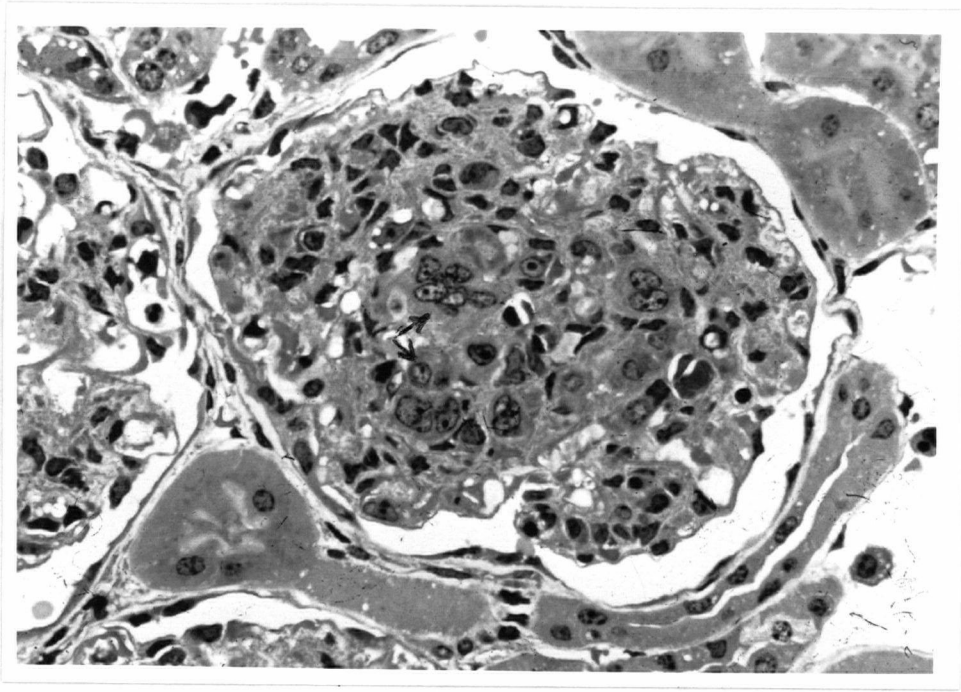


Figure 26:

A kidney section from the HCHP group at day 70 stained with H&E. Note the presence of megakaryocytes (arrows) indicating extramedullary haematopoiesis (H&E x 400).

Figure 27:

A kidney section from a cholesterol-fed guinea pig at day 70 showing the presence of Perl's Prussian blue-positive deposits in the epithelial cells of the renal tubules. No positive reaction is present in the affected glomerulus (Perl's Prussian blue x 250).



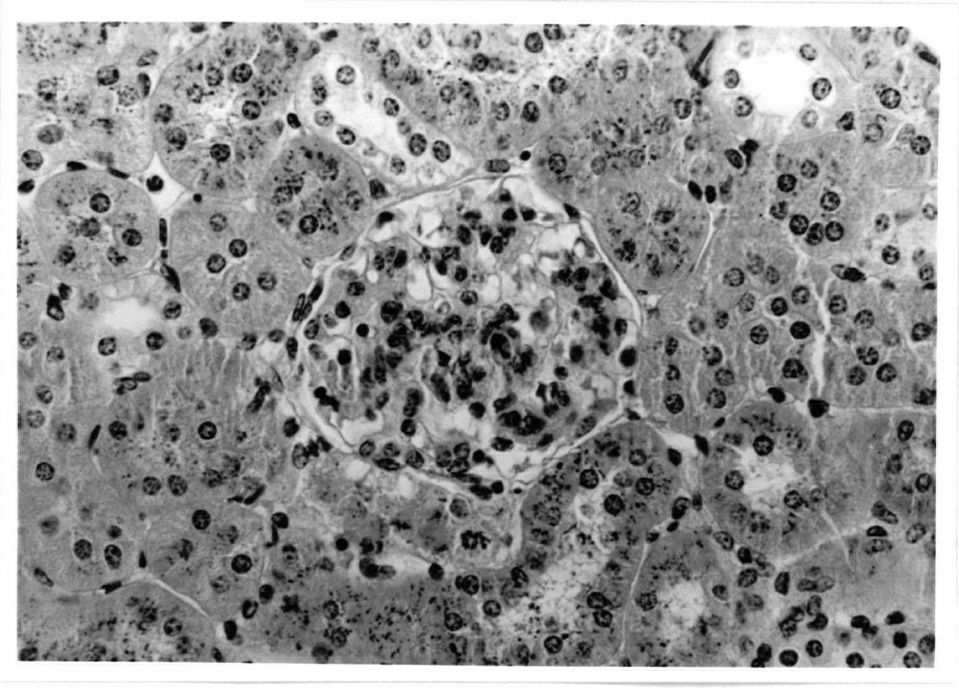
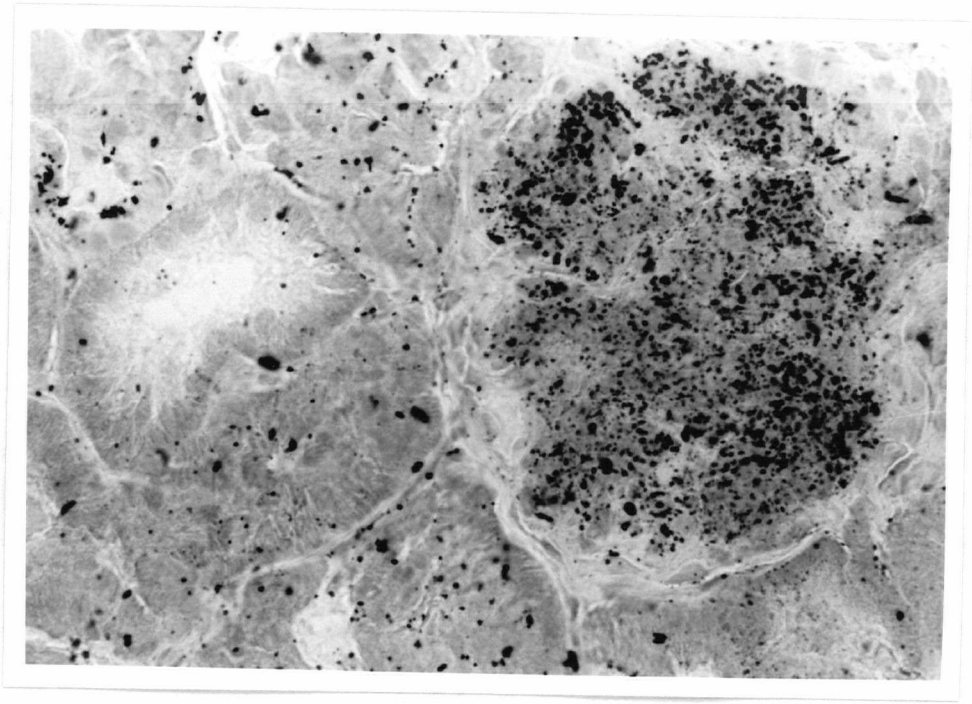


Figure 28:

A frozen kidney section from a cholesterol-fed animal at day 70. The glomerulus is filled with Oil-Red O positive multi-size particles (ORO Stain x 250).

Figure 29:

A frozen kidney section from the HCHP group at day 70 depicts renal tubules filled with Oil-Red O positive particles which are found in the epithelial cells and inside the tubular lumen (ORO Stain x 250).



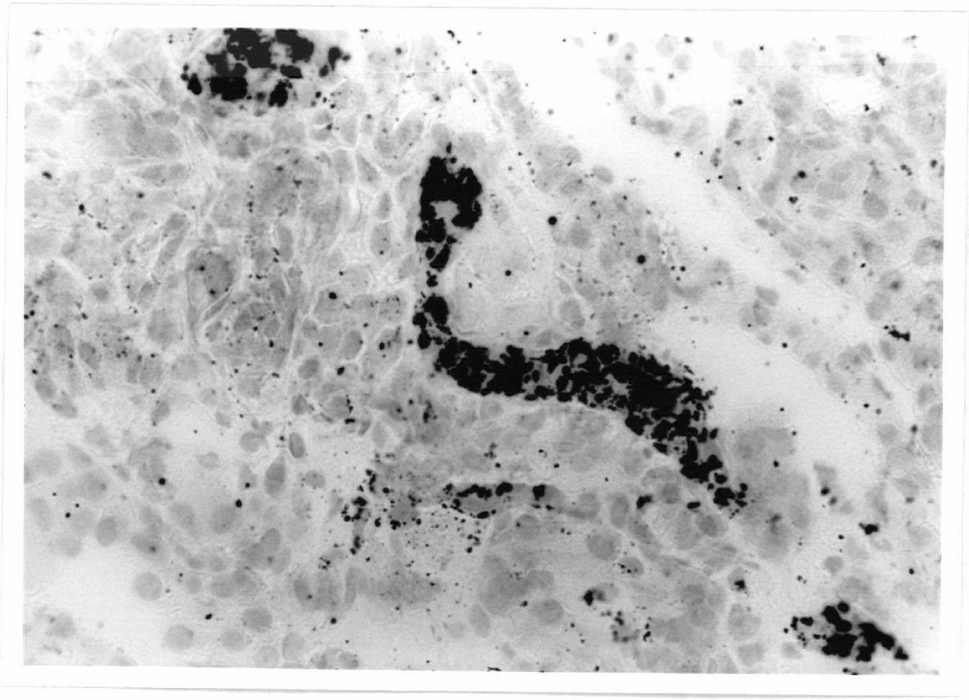


Figure 30:

A glomerulus with NSE positive cells in the kidney of a cholesterol-fed guinea pig at day 70. One of these cells appears in the center with two intracytoplasmic vacuoles (arrow) (NSE x 400).

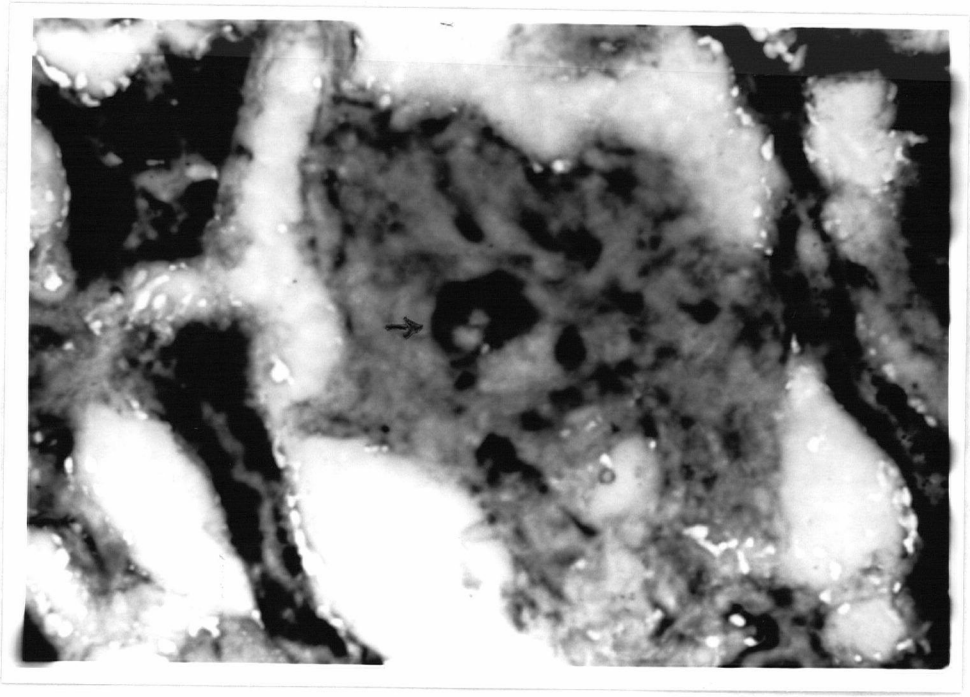
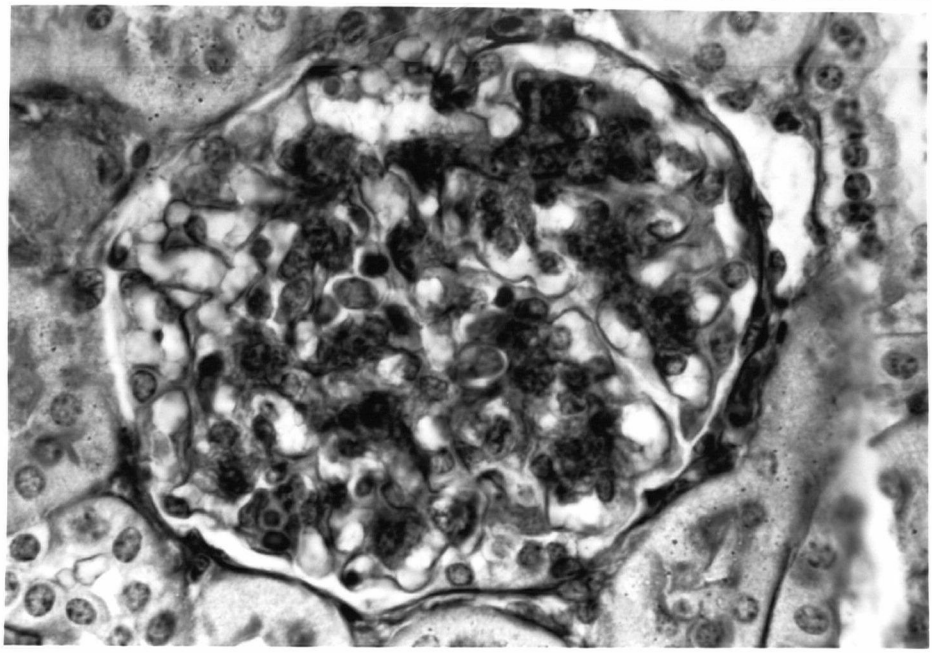


Figure 31:

A kidney section from the HCHP group at day 70 stained with PAS stain. The mesangium is expanded (PAS x 400).



increase in the number of intraglomerular NSE-positive cells at day 30 in either of HC or HCHP groups. More extensive PPb-positive deposits were found in the renal tubular epithelium of the HCHP group than in the HC group at day 70. No such deposits were present at day 30. The PAS stain revealed an increase in mesangial matrix in the HCHP and HC groups compared to other groups at day 70 (Figure 31). No von Kossa-positive lesions were detected in either HC or HCHP groups.

The histoimmunofluorescence study showed that there was no staining for guinea pig IgG in the kidneys of the HC and HCHP groups.

The normal glomerular ultrastructure of a CONT animal is depicted in Figure 32. At day 70 in both HC and HCHP groups (Figures 33-37), clear vacuoles were present inside intracapillary monocytes, endothelial cells and the mesangial cells. Some of these mesangial cells had the cytoplasmic characteristics of monocytes (lysosomes, rough endoplasmic reticulum and vesicles). The increase in the mesangial matrix was variable in the HC and HCHP groups. There were no electron dense deposits nor was there any significant change in the glomerular basement membranes (GBM) in the test groups. However, fusion of foot processes was evident. Some intracapillary mononuclear cells had large number of ribosomes but no rough endoplasmic reticulum, lysosomes, vesicles, or filaments. They were most likely lymphoid or haematopoietic cells.

2. Morphometry:

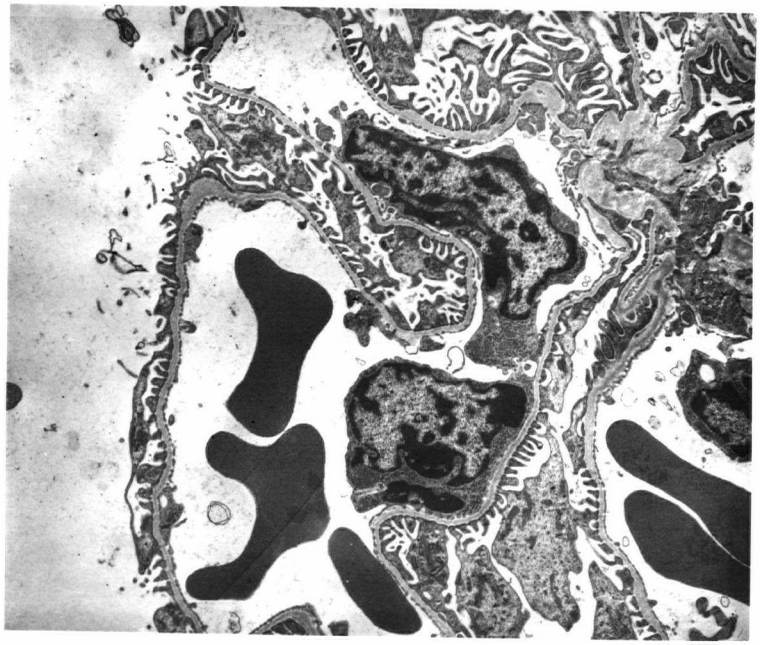
The data of the morphometric analyses of all groups throughout the different experimental periods are presented in Table 17. Mesangial area/ glomerular tuft area (MA/GTA) ratio was significantly higher in the HC and

Figure 32:

A glomerulus of a control animal at day 70. Capillary loops are widely patent and foot processes are intact. There was no increase in mesangial matrix. (EM x 3000).

Figure 33:

A glomerular endothelial cell with multi-sized lipid droplets from a cholesterol-fed animal at day 70. There is focal fusion of the foot processes. (EM x 4400).



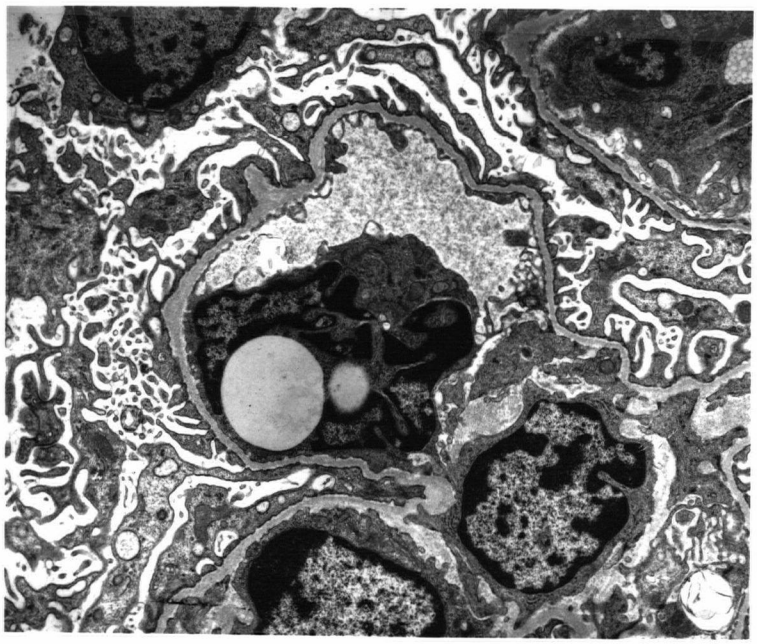


Figure 34:

A glomerulus from a cholesterol-fed animal at day 70. Note the intracapillary monocytes each of which contains some clear vacuoles. (EM x 3000).

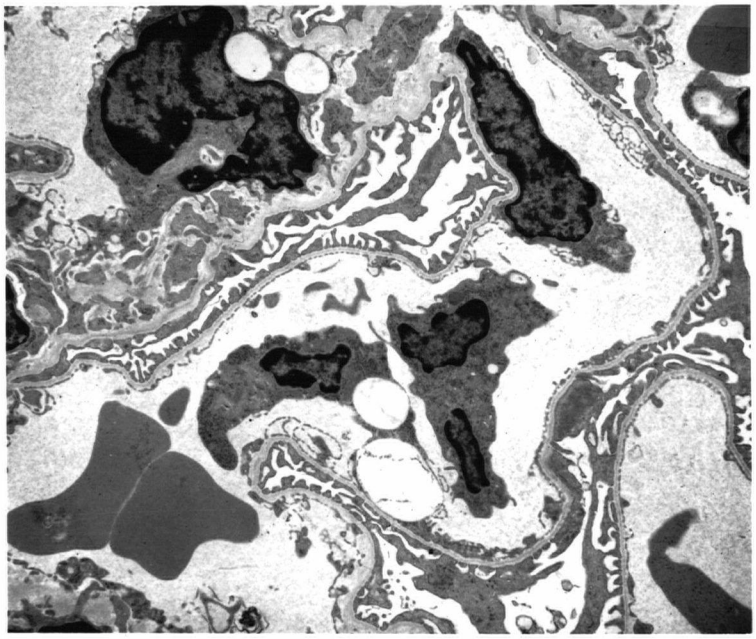


Figure 35:

Ultrathin section from kidney of the HC group at day 70 reveals the expanded mesangium. (EM x 3000).

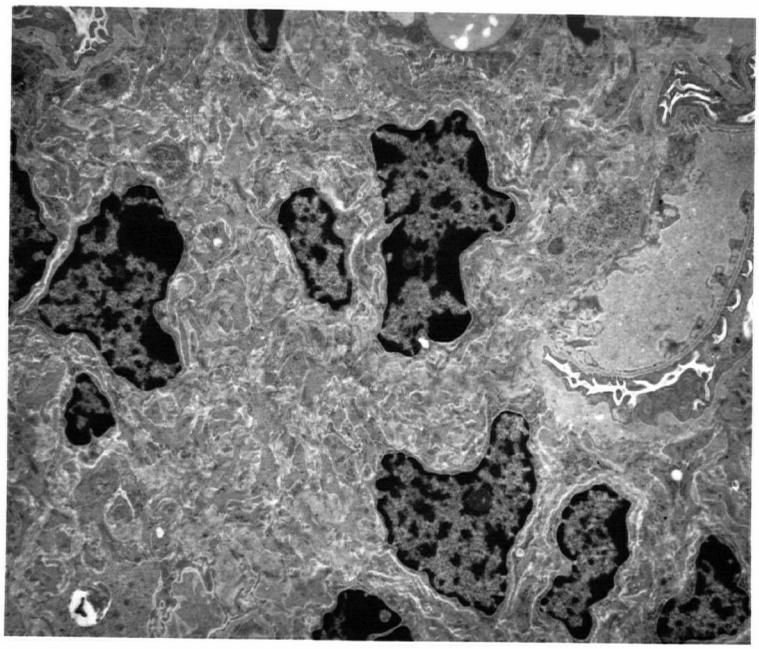
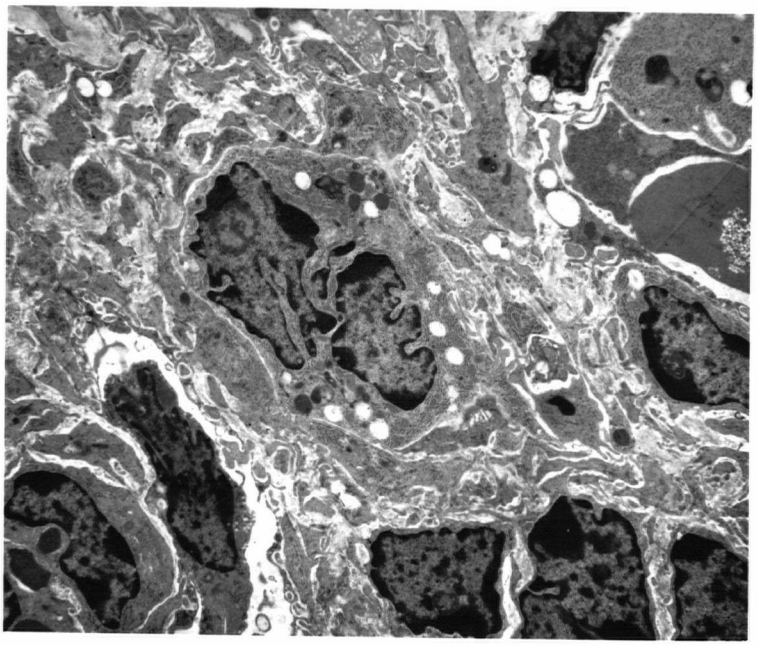


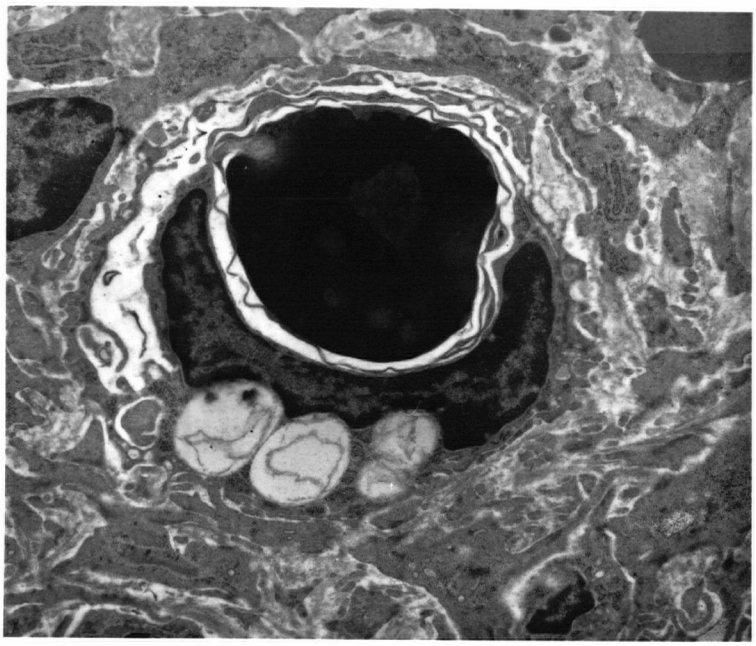
Figure 36:

An electron micrograph reveals a vacuolated cell with some of the characteristics of a monocyte in the expanded glomerular mesangium in a HCHP animal at day 70. (EM x 7000).

Figure 37:

Erythrophagocytosis in the mesangium of an animal in the HCHP group at day 70. Note a macrophage with at least three lipid droplets engulfing an erythrocyte. (EM x 7000).





HCHP groups than in the CONT and HP groups at day 70 ($p < 0.001$). The (MA/GTA) ratio in the HCHP group was higher than in the HC group at day 70 ($p < 0.05$). There was a good correlation between MA/GTA and the number of intraglomerular NSE-positive cells ($r = 0.719$, $p < 0.001$) over the entire experimental period. At day 30, the MA/GTA ratio was significantly increased in the HC group compared to the CONT group ($p < 0.01$). Also, this ratio was higher in the HCHP group than in the HC group at day 30 ($p < 0.01$). No significant increase in MA/GTA ratio was detected prior to day 30 in all the groups.

In both HC and HCHP groups, the number of mesangial cells was significantly increased compared to the CONT and HP groups ($p < 0.01$) at day 70 (Table 17). A significant increase was also found at day 30 in the HCHP group ($p < 0.001$) and HC group ($p < 0.05$).

The MA/GTA ratio correlated individually with serum TC ($r = 0.790$, $p < 0.001$), tissue TC ($r = 0.792$, $p < 0.001$), and cortical CE% ($r = 0.409$, $p < 0.01$). However, with multiple regression analysis, only correlations of MA/GTA with tissue TC ($r = 0.490$, $p < 0.01$) and NSE-positive cells ($r = 0.239$, $p < 0.05$) were significant. The level of proteinuria correlated individually with tissue TC ($r = 0.756$, $p < 0.001$), MA/GTA ($r = 0.785$, $p < 0.001$) and NSE-positive cells ($r = 0.519$, $p < 0.05$). None of the latter three parameters showed significant independent correlation with urinary protein, using multiple linear regression analysis. The animals injected with APH did not show any morphometric change compared to the values found in the animals who received normal diet and were not subjected to this treatment (Table 18).

Table 17

[Kidney Morphometry at Different Experimental Periods. Mean ± SD]

Group	Day	MA/GTA	[2]MC	[3]NSE/glomerular section
CONT	5-10 n=15	.16 ± .05	10.3 ± 1.5	--
	30 n=16	.17 ± .04	10.1 ± 2.7	.11 ± .07
	*70 n=48	.17 ± .04	18 ± 5	.45 ± .07
HC	(5-10) n=30	.17 ± .03	12.8 ± 1.9	.28 ± .1
	30 n=25	.20 ± .03[b]	13 ± 2.8[a]	.41 ± .27
	*70 n=56	.28 ± .08[e]	32 ± 8[b]	1.30 ± 0.83
HCHP	5-10 n=31	.15 ± .04	11.5 ± 2.8	.37 ± .21
	30 n=24	.25 ± .07[b]	19.8 ± 2.9[e]	.32 ± .19
	*70 n=55	.32 ± .11[e]	39 ± 17[b]	1.90 ± 0.61
HP	*70 n=30	.18 ± .03	21 ± 7	.40 ± 0.08

(1) Mesangial area/glomerular tuft area

(2) Number of mesangial cells/glomerular section

(3) Number of (Non-specific esterase)-positive cells/glomerular section

* = Data from Experiment III

n = Number of Glomeruli Examined

a = Significantly different than CONT ($p < 0.05$)

b = Significantly different than the control groups ($p < 0.01$)

e = Significantly different than CONT ($p < 0.001$)

3. Kidney Lipid Analysis:

The lipid content of whole-kidney tissue (TC, FC, CE%, TPL, TG) in the various groups of animals at day 70 (experiment I) is shown in Table 19. The HC and HCHP groups showed a significant increase ($p < 0.05$, < 0.01, respectively) in TC and CE% compared to the CONT and HP groups. Except for the HCHP group, FC did not significantly increased in other groups. Similarly, TPL was increased at $p < 0.05$ level in the HCHP group relative to the CONT groups, while no significant changes were found in these groups, although there was a tendency to higher values in the HC group.

Representative pieces of renal cortex in experiment III were analysed for lipid content. Cortex was chosen to exclude the possible lipid contribution by the medullary portion of the renal tubules. The lipid changes in cortex paralleled those in the whole-kidney tissue (experiment I). There was a 10-20% reduction in the levels of the different lipid components - a finding which demonstrates that the bulk of the lipid content was in the cortex (Table 20).

As summarized in Table 20, lipid profile of the renal cortical tissue did not significantly change after 5 and 10 days on the respective diets. Both HCHP and HC groups had an increased percentage of CE relative to the CONT group ($p < 0.05$) at day 30. On the other hand, although there was a trend toward an elevation in FC and TPL in the HC and HCHP groups, this increase was not statistically significant ($p > 0.1$).

Table 18 shows the lipid contents of the whole-kidney extract in the APH-treated animals (experiment II). No significant change in any of the lipid parameters (examined at day 40) was found compared to the levels in the CONT group.

Table 18

APH-Induced Anaemia Experiment

[Kidney Lipid Profile (ug/mg protein) and Kidney Morphometry at Day 40. Mean ± SD]

n = 6

TC	FC	CE%	PL	TG
4.4 ± 0.3	4.1 ± 0.3	7 ± 1.1	27.2 ± 1.7	7.4 ± 1.8

Total cell count (GC)	Mesangial Cell Count (MC)	MA/GTA
32.3 ± 7.9	15.3 ± 5	0.14 ± 0.03

N.B. Data are compared to those of the control group in tables 17 and 20.

Table 19

[Kidney Lipid Profile at Day 70. Mean ± SD]

Groups	TC[1]	FC[2]	CE%[3]	TPL[4]	TG[5]
CONT (n=6)	4.3 ± 1.1	3.8 ± 1.2	10 ± 5.3	31.4 ± 4.6	10.9 ± 3.3
HP (n=6)	5.4 ± 0.5	4.9 ± 0.4	10.1 ± 3.8	32 ± 10.9	14.6 ± 7.1
HC (n=6)	6.2 ± 1.5	4.7 ± 1.6	23.6 ± 11.6[b]	37.7 ± 5.1	16.7 ± 9.5
HCHP (n=6)	8.1 ± 1.7	6.5 ± 1.5[a]	20 ± 5.3[b]	40.5 ± 6.7[a]	8.8 ± 3.7

(1) TC = Total Cholesterol (ug/mg protein)

(2) FC = Free Cholesterol (ug/mg protein)

(3) CE% = Percentage of Cholesteryl Ester

(4) TPL = Total Phospholipids (ug/mg protein)

(5) TG = Triglycerides (ug/mg protein)

a = Significantly different than CONT ($p < 0.05$)
b = Significantly different than the control groups ($p < 0.01$)

Table 20

[Lipid Profile of Renal Cortex (ug/mg protein).

At Different Periods. Mean ± SD]

Group	Day	TC	FC	CE%	TPL
CONT**	(n=13)	3.0 ± 0.5	2.7 ± 0.5	10 ± 5	20.6 ± 6.1
HC	5(n=5)	3 ± .7	2.7 ± .5	9.6 ± 4.1	20 ± 7.1
	10(n=5)	3.5 ± .4	3.1 ± .6	10.9 ± 6.1	23 ± 7
	30(n=5)	4 ± 1	3.15 ± 1.1	21.1 ± 9.8[a]	24 ± 7.7
	*70(n=6)	5.4 ± 1.2	4.0 ± 1.4	26.8 ± 10.9[b]	29 ± 6.2
HCHP	5(n=5)	2.8 ± .4	2.5 ± .3	10.3 ± 3.5	19.5 ± 6
	10(n=5)	3.8 ± .7	3.35 ± .4	11 ± 6	25 ± 4.9
	30(n=5)	5 ± 1.2	3.93 ± 1.5	20 ± 7.1[a]	25 ± 9
	*70(n=6)	7 ± 2	5.6 ± 1.6[a]	21 ± 5.6[b]	31 ± 7[a]
HP	*70(n=5)	4 ± 1.1	3.7 ± 1.2	10.2 ± 4	24.6 ± 9

n = Number of animals/group
* = Data from Experiment III
TC = Total Cholesterol (ug/mg protein)
FC = Free Cholesterol (ug/mg protein)
CE% = Percentage of Cholesteryl Ester

TPL = Total Phospholipids (ug/mg/protein)
a = Significantly different than CONT ($p < 0.05$)
b = Significantly different than CONT ($p < 0.01$)

** = Data of days 5,10,30 and 70 were combined due to the absence of significant changes among the animals of the control group.

VIII Renal de novo Cholesterol Synthesis

As described in the "Methods", two different procedures (assay of HMG COA reductase and incorporation of ^{3}H-water into cholesterol) were used. The purpose of these experiments was to assess tissue cholesterol synthesis in the control animals and compare it with that in the HP, HC, and HCHP groups. Table 21 summarizes the findings.

In comparison to the CONT group, HMG COA reductase activity at day 70 was significantly decreased in the HC group ($p < 0.001$), while that of the HCHP group decreased less markedly ($p < 0.05$). The HCHP group's de novo cholesterol synthesis was higher, by both methods, than that of the HC group ($p < 0.001$). Kidneys from the HP group had lower HMG-COA reductase activity than the CONT or HCHP groups ($p < 0.001$ and $p < 0.01$, respectively). These findings were in good agreement with the data from a separate experiment in which the rate of incorporation of tritiated water into cholesterol was measured. In general, there was a significant positive correlation ($r = 0.91$, $p < 0.05$) between the data obtained by the two methods. These findings indicated that cholesterol feeding suppressed de novo cholesterol synthesis in renal tissue. This suggests that the bulk of the accumulated lipid (as described in the previous section) is derived from plasma. This observation was strongly supported by the poor correlation between the parameters of de novo synthesis and those of the cortical lipid profile.

Table 21

*[de novo Cholesterol Synthesis in renal tissue at Day 70]

Group	HMG-CoAR.[1]	$T.H_2O$ Inc.[2]
CONT	40.8 ± 2.7 (n=3)	82.9 ± 6.3 (n=2)
HC	29.9 ± 4.1 (n=5)[e]	18.5 ± 3.6 (n=3)
HP	24.4 ± 2.7 (n=3)[e]	42.5 ± 3.9 (n=2)
HCHP	36.7 ± 2.5 (n=5)[a]	44.1 ± 6.3 (n=3)

(1) HMG-CoAR. = ß-hydroxy methyl glutaryl COA reductase (Pmol/mg protein/min)

(2) $T.H_2O$ inc. = Tritiated water incorporation in cholesterol (nmol/gm/hr)

* = Mean ± SD

n = Number of animals/group

a = Significantly different than CONT ($p < 0.05$)

e = Significantly different than CONT ($p < 0.001$)

DISCUSSION

DISCUSSION

I. General Observations:

The groups of animals receiving a cholesterol-rich diet grew at an equal rate to that of the control group for 30 days. Afterwards they failed to maintain a normal weight gain. This failure was quite marked at day 70. These data agree with those obtained by other investigators (66,77,98,99). However, in none of the previous studies were the changes in body weight recorded before day 60, except for Matin and Ostwald (1975) who reported that cholesterol-fed guinea pigs showed a significant reduction in weight gain at the end of a 33-day experiment (100).

Although there was no marked change in erythrocyte count at day 30, the present study and others (66,77) provided good evidence for the correlation between the decrease in weight gain and the severity of anaemia particularly after 60 days of feeding. In this investigation marked anaemia was noticed at day 70. However, morphological changes in erythrocytes were already present at day 30. Such changes might have the potential to decrease the functional efficiency of erythrocyte which could, in part, contribute to the failure to thrive. Moreover, acetyl phenylhydrazine-induced anemia was also accompanied by a significant decrease in weight gain. Additional evidence for the possible correlation between haemolytic anaemia and the decrease in weight gain comes from the data on protein supplementation. High protein supplements resulted in increased severity of haemolysis in the cholesterol-fed animals; these animals also gained the least weight. The dual effect of high protein supplementation in cholesterol-fed animals on weight gain and the degree of haemolysis has not been previously thoroughly investigated.

However, it has been reported that neither anaemia nor reduction in weight gain of cholesterol-fed guinea pigs were improved by casein enrichment of diet (101). Another report pointed out that a casein-rich diet suppressed the ability to gain weight in rabbit (102). Our data suggest that high casein supplementation per se does not significantly affect the ability to gain weight. In addition, since there was no detectable change in either erythrocyte morphology or count in the group receiving high protein diet alone (HP), it is tempting to attribute the failure to thrive to haemolytic anaemia as a major cause in both HC and HCHP groups.

Other causes for the reduction in weight gain in cholesterol- or cholesterol/protein-fed animals can be postulated. At least one report has pointed out a reduction of food intake after cholesterol feeding in guinea pigs (73), although the workers did not elaborate on that observation. Such a finding, again, may be attributed to anaemia which is known to cause anorexia. Also, anorexia in uraemic rats fed high protein diet has been suggested as a cause for stunted growth (103). This possibility is unlikely in our animals, since they did not develop uraemia. In conclusion, the data presented in this study are in favor of the causal relationship between haemolytic anaemia and failure to thrive.

The liver and spleen of the experimental groups were markedly affected by both cholesterol and cholesterol/protein feeding. A gradual increase in the weight of liver and spleen from day 30 to day 70 was noticed in animals kept on these two dietary regimens. The liver enlargement has been described in several previous reports (23,66,77,99,101) and has been attributed to massive fatty infiltration. Macroscopically, the liver appears pale yellow and greasy. Unlike an earlier report (66) we did not

observe necrotic areas under gross examination. The enormous enlargement of spleen can be explained by the destruction of the modified erthrocytes. The enlargement of spleen in animals fed either a cholesterol-rich diet alone or with a protein supplement was noticed for the first time at day 30 concommitantly with the morphological changes in erythrocytes. The early splenomegaly was modest and probably represented the early stages of erythrophagocytosis and extramedullary haematopoiesis. These findings are in agreement with other studies (66,77), although in those studies the gross changes were not monitored in the early stages of the experiment.

A mortality rate of approximately 10% was recorded among the cholesterol-fed guinea pigs. Earlier reports on such animals mentioned different mortality rates. Approximately 30% of experimental animals died within 8-10 weeks of cholesterol/fat feeding in Ostwald and Shannon's study (66); other workers found that the animals became more susceptible to infection (77). On the other hand, Drevon and Hovig (1977) reported no deaths among albino guinea pigs kept on 1% cholesterol-rich diet (99). Mortality in cholesterol-fed guinea pigs might be attributed to haemolytic anaemia which has been consistently reported in this experimental animal model. Consistent with this is the observation that the mortality rate was higher in the group that received a high protein supplement in addition to the cholesterol diet. In this group, the haemolysis is more severe suggesting that the differences in the mortality rates between the two groups were related to the severity of haemolysis. However, another possible cause for mortality in our animals is infection, but on necropsy, no gross pathological lesions were detected in the viscera of the dead animals. Although this finding decreases the likelihood of infection as a leading cause of death, it does not exclude

such a possibility from contributing to the increased mortality rate. Thus, we conclude that anaemia was most likely the main cause of death with the possibility of infection as a contributory factor.

II. Plasma Lipid Abnormalities

1. Effect of the cholesterol-rich diet:

As in other studies (98,67,104), the cholesterol-fed guinea pigs showed high levels of plasma TC and FC compared to the control group. A gradual increase in plasma TC was found at days 5,10,30 and 70 of the experiment. Earlier it had been pointed out that cholesterol-fed guinea pigs develop hypercholesterolaemia accompanied by the appearance of new lipoprotein species rich in unesterified cholesterol (50,69,76). In these reports of the abnormal lipoproteins VLDL and LDL were the most thoroughly studied. It has been suggested that hypercholesterolaemia might be brought about in part by dietary cholesterol suppression of LDL receptors (105,106). This suggestion is supported by the data in cholesterol-fed rabbits (107) and dogs (108) which showed an elevation in IDL and LDL due to down regulation of LDL receptors. In this regard, Terpstra and Beynen (1984) reported that most of the cholesterol in cholesterol-fed guinea pigs was found in the LDL fraction (104). The appearance of abnormal LDL and VLDL was observed in the hypercholesterol-anemic animals (discussed further, below, in this section). Moreover, in agreement with previous findings (104, 108), HDL-cholesterol was increased in our animals. Thus, our results confirm that cholesterol-rich LDL is the main source of the elevated plasma TC. This may be due to suppression of LDL uptake in addition to increased intake of cholesterol. However, since the percentage of plasma unesterified

cholesterol (FC%) in the HC group was higher than in the CONT group, the increase in serum FC and subsequently TC cannot be explained by merely the retention of lipoproteins in circulation. Rather, the explanation should include the esterification activity. Despite the possible intracellular esterification of FC in jejunum (109) and liver (98) by acyl COA:cholesterol acyltransferase (ACAT), the FC% was high in HC group. This observation suggests a reduction in cholesterol esterification in plasma.

Our data showed that LCAT activity (as FER) was variably affected depending on the type of substrate used for the assay. Using endogenous substrate (whole plasma), FER was remarkably reduced in HC group, while there was no significant change in MER. This is in agreement with the findings of Ostwald et al. (1979) who showed an eightfold reduction in the rate of free cholesterol esterification in cholesterol-fed guinea pigs using autologous plasma in the enzyme assay (101). Also, they demonstrated no reduction in MER, a finding which was also confirmed in Drevon's report (98). Our data and these reports indicate that absence of a significant reduction in MER could be due to an increased level of FC in plasma lipoproteins. Such an increase in FC has been described as an inhibitory factor for LCAT activity in humans and rat plasma due to decreased phospholipids: FC ratio in the lipoproteins (110). In this regard, the issue of LCAT substrate in guinea pigs is of a special interest. Due to the virtual absence of HDL in normal guinea pig (69), and the ability of apo-C1 in VLDL to activate LCAT (44), it was suggested that VLDL normally acts as a substrate for LCAT in guinea pigs (109). However, a lipoprotein species with a hydrated density of 1.063-1.21 gm/ml was detected in their circulation (97). In cholesterol-fed guinea pigs, both VLDL and this

lipoprotein species are cholesterol-rich (69). Thus, neither of the two lipoproteins is a good substrate for LCAT activity. This indicates that using an endogenous substrate (whole plasma) in our study may not be the optimal way to measure LCAT activity, since free cholesterol-rich lipoproteins were found in our guinea pigs (discussed below). Therefore we used an exogenous (artificial) substrate to measure LCAT activity. This assay has also been generally accepted as an approximation of LCAT mass (111,112). Employing this method, LCAT activity (FER and MER) was significantly reduced in the HC group at day 70. Although the reduction was moderate, it likely reflected a reduction in the enzyme mass. This could be due to a massive fatty degenerative change of the liver (113). On the contrary, a misleading estimate of the enzyme mass is achieved by applying an endogenous-substrate assay. Our data on LCAT activity in the CONT and HP groups using an endogenous-substrate assay showed a slight reduction in FER in the absence of detectable liver lesions. This observation indicates a poor correlation between the reduction in FER and the enzyme mass, and suggests that the type of substrate used affected the enzymatic reaction. In fact, plasma TC at day 70 was higher in the HP group than in the CONT group (see the second part of this section for further discussion), and was slightly increased in the CONT group at day 70 compared to day zero. A similar elevation in plasma cholesterol with age has been previously reported (114-117).

In summary, the slight increase in FC% in plasma at day 70 is apparently due to an absolute increase in plasma FC and a moderate reduction in LCAT activity which might be due to the fatty degenerative change found in the liver of cholesterol-fed animals. We recommend using an artificial substrate

for measurement of LCAT activity to obviate the variability in the nature of substrates in autologus plasma.

Different alterations were observed in the lipoproteins of HC group at different experimental periods. Plasma cholesterol of HDL (HDL-C) was gradually elevated from day 30 to day 70. This finding is in agreement with the data obtained by other groups (104,118). Terpstra and Beynen (1984) suggested that cholesterol feeding to guinea pigs increased the density of HDL which led to the appearance of HDL_C particles rich in apo E (104). In this regard, our data on the HC group show a protein fraction with a molecular weight close to that of apo E found in the lipoprotein species with a hydrated density 1.063-1.21 gm/ml. The same finding has been described earlier (69,119).

In the present investigation, the VLDL fraction showed the earliest electrophoretic alterations in the HC group. A slow prebeta band appeared by day 10 on agarose gel. This abnormally migrating prebeta has been described earlier in cholesterol-fed guinea pigs (68,69), and recently in rabbits, dogs, rats, and monkeys fed cholesterol-rich diets (120). It has an increased amount of apo E and a decreased level of C-apoproteins (119). These protein changes correlate with the concentration of plasma unesterified cholesterol (119). It has been suggested that changing proportions of apoprotein and cholesterol content affects the net surface charge of VLDL and causes slow prebeta migration of this lipoprotein (121). Our data which showed a gradual decrease in the anodic mobility of the prebeta band throughout the different experimental periods supports the speculation that the surface charge of VLDL might be changed in parallel with the hyper-cholesterolaemic state. This argument seems to be in accordance with the

reports that slowly migrating prebeta lipoprotein is rich in unesterified cholesterol (50,69,104).

The electrophoretic mobility of LDL in the HC group showed an interesting change. At day 70, a slowly migrating beta band was detected in the HC group compared to CONT group. This electrophoretic behavior which was noticed in all animals of the HC group paralleled the marked increase in plasma TC. Puppione et al (68) and Sardet et al (69) previously reported a normally migrating beta band in cholesterol-fed guinea pigs. This discrepancy may be due to a difference in the proportion of cholesterol supplement in the experimental diet, since we used 2% cholesterol-rich diet versus their 1% fatty diet regimen. Our finding might be related to a high cholesterol content of LDL particles determining their electrophoretic mobility.

In conclusion, the cholesterol feeding to guinea pigs induced hypercholesterolaemia which might be accounted for by the following mechanisms: first, intake of a cholesterol-rich diet. Second, decreased uptake of LDL due to cholesterol-mediated suppression of LDL-receptors. Third, a slight increase of the percentage of unesterified cholesterol in plasma due to a moderate reduction in LCAT activity. The increased plasma cholesterol might modify the electrophoretic migration of the different lipoprotein species. The relevance of the lipoprotein abnormalities to the histological changes will be discussed in the following sections.

2. Effect of the cholesterol/protein-rich diet:

Enrichment of the cholesterol-rich diet with high protein supplements increased plasma TC and FC. At day 70 FC was higher in the HCHP group than in the HC group. This marked increase is attributed, in addition to the factors discussed in the first part of this section, to the protein supplement. It has been found that rabbits fed cholesterol-free diets of carbohydrate and casein had suppressed hepatic LDL receptors which led to a decreased rate of LDL clearance from circulation (122). The hypercholesterolaemic effect of different types of protein has also been investigated. It has been pointed out that animal protein is more effective than vegetable protein (102,123). The plasma cholesterol level in rabbits fed a casein-rich diet was double that seen with a soybean diet (124). Further study of the hypercholesterolaemic effect of casein in the rabbit model has suggested that cholesterol level increased in LDL, IDL, and VLDL (125). The findings of Terpstra et al. (1982) also indicated that the bulk of the increased serum cholesterol after feeding 40% casein-rich diet was in the LDL fraction (118). In addition to the theory of the suppressive effect of casein on the LDL receptors, there are other possible explanations for the hypercholesterolaemic effect of casein. Van der Meer (1983) hypothesized that the hypercholesterolaemic effect of casein is related to its phosphorylation state. This hypothesis implies that casein and its phosphopeptides compete with bile acids and/or biliary micelles to bind insoluble calcium phosphate increasing the availability of bile acids for lipid digestion and reabsorption (126). Recently, Van der Meer et al. (1985) proved this effect in rabbits fed casein (127). They found that increased amount of dietary calcium inhibited casein-induced

hypercholesterolaemia. Valhouny et al. (1985) pointed out that rates of clearance of chylomicrons and VLDL were decreased in rats fed a semipurified diet containing casein (128). Moreover, it has been reported that hepatic secretion of lipoproteins is greater with casein-based diets than with soy protein-based one (129). Contrary to these reports, dietary experiments in chickens showed that hypercholesterolaemia did not result from feeding casein or soybean alone but rather after supplementing the protein diets with 1% cholesterol (130). Our data do not agree with this report, since there was a slight increase in plasma total cholesterol in the control HP group at day 70. However, we confirm that hypercholesterolaemia is more pronounced with a mixture of casein/cholesterol-rich diet. Thus, from the available reports, it seems that casein may aggravate hypercholesterolaemia induced by cholesterol-rich diet, mainly, by increasing fat absorption and decreasing LDL uptake through suppressing LDL receptors.

Although plasma FC was higher in the HCHP group than in the HC group, no significant difference was noted in FC% between the two groups. To our knowledge, the effect of high protein on cholesterol esterification in plasma has not been investigated. Our results using either artificial or endogenous substrate indicate that there was no significant difference in LCAT activity (FER and MER) between the HCHP and HC groups. The absence of such a difference in enzyme activity using endogenous substrate might be attributed to the low sensitivity of the assay where differences at the low level could not be detected. Alternatively, due to the higher level of the FC in HCHP group than HC group, it might be expected to have lower FER in the first one due to the adverse effect of FC on appropriateness of the substrate (discussed above). The slight reduction in FER found in the HP

group where there was a moderate increase in plasma cholesterol supports this argument. However, we based our conclusion, that no difference in LCAT activity was found between HCHP and HC groups on the data obtained with the artificial-substrate.

No significant differences in HDL-C, protein composition, or electrophoretic mobility were observed between the HCHP group and HC group. This issue has not been studied thoroughly by other investigators in guinea pigs. Mol et al. (1982) found no differences in the density profile and lipoprotein composition between groups of chicken receiving cholesterol-free diets containing either casein or soybean protein (130). Our data suggest that high casein supplementation did not alter the chemico-physical properties of lipoprotein species in the animals. This might be due to the slight hypercholesterolaemic effect of the casein-enriched diet found in our study.

In conclusion, high protein supplementation in either CONT or HC groups had a hypercholesterolaemic effect which might be due to an increased fat absorption and/or decreased catabolism of lipoproteins, particularly LDL. The high protein supplement did not further impair cholesterol esterification compared to the HC group. In addition, no remarkable changes in the lipoprotein composition and electrophoretic behavior could be attributed to the high protein supplement per se.

III. Haematological Changes

1. Effect of cholesterol-rich diet:

Similar to several other studies (23,66,67,69,77) the cholesterol-fed guinea pigs in our study developed anaemia. Compositional, morphological and functional alterations in erythrocytes will be discussed in an effort to

elucidate the mechanism(s) responsible for anaemia in these animals.

Already after thirty days on the cholesterol-enriched diet erythrocyte content of cholesterol increased. This finding is in agreement with the data obtained by others (66,131). A role for the abnormal lipoproteins observed in cholesterol-fed guinea pigs has been postulated in delivering cholesterol to erythrocyte membranes (EM). Thus, the appearance of new species of HDL and changes in LDL in the plasma of these animals correlate with the severity of haemolysis (69). Our findings of the lipoprotein alterations (discussed in the previous section) agree with these observations. In an in vitro study, it has been reported that cholesterol is transferred mainly from HDL to EM when normal erythrocytes were incubated with hypercholesterolaemic plasma of cholesterol-fed guinea pigs (131). In the same way, cholesterol-enriched erythrocytes obtained from cholesterol-fed guinea pigs lost their cholesterol to plasma when they were incubated with normal plasma (131). These observations demonstrate that plasma is the main source of cholesterol found in excess in EM. This effect can be accounted for by plasma lipoproteins contributing cholesterol to cells either by receptor-mediated endocytosis (132) or by isolated surface transfer of free cholesterol. In addition to cholesterol transfer, it has been well documented that there is an equilibration between plasma and erythrocyte phospholipids (133). Our findings of increased plasma total phospholipids in the HC group suggest that part of phospholipids in EM of this group may originate from plasma in an equilibration process similar to that described previously (131,133). This suggestion is in accordance with another observation in cholesterol-fed guinea pigs of an increased level of phospholipids in EM (66). In summary, the lipid content of EM found in the HC group is most likely derived from plasma.

Studies of physical properties of lipids in membranes have shown that incorporation of cholesterol molecules into the lipid bilayer increased order in the hydrocarbon region which decreases water permeability (134). Also, it has been suggested that insertion of cholesterol molecules into the phospolipid array of EM facilitates a closer fit and interaction between the long acyl groups of the adjacent lipid molecules (135). This alteration causes a contraction of membrane surface referred to as a "condensation effect" (135). This effect might explain the slight decrease in mean corpuscular volume (MCV) of erythrocytes in the HC group at day 30. Other studies have reported that the increased cholesterol content of EM leads to an increase in local viscosity and rigidity of membranes (136). Also, increased membrane deformity and fragmentation were reported in these cells (137). Relevant to these observations is the finding that the increase in erythrocyte cholesterol content was accompanied by the appearance of spiked erythrocytes (69). We found echinocytes to be the predominant erythrocyte form in the peripheral blood at day 30 in the HC group. Interestingly, Owen et al. (1985) reported that echinocyte form developed in seconds when normal erythrocytes were incubated with plasma from jaundiced patients having abnormal HDL (138). They mentioned that formation of these cells did not involve cholesterol transfer, but rather attachment of the abnormal HDL which contains apo E to specific membrane receptors on erythrocytes. Our findings do not exclude the possibility of echinocytes developing through a mechanism similar to that described by Owen et al. In any event, this mechanism does not substitute for the cholesterol-mediated mechanism which we adopt. Whatever the mechanism, we found these cells accompanied by a decrease in erythrocyte fragility, a finding which has been reported by

several investigators (131,135,137). A similar decrease in osmotic fragility has been described in patients with abetalipoproteinemia who presented with acanthocytes in their blood (136). Again, this could be attributed to the "condensation effect" which decreases membrane permeability (135).

The deformed erythrocytes on entry of the blood through the splenic cords are either engulfed and destroyed by the splenic macrophages on their first pass or their spikes are amputated first (pitting process) to be engulfed on the following process (139). We speculate that pitting off the echnicoyte spikes could affect EM permeability and lead to spilling out some of the haemoglobin into plasma. These hypochromic cells (HCC) with corrugated membrane (on microscopy) appeared at day 70 in our study. Such membrane corrugation could indicate the sites of the "pitting process". In accordance with our speculation, the splenic "remodeling" or "conditioning" of "spikey" erythrocytes has been reported by several investigators (141,140,141). We suggest that HCC are osmotically unstable cells due to their defective membrane which could be subjected to intravascular haemolysis. Thus, these cells might have a shorter life span than normal cells.

In summary, hypercholesterolaemia affected erythrocyte composition - namely, increased cholesterol content of EM. Cholesterol enrichment of erythrocytes might, in part, render their membranes rigid (echinocytes) and thus highly susceptible to splenic erythrophagocytosis. It seems that haemolytic anaemia in cholesterol-fed guinea pigs occurs both intra and extravascularly.

By day 70 vacuolated white blood cells were found in the peripheral blood of the HC group. Using ORO and NSE stains, it was shown that these

cells were lipid-laden monocytes (LLM). The presence of these cells has earlier been reported in hyperlipidaemic rats (142;143). The appearance of lipophages in peripheral blood has been an intriguing subject in the studies of atherogenesis and atherosclerotic plaque regression. Gerrity (1981) presented histochemical evidence that macrophages detected in swine atherosclerotic plaques were blood-borne monocytes (144). He showed an important role for these cells in scavanging lipid deposits in the arterial intima. Gerrity was able to demonstrate a "monocyte clearance system" in which the circulating monocytes penetrate the arterial intima to be laden by lipid; these foam cells migrate back into the blood stream by crossing the arterial endothelium (145). Later, these observations were supported by Faggiotto et al. (1984) in a sequential follow-up study of atherogenesis in hypercholesterolaemic non-human primates. In this model, after three months on a cholesterol-enriched diet, the endothelial continuity was interrupted over the lipid-laden macrophages in atherosclerotic plaques. The exposure of the foam cells to the circulating blood may lead to their appearance in blood (146). In the present study, circulating LLM were not seen before day 70, although hypercholesterolaemia was noticed in the HC group as early as day 5 of the cholesterol-rich diet regimen. There is some analogy to the observations of Faggiotto et al. who showed a time-dependent appearance of these cells in circulation. Interestingly, unlike their findings, we did not observe any detectable atheroma in the aortas of the guinea pigs during the 70-day experiment. This suggests that there may be a different source of the circulating LLM in our animals (other than arterial fatty lesions). We hypothesize that these cells might originate from the reticuloendothelial system. Data of other investigators as well as our own (as discussed in the

following paragraphs) support this hypothesis. Earlier, Ostwald and Shannon found a significant increase in the lipid content of the spleens of cholesterol-fed guinea pigs (66). Also, it has been noticed that this lipid accumulation was distributed in the splenic interstitial tissue and inside macrophages; the hepatic sinusoidal macrophages were also depicted as lipophages (77). Moreover, as discussed above, erythrocytosis and the "pitting process" of the spikes of echinocytes was most likely caused by the macrophages of splenic cords. Since echinocytes have lipid-rich membranes, splenic macrophages would have been overloaded with lipid materials. Based on all these observations we postulate that lipid-laden splenic macrophages might be released from the lining of the splenic cords and gain access to the circulation. Another possible source of fat-loading macrophages of the reticuloendothelial system is plasma lipoproteins. In an in vitro study, peritoneal macrophages and monocyte-derived macrophages have been shown to accumulate massive amounts of sterol when incubated with chemically modified lipoproteins or with beta migrating VLDL isolated from hypercholesterolaemic animals (147-150). Beta migrating VLDL isolated from hypercholesterolaemic rabbit plasma has been shown to react with specific membrane receptors on macrophages (147). We found beta migrating VLDL at day 70 when a significant number of LLM was detected in circulation. However, beta migrating VLDL was also demonstrated at day 30 without LLM presence in peripheral blood. This might suggest that loading macrophage with this abnormal lipoprotein is a time-dependent process. First, macrophage must be overloaded with lipid and then released from its tissue of origin into the circulation. In addition to beta-VLDL, HDL_c has also been implicated in delivery of cholesterol to different tissues in the body. Andersen and Dietschy (1981)

demonstrated preferential uptake of cholesterol from HDL by rat adrenal (151). The preferential degradation of the cholesterol moiety of HDL was also reported in different cells by other investigators (152,153). In our experiment a remarkable increase in the percentage of circulating LLM was paralleled by the appearance of an alpha-migrating band upon agarose gel electrophoresis. Based on the above reports and our observations, it is tempting to suggest that alpha-migrating lipoproteins might be another source of macrophage lipid.

In summary, it is unlikely that in our experimental model circulating LLM originated from an atherosclerotic lesion. We suggest that these cells are macrophages of the reticuloendothelial system loaded with lipid materials and released into the circulation. The source(s) of this lipid material might be, in part, the lipid-rich membranes of echinocytes, and beta-migrating VLDL and/or alpha-migrating lipoprotein or all of these.

Our study showed a concomittant increase in the percentage of LLM in the peripheral blood with the number of glomerular NSE-positive cells. This suggests a possible role for LLM in the pathogenesis of the glomerulo-sclerotic lesion found in the cholesterol-fed guinea pigs.

In conclusion, anaemia found in the cholesterol-fed guinea pigs may be explained as follow: cholesterol feeding to these animals led to an increase in EM cholesterol content which accounts for many of the changes in erythrocyte morphology. The decrease in erythrocyte osmotic fragility was most likely attributed to the increase rigidity of EM (morphologically shown as echinocytes). Echinocytes were either cleared directly from blood circulation by erythrophagocytosis in spleen or their spikes were removed in a "pitting process" by splenic macrophages, where they became susceptible to

intravascular haemolysis. We speculate that splenic macrophages, after being loaded with lipid materials from different sources, were released into circulation and were detected as LLM.

2. Effect of Cholesterol/Protein-Rich Diet:

The effect of high protein supplementation on dietary cholesterol-induced anaemia has not been investigated previously except for the work of Ostwald et al. (1971). They reported that neither 20% nor 30% casein-rich diets protected cholesterol-fed guinea pigs from anaemia (101). In our study there were several haematological changes in the animals fed the cholesterol/protein-rich diet (HCHP group).

As discussed in the previous part of the section HCC (hypochromic cells) appeared at day 70 possibly as a result of the "pitting process" of spikes of echinocytes in splenic cords. HCC were seen in a higher percentage in the HCHP group than in the HC group. This might be due to an increase in the rate of the "pitting process" which could be attributed to an increase in the degree of erythrocyte deformity. To discuss this hypothesis the plasma lipid profile in the HCHP group should be examined. In this group a higher level of mainly free cholesterol concentration was found as compared to the HC group. Having considered the equilibrium state between plasma and EM cholesterol (131), it seems likely that EM in the HCHP group had accepted more cholesterol than those of the HC group. The increase in the lipid content of EM is one factor determining the deformity of erythrocytes. These changes might lead to more pitting of the spikes of echinocytes. Supporting this speculation is the finding that the cholesterol content of EM in the HCHP group was comparable to that in the CONT group at day 70

where HCC were the predominant erythrocyte form in peripheral blood. We believe that such a reduction in the EM cholesterol content was most likely due to the pitting of the echinocyte spikes by splenic macrophages.

Unlike in the HC group, erythrocyte osmotic fragility in the HCHP group was within normal CONT-group level at day 70. Since osmotic fragility is inversely related to the amount of cholesterol in EM, this fits with the normal cholesterol content of EM. Thus, with predominance of HCC in the peripheral blood, there are at least two possibilities which may be accounting for the elevation of osmotic fragility from the level found in the HC group to the CONT-group level: first, a decreased amount of erythrocyte cholesterol leads to decreased rigidity and increased permeability of EM (134,136). Second, the "pitting process" of the echinocyte spikes most likely leads to decrease osmotic stability of erythrocyte, hence we speculate that HCC are vulnerable cells with shortened life span.

In general, although there was no significant difference in erythrocyte count, haemoglobin concentration, and haematocrit between the HCHP and HC groups, we believe that haemolysis was more severe in the HCHP group for the following reasons: first, the presence of HCC in a larger percentage in the HCHP group than in the HC group. This might indicate that the animals of the HCHP group had more vulnerable erythrocytes (see above) which are likely to be subjected to intravascular haemolysis. In this regard, our results described HCC as bigger-than-normal cells with a remnant of haemoglobin at the cell periphery. However, there was no significant difference in haemoglobin concentration between HCHP and HC groups. This might be due to compensatory reticulocytosis which appeared in a larger degree in the HCHP

group. Second, serum indirect bilirubin was higher in the HCHP group than in the HC group at day 70. This observation indicates either a deterioration in liver function (particularly the conjugating ability) or a higher degree of haemolysis. In fact, there was no significant difference in liver function values between HCHP and HC groups, besides, a slightly higher level of serum direct bilirubin was found in the HCHP group (see the following section for further discussion). Thus, it is more likely that increased haemolysis accounted for the elevated serum indirect bilirubin in the HCHP group. In summary we speculate that greater lipid enrichment of EM in the HCHP group than that in the HC group might be the potential cause for the increased rate of "pitting process" which led to the appearance of more percentage of HCC in circulation. For at least two reasons we believe that the degree of haemolysis was higher in the HCHP group than in HC group.

In the first part of this section, it was postulated that the circulating LLM in the HC group might originate from macrophages in the reticuloendothelial system. Further we speculated that the lipid content of these cells could be from at least two sources - the lipid-rich membranes of echinocytes and plasma lipoproteins. The findings observed in the HCHP group support this argument. There was a higher percentage of LLM found in the HCHP group compared to the HC group at day 70 (this correlated well with the percentage of HCC). Since the percentage of HCC in the circulation, as discussed above, might be considered an indirect indicator for the rate of "pitting process", it seems likely that the appearance of LLM is related to the "pitting process". Moreover, since plasma FC in the HCHP group at day 70 was higher than in the HC group, and since there was a paralleled increase in the plasma FC to the increase in the percentage of LLM in the

circulation, plasma lipid may be another possible source for the lipid content of LLM.

In conclusion, high protein supplementation of the cholesterol-fed animals led to more severe haemolysis than that observed in animals on the cholesterol-rich diet alone. We speculate that the rate of the "pitting process" of echinocyte spikes by spleen macrophages was higher in the HCHP group than in the HC group. We believe this might be related to the EM overloading with, mainly, cholesterol derived from plasma since plasma FC was higher in the HCHP group than in the HC group. Also, HCC may be the echinocyte remnant after the "pitting process". Hence, the high number of HCC in circulation in the HCHP group was concomittant with lower erythrocyte cholesterol and higher fragility than in the HC group.

IV. Hepatic Changes

While the gross and histopathological changes in the livers of cholesterol-fed guinea pigs have been previously described in detail (23,66,77,99), these investigations have not included liver function tests. In the present study, serum direct and indirect bilirubin, asparate transaminase (AST), albumin, and total protein level were monitored throughout the experimental periods. The animals of the HC and HCHP groups had severe gross liver fatty infiltration which increased steadily from day 30 to day 70 of the experiment. In humans, the exact pathogenesis of fatty liver is not fully understood but it seems to be due to any interference with the different steps in fat metabolism which can lead to fat accumulation in hepatocytes (154). In the fatty-diet experiments, fatty liver is most likely due to the high level of chylomicron remnants in plasma

which reach hepatocytes. Cholesterol is stored after being esterified by ACAT (155). This is applicable to the fatty liver found in the HC and HCHP groups in our experiments.

Regarding cellular changes, aminotransferases are known as indicators of hepatocellular damage. They are released from hepatic cells when cellular damage occurs or when there is an increase in cell membrane permeability (156). In this study, an increase in AST was noticed as early as 10 days in both HC and HCHP groups. The AST activity showed a marked increase by day 70. Beynan et al. (1985) demonstrated an increase in serum AST and alanine amino transferase (ALT) in rats on a cholesterol-rich diet (157). Also, hyperlipaemic obese rats have increased AST activity (158). On the other hand, Laitnen et al. (1982) reported that a cholesterol-free diet labilized the membranes of rat hepatocytes and facilitated release of AST in blood (159). However, their conclusion was based on the observation that AST activity did not change during the 6-week experiment of cholesterol-rich diet feeding while it increased after withdrawal of cholesterol from the diet. Likely, the cellular injury in the Laitnen et al. experiment had already been induced by lipid deposits during the cholesterol-feeding period; thus, withdrawal of cholesterol could not preclude release of AST in serum. Based on these reports and our findings, it seems that there is a good correlation between plasma cholesterol and serum AST in cholesterol-fed guinea pigs. However, this correlation should be viewed with caution, since it rarely, if ever, exists in patients with hypercholesterolaemia without hepatocellular lesions. Thus, it would be more appropriate to examine the correlation between the hepatic fatty infiltration and serum AST. As discussed above, there is a causal

relationship between hypercholesterolaemia in cholesterol-fed animals and liver lesions. It is this relationship that results in a positive correlation between AST levels and plasma cholesterol in these animals. Our results (as mentioned above) indicate that an elevated level of AST in serum was detected before the gross hepatic fatty infiltration. This might be attributed to the microscopic changes occurring prior to day 30 of the experiment and not evident macroscopically. In addition to the data derived from animal studies, there are some reports in human pathology supporting our findings. In one study of patients with fatty liver, a slight elevation of serum aminotransferases has been demonstrated (160). Also, in Reye's syndrome where there is fatty degeneration of liver, the serum AST is high (161).

Interestingly, there was also a gradual mild increase in AST in serum of the CONT and HP groups. Although there was a slight elevation in plasma cholesterol in these two groups toward day 70 of the experiment, it is unlikely that this leads to hepatocellular damage. However, it has been reported that hepatocytes show a reduced ability to synthesize cholesterol with aging (162,163). Furthermore, in aged rats, physiological regeneration of hepatic cells is reduced compared to that in young animals (164). These findings suggest a defective cell membrane due to the aging process which leads to leakage of the intracytoplasmic enzymes. Our interpretation of these reports is supported by the findings of Nagy _et al_. (1982) who pointed out that aging may cause deterioration of cellular functions by altering the cell membrane structure and function (165). Thus the changes in AST in the CONT and HP groups could perhaps be attributed to the age-related cell membrane changes.

In summary, fatty infiltration of the liver in both groups kept either on cholesterol-rich diet alone or cholesterol/protein-rich diet might be brought about by hypercholesterolaemia with fatty infiltration of the liver.

The increase in serum indirect (unconjugated) bilirubin in both HC and HCHP groups at day 70 correlated well with the severity of haemolysis. Also, serum indirect bilirubin was higher in the HCHP group than HC group. As discussed in the previous section, the increase in this parameter in our animals, most likely, reflects the degree of haemolysis more than the hepatic ability of conjugation. This interpretation is supported by the relative increases of direct (conjugated) bilirubin found in the HC and HCHP groups. Moreover, at day 70, serum direct bilirubin in the HCHP group was higher than in HC group. These two observations indicate that the conjugating ability of hepatocytes suffered no major deterioration and was responding to increased levels of serum indirect bilirubin. However, intrahepatic cholestasis might occur due to the extensive fatty degeneration found at day 70. In any event, this does not rule out the fact that the hepatic conjugating system is still effective at day 70. Thus, the changes in both direct and indirect bilirubin found in the HC and HCHP groups, most likely, were related to the haematological rather than hepatic abnormalities.

Albumin concentration in serum was not reduced in the HC and HCHP groups throughout the experimental period. This indicates that the synthetic ability of the liver was not affected (see further discussion on the liver as a synthetic organ, below). On the other hand, total protein was increased in both HC and HCHP groups at days 10,30 and 70. This finding might be attributed to an increase in the production of immunoglobulins by the cells of immune system in a response to retained antigens in the

circulation. In this regard, several observations have suggested that Kupffer cells may fail to sequester antigens absorbed from the gut, either because of functional impairment or as a result of shunting of portal blood due to the hepatic lesion (166-168). These two possibilities are unlikely at least at the experimental periods prior to day 70. Nonetheless, Kupffer cell ability for sequestering circulating antigens might be affected at day 70 due to the marked fatty degenerative lesion detected in the liver of the HC and HCHP groups. Another potential source of increasing total protein in serum is the acute phase reactant proteins which could be increased due to the hepatic changes found in the HC and HCHP groups.

Liver function tests were performed to answer a critical question - namely, was the decrease in hepatic function severe enough to cause secondary renal failure as in the hepato-renal syndrome? The hepatorenal syndrome is an incompletely explained renal failure in patients with liver disease in the absence of clinical, laboratory, or anatomical evidence of other known causes of renal failure (169). The renal lesions have been described with biliary cirrhosis (170). The renal lesion has no correlation with the presence or absence of proteinuria (169). There is a reduction in glomerular filtration rate and renal plasma flow. Our findings exclude the presence of any type of hepatic cirrhosis. Moreover, the glomerular filtration rate was not reduced in any of the experimental groups. In agreement with our data, Papper (1983) stated that although there are examples of animal liver disease, none of them is accompanied by renal failure (171). Further, our results indicate that the synthetic ability of liver in the HC and HCHP groups was retained (the albumin level in serum was normal). Also, urea production was increased compared to the CONT group

particularly in the HCHP group. Moreover, the conjugating ability of the liver in the HC and HCHP groups was not severely affected. These data indicate that the possibility of involvement of the hepatic lesion in induction of renal changes is unlikely.

V. Renal Structural and Functional Alterations

1. Effect of cholesterol-rich diet: a - Histological Changes:

A proliferative sclerotic glomerular lesion was induced in the guinea pigs kept on cholesterol-rich diet for seventy days. The main histopathological features in this group encompassed moderate glomerular cellularity and expansion of mesangial areas, in addition to the presence of NSE-positive cells and ORO-positive deposits in the glomerular tissue. In agreement with our findings, French *et al*. have previously reported similar observations in cholesterol-fed guinea pigs (23). On the other hand, Drevon and Hovig (1977) did not find any significant histological changes in kidneys of the same experimental model (99). This might be due to guinea pig strain differences and/or dietary regimen variation. Nonetheless, there has been another report which disagreed with Drevon and Hovig's observations; it demonstrated, in addition to the glomerular lesion, haemosiderin in the proximal convoluted tubules of cholesterol-fed guinea pigs (77). Our results confirmed the presence of Perl's Prussian-blue positive (PPb) materials in the epithelial cells of the cortical tubules. The PPb-positive materials likely represent the reabsorbed dimers of haemoglobin molecules by tubular cells during an intravascular haemolytic process (139).

The histochemical studies demonstrated expanded mesangia in the affected glomeruli in the HC group. A previous report (23) is in agreement with this

finding. In this study, the ultrastructural findings in kidneys of the HC group at day 70 are similar to those of French et al. (78). These authors reported fusion of foot processes of the glomerular epithelial cells and intravascular erythrophagocytosis. In addition to these observations, we detected different sized lipid droplets inside the cytoplasm of the endothelial cells. Lipid was noted in mesangial cells and infiltrating monocytes.

The morphometric assessment of the glomerular lesion, in this study, showed a significant increase in MA/GTA ratio and MC in the HC group at day 70 compared to the CONT group. Although a small but significant increase in MA/GTA ratio was observed at day 30, there were no significant changes in these parameters at days 5 and 10. No previous study has dealt with a morphometric assessment of glomerular lesions found in cholesterol-fed guinea pigs.

In summary, the cholesterol-rich diet induced a glomerulosclerotic lesion in guinea pigs. This lesion included mesangial cellular proliferation, mesangial expansion, monocyte/macrophage infiltration of the glomerulus and the mesangium and lipid deposits. These histological changes led to an increase in mesangial area.

b. Renal functional alterations:

24-hour urinary protein (UPr) significantly increased at day 70 in the HC group compared to the CONT group. Changes in renal function have not been monitored in previous studies of cholesterol-induced kidney diseases in animals. However, in humans, proteinuria has been reported with hyperlipidaemia and hypoalbuminaemia in cases of nephrotic syndrome (10).

Also, in LCAT deficient patients, proteinuria is common (1). In both these situations there are quantitative and qualitative lipoprotein changes accompanied by variable degrees of glomerular changes in some cases. The proteinuria can be either glomerular or tubular. The deterioration of the tubular reabsorptive capability due to protein overload in glomerular filtrate has been mentioned previously (172,173). Our study did not demonstrate marked tubular disease in either of the HC or HCHP groups. Thus, proteinuria found in the HC group seems due, mainly, to a decrease in glomerular permselectivity.

Mild haematuria was noticed in the HC group at day 70. These findings are in agreement with those reported by French et al (23). They found erythrocyte aggregations in different portions of renal tubules. Haematuria in the cholesterol-fed animals could be attributed to the factors which cause damage of GBM. The mechanism of glomerular damage is discussed in the third part of this section. However, there is always a possibility of confusion in tracing the origin of haematuria. There are, at least, three possible causes of haematuria: glomerular damage, interstitial injury, and any injury to the post-renal urinary tract. In the present study, there was no evidence of tubulointerstitial injury. Since lower urinary tract injury is sporadic, it is unlikely that this would be a cause of haematuria in HC since all animals demonstrated this change. Thus glomerular damage is the most likely cause of the haematuria.

The levels of both UPr and haematuria in the HC and HCHP groups at day 70 (see the "effect of cholesterol/protein-rich diet" in this section), correlated well with the number of the glomerular NSE-positive cells and

MA/GTA ratio. This suggests a direct relationship between renal structural alteration and functional deterioration.

Blood urea nitrogen (BUN) was moderately increased in the HC group at day 30 and became higher at day 70. This gradual increase is unlikely to be accounted for by impairment of glomerular filtration since creatinine clearance was normal. It might be attributed to haemolytic anaemia and decreased weight gain. It is known that urea synthesis increases in cases of tissue breakdown and decreased protein synthesis (174).

Serum creatinine level in the HC group did not increase significantly throughout the experimental period, indicating a lack of serious renal functional impairment (the creatinine clearance was normal in the HC group). However, the serum creatinine levels were in parallel to those of weight gain in both groups. This observation suggests that changes in serum creatinine were related to the changes in body muscle mass. Such a correlation has been established earlier (175). As mentioned above, creatinine clearance in the HC group at day 70 was unchanged from that in the CONT group. Such findings indicate that the glomeruloproliferative lesion was not severe enough to decrease GFR.

2. Effect of Cholesterol/Protein - Rich Diet:

a. Histological Changes:

No available reports have described the combined effect of cholesterol- and protein-rich diets on the progression of the glomerular lesion in experimental animals. Our data revealed a similar pattern of histopathological changes in both HC and HCHP groups. However, the renal changes were more pronounced in the HCHP group than in the HC group.

The strength of the Perl's Prussian-blue positive reaction was more prominent in the tubular epithelial cells of the HCHP group at day 70 than in the HC group. Since this change is probably due to the renal tubular reabsorption of dimers of haemoglobin molecules, it is consistent with greater haemolysis observed in the HCHP group.

In the HCHP group there was an increase in the glomerular NSE-positive cells compared to the HC group at day 70. Further, there was a significant but weak correlation between the number of the glomerular NSE-positive cells and MA/GTA ratio found among the groups throughout the experimental period. These observations suggest a causal relationship between NSE-positive cells and the mesangial expansion in the HC and HCHP groups (see the third part of this section for detailed discussion).

b. Renal functional alterations:

The UPr and haematuria were markedly higher in the HCHP group at day 70 than in the HC group and other control groups. This likely reflects a greater glomerular damage in the HCHP group. Supporting this conclusion is the presence of higher MA/GTA ratio in the HCHP group than in the HC group.

Serum BUN was markedly higher in the HCHP group than in the HC, HP and CONT groups. In addition to haemolytic anaemia and decreased weight gain, the higher BUN level in the HCHP group compared to the other groups is likely due to the high protein supplement, resulting in more amino acids being metabolized to urea in the liver (176). This is the likely explanation for the HP group's increased level of serum BUN.

In conclusion, addition of high protein supplement to a cholesterol-rich diet aggravated the renal functional and structural abnormalities found in

the animals kept on a cholesterol-rich diet alone. The major abnormalities consisted of proteinuria and haematuria.

Previously, it has been suggested that glomerulosclerosis in cholesterol-fed guinea pigs might be induced by haemolytic anaemia (23). Our data showed no Perl's Prussian blue-positive deposits in the glomerular lesions in the HCHP and HC groups. Moreover, after induction of haemolytic anaemia by acetylphenylhydrazine injections, there was no glomerular change. However, there was a massive infiltration of Perl's Prussian blue-positive material in the epithelial cells of renal tubules, a finding consistent with haemolytic anaemia. These data make the proposed glomerulosclerotic role of haemolysis very unlikely. However, by different mechanisms, glomerular lesions were reported in sickle cell disease (177) and malaria (178).

The normal serum phosphate in all groups and the absence of tissue calcification indicate that difference in diet phosphorus (in casein-rich diet) cannot account for the glomerular lesions found in the HCHP group. The absence of glomerular lesions in the HP group also supports this conclusion.

The next section deals with the biochemical-histological interactions which might take place in kidney of this animal model.

3. Kidney Lipid Content: Origin and Cellular Interaction

In kidney tissue, CE% was significantly increased in the animals kept on a cholesterol-rich diet for 30 and 70 days. No significant changes found at days 5 and 10 of the experiment. Previous studies dealing with cholesterol-fed rabbits (179) and guinea pigs (109,99,155) have also shown increased cholesterol content of kidney tissue. Our results confirm the

observations of Drevon and Hovig (99) in that there was no significant increase in the content of kidney FC, TPL and TG in the cholesterol-fed guinea pigs. However, we noticed a tendency to an increase in FC and TPL in these animals. In contrast, FC content in kidney tissue was significantly increased in the HCHP group at day 70 of our study compared to the CONT group.

What is the source of the increased lipid content in kidney tissue? In general, there are three possible sources of tissue lipid: first, direct delivery from plasma, second, cell-mediated delivery of lipid, and third, kidney cholesterol de novo synthesis. Regarding the first source, lipids could be delivered either selectively or as a part of an intact lipoprotein to the cells of different tissues (10,24,147,180). Cholesterol flux between surfaces of lipoproteins and cells has been reported (180) such that free cholesterol is delivered to peripheral cells from abnormal cholesterol-loaded lipoproteins. Moreover, preferential degradation of cholesterol of HDL by different cells has been demonstrated by several investigators (151-153). In our study, alpha-migrating lipoprotein was detected at day 70 in the HC and HCHP groups and HDL-C was increased, as well. This might suggest a similar mechanism of cholesterol degradation by glomerular endothelial cells. This speculation is supported by the presence in the HC and HCHP groups of slowly migrating VLDL found a lipoprotein species which was shown to be cholesterol-rich VLDL (see discussion, section II). More evidence in support of this concept is derived from the findings of the present study that revealed some of the endothelial cells of the glomerular capillaries contained clear vacuoles, presumably lipid. Nevertheless, the presence of these droplets in the lining of glomerular capillaries could

also indicate incorporation of intact lipoproteins into the endothelial lining. Other workers have addressed this possibility, too. Iverius (1972) stated that VLDL and LDL could bind to polyanionic glycosaminoglycans of the GBM, such that these lipoproteins might alter the permeability of the glomerular barrier (24). Later, Moorhead et al. (1982) included these findings in their hypothesis of the role of lipid in chronic progressive glomerulo-tubulo-interstitial disease (10). Thus, the increased lipid content of glomerular tissue might be selectively derived from cholesterol-rich lipoproteins and/or binding of intact lipoproteins to GBM and glomerular endothelial cells. Delivery of lipid to kidney tissue could be further increased by blood-borne monocytes which infiltrate glomerular mesangium and protein-rich dietary supplementation (discussed later in this section).

The other source of the fatty accumulation in kidney tissue is cell-mediated delivery of lipid. Monocyte-macrophage and erythrocyte are the most likely cells which could be implicated in this process. Kreisberg et al. (1979) pointed out that monocytes participate directly in inducing glomerular injury through release of lytic enzymes that alter GBM (181). This effect could promote the lipid-induced damage of GBM and enhance permeation of intact lipoproteins into mesangium and to renal tubules. The circulating LLM might have contributed to the lipid delivery to the mesangium. Nevertheless, we could not provide conclusive evidence that circulating LLM infiltrate the mesangial tissue where it appears as NSE-positive cells. In other words, although LLM might infiltrate GBM to the mesangium as other monocytes do, it is unlikely that LLM can be the main source of the intraglomerular NSE-positive cells. This conclusion is

justified by our observation that not all the intraglomerular NSE-positive cells were lipid-laden. Thus, it is unlikely that circulating LLM are an important source of the glomerular lipid. The infiltrating blood-borne monocyte into glomerular mesangium might engulf the permeating lipoprotein species. In this regard, Goldstein et al (1980) demonstrated a high affinity binding site on the surface of mouse peritoneal macrophages which recognizes beta-VLDL isolated from hyperlipidaemic dogs (147). They reported macrophage uptake of the beta-VLDL, LDL, and HDLc; however, beta-VLDL was the most efficient stimulator of cholesterol esterification inside macrophage. In agreement with this report, in vivo studies demonstrated that accumulation of beta-VLDL in the blood stream of cholesterol-fed animals is associated with cholesteryl ester deposition in macrophages in a variety of tissues (182,183). Since we found beta-VLDL and cholesterol-rich alpha migrating lipoprotein in the HC and HCHP group at day 70, we speculate that intramesangial monocyte-macrophage might take up these abnormal lipoproteins by a mechanism similar to that described above.

In brief, monocytes could mediate lipid accumulation in the glomerular mesangium mainly by aggravating damage of GBM which enhances filtration of intact lipoproteins. Also, blood-borne monocytes could infiltrate into mesangium and engulf the filtered lipoproteins.

The other possible cellular source of the accumulated kidney lipid is the erythrocyte membrane (EM). The importance of this source is suggested by two observations reported in our study: first, the EM was cholesterol rich in the HC and HCHP groups, and second, the presence of intraglomerular erythrophagocytosis. The latter phenomenon was a rare observation even in the HCHP group. Moreover, there was no indication of intramesangial

haemolysis in both HC and HCHP groups. These findings argue against EM as an important source of the lipid accumulated in kidney tissue.

The third possible source of lipid in kidney tissue is de novo cholesterol synthesis. The present study demonstrates suppression of de novo cholesterol synthesis in the HC group. This finding agrees with those reported by several other investigators (184-186). Brown and Goldstein (1979) demonstrated that increased cholesterol uptake suppresses synthesis of endogenous cholesterol at the level of HMG-COA reductase (184). Also, HMG-COA reductase activity is reduced in fibroblasts cultured in an LDL-rich medium (185). Furthermore, using tritiated water incorporation into cholesterol as a means to measure the rate of cholesterol synthesis, it has been demonstrated that the rate of tritiated water incorporation was reduced in cholesterol-fed rats (166). Our findings in the cholesterol-fed animals confirm the previously reported suppressive effect of cholesterol feeding on de novo cholesterol synthesis. However, protein supplementation of the cholesterol-rich diet resulted, in our study, in an increase in the de novo synthesized cholesterol compared to the level found in the HC group (albeit significantly less than that in the CONT group). This relative increase in de novo synthesis might be due to the increase in glomerular cellular proliferation (requiring more membrane synthesis) found in the HCHP group compared to the HC group at day 70. Interestingly, the HP group showed a reduced rate of de novo cholesterol synthesis compared to the CONT group at day 70. This finding might be explained, in part, by the hypercholesterolaemic effect of protein which was shown in experiment I and agreed with findings of other investigators (102,122,123,124,125). Also, concomittant with the slight increase in plasma cholesterol in the HP group,

we found, in the chemical analysis of kidney lipid, a tendency to an increase in kidney cholesterol content. These findings indicate that suppression of de novo cholesterol synthesis occured due to a relative increase in cholesterol delivery to kidney tissue. However, we believe that other mechanism(s) besides the one we propose might be involved to account for the level of reduction in kidney. Thus, further investigation is required to explain this observation. Our data indicate the increase in the lipid content of kidney tissue found in the HC and HCHP groups is unlikely to be synthesized in situ, except for that needed for mesangial cellular proliferation.

Altogether, the present findings suggest that the accumulated kidney lipid content found in the HC and HCHP groups is of plasma origin. Our observations backed by data of other investigators suggest that plasma lipid could be delivered to glomerular tissue either as separate components (e.g. cholesterol) or as intact lipoproteins.

Our results show a significant correlation between the lipid content of kidney tissue and the degree of mesangial expansion. This finding is consistent with a previous report which suggested that binding of plasma lipoproteins to the glomerulus may stimulate production of matrix by mesangial cells (187). On the other hand, the early work of French et al indicated no correlation between the presence of fatty deposits in glomeruli and the degree of glomerulosclerosis (23). This seems to be due to the fact that they derived their correlation from qualitative assessment of both lipid deposits and histological lesions. However, no significant difference at day 30 in the intraglomerular NSE-positive cells was detected between HCHP and HC groups. This indicates that a glomerular proliferative lesion

could be induced by accumulated lipid materials in absence of a significant increase in the number of NSE-positive cells. There is no direct evidence in the literature which supports a mitogenic role for lipid in the development of glomerulosclerotic lesions. However, in a recent editorial, Kashgarian (1985) stated that proteinaceous macromolecules engulfed by mesangial cells by endocytosis may activate these cells to proliferate and/or produce matrix (188). This suggestion may provide at least a partial explanation of our finding. High protein supplement induces hypercholesterolaemia (102,118,122,123,124,125) and increases GFR (31,189,190) and glomerular plasma flow (191). Thus, at day 30 when there was no significant increase in intraglomerular NSE-positive cells in the HCHP group compared to HC group more lipid deposition could be induced in the HCHP group by virtue of the two mentioned effects of protein. The significant increase in MA/GTA in the HCHP group compared to the HC group at day 30 was most likely due to the increase of lipid deposition in the HCHP group compared to the HC group.

In addition to the significant increase in the kidney lipid content in both HC and HCHP groups which correlated well with MA/GTA ratio, there was a significant correlation at day 70 between the degree of monocytic infiltration and the MA/GTA ratio. Since there were no significant changes in the lipid contents between days 30 and 70, in neither the HC nor the HCHP groups, it is reasonable to conclude that monocytic infiltration augmented the lipid-induced glomerular lesion. The involvement of the monocyte in the pathogenesis of glomerulosclerosis is consistent with the results of previous investigations. In humans, using NSE stain and/or electron microscopy, Monga _et al_. (1985) reported monocyte infiltration of glomeruli

in various types of glomerulonephritis (192). Also, recently, the involvement of monocyte in various types of experimental glomerulonephritis has been reviewed (193). In this regard, a histochemical study has shown that monocytes constituted the major proportion of the cells in glomerular crescents in rabbit and sheep (194). Furthermore, in an in vitro study, it has been suggested that blood monocytes might play a role in the pathogenesis of mesangial cell proliferation in glomerulonephritis (195). Recently, Ferrario et al. (1985) reported a good correlation between proteinuria and intraglomerular monocytic infiltration (196).

We have demonstrated that the accumulated lipid material found in kidney in the HC and HCHP groups originated mostly from plasma. It has been reported that rats fed a high-protein diet had higher glomerular plasma flow than that found in rats kept on low-protein diet (190). It is possible that in the earlier stages of our experiment (prior to the development of glomerulosclerosis), plasma flow was increased in the HCHP group, resulting in more lipoprotein particles delivered to the glomeruli. However, we could not provide conclusive evidence for such an increase in the plasma flow. On the other hand, our findings in the HP group indicate that no glomerular lesions were induced in the absence of abnormal lipoproteins. This suggests that a synergistic effect of increased plasma flow and abnormal lipoproteins in mesangial tissue induces a glomerular proliferative lesion in the HCHP group.

Alterations in glomerular haemodynamics due to high-protein diet have been implicated in the pathogenesis of mesangial injury (197). However, the mechanism is still unknown. Raij et al. (1983) provided experimental evidence which suggested that alterations in glomerular haemodynamics act

synergistically with mesangial entrapment of macromolecules (198). Their findings are in agreement with our hypothesis that increased glomerular plasma flow leads to increased delivery of plasma lipoproteins to the glomerular tissue where the abnormal lipoproteins are trapped. The basis of our understanding of the mechanism of aggravating cholesterol-induced glomerulosclerosis by high protein supplement can be explained by the following scenario:

In the cholesterol-fed guinea pigs, the increase in plasma lipids led to an increase in renal tissue lipid which was accompanied by increased mesangial expansion. The initiation of the glomerular lesion by lipid seems to be aggravated by the presence of monocyte-macrophage. The blood-borne monocytes infiltrate the glomerular mesangium and participate in induction of a glomerular lesion by an as-yet-unidentified mechanism (200,201). However, it has been proposed that the monocyte stimulatory effect on mesangial proliferation could be due, in part, to enhancement of endogenous mesangial cell prostaglandin E production (202). Once the mesangial cells are stimulated by a mitogenic factor(s) released by infiltrating monocyte-macrophage, mesangial cells proliferate and produce another growth factor (203). In this regard, Lovett _et al_. (1986) purified and characterized a protein derived from cultured glomerular mesangial cells. They found that this cytokine has a close resemblance to interleukin 1 produced by macrophage and acts as an autocrine or paracrine growth factor (203). A similar finding has been reported by the same group showing a mesangial cell-derived thymocyte-activating factor which induces thymocyte proliferation and enhances production of interleukin-2 by peripheral lymphocytes (204). These workers have suggested that the local release of

cytokine by glomerular mesangial cells might be an important factor in enhancing mesangial proliferation and matrix expansion. Thus, monocyte infiltration into glomerular mesangium may induce an accelerated propagation of mesangial cells exerted by a cascade of actions started by a monocyte-derived growth factor which stimulates mesangial cell proliferation. Since it has been demonstrated that cultured mesangial cells produce different connective tissue components (205), we believe that the expanded mesangial matrix in our study is due to proliferating mesangial cells. Supporting this conclusion, the magnitude of the PAS-positive lesions we noticed in the HC and HCHP groups was in accordance with the values of MA/GTA ratios.

We did not study the chemotactic mechanism which induces monocytic infiltration into the mesangial tissue. However, different chemotactic factors might be implicated, among them the platelet-derived growth factor (PDGF) (199) and a factor produced by endothelial cells previously exposed to beta VLDL (206). These need to be studied in detail.

The high-protein supplementation might lead to increase renal plasma flow prior to the development of glomerulosclerosis in the HCHP group (as discussed above). This effect could cause more delivery of blood monocytes to the glomerular tissue, a suggestion which is supported by the presence of a significant correlation between mesangial expansion and the number of intraglomerular monocytes. With this in mind together with the presence of greater lipid deposition in the glomerular tissue of the HCHP group compared to the HC group, one could explain the aggravating effect of the high protein diet on the cholesterol-induced glomerular lesion.

VI. General Conclusions and Postulated Mechanisms of Anaemia and Glomerulosclerosis

Cholesterol feeding induced haematological and renal changes in guinea pigs which are related to plasma lipid and lipoprotein abnormalities. Cholesterol may transfer from cholesterol-laden lipoproteins to erythrocyte membrane driven by its concentration gradient. Cholesterol-enriched erythrocytes acquires echinocyte morphology which would, likely, be subjected to phagocytosis and the "pitting process" by splenic macrophages. The net sequalae of these two processes are extra- and intravascular haemolytic anaemia. Similarly, cholesterol could selectively transfer from the cholesterol-laden lipoproteins to glomerular tissue; in addition, these lipoproteins might bind to the glomerular endothelial lining and GBM. The blood-borne monocyte-macrophages infiltrated into the mesangial tissue could stimulate mesangial cellular proliferation by an as-yet-unidentified mitogenic factor, possibly interleukin-1. There is evidence that proliferating mesangial cells may release a growth factor which could amplify the cellular proliferation and production of cellular matrix.

Enrichment of the cholesterol-rich diet by a high protein supplement aggravated the hypercholesterolemia which, apparently, worsened haemolysis and glomerulosclerosis. In addition to the hypercholesterolaemic effect, the high protein supplement may have increased delivery of lipoproteins and monocytes to the glomeruli by increasing glomerular plasma flow prior to the development of glomerulosclerosis. The increase in monocyte infiltration may be partly responsible for the augmented mesangial expansion seen in the HCHP group. However, in the early stage (day 30), no significant correlation between intraglomerular monocytes and MA/GTA ratio was detected;

thus, the mesangial lesion found in the HC and HCHP groups at that time was mainly attributed to the lipid effect on mesangial cells. It is these findings that permit our conclusion that cholesterol-induced glomerulo-sclerosis could be accounted for by both lipid and monocytic infiltration into mesangial tissue.

The significant correlation between proteinuria and haematuria and the glomerular changes indicate a good relationship between the structural and functional alterations found in the HC and HCHP groups.

Our data did not support the concept of a relationship between either haemolytic anaemia or abnormalities of liver function and the glomerulosclerotic lesion, nor was the lesion an immune-mediated one.

New Findings in this study:

1. The sequential studies at 5, 10, 30, and 70 days showed that:

   a - The lipid deposits in the glomerular tissues of cholesterol-fed guinea pigs initiate the glomerulo-sclerosis. The presence of abnormal lipoproteins in plasma is a prerequisite for these deposits.

   b - The monocytic infiltration into the glomerular tissue follows the lipid deposits and augments the glomerulosclerotic process. We postulate that a process resembling atherosclerosis is taking place at the capillary level.

2. The high protein suppplementation aggravated the cholesterol-induced glomerulosclerosis, most likely by elevating plasma cholesterol. In addition, monocytic infiltration into lipid-infiltrated glomeruli was increased in animals on high cholesterol diet with protein supplement.

3. We ruled out haemoloysis as a possible cause of the glomerulosclerotic lesion found in cholesterol-fed guinea pigs.
4. Lipid-laden monocytes were observed in the circulation of cholesterol fed guinea pigs. The number of these cells increased after a high-protein supplementation.

VII. Further Work

Several aspects of our findings need to be further investigated in greater detail:

1. Confirmation, in *in vitro* and may be *in vivo* studies, that mesangial cell proliferation could be induced by lipid materials in the absence of monocytes or other cellular types which can release mitogenic factors (e.g. platelets).
2. Investigation of the possible chemotactic factors which might account for the monocytic infiltration into the mesangial tissue.
3. Studying the isolated effect of each lipoprotein species (from normal and hyperlipidaemic animals) and free cholesterol on the induction of mesangial cell proliferation.
4. Elucidation of the mechanism accounting for the appearance of lipid-laden monocytes in the circulation.

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