THE ANTI-PROTEINURIC EFFECTS OF UNSATURATED FATTY ACID DIETS IN HEALTHY RATS

by

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Declaration

This thesis was compiled, written and typed entirely by me alone and does not contain extracts from other works or authors unless otherwise stated. The laboratory work was performed by me in the University Department of Medicine, Glasgow Royal Infirmary unless otherwise stated and parts have been published or presented as listed below.

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Abbreviations

Å	Angstrom (10^{-10} m)
AA	Arachidonic acid
ACE	Angiotensin converting enzyme
ACE-I	Angiotensin converting enzyme inhibitor
ADR	Adriamycin
AER	Albumin excretion rate
AI	Angiotensin I
AII	Angiotensin II
ALA	α-linolenic acid
CD	Control diet
CI	Confidence interval
COI	Cyclooxygenase inhibitors
DGLA	di-homo γ linolenic acid
DA or D	DA rat
DHA	Docosahexaenoic acid
EFA	Essential fatty acid(s)
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPA	Eicosapentaenoic acid
EPO	Evening primrose oil
EPOD	Evening primrose oil diet
FA	Fatty acids
FC	Fractional clearance
FO	Fish oil
FOD	Fish oil diet
GAG	Glycosaminoglycans
GBM	Glomerular basement membrane
GC-MS	Gas Chromatography - Mass spectrometry
GCW	Glomerular capillary wall
GFR	Glomerular filtration rate
GLA	γ-linolenic acid
GN	Glomerulonephritis
HDL	High density lipoprotein
HRP	Horseradish peroxidase
HSA	Human serum albumin
HSPG	Heparan sulphate proteoglycan
ICGN	Immune complex glomerulonephritis

IDDM	Insulin-dependent diabetes mellitus
IF	Immunofluorescence
JGA	Juxtaglomerular apparatus
kD	kilo Dalton
Kf	Ultrafiltration coefficient
Lew or L	Lewis rat
LA	Linoleic acid
LDL	Low density lipoprotein
MCN	Minimal change nephropathy
M_r	Molecular radius
MWt	Molecular weight
NSAID	Non-steroidal anti-inflammatory drugs
NSN	Nephrotoxic serum nephritis
OO	Olive oil
OOD	Olive oil diet
PAN	Puromycin aminonucleoside nephrosis
PBS	Phosphate buffered saline
Pgc	Glomerular hydrostatic pressure
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
PHN	Passive Heymann nephritis
pI	Isoelectric point
PUFA	Polyunsaturated fatty acid(s)
QC	Quality control
RPF	Renal plasma flow
RSA	Rat serum albumin
SC	Sieving co-efficient
SD	Standard deviation
SNGFR	Single nephron glomerular filtration rate
SO	Safflower oil
SOD	Safflower oil diet
STZ	Streptozotocin
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2
UFA	Unsaturated fatty acid(s)
VLDL	Very low density lipoprotein

Summary

Proteinuria is the hallmark of impaired glomerular permselectivity and is associated with a poor prognosis in renal disease. It is widely acknowledged that a fall in urine protein excretion may indicate an improvement in renal function or retardation of disease progression. The finding that diets rich in polyunsaturated fatty acids (PUFA), reduced urine albumin excretion in two healthy strains of rat (Lewis and DA) with differing susceptibilities to glomerular disease, was therefore deemed worthy of further investigation.

Each successive study in this thesis is an attempt to unravel the mechanism behind the apparent anti-proteinuric actions of PUFA diets. However, establishing a relationship between PUFA diets and albumin excretion is complicated by not only the many and varied functions of PUFAs which include eicosanoid biosynthesis, cell membrane structure and function and lipid metabolism but also the various factors which control the filtration of albumin such as glomerular capillary wall structure and charge, glomerular haemodynamics and the physico-chemical characteristics of the filtered molecule.

As precursors of prostaglandins and thromboxane, polyunsaturated fatty acids can regulate glomerular blood flow and pressure and modulate the activity of the renin-angiotensin system but ultimately, the observed fall in albumin excretion rate in Lewis and DA rats could neither be attributed to changes in glomerular or circulating levels of prostaglandins, thromboxane or renin levels in the PUFA diet groups. That PUFA diets might effect changes in the intrinsic permeability properties of the glomerular capillary wall in such a way as to reduce the filtration of albumin was explored by assessing the magnitude of the negative charge on the glomerular capillary wall (glomerular polyanion). Lewis rat glomeruli sequestered significantly less of a radiolabelled cationic protein probe than DA rat glomeruli and this was taken as confirmation of a diminished glomerular polyanion in Lewis rats relative to DA rats but overall, PUFA diets had little effect on glomerular capillary wall charge.

The binding of fatty acids to serum albumin can alter the electrical charge of the albumin molecule and it was shown that the isoelectric point of Lewis rat serum albumin was reduced in all the experimental PUFA diet groups compared to that from rats fed a standard laboratory diet. Structural modification of the serum albumin molecule, induced by the binding of unsaturated fatty acids, may have further increased its anionic charge and thereby reduced its filtration across the glomerular capillary wall.

The PUFA diet-induced fall in albumin excretion rate in healthy Lewis and DA rats prompted a study to investigate the capacity of evening primrose oil diet to prevent an anticipated rise in albumin excretion rate in diabetic Lewis rats. Although it reduced both total protein and albumin excretion in Lewis rats during the non-diabetic phase of the study, evening primrose oil diet did not prevent a rise in albumin excretion rate in diabetic Lewis rats.

It is unlikely that the anti-proteinuric effects of PUFA diets in healthy Lewis and DA rats were mediated by either changes in glomerular prostanoid or renin levels or the intensity of the glomerular polyanion and is more likely to be related to a change in the isoelectric point (pI) of the albumin molecule as a result of binding fatty acids. The failure to demonstrate a fall in albumin excretion rate in diabetic Lewis rats fed evening primrose oil diet could be due to competition between glucose and fatty acid molecules for binding sites on the albumin molecule.

Section 1

INTRODUCTION

1.1.1 Background and aims

It is still unclear why some individuals develop the more serious or progressive forms of renal disease than others with identical symptoms (Hood *et al*, 1981, MacTier *et al*, 1986). Differences in susceptibility to nephropathies such as IgA, membranous or diabetic nephropathy might best be explained by the inheritance of a particular glomerular characteristic such as a weakened glomerular polyanion or impaired mesangial clearance function, in association with a particular tissue type (Rees *et al*, 1984, Klouda *et al*, 1979, Kashiwabara *et al*, 1982, Weening *et al*, 1986) or defective sulphation enzyme activity which results in a diminished negative charge on the GCW (Deckert *et al*, 1989). These properties of the glomerulus may determine how it responds to a change in blood flow or pressure or eliminates macromolecules or immune complexes and the subsequent course of disease development (O'Donnell *et al*, 1985, Remuzzi *et al*, 1988, Bertani *et al*, 1989).

Lewis and DA are two healthy strains of rat with different inherited glomerular properties which make them useful models in which to study differences in disease progression (Boulton-Jones *et al*, 1986). The Lewis rat strain is more susceptible to Heymann nephritis, an animal model of membranous nephropathy to which the DA rat strain is resistant and when diabetes is induced by STZ in Lewis rats, they develop more pronounced proteinuria and extensive mesangial expansion than DA rats (Payton and Boulton-Jones, 1989). Compared to DA rats, Lewis rats also have a weaker glomerular polyanion, exhibit reduced rates of mesangial uptake of circulating macromolecules and higher levels of proteinuria following administration of the polycation hexadimethrine (Chandrachud and Boulton-Jones, 1988). Inherited variations in glomerular properties was the most plausible explanation for the differences in susceptibility to diabetic nephropathy between Lewis and DA rats (Payton and Boulton-Jones, 1989).

The work carried out in this thesis attempts to develop the concept of inherited differences in glomerular properties and disease susceptibility in relation to the feeding of polyunsaturated fatty acids (PUFA) diets to Lewis and DA rats with particular reference to an apparent lowering of urine albumin excretion. PUFAs are recognised as precursors of prostaglandins, thromboxane and leukotrienes, structural and functional components of cell membranes and lipid-lowering agents linked to a reduced incidence of cardiovascular risk (see section 1.3) and their effects have been studied in various experimental and human renal diseases (see section 1.4).

1.1.2 Factors associated with glomerular disease progression

Renal disease progresses in association with systemic and glomerular hypertension and renal hypertrophy, mesangial overloading and glomerulosclerosis and derangements of coagulation, lipid metabolism and the immune and inflammatory responses. Proteinuria has long been recognised as the clinical hallmark of glomerular injury, denoting an increased permeability of the glomerular capillary wall to plasma proteins that often mirrors the extent of disease severity (Bright, 1836, Hulme and Hardwicke, 1968, Brenner *et al*, 1978, Winetz *et al*, 1981a, Bertani *et al*, 1986a, Grond *et al*, 1986, Anderson *et al*, 1988, Rennke and Klein, 1989). However, as a reflection of large amounts of protein filtering both across the glomerular capillary wall and through the mesangium, proteinuria is now regarded as an important factor associated with the precipitation and persistance of glomerular injury (Klahr *et al*, 1988, Eddy *et al*, 1989, Remuzzi and Bertani, 1990). Many studies have shown that proteinuria precedes the onset of glomerulosclerosis (Olsen *et al*, 1982, Weening *et al*, 1983, Zatz *et al*, 1986, Anderson *et al*, 1986; 1989, Fogo *et al*, 1988) although nearly half of experimental adriamycin nephrosis (ADR) rats with extensive epithelial cell damage and proteinuria did not develop glomerulosclerosis (Bertani *et al*, 1986a) and there was little sign of glomerulosclerosis in rats with Heymann nephritis in which large quantities of protein filter across the glomerular capillary wall (Barabas *et al*, 1985).

Further evidence of the pernicious nature of proteinuria is the slow incipient rise in urine albumin excretion (microalbuminuria) that predicts of the onset of renal disease in diabetes (Parving *et al*, 1982, Mogensen, 1987) and its association with increased mortality and morbidity in chronic heart disease, perhaps as indicative of more widespread vascular damage (Borch-Johnsen *et al*, 1985, Borch-Johnsen and Kreiner, 1987, Deckert *et al* 1989, Jensen *et al*, 1989). Diabetic patients with microalbuminuria are not only at greatly increased risk of developing nephropathy but are also much more likely to suffer other vascular complications such as ischaemic heart disease (Jensen *et al*, 1987). Established cardiovascular risk factors such as elevated blood pressure and heightened plasma lipid and fibrinogen levels are common in diabetic patients with microalbuminuria (Jensen *et al*, 1988). A fall in albuminuria in diabetic patients as a result of antihypertensive treatments, glycaemic control or low protein diets is taken to indicate an improvement in disease activity (see section 2.5.1)

Several theories have been forwarded to explain the initiation and progression of glomerular disease but the most widely favoured is that glomerular injury is precipitated and perpetuated by changes in glomerular haemodynamics (Anderson *et*

al, 1985a, Zatz and Brenner, 1986, Blantz *et al*, 1987, van Hooft *et al*, 1991). When a critical amount of functioning renal tissue is removed either surgically, as in experimental renal ablation or clinically, as a result of disease, there is a sustained, compensatory rise in blood flow and pressure (hyperperfusion and hyperfiltration) which sets up a cycle of destruction within the remaining healthy nephrons (Deen *et al*, 1974, Shimamura *et al*, 1975, Brenner *et al*, 1981; 1982; 1983, Hostetter *et al* 1981; 1982, Provoost *et al*, 1991). Hyperperfusion not only damages the glomerular endothelium, weakening its natural thromboresistance and increasing the incidence of intraglomerular thrombosis and coagulopathies but also increases transglomerular protein flux (Purkerson *et al*, 1982; 1985, Olsen *et al*, 1984, Schiepatti *et al*, 1984, Zoja *et al*, 1990a, Lianos and Zanglis, 1990).

Hypertrophy is yet another consequence of glomerular hyperfunction and is the kidneys response to a heightened functional demand for reabsorption of an increased filtered solute load in which all the component parts of the nephron are enlarged to increase its reabsorptive and secretory capacity (Fine, 1986, Schweiger and Fine, 1990). Glomerular hypertrophy can cause epithelial cell to detach from the underlying GBM and is associated with heavy proteinuria and progressive glomerulosclerosis (Brenner *et al*, 1985, Fries *et al*, 1989, Yoshida *et al*, 1989, Miller *et al*, 1990). Many groups challenge the importance of haemodynamic factors in the initiation of glomerular injury (Bank *et al*, 1987, Fogo *et al*, 1988, Remuzzi *et al*, 1988, Scholey *et al*, 1989, Yoshida *et al*, 1989; 1989a).

The primary glomerular injury is often precipitated by immune mechanisms (Couser *et al*, 1985, Glassock *et al*, 1992). Immune complexes can cause the glomerular capillary wall to leak either through structural damage or complement-mediated release of cellular and humoral mediators (Cavallo *et al*, 1983, Groggel *et al*, 1985, Cybulsky *et al*, 1986, Salant *et al*, 1989, Stahl *et al*, 1990). Alternatively, agents such as cytokines (Bevilaqua *et al* 1985, Tipping *et al*, 1991), growth factors (Bertani *et al*, 1987, Shultz *et al*, 1988: McNamara *et al*, 1989: Border *et al*, 1989, Zoja *et al*, 1990), hydrolytic enzymes (Bolton *et al*, 1987), proteases and reactive oxygen species released by native or infiltrating immune and inflammatory cells can alter GCW and damage the glomerular capillary wall and mesangium (Fantone *et al*, 1982, Nathan, 1987).

1.1.3 Retarding the progression of glomerular disease

Hypertension not only increases the risk of developing renal disease but also accelerates the rate of decline in renal function in nephrotic patients (National High Blood Pressure Education Program, 1991). Animal (Yoshida *et al*, 1989),

epidemiological (Miller *et al*, 1991) and clinical (Payton *et al*, 1988) studies suggest that reducing systemic blood pressure to within normal limits, not only retards the rate of progression of renal disease but lowers the risk and incidence of cardiovascular disease in susceptible subjects (Neugarten *et al*, 1982, Blantz *et al*, 1987a, ter Wee and Donker, 1992). Although many anti-hypertensive therapies do slow the rate of disease progression (Neugarten *et al*, 1985, Meyer *et al*, 1987, Dworkin *et al*, 1989) some are less effective than others (Dworkin *et al*, 1987). This discrepancy is most likely to be related to the fact that a reduction in systemic hypertension is not always accompanied by a corresponding fall in *glomerular* blood pressure, the latter being a critical factor in reducing proteinuria and glomerulosclerosis. ACE inhibitors, which selectively reduce P_{gc}, afford superior protection from glomerular injury in both animal (Anderson *et al*, 1985; 1986; 1989, Meyer *et al*, 1985, Zatz *et al*, 1986, Brunner *et al*, 1987) and human (Heeg *et al*, 1987, Mann *et al*, 1987, Marre *et al*, 1988, Ruilope *et al*, 1989, Praga *et al*, 1991) glomerulopathies albeit the anti-proteinuric effects of ACE inhibitors in humans vary from significant (Marre *et al*, 1987, Taguma *et al*, 1985, Parving *et al*, 1988) to modest (Hommel *et al*, 1986a, Valvo *et al*, 1988) to non-existent (Bjorck *et al*, 1986).

Dietary protein restriction has been shown to be as effective as antihypertensive therapy in lowering glomerular blood pressure (P_{gc}) in animal models particularly (Neugarten *et al*, 1983, Diamond *et al*, 1987, Woods, 1993). High protein diets increase GFR, protein excretion and histological damage in normal and nephrotic animals (Hostetter *et al*, 1986) and humans (Bosch *et al*, 1986, Ilhe *et al*, 1989) while protein restriction diets retard the progression of renal insufficiency (Brenner *et al*, 1982, El Nahas *et al*, 1984, Wen *et al*, 1985, Remuzzi *et al*, 1985a, Nath, 1986a, Cohen D *et al*, 1987, Okuda *et al*, 1987, Wiseman *et al*, 1987, Evanoff *et al*, 1988, Castellino *et al*, 1989). Increased dietary protein is thought to impair glomerular function by inducing glomerular hypertension as a result of afferent arteriolar vasodilation; however, changes in protein intake are not always accompanied by shifts in P_{gc} (Ichikawa *et al*, 1980, Mauer *et al*, 1985, O'Donnell *et al*, 1990, Weigmann *et al*, 1990). The vasodilation has been linked to activation of the renin-angiotensin system (Rosenberg *et al*, 1987; 1990) or heightened prostaglandin (Levine *et al*, 1986, Stahl *et al*, 1987a, Krishna *et al*, 1988), hormone (Kontessis *et al*, 1990) or nitric oxide (King *et al*, 1991) activity. Protein loading may also induce hypertrophy by increasing the renal work load required to excrete urea (Ichikawa *et al*, 1980a). While both enalapril and low protein diets reduced protein excretion and early sclerotic lesions in experimental renal disease (Marinides *et al*, 1990) only enalapril markedly lowered proteinuria in patients with chronic glomerular injury - protein restriction did

not (Don *et al*, 1991). Neither did protein restriction improve renal function nor size selectivity in patients with membranous nephropathy (Remuzzi *et al*, 1991).

Hyperlipidaemia is implicated in both the initation and progression of renal disease (Kasiske *et al*, 1990, Keane *et al*, 1990; 1991: Diamond, 1989, Kaysen *et al*, 1986; 1991, Klahr and Harris, 1989). Accelerated rates of glomerulosclerosis were observed in rats fed cholesterol-rich diets (Diamond and Karnovsky, 1987a) while lipid lowering drugs such as clofibric acid and lovastatin improved renal function in obese Zucker rats and rats with reduced renal mass (Kasiske *et al*, 1985; 1988; 1988a), PAN rats (Harris *et al*, 1990) and nephrotic humans (Rabelink *et al*, 1990, Chan *et al*, 1992). Hyperlipidaemia is thought to be the result of diminished clearance and heightened synthesis of lipids and lipoproteins by the liver, the latter in reponse to a low serum oncotic pressure (Appell *et al*, 1985, Kaysen *et al*, 1987). Loss of liporegulatory proteins via the urine may also contribute to hyperlipidaemia (de Mendoza *et al*, 1976, Staprans *et al*, 1981).

The link between hyperlipidaemia and proteinuria has been attributed to both abnormal lipid deposition which can impair the permeability of the glomerular capillary wall (Iverius, 1972, Grone *et al*, 1989, Moorhead *et al*, 1991, Takemura *et al*, 1993) and glomerular hypertension due to raised plasma viscosity (Keane *et al*, 1991). Glomerulosclerosis and atherosclerosis have similar pathogenetic origins (Diamond and Karnovsky, 1988). An increased uptake of low density- and oxidised lipoproteins by the mesangium may stimulate mesangial proliferation and expansion (Coritsidis *et al*, 1991). However, the relative infrequency of renal disease in patients with common forms of hyperlipidaemia does suggest that lipids induce glomerular injury mainly in conjunction with other predisposing factors. The lipid-lowering activity of PUFA diets (Harris *et al*, 1983, Nestel *et al*, 1984; 1990, Barcelli *et al*, 1988, Mensink *et al*, 1989) may contribute to improvements in blood viscosity, pressure and flow (Terano *et al*, 1983, Cartwright *et al*, 1985, Rogers *et al*, 1987) and lipid deposition within the glomerular and systemic vasculature (Phillipson *et al*, 1985, Boberg *et al*, 1986).

The kidney functions as a molecular sieve, filtering, reabsorbing and metabolising proteins while conserving serum albumin stores which are essential for maintaining up to 80% of the oncotic pressure of the blood. The glomerular capillary wall was defined as the major structural barrier to the filtration of proteins, after minute quantities of the glomerular ultrafiltrate were aspirated directly from Bowmans capsule or the proximal tubule and found to contain negligible amounts of protein (Walker *et al*, 1941). Solutes and low molecular mass proteins which do pass from the plasma into the glomerular ultrafiltrate are reabsorbed to varying degrees by endocytosis and degraded in the renal proximal tubules; normally, more than 90% of any albumin which is filtered is reabsorbed (Landewehr *et al*, 1977, Mogenesen and Solling, 1977).

Proteinuria occurs when the filtered load of albumin or other proteins overwhelms the reabsorptive capacity of the tubules. The passage of an albumin molecule across the glomerular capillary wall is a function of

- the structure and electrostatic charge of the glomerular capillary wall
- blood flow and pressure within the glomerular capillary
  and
- the size, charge and shape of the filtered molecule

but the amount of protein which appears finally in the urine is dependent also upon its plasma concentration and the rates of both glomerular filtration and tubular reabsorption.

1.2.1 Glomerular capillary wall (GCW) structure

Each glomerulus is a network of anastamosing capillaries across which blood filters passively but under pressure, to form a virtually protein-free ultrafiltrate of the plasma (Pappenheimer, 1951). The glomerular capillary wall (GCW) allows solutes and low molecular weight macromolecules or proteins to pass through unimpeded while molecules the size of albumin (36 Å) and larger are restricted to varying degrees.

From the lumen outwards, the GCW comprises three layers - (i) a vascular endothelium, (ii) an extracellular glomerular basement membrane (GBM) and (iii) a visceral epithelium. For a plasma protein such as albumin (36 Å) to reach the glomerular ultrafiltrate, it must overcome all three layers.

The glomerular endothelium, which is perforated with numerous pores of up to 100 Å in diameter, is unlikely to offer much resistance to circulating macromolecules although a layer of anionic sialic acid residues on the endothelial cell surface, known as the glycocalyx, will repel anionic molecules like albumin to some degree (Blau and Haas, 1973).

The glomerular basement membrane (GBM) is a three-layered extracellular gel matrix comprising a central *lamina densa* sandwiched between the sub-endothelial *lamina rara interna* and sub-epithelial *lamina rara externa.* Strong, tensile perpendicular fibrils tether these two outer layers to the *lamina densa* (Batsford *et al*, 1987, Caulfield *et al*, 1987). The GBM is approximately 300 nm wide in humans and 150 nm in the rat or mouse (Takami *et al*, 1991). Extensively crosslinked by disulphide bonds, the GBM can be separated into a collagenous glycopeptide fraction containing hydroxylysine, hydroxyproline and glycine and a more polar proteoglycan fraction. The collagen backbone of the GBM complexes with (i) glycoproteins such as fibronectin, laminin (Ekblom *et al*, 1991), podocalyxin (Kerjaschki *et al*, 1984) and the entactin-nidogen complex (Katz *et al*, 1991) and (ii) various large polyanionic proteoglycans (95% polysaccharide and 5% protein) which bind water and cations (Gallagher *et al*, 1986, Lelongt *et al*, 1987b). Glycosaminoglycans (GAG) are the variable disaccharide units of glucosamine and galactosamine, the sulphate and carboxylate groups of which give proteoglycans their negative charge (Lindahl and Hook, 1978, Kanwar *et al*, 1981). Heparan sulphate, chondroitin sulphate and keratan sulphate, hyaluronic acid and heparin are the principal GAGs of the glomerular polyanion and are abundant particularly in the *lamina rara externa* (Caulfield *et al*, 1976, Kanwar and Farquhar, 1979a; 1984, Timpl,1986). These anionic groups were first identified using cationic dyes or tracers such as ruthenium red (Kanwar and Farquhar, 1979), alcian blue (Caulfield *et al*, 1979), cationic ferritin (Danon *et al* 1972, Rennke *et al*, 1975) and polyethyleneimine (Schurer *et al*, 1978). Glomerular permeability to anionic ferritin (Kanwar *et al*, 1980) and albumin (Rosenzweig and Kanwar, 1982) was shown to rise following removal of heparan sulphate with heparinase. In contrast, chondroitinase and neuraminidase, enzymes which hydrolyse only sialic acid residues, did not have the same potency (Vernier *et al*, 1983). The GAG and sialic acid content of the GCW is altered or diminished in many glomerulopathies and experimental diabetes (Cohen and Surma, 1981, Brown DM *et al*, 1982, Parasarathy and Spiro, 1982, Wahl *et al*, 1982, Rohrbach *et al*, 1986, Groggel *et al*, 1988).

The GBM becomes thickened in a variety of glomerulopathies but whether as a result of increased synthesis, or diminished degradation of GBM glycoproteins is not clear (Sternberg *et al*, 1983, Reddi *et al*, 1985, Cohen, 1987, Nimni and Harkness, 1988). Reduced collagenase and lysosomal enzyme activity is reflected by increased GBM glycan levels in established diabetic nephropathy (Monahan and Bose, 1983). In diabetes, endogenous GBM proteins with a slow turnover rate, are susceptible to non-enzymatic glycosylation (Cohen and Ku, 1984, Sternberg *et al*, 1985, Tarsio *et al*, 1985) and although early glycation products are relatively harmless and their production can be reduced by insulin, gradual accumulation of advanced glycation products is more persistent and destructive (Brownlee *et al*, 1988, Ellis and Good, 1991). Glycosylation can alter the structure and binding properties of GBM proteins and phospholipids (Brownlee *et al*, 1985, Abrahamson, 1986, Klein *et al*, 1986, Vishwananth *et al*, 1986, Silbiger *et al*, 1993) but to date, the evidence suggesting that non-enzymatic glycosylation of GCW components is responsible for increased glomerular permeability in diabetes is still rather weak (Copeland *et al*, 1987). GBM thickening normally starts during the incipient phase of diabetic nephropathy (and presents about two years after diagnosis) but does not correlate with declining renal function (Osterby, 1975, Falk *et al*, 1983, Mauer *et al*, 1984, Thompsen *et al*, 1984, Ellis *et al*, 1986, Tarsio *et al*, 1988).

The third and outermost layer of the glomerular capillary wall is the visceral epithelium with its characteristic interdigitating foot processes, known also as podocytes. The passage of a molecule through this layer is limited by thin filtration slit diaphragms (4Å - 6Å thick) which span the narrow filtration slits (20Å - 30Å wide) between the foot processes. Viewed face on, the slit diaphragm comprises an ordered zipper-like array of rectangular pores which have dimensions similar to those of an albumin molecule (40Å x 140Å) (Furukawa *et al*, 1991). Colloidal iron staining identified a substantial glycocalyx extending over the surface of the epithelial foot processes and into the filtration slits, terminating deep within the *lamina rara externa* (Caulfield, 1979). Glomerular epithelial cells also synthesise not only constituent glycoproteins and proteoglycans of the underlying GBM but secrete heparin or heparin-like substances which may inhibit mesangial cell proliferation and glomerular hypertrophy (Castellot *et al*, 1985). Glomerular epithelial cell damage, in particular, is associated with nephrotic range proteinuria in animal and human nephropathies (Grishman and Churg, 1975, Bertani *et al*, 1982, Weening and Rennke, 1983, Rollason and Brewer, 1984, Messina *et al*, 1987, Fries *et al*, 1987). Being unable to replicate in response to a proliferative stimulus, podocytes become distorted and detached from the underlying GBM, giving rise to 'bald' patches along the glomerular capillary wall through which proteins can pass freely into Bowman's

capsule (Kanwar and Rosenzweig, 1982, Fries *et al*, 1989). Glomerular epithelial cells may also function as antigen presenting cells in immune mediated glomerular injury (Mendrick *et al*, 1991).

A large proportion of the charge on the GCW (glomerular polyanion) is invested in the glomerular epithelium and depletion of this charge characterises a variety of glomerulopathies (Rohrbach *et al*, 1983, 1986, Chakrabarti *et al*, 1989, Templeton *et al*, 1989). The increased proteinuria resulting from epithelial cell damage caused by cationic protamine sulphate (Seiler *et al*, 1975) or hexadimethrine (Hunsicker *et al*, 1981) was reversed by the polyanion, heparin (Seiler *et al*, 1977).

Intravenous administration of electron dense tracer molecules has been used to locate the ultrastructural barriers within, and follow the progress of proteins or macromolecules through the glomerular capillary wall. Although in early studies, the significance of the isoelectric point of the tracer molecules used was not fully appreciated, the results of experiments shown in Table 1.2.1 show that larger molecules such as ferritin (61Å) localise more readily in the endothelium and *lamina rara interna* whereas smaller, cationic molecules penetrate more deeply into the *lamina rara externa* and epithelial slit diaphragms. These studies gave rise to the early hypotheses that both the size and electrical charge of a molecule are important in determining its passage across the glomerular capillary wall.

Table 1.2.1 Ultrastructural tracer molecules

Tracer	pI	Molecular radius (Å)	Location
Ferritin (native) (Farquhar *et al*, 1961)	4.5	61	endothelium and LRI
Serum albumin (Ryan *et al*, 1978)	4.7	36	endothelium and LRI
Catalase (Venkatachalam *et al*, 1970)	5.7	52	GBM and slit diaphragm
Neutral dextrans (Caulfield *et al*, 1974)	7.4	Polydisperse	GBM
Horseradish peroxidase (Graham & Karnovsky, 1966) (Venkatachalam *et al*, 1970)	7.4	30	Urinary space and GBM
Ferritin (cationic) (Rennke *et al*, 1977)	>8.0	61	GBM and slit diaphragm
Lactoperoxidase (Graham and Kellermeyer, 1968)	8.0	38	Slit diaphragm
Myeloperoxidase (Graham & Karnovsky, 1966)	10.5	44	Slit diaphragm

1.2.2 The mesangium

The mesangium, which is centred axially deep within the glomerulus, comprises mesangial cells embedded in an extracellular glycoprotein matrix (Kriz *et al*, 1990). Circulating macromolecules can enter the mesangium directly, via the glomerular endothelium and at various points, the mesangium reflects on to the glomerular basement membrane (see plate 1.1). There are two types of mesangial cell, the predominant one being smooth muscle-like with contractile properties while the remainder (≈5%) are phagocytic (Michael *et al*, 1980; 1984, Schlondorff, 1987, Badr *et al*, 1989, Ishino *et al*, 1991). Mesangial cells synthesise their own extracellular matrix and GBM components plus a variety of regulatory peptides, prostaglandins (Lovett *et al*, 1987a), growth (Yoshimura *et al*, 1991) and platelet activating (Abboud *et al*, 1987) factors and cytokines and indeed, receptors for many of these same mediators are expressed on the surface of mesangial cells (Foidart *et al*, 1980, Cosio *et al*, 1990, Ray *et al*, 1991).

Mesangial cells are fairly quiescent under normal conditions but they do proliferate in response to injury (Lovett *et al*, 1987, Kitamura *et al*, 1991). Mesangial hypercellularity and matrix expansion, which are the histological hallmarks of glomerulosclerosis, may occur in response to the action of growth factors and cytokines released by either native or infiltrating cells (Abbott *et al*, 1991, Shultz *et al*, 1988, Ross *et al*, 1986b, Schriener *et al*, 1984, Striker *et al*, 1985, Floege *et al*, 1991, Shultz and Raij, 1991). Heparin, heparan sulphate and heparin-like molecules secreted by glomerular epithelial and endothelial cells can exert anti-proliferative effects on mesangial cells (Guyton, 1980, Castellot, 1984; 1985, Floege *et al*, 1993). Acting as a local reticuloendothelial system, the mesangium helps to maintain the patency of the GCW (Kanwar and Rosenzweig, 1982) but plasma proteins and circulating inflammatory cells percolating through, or their accumulation within the mesangium is yet another potent stimulus for glomerulosclerosis (Raij *et al*, 1984, Striker *et al*, 1984, Grond *et al*, 1985, Keane and Raij, 1985). The rate of mesangial clearance of molecular debris influences the rate of progression of glomerulosclerosis: sclerosis progressed more rapidly in PAN rats with diminished mesangial clearance rate compared to ADR rats in which mesangial function is unchanged (Grond *et al*, 1984). In diabetes, expansion of the mesangial matrix is evident usually after around five years of diabetes and is more extensive in those diabetics who develop nephropathy than those who do not: however, unlike GBM thickening, mesangial expansion *does* correlate with renal functional impairment by occluding capillaries and reducing capillary surface area (Mauer *et al*, 1984, Osterby *et al*, 1988).

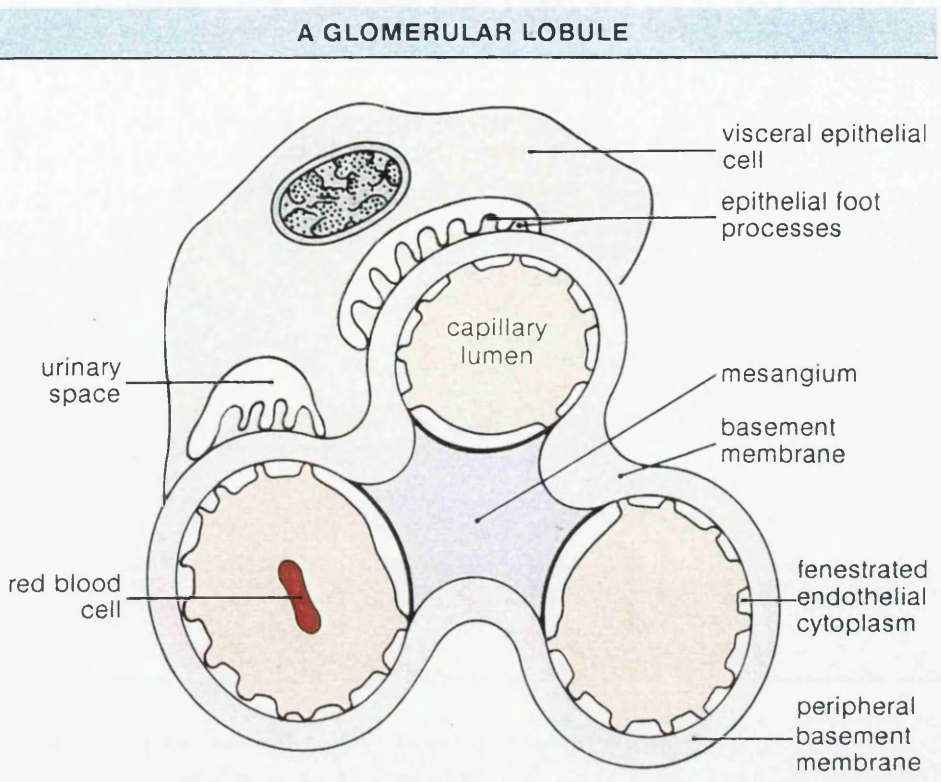


Plate 1.1 A glomerular lobule (adapted from Clinical Diabetes, Besser *et al*)

The mesangium can also regulate glomerular filtration, independently of blood flow, by contracting and relaxing to alter the glomerular capillary surface area across which filtration takes place. The contractile effects of angiotensin II and arginine vasopressin (Ausellio *et al*, 1980, Foidart *et al*, 1980, Ardaillou *et al*, 1987), endothelin (Badr *et al*, 1989, Zoja *et al*, 1990) bradykinin and platelet activating factor (Schlondorff *et al*, 1984) or leukotrienes (Barnett *et al*, 1986) on the mesangium are attenuated by prostaglandins (Ardaillou *et al*, 1983; 1984, Yamamoto *et al*, 1991).

1.2.3 Mechanics of glomerular filtration

Although there is no histological evidence of any physical pores, the glomerular capillary wall was conceptualised originally as a "membrane" perforated by identical cylindrical pores of radius 50-55 Å, each with a uniform distribution of intrinsic anionic charges. The most popular hypothesis is that the glomerular capillary wall is *heteroporous*, with up to 99% of its surface area invested with standard 55 Å pores through which most of the ultrafiltrate flows: the remainder of the capillary wall comprises larger, non-restrictive pores with an effective radius in excess of 70Å and is termed the *shunt pathway* (w_o) (Deen *et al*, 1980; 1981; 1985, Myers *et al*, 1982).

Physical properties of the GCW such as

- the ultrafiltration coefficient (Kf), which is a function of the permeability (hydraulic conductivity (*k*) and capillary surface area (*s*))
- effective pore radius (r_o)
  and
- density of fixed negative charges (Cm)

act in concert with the forces of plasma water flux (convection) and solute concentration (diffusion) to regulate the transglomerular passage of molecules (Bohrer *et al*, 1977).

The rate of filtration across a single nephron (SNGFR) is dependent upon

- renal plasma flow (RPF) or initial glomerular plasma flow rate (Q_A)
- oncotic pressure in the afferent arteriole (π_a)
- hydrostatic pressure within the glomerular capillary (P_{gc}).

Glomerular blood flow and pressure are regulated by changes in afferent and efferent arteriolar resistance. The ultrafiltration pressure (P_{uf}), is the difference between the hydraulic (ΔP) and colloid osmotic pressure (Δp) across the glomerular capillary wall. The rate of filtration across a single nephron (SNGFR) is a product of P_{uf} and Kf.

1.2.4 The renin-angiotensin system and the glomerulus

The renin-angiotensin system (RAS) controls blood pressure and fluid and electrolyte balance. Renin is synthesised, stored in granules and released from cells in the afferent arteriole of the glomerulus but has no direct physiological function other than to convert its plasma substrate *angiotensinogen*, produced in the liver, to the non-pressor decapeptide angiotensin I (AI). Subsequently, AI is converted by angiotensin I converting enzyme (ACE) to the potent pressor, angiotensin II (AII) - a step which is blocked by ACE inhibitors. Renin also promotes sodium reabsorption by stimulating aldosterone production (Laragh, 1981).

Angiotensin II has marked effects on glomerular function through its actions on both the afferent and efferent arterioles and the mesangium (Blantz *et al*, 1976, Ichikawa and Brenner, 1979, Blantz and Gabbai, 1987). The normal kidney responds to a fall in blood pressure through dilation of the afferent, and constriction of the efferent arteriole, which raises the hydrostatic pressure within the glomerulus (P_{gc}) and maintains filtration fraction (FF = GFR ÷ RPF; Icikawa *et al*, 1983). Although it is widely held that AII constricts the efferent arteriole preferentially, to preserve both GFR and filtration fraction when glomerular perfusion falls, filtration is likely to be compromised due to a fall in glomerular blood flow and pressure if AII constricts the afferent arteriole.

Angiotensin II can also impair glomerular filtration through contractile and proliferative effects on the mesangium (Kaizu *et al*, 1985, Yoshida *et al*, 1989). AII stimulated mesangial uptake of large ferritin molecules (Raij *et al*, 1982) and IgG complexes (Singhal *et al*, 1990) *in vitro* while saralasin (an AII receptor antagonist) blocked the increased mesangial uptake of IgG in PAN rats (Mauer *et al*, 1985). ACE inhibitors have been shown to curtail the progression of sclerosis (Grond *et al* 1985, Bertani *et al*, 1986, Klahr *et al*, 1988). With its growth-promoting properties, AII stimulates mesangial cell proliferation and hypertrophy (Fogo *et al*, 1990, Ardaillou *et al*, 1990, Ray *et al*, 1991, Wolf *et al*, 1991) and although AII appears to have no mitogenic potential in vascular smooth muscle cells (Geisterfer *et al*, 1988) it may cause them to hypertrophy (Berk *et al*, 1989) and evidence that enalapril inhibits hyperplasia in nephrectomised rats implicates AII in the hypertrophic response to systemic hypertension (Wang *et al*, 1990, Wight *et al*, 1990).

1.2.5 Normal glomerular permselectivity

In practical terms, it is not easy to characterise the filtration of a molecule across the glomerular capillary wall. The sieving co-efficient (SC) can only be measured in animals because it requires micropuncture of Bowmans space or the first part of the proximal tubule to establish the concentrations of a solute in both the glomerular ultrafiltrate and blood. The fractional clearance (FC) of a molecule on a timed urine collection is more convenient measurement because it compares the clearance of a test molecule to that of the small polysaccharide inulin (MWt = 5200 kD; M_r = 14Å) which passes freely across the GCW and is neither secreted nor reabsorbed by the proximal tubule. The clearance rate of inulin is an acceptable approximation to GFR. A FC value of 1 (or 100%) implies unrestricted filtration whereas a FC value close to zero suggests filtration is impeded. Implicit in the finding of a SC of 0.0001 and FC of 0.01 is that 99% of the filtered albumin is reabsorbed in the proximal tubule (Maack *et al*, 1992). The selectivity index (SI), which compares the clearance of a large, neutral molecule such as IgG (M_r = 55Å; MWt = 155,000 kD; pI = 7.4-7.6) to that of a smaller, more anionic molecule like albumin (MWt = 69, 000 kD; M_r = 36Å; pI = 4.7 - 4.9) can also be used to assess glomerular permselectivity. A high SI denotes impaired size selectivity (although a concommitant loss of charge selectivity cannot be overlooked) whereas a low SI implies that charge although size selectivity may also be impaired. A SI value equal to or below 0.2 characterises diabetic humans with varying degrees of microalbuminuria (Viberti and Keen, 1984). The isoamylase clearance ratio (ICR) is yet another, but perhaps more appropriate index of glomerular permselectivity because it compares the clearance of two differently charged isoforms of the same protein (Fox *et al*, 1993). The *pancreatic* isomer of amylase (M_r = 29Å; MWt = 56, 000 kD; pI = 7) is more cationic than the anionic *salivary* isomer of amylase (pI = 5.9 - 6.4) of the same molecular weight (Wetzels *et al*, 1988). A low isoamylase clearance ratio (P_{AM} / S_{AM}) is associated with depleted glomerular charge.

Graded neutral dextrans and polyvinylpyrrolidone have been used mainly in animal, but also in some human experiments to study the relationship between the size or dimensions of a molecule and its passage through the GCW (Caulfield and Farquhar, 1979). Homogeneous in chemical structure, molecular configuration and charge, their molecular weight and size can be varied, and like inulin, they are neither secreted nor reabsorbed by the renal tubules. Early experiments characterising glomerular permselectivity showed that neutral dextrans with a molecular radius up to 20Å passed virtually unhindered across the glomerular capillary wall (SC = 1 or FC =100%) but clearance tailed off sharply towards zero as their molecular radius increased from 20Å to 42Å (Chang *et al* 1975; 1975a; 1975b).

For any given molecular size, the glomerular capillary wall restricts the passage of anionic molecules more strongly than neutral ones while facilitating the transport of cationic molecules. Interaction between molecules of differing electrostatic charge and the glomerular capillary wall was further investigated by comparing the clearance of neutral dextrans (Chang *et al*, 1975a), anionic (-) dextran sulphate (Bennett *et al*, 1976) and cationic (+) diethylaminoethyl dextrans (Bohrer *et al*, 1978). Cationic dextrans, the same size as albumin, were filtered more readily (FC = 0.42) than anionic dextrans (FC = 0.01), the clearance of which was retarded relative to neutral dextrans (FC = 0.15) (Brenner *et al*, 1977), suggesting that the negative charge on the albumin molecule was responsible for the significant reduction in its clearance. Rennke et al (1978) compared the filtration rates of anionic, cationic and neutral forms of the smaller horseradish peroxidase protein (M_r = 30Å).

The shape or conformation of a molecule is another important determinant of its filtration. A rigid globular protein like albumin is restricted more than dextran molecules which are relatively flexible (Rennke *et al*, 1979). To establish that the difference in filtration was not due to differences between proteins and polysaccharides, Bohrer *et al* (1979) compared the clearance of two polysaccharides and showed that coiled flexible dextrans were filtered more readily than rigid, cross-linked spherical ficoll molecules. The fractional clearance of a rigid ficoll molecule, the same size as albumin, was about half that of a dextran molecule of comparable radius while the clearance of neutral dextrans was increased seven-fold relative to native globular horseradish peroxidase (M_r = 28Å; pI = 7.4). Both studies confirmed the conformational differences between proteins and non-proteins of similar dimensions and charge.

1.2.6 Impaired glomerular permselectivity

Usually, high levels of protein in the urine is evidence that the normal permselective properties of the glomerular capillary wall are defective (Waller *et al*, 1989). Glomerular permeability to proteins is related to the size, number and electrostatic charge of the GCW pores which, in turn, are influenced by glomerular blood flow and pressure and the hydraulic conductivity of the glomerular capillary wall.

Although a loss of glomerular size selectivity might initially suggest increased pore diameter, the size of a GCW pore is also affected by its charge, intraglomerular blood pressure and mesangial expansion or GBM thickening. When glomerular pores become enlarged or stretched, by whatever means, their inherent charge may be diminished. Enlarged pores is the only plausible explanation for a simultaneous

increase in the filtration of both large cationic and anionic molecules (Bertolatus and Hunsicker, 1985, Bertolatus *et al*, 1987, Groggel *et al*, 1988).

On the other hand, a fundamental loss of charge selectivity has been cited as being responsible for the impaired permselectivity in many forms of experimental glomerular injury (Michael *et al*, 1970, Lelongt *et al*, 1987a, Bennett *et al*, 1976, Bohrer *et al*, 1977, Carrie and Myers, 1980) and MCN patients (Carrie *et al*, 1981, Bridges *et al*, 1982). However, diminished GCW charge can also weaken the electrostatic interactions which maintain pore structure and so impair size selectivity (Olsen *et al*, 1981, Weening and Rennke, 1983, Barnes *et al*, 1984, Bertolatus *et al*, 1985, 1987). Raised blood pressure or plasma volume expansion too, can cause a generalised increase in pore diameter and increase the volume of ultrafiltrate passing through the shunt pathway or alternatively, aggravate pre-existing size selective defects within the GCW (Carrie *et al*, 1980, Shemesh *et al*, 1986, Alfino *et al*, 1988). The shunt pathway allows large and small molecules, irrespective of charge, to pass unimpeded into the glomerular filtrate and its utilisation may be heightened when blood pressure is raised (Bohrer *et al*, 1977, Alfino *et al*, 1988, Neugarten *et al*, 1985: 1988, Yoshioka *et al*, 1986;1987, Shemesh *et al*, 1986. It was presumed that anti-hypertensive drugs or aortic ligation reduced proteinuria by limiting the contribution of the shunt pathway (Neugarten *et al*, 1985; 1988, Alfino *et al*, 1988). In relation to the pressor effects of dietary protein intake, a high protein diet altered glomerular haemodynamics in ADR rats but size selectivity was not affected directly (Remuzzi *et al* (1985) and neither did a low protein diet improve size selectivity in membranous nephropathy (Remuzzi *et al*, 1991).

The hydraulic conductivity of the GCW is affected by GBM thickening, mesangial expansion or foot process fusion (Robson *et al*, 1974) all of which can alter effective pore size, density and charge (Chang *et al*, 1976, Bohrer *et al*, 1977a, Bridges *et al*, 1982). The effective number or density of GCW pores is directly proportional to total pore surface area but indirectly proportional to pore length: thus GBM thickening reduces pore density by increasing effective pore length while mesangial expansion effectively reduces the number of functional pores.

Angiotensin II can heighten glomerular permeability to proteins by increasing filtration fraction as a result of a fall in glomerular plasma flow (Eisenbach *et al*, 1975, Bohrer *et al*, 1977a, Carrie *et al*, 1980, Olivetti *et al*, 1984, Yoshioka *et al*, 1986) although Loon *et al* (1989) proposed that angiotensin II actually lowers proteinuria by the same mechanism. Despite substantial experimental evidence of a link between angiotensin II and proteinuria in animals, the data for humans is less

persuasive (Marinides *et al*, 1987, Scholey *et al*, 1987; 1989, Beukers *et al* 1988, Heeg *et al*, 1991, Gansevoort *et al*, 1993). The anti-proteinuric properties of ACE inhibitors are attributed to their ability to reduce glomerular hypertension specifically (Anderson *et al*, 1986, Zatz *et al*, 1986, Hommel *et al*, 1986, Parving *et al*, 1988) but may also be related to an ability to reduce the radius of the largest membrane pores and thus diminish flow through the shunt pathway (Yoshioka *et al*, 1987, Morelli *et al*, 1990, Heeg *et al*, 1987, Remuzzi *et al*, 1990, 1991a). Some reports have gone as far as to suggest that ACE inhibitors reduce only large pores in IgA nephropathy (Remuzzi *et al*, 1991) but both large and small pores in diabetic nephropathy (Morelli *et al*, 1990)

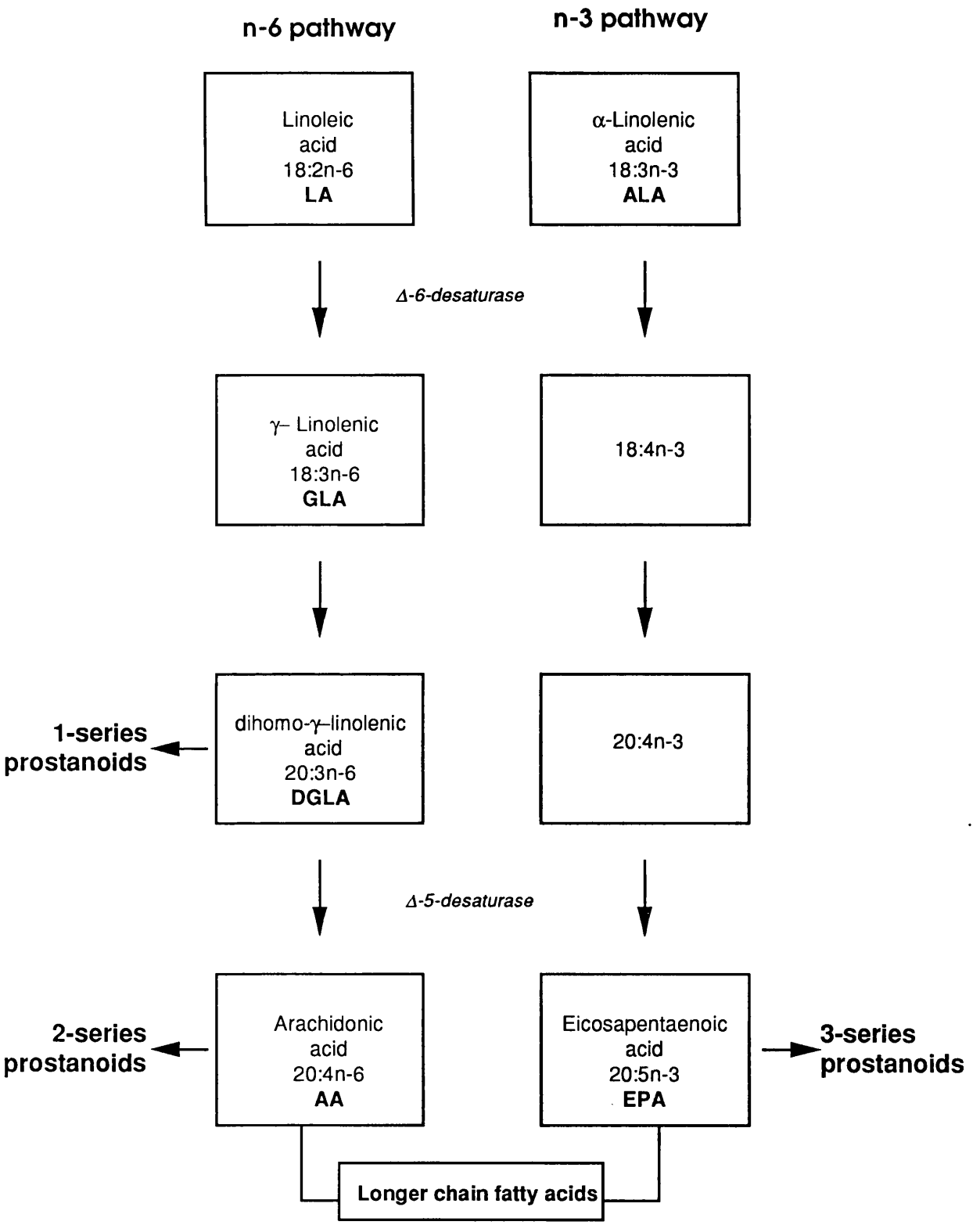
In diabetes, the GCW is subject to a variety of biochemical changes (see section 1.2.1) and changes in glomerular permselectivity may be governed by different determinants at different stages of nephropathy (Carrie *et al*, 1980, Michels *et al*, 1982). Although by no means definitive, early changes in permeability have been associated with glomerular hypertension and subsequent loss of size and charge selectivity (Viberti *et al*, 1984). In advanced diabetes, the decline in GFR is associated with loss of ultrafiltration capacity related to GBM thickening and mesangial expansion which affects pore characteristics and utilisation of the shunt pathway becomes more marked (Myers *et al*, 1982, Winetz *et al*, 1982, Friedman *et al*, 1983, Tomlanovich *et al*, 1987, Nakamura *et al*, 1988).

Polyunsaturated fatty acids are not only a source of metabolic energy and inherent components of cell membrane phospholipids but certain *essential* polyunsaturated fatty acids (EFA: see below) can be oxygenated to generate prostaglandins and thromboxane (prostanoids) and leukotrienes, known collectively as eicosanoids. This section will be limited to the effects of prostaglandins and thromboxane on glomerular function in relation to vascular reactivity (blood pressure and coagulation), the renin-angiotensin system, mesangial function and to a lesser extent, more general aspects of cellular function including the immune and inflammatory responses, proliferation and hypertrophy.

1.3.1 Essential fatty acid metabolism and prostanoid biosynthesis

The ω-6 and ω-3 pathways of EFA metabolism are probably the most important Linoleic acid (LA: 18: 2ω-6) is converted to arachidonic acid (AA: 20: 4ω-6) along the ω-6 pathway and α-linolenic acid (ALA: 18:3ω-3) is converted to eicosapentaenoic acid (EPA: 20:5ω-3) down the parallel ω-3 pathway (Figure 1.3.1). Fatty acids are classified according to the number and position of double bonds contained within the molecule: linoleic acid, for example, is denoted as C18: 2ω-6 because it contains eighteen carbon atoms and two double bonds, the first of which is located six carbon atoms from the ω end of the molecule. Although not all PUFAs are essential fatty acids (EFA), all EFAs are polyunsaturated and the double bonds must be in the *cis* conformation to confer prostanoid activity.

The three main series of prostanoids (*mono-, di- and tri-* enoic) with varying degrees of potency, are derived from γ-linolenic acid (DGLA), arachidonic acid (AA) and eicosapentaenoic acid (EPA), respectively. Dienoic (or 2- series) prostanoids which include PGE_2, PGI_2, and TxA_2 are regarded as being the most abundant and biologically active. However, prostanoid biosynthesis cannot occur without a supply of free unesterified AA which must be acquired either from (i) hydrolysis of membrane phospholipids through the actions of phospholipases or (ii) by dietary supplementation with essential fatty acid (EFA) which cannot be synthesised *de novo* by humans. Oils from the seeds of the evening primrose or safflower are rich in linoleic acid (LA) and GLA whereas leaves and grasses contain high levels of α-linolenic acid (ALA) and it is the high levels of α-linolenic acid in phytoplankton which makes oils from marine animals a rich source of eicosapentaenoic acid (EPA). The relative amounts of a particular prostanoid series generated, depends upon the variable rates of the reaction steps within the EFA pathways. The initial step in the



Essential Fatty Acid metabolism

Figure 1.3.1

ω-6 pathway, which is rate-limiting and catalysed by Δ- 6 desaturase, is the slow conversion of LA to γ-linolenic acid (GLA: C18: 3ω-6) and because GLA is then elongated rapidly to dihomo γ linolenic acid (DGLA: C20: 3ω-6), tissue levels of GLA are low under normal conditions: DGLA then undergoes another slow desaturation step to arachidonic acid (AA: C20: 4ω-6). Depending upon the abundance or availability of GLA, DGLA is converted to either arachidonic acid and 2- series prostanoids or 1- series prostanoid derivatives such as PGE_1 and 15 OH-DGLA.

The ω-3 pathway utilises the same desaturase and elongase enzymes to convert α-linolenic acid to EPA and 3- series prostanoids, which have modified activity and potency relative to the 2- series prostanoids (Marshall *et al*, 1983; see Figure 1.3.1). Essential fatty acids surplus to requirements, may be reincorporated into cell membrane phospholipids.

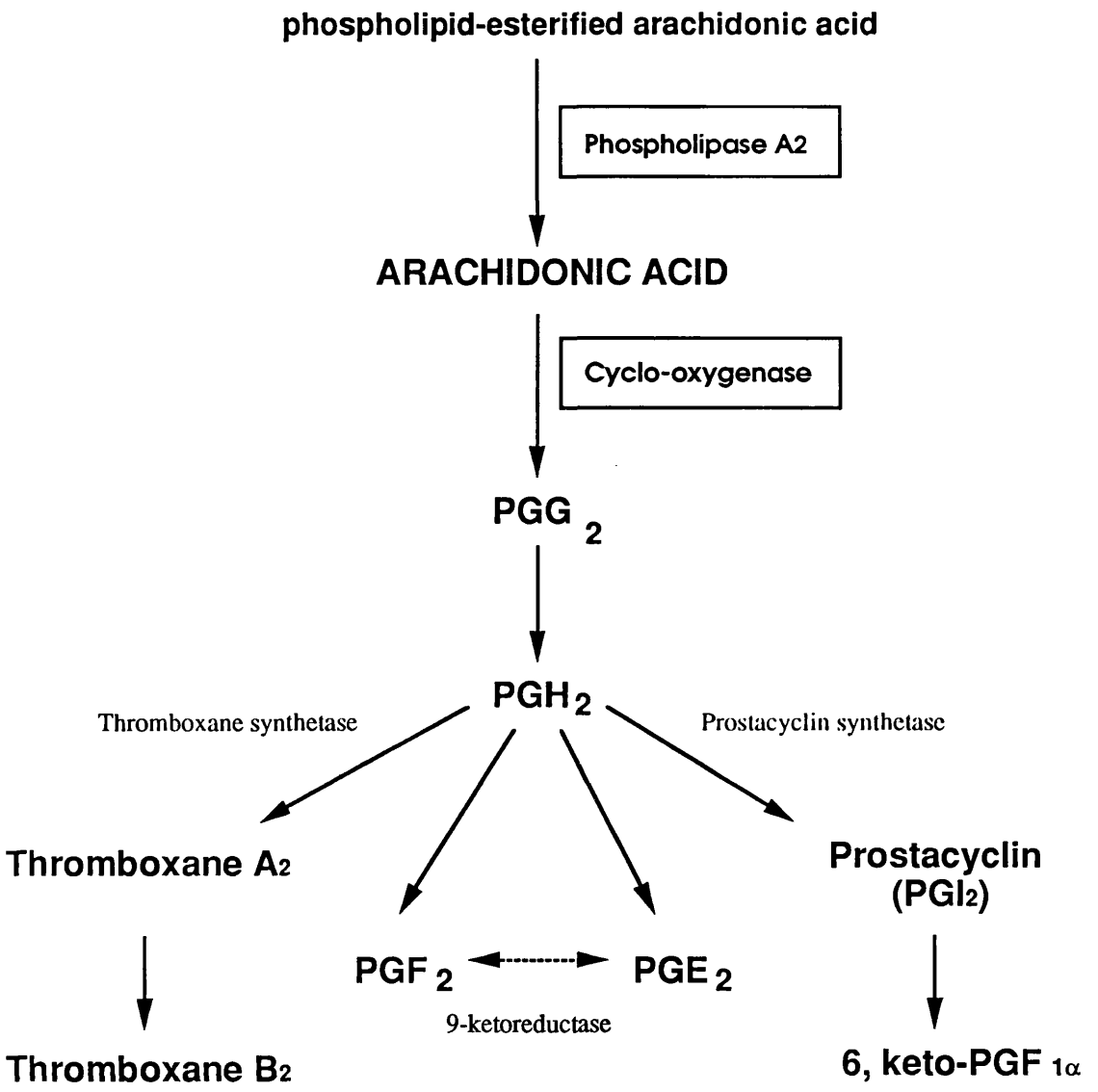
1.3.2 Conversion of EFA to prostanoids

Prostaglandins comprise a pentane ring with two acyl side chains containing a number of double bonds which determine the prostanoid series formed.

- 1- series prostanoids with one double bond are derived from DGLA
- 2- series prostanoids with two double bonds are derived from AA
- 3- series prostanoids with three double bonds are derived from EPA.

Prostaglandins are classified (A, B, C, D, E or F) according to the substituents on the pentane ring. Prostacyclin (PGI_2) is not an authentic prostaglandin because of the presence of an oxygen bridge between carbon atoms 6 and 9 and likewise, thromboxane A_2 has an unstable bicyclic oxane-oxetane ring structure.
The first step in the conversion of arachidonic acid to a prostanoid is its oxygenation to the unstable prostaglandin endoperoxides, PGG_2 and PGH_2 by cyclooxygenase (Hamberg *et al*, 1975, Gryglewski *et al*, 1976) - a step which can be inhibited by indomethacin and other non-steroidal anti-inflammatory drugs (Vane, 1971, Carmichael and Shankel, 1985, Schlondorff, 1993). Specific enzymes then convert PGG_2 to a variety of prostaglandins and thromboxanes (Figure 1.3.2).

The alternative lipoxygenase enzyme pathway can convert EFAs to pro-inflammatory and vasoconstrictory leukotrienes and DGLA has anti-inflammatory properties because its metabolites PGE_1 and 15 OH-DGLA inhibit lipoxygenase activity (Lagarde *et al*, 1987, Lewis *et al*, 1990). Not only is there competition between the ω-6 and ω-3 EFA pathways for the elongase and desaturase enzymes but EPA and



Dienoic Prostanoid Synthesis

Figure 1.3.2

AA compete for the cyclooxygenase and lipoxygenase enzymes (Culp *et al*, 1979). When EPA replaces AA as a substrate for cyclooxygenase, the trienoic prostanoids, PGI_3 and TxA_3 are generated and although PGI_3 and PGI_2 have equally potent vasodilatory properties and abilities to inhibit platelet aggregation, TxA_3 is a weaker vasoconstrictor and platelet aggregating agent than TxA_2 (Dyerberg and Bang, 1979, Fischer and Weber, 1983; 1984; 1985). Similarly, when EPA replaces AA as a substrate for the lipoxygenase enzyme, 5- series leukotrienes with attenuated inflammatory properties relative to 4- series leukotrienes derived from arachidonic acid, are formed and this explains the states of reduced vascular resistance, thrombogenicity and inflammation induced by fish oils (Culp *et al*, 1979, Jim *et al*, 1982, Sraer *et al*, 1983, Lee *et al*, 1984, Terano *et al*, 1984; 1986, Leaf *et al*, 1988, Endres *et al*, 1989).

1.3.3 General prostanoid function

The kidney, along with the lungs and liver, is particularly active in relation to prostanoid biosynthesis, and activity increases progressively from the cortex through the medulla to the papilla. At various sites within the kidney, different prostanoids are synthesised to perform specific functions (Shryver *et al*, 1984, Eriksen *et al*, 1987, Bonventre and Nemenoff, 1991). For example, in humans, PGI_2 is the principal prostanoid in the highly vascular renal cortex although in rats, PGE_2 is the predominant prostanoid (Whorton *et al*, 1977). In the renal medulla, PGE_2 is the main prostanoid in association with its role in the control of salt and water excretion and its modulatory effects on the actions of ADH (Grantham and Orloff, 1968, Muirhead *et al*, 1972, Larsson and Angaard, 1974, McGiff and Wong, 1979).

Native and infiltrating cell types contribute to the glomerular prostanoid profile (Folkert and Schlondorff, 1979, Hassid *et al*, 1979). Glomerular endothelial cells synthesise prostacyclin (Bunting *et al*, 1976, Moncada and Vane, 1979; 1979a) whereas epithelial and mesangial cells produce large amounts of PGE_2 (Sraer *et al*, 1979, 1982, Petrulis *et al*, 1981, Kreisberg *et al*, 1982). Platelets are the principal source of thromboxane (Hamberg *et al*, 1975, Needleman *et al*, 1976; 1979) but peripheral blood mononuclear cells (Goldstein *et al*, 1978) and macrophages (Brune *et al*, 1978) also produce significant quantities of prostanoids.

1.3.4 Prostanoids and glomerular function

Perhaps the most significant distinction between prostaglandins and thromboxane is that prostaglandins are vasodilators and inhibit platelet aggregation whereas thromboxane is a vasoconstrictor and potent platelet aggregating agent. Prostacyclin produced by endothelial cells in vessel walls, opposes the effects of thromboxane A_2 released by activated platelets following endothelial damage or immune injury and so the synthetic balance between these two prostanoids is important in maintaining normal thromboresistance and systemic and glomerular blood pressure and flow (Grylewski *et al*, 1976, Moncada and Vane, 1979, Perico *et al*, 1982, Chang *et al*, 1984).

It is widely accepted that prostanoid activity is dormant in kidneys which are functioning normally but prostaglandin levels, in particular, often rise to maintain glomerular function when it is threatened: thromboxane, on the other hand, is perhaps more likely to be generated when the kidney is damaged (Finn and Arendshorst, 1976, Baylis, 1980, Zipser *et al*, 1985, Benigni *et al*, 1986).

Prostaglandins maintain glomerular filtration when blood flow is reduced (see Table 1.3.1) and modulate the effects of vasoconstrictors such as angiotensin II, antidiuretic hormone (ADH) and epinephrine (Baylis and Brenner, 1978, Gerber *et al*, 1978, Ichikawa and Brenner, 1980a, Yarger *et al*, 1980, Myers *et al*, 1981, Patrono *et al*, 1982, Podjarny *et al*, 1986). A close relationship between prostanoids and various components of the renin-angiotensin system was first recognised when McGiff *et al* (1970) observed that prostaglandin levels rose in response to constriction of the renal artery or an infusion of angiotensin II (Aiken and VAne, 1973, Oates *et al*, 1979, Schlondorff *et al*, 1980, Stahl *et al*, 1984a). Moreover, specific prostaglandins may control renin release from different parts of the glomerulus (Ito *et al*, 1989). Prostaglandins may also modulate blood pressure by altering both vascular reactivity and extracellular volume in association with their diuretic and natriuretic actions (Laragh, 1981: Axelrod, 1982, Dusing *et al*, 1983, Dunn and Groene, 1985).

The use of cyclooxygenase inhibitors (COI) has reinforced the importance of prostaglandins in maintaining renal function (Kimberly and Plotz, 1977, Dunn and Zabramski, 1980, Clive and Stoff, 1984, Brater *et al*, 1985, Carmichael *et al*, 1985, Patrono and Dunn, 1987, Schlondorff, 1993) and have been shown to abolish compensatory increases in GFR in STZ rats (Craven and De Rubertis, 1989) and impair renal function in patients with chronic glomerulonephritis (Ciabattoni *et al*, 1984), lupus nephritis (Kimberly *et al*, 1978) and nephrotic syndrome (Arisz *et al*, 1976).

Nephrotoxic serum nephritis	Lianos *et al*, 1983 Kaizu *et al*, 1985
Immune complex glomerulonephritis	Rahman *et al*, 1987 Stahl *et al*, 1987
STZ diabetes	Jensen *et al*, 1986
Obstructive nephropathy	Yarger *et al*, 1980 Folkert and Schlondorff, 1981 Yanagasawa *et al*, 1990
2-Kidney, 1-Clip hypertension	Stahl *et al*, 1984
Nephrotic syndrome	Donker et al, 1978
Glomerulonephritis	Donadio *et al*, 1984 Stork and Dunn, 1985

Table 1.3.1 Compensatory increases in prostanoid synthesis in various glomerulopathies

Thromboxane appears to be more damaging than prostaglandins in terms of glomerular functional deterioration and impaired permselectivity (see Table 1.3.2). In relation to its ability to raise intraglomerular blood pressure (P_{gc}), reduce glomerular blood flow and stimulate renin production, thromboxane is a strong contender in the pathophysiology of hypertension (Purkerson *et al*, 1986, Baylis *et al*, 1987, Mistry *et al*, 1990). Glomerular thromboxane levels were shown to be raised in genetically hypertensive SHR (Shibouta et al, 1979) and Lyon rats (Geoffroy et al, 1989) although the hypertension which developed in Sabra rats after DOCA-salt treatment was attributed to a deficiency of prostacyclin rather than any increase in thromboxane production (Geoffroy et al, 1988). However, improvements in renal function in hypertensive Dahl-S rats (Yamashita *et al*, 1988) and normotensive Milan rats following thromboxane inhibition occurred independently of any change in blood pressure (Pugliese *et al*, 1986, Salvati *et al*, 1990). It has been suggested that thromboxane mediates the renovascular effects of angiotensin II (Shibouta *et al*, 1979, Geoffroy *et al*, 1989, Wilcox *et al*, 1990; 1991).

The hypercoagulable state of the nephrotic syndrome and other glomerulopathies is likely to be associated with raised thromboxane levels (Perico *et al*, 1982, Mortensen *et al*, 1983, Socini *et al*, 1985, Purkerson *et al* in 1985, Schlondorff *et al*, 1986, Morrison *et al*, 1986, Yoshida *et al*, 1978, Patrignani *et al*, 1982, Donadio *et al*, 1988) although a deficiency in prostacyclin production cannot be discounted. The benefits of thromboxane synthesis inhibition in rats with reduced in renal mass were attributed to attenuated platelet activity, intraglomerular thrombosis and hypertension. However, comparisons between the effects of low dose aspirin, which suppresses only platelet TxA_2 and a Tx-receptor antagonist, which blocks both platelet and vascular receptors for TxA_2, and neither of which had any effect on blood pressure, led to the conclusion that platelet activation and thromboxane production made no substantial contribution to renal functional deterioration in patients with lupus nephritis (Pierucci *et al*, 1989) or type I diabetes (Allesandrini *et al*, 1988).

Many aspects of mesangial function are influenced by prostanoids (Mene *et al*, 1990). A fall in GFR, secondary to the contractile actions of angiotensin II (Kreisberg *et al*, 1984, Schlondorff *et al*, 1985), thromboxane (Mene *et al*, 1986), leukotrienes (Simonson *et al*, 1986) or endothelin (Zoja *et al*, 1990) may be offset by prostaglandins and cyclooxygenase inhibitors have been shown to intensify the effects of mesangial contraction on glomerular filtration (Dworkin *et al*, 1983, Scharschmidt *et al*, 1986, Dunn and Scharschmidt, 1987). In addition, the growth-promoting and proliferative effects of prostanoids are implicated in mesangial expansion and glomerulosclerosis (Levine *et al*, 1977, Taylor and Polgar 1980,

Ureteral obstruction	Okegawa *et al*, 1983 Stahl *et al*, 1986
Renal ablation	Purkerson *et al*, 1985 Zoja *et al*, 1990a
Adriamycin nephrosis	Remuzzi *et al*, 1985
Progressive kidney disease (Milan normotensive model)	Salvati *et al*, 1990
STZ diabetes	Craven *et al*, 1992
Nephrotoxic serum nephritis	Lianos *et al*, 1983
Immune complex glomerulonephritis	Saito *et al*, 1984 Rahman *et al* 1987 Yamashita *et al*, 1988 Stahl *et al*, 1990 Thaiss *et al*, 1989
Unilateral glomerulonephritis	Cook *et al*, 1986
Mercuric chloride (ICGN)	Papanikalou, 1987
Anti-GBM nephritis	Suzuki *et al*, 1987
Murine lupus	Kelley *et al*, 1986
Systemic lupus erythymatosis	Patrono *et al*, 1985 Pierucci *et al*, 1989
Haemolytic uraemic syndrome	Tonshoff *et al*, 1990

Table 1.3.2 Raised thromboxane levels in experimental and human glomerulopathies

Habenicht *et al*, 1985, Mene *et al*, 1990, Floege *et al*, 1990, Ardaillou *et al*, 1990) and may relate to changes in intracellular message transmission or an increased glomerular work load (Nath *et al*, 1986, Mene and Dunn, 1990). Indomethacin suppressed DNA synthesis and renal function in uninephrectomised rats (Logan *et al*, 1986). Thromboxane is also recognised as a strong chemoattractant (Saito *et al*, 1984) and may act as a growth factor, stimulating mesangial cell proliferation and matrix expansion (Ishimtsu *et al*, 1988, Shultz *et al*, 1988, McNamara *et al*, 1989, Border *et al*, 1989, Yamamoto *et al*, 1991, Bruggeman *et al*, 1991). Both indomethacin and a thromboxane synthetase inhibitor preserved renal function in an experimental model of mesangial cell injury (Stahl *et al*, 1990).

Prostanoids are involved also in regulation of the immune response (Goodwin, 1980, Herbert, 1978, Eriksen *et al*, 1986, Couser *et al*, 1985) and have potent anti-inflammatory properties (Zurier *et al*, 1977, Winkelstein and Kelley, 1981, Kunkel *et al*, 1986, Salmon and Higgs, 1987, Goodwin, 1991, Lefkowith *et al*, 1992).

1.3.5 Prostanoids and altered glomerular permselectivity

The anti-proteinuric effects of cyclooxygenase inhibitors (COI) implicate prostaglandins, in particular, in the impaired permselectivity of many animal and human glomerulopathies (Kimberly *et al*, 1978a, Kirschenbaum and Serros, 1981, Plotz and Kimberly, 1981, Zatz *et al*, 1985, Benigni *et al*, 1986). Although the mechanism is unclear, it is most likely that COI reduce proteinuria by lowering prostaglandin levels (Arisz *et al*, 1976, Donker *et al*, 1983, Alavi *et al*, 1986, Vreisendorp *et al*, 1985, 1986). Indomethacin reduced proteinuria, without any further fall in glomerular function, in patients with heavy proteinuria (Tiggler *et al*, 1979), experimental passive Heymann nephritis (Zoja *et al*, 1987) and autologous immune complex glomerulonephritis (Kirschenbaum *et al*, 1985). However, Kirschenbaum questioned whether the effects of indomethacin were related to prostaglandin inhibition after showing that aspirin did not lower proteinuria, despite inhibiting PGE_2 to a similar degree. Alternatively, COI may lower proteinuria by suppressing the activity of the renin-angiotensin system (Eisenbach *et al*, 1975, Donker *et al*, 1978), a direct effect on the glomerular capillary wall (Sessa *et al*, 1973, Suzuki *et al*, 1984), redirection of blood flow from outer to less permeable inner cortical nephrons (Chang *et al*, 1975) or stimulation of tubular reabsorption of proteins (Brater, 1979). Nevertheless, indomethacin did not reduce proteinuria in either the NSN rat model of anti-GBM nephritis (Kurokawa *et al*, 1982) or autologous immune complex nephropathy (Donker *et al*, 1983) which suggests that it may only be an effective anti-proteinuric agent in conjuction with an activated renin-angiotensin system (Arisz *et al*, 1976, Alavi *et al*, 1986).

Yet another possibility is that cyclooxygenase inhibitors reduce glomerular pore size directly. The reduced clearance of proteins and large (> 42Å) neutral dextrans in NSN rats given indomethacin, correlated with a fall in both GFR and filtrate volume but glomerular hypertension was unchanged (Neugarten *et al*, 1989). Golbetz's group (1989) also concluded that the anti-proteinuric effects of indomethacin in NS patients was related to restoration of impaired glomerular permselectivity despite a concommitant reduction in GFR.

The substantial variability and many inconsistencies in the anti-proteinuric effects of cyclooxygenase inhibitors, suggests that prostaglandins do not play a major role in the pathogenesis of proteinuria. On the other hand, heightened thromboxane levels often correlate with rising proteinuria in animal and human glomerulopathies (see Table 1.3.3) but thromboxane is regarded as being more likely to induce proteinuria either in reponse to, or in conjunction with other mediators of glomerular injury. In immune-mediated glomerulopathies, the association between thromboxane and

Streptozotocin diabetes	Collins *et al*, 1989 Hora *et al*, 1990 de Rubertis *et al*, 1992 Craven *et al*, 1992
Renal ablation	Purkerson *et al*, 1985
Spontaneous progressive renal disease	Yamashita *et al*, 1988 Salvati 1990
Adriamycin nephrosis	Remuzzi *et al*, 1985
Nephrotoxic serum nephritis	Lianos *et al*, 1983 Takahashi *et al*, 1990
Heymann nephritis	Cybulsky *et al*, 1986
Insulin-dependent diabetes	Barnett *et al*, 1984 Esmatjes *et al*, 1990
Systemic lupus erythematosus	Pierucci *et al*, 1989

Table 1.3.3 Raised thromboxane levels correlate with protein excretion in various glomerulopathies

proteinuria may be linked to complement-mediated generation of thromboxane, proteolytic enzymes or inflammatory agents which impair the permselectivity of the glomerular capillary wall (Camussi *et al*, 1984; 1986, Brown *et al*, 1987, Cybulsky *et al*, 1987, Lianos *et al*, 1990). Nevertheless, blocking thromboxane production neither prevented nor lowered proteinuria in rats with NSN (Lianos *et al*, 1983), immune complex glomerulonephritis (Rahman *et al*, 1987a) or experimental membranous nephropathy (PHN) although in the latter case, complement depletion did (Stahl *et al*, 1987). A broad spectrum platelet inhibitor (Zoja *et al*, 1990a) and triple action inhibitor of thromboxane and leukotriene synthesis and lipid peroxidation (Shibouta *et al*, 1991) were more effective than thromboxane inhibitors in terms of improving disease progression in non-immune-mediated glomerular disease.

1.3.6 PUFAs as membrane components

Polyunsaturated fatty acids are structural and functional components of all cell membranes, having undergone transacylation to glycerolipids and conversion to various phospholipids which associate spontaneously to form the characteristic membrane lipid bilayer (Kummerow, 1988, Kinsella, 1990). The three major lipid components of eucaryotic cell membranes are phosphoglycerides (or glycerophospholipids), sphingolipids and cholesterol. Phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and phosphatidylserine are the most abundant phospholipids, containing myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and arachidonic (C20:4) acids: usually, a saturated and unsaturated fatty acid are bound to the first and second carbon atom of the glycerol molecule, respectively. Phosphatidylethanolamine, for example, has palmitic or oleic acid esterified to the first, but one of the longer chain polyunsaturated or essential fatty acids such as arachidonic acid to the second carbon atom.

A constant turnover of unsaturated fatty acids, in particular, within phospholipids maintains the membrane fluidity and flexibility of cell (Schacter *et al*, 1984, Stubbs and Smith, 1984, Cartwright *et al*, 1985). Membrane fluidity affects a host of cellular functions including:

- the lateral movement and rotational diffusion of integral membrane proteins (glycoproteins) and enzymes
- selective ion exchange across the cell membrane
- hormone receptors and intracellular signalling
- mobility and adhesion, secretion, endocytosis and phagocytosis, lymphocyte capping

Membrane fluidity is related to both the degree of fatty acid unsaturation and acyl chain length and replacement of a saturated, with an unsaturated fatty acid is likely to improve membrane fluidity. Saturated and *trans* unsaturated fatty acids are straight chain molecules but the presence of a *cis* double bond creates a bend in the hydrocarbon chain of the fatty acid which prevents crystallisation and maintains fluidity. Saturated acyl chains form highly ordered membranes with low fluidity (Kamada and Otsuji, 1983) and incorporation of cholesterol reduces membrane fluidity (Huang *et al*, 1986: Kinsella, 1990). Differences in melting points between saturated and unsaturated fatty acids effect transitions between the fluid and crystalline gel states of the membrane (Le Grimellec *et al*, 1992). The concept of membrane polarity is a function of differences in fluidity between the inner and outer

leaflets of the lipid bilayer in relation to the proportions of saturated, or *trans* or *cis* unsaturated fatty acids with different melting points.

Changes in the PUFA content of the membrane may influence various aspects of cellular activity such as:

(i) the orientation and conformation of cation transport mechanisms such as the Na^+-K^+ ATPase, Ca^{++}- ATPase or sodium-lithium countertransport system (Almeida *et al*, 1984, Pagnan *et al*, 1989). Ion transport across the red blood cell membrane may be abnormal in hypertensive patients (Canessa *et al*, 1980, Carr *et al*, 1990, Dominiczak *et al*, 1991) and indeed, linoleic acid had favourable effects on blood pressure in normotensive volunteers in relation to changes transmembrane sodium flux (Heagarty *et al*, 1986).

(ii) the expression and/or affinity of a variety of receptors including those for insulin or low density lipoproteins (Kinsella, 1990). Heightened membrane fluidity in Friend erythroleukaemia cells grown in medium supplemented with linoleic, as opposed to oleic acid, was associated with an increased number, and decreased affinity of insulin receptors (Ginsberg *et al*, 1981). Hormones and polypeptides bind to cell membrane receptors and transmit their signal through intracellular messengers such as cAMP and the protein kinase A pathway, phophatidylinositol 4,5-bisphosphate (PIP_2), the protein kinase C pathway and the inositol 1, 4, 5 triphosphate (IP_3) - calcium pathways or cGMP and activation of protein kinase G pathway, embedded in the cell membrane. A greater degree of membrane fatty acid unsaturation reduces the binding of various steroids and hormones and so a deficiency of UFAs may exaggerate the response to normal peripheral levels of a particular agonist.

(iii) the deformability and passage of erythrocytes through capillary beds (Terano *et al*, 1983, Popp-Snijders *et al*, 1986). ω-3 fatty acids improved the impaired fluidity of erythrocyte membranes in diabetic patients (Kamada *et al*, 1986).

(iv) the response of platelets to ADP and thrombin, their subsequent aggregation and release of growth and mitogenic factors and their interactions with endothelial cells to promote coagulation and thrombosis (Dyerberg *et al*, 1979, Berlin *et al*, 1980, Seiss *et al*, 1980, Sanders *et al*, 1983, Fischer and Weber, 1983, Croft *et al*, 1984, von Schacky *et al*, 1985, Heemskerk *et al*, 1989).

(v) the locomotion and migration, phagocytosis and endocytosis by neutrophils (Ziboh *et al*, 1986, Prescott *et al*, 1984) and macrophages (Mahoney *et al*, 1977), the secretory functions of platelets and lymphocytes (Singh *et al*, 1988, Meydani *et al*, 1991). The incorporation of ω-3 fatty acids into the membrane of neutrophil and monocytes reduces the production of pro-inflammatory mediators such as leukotrienes and cytokines (Lee *et al*, 1984; 1985, Kremer *et al*, 1987, Endres *et al*, 1989).

(vi) capping of antigens on the surface of lymphocytes (Kinsella, 1990).
(vii) the assembly and activity of membrane-bound complement with significant implications for glomerular epithelial cells and glomerular permselectivity (Cybulsky *et al*, 1986; 1989; 1990: Clarke *et al*, 1993).

1.3.7 Lipid-lowering effects of PUFAs

Disturbances in plasma lipid and lipoprotein metabolism contribute to excess risk of atherosclerosis and coronary heart disease (Jensen *et al*, 1987, Scanu, 1991). Cholesterol is transported in low density lipoproteins (LDL) and removed from cells by high density lipoproteins (HDL) which transport it to the liver for excretion in the bile. The cholesterol-lowering effects of ω-6 fatty acids have been reported widely (Huang *et al*, 1987, Kissebah and Schectman, 1988) and the effects of PUFA diets on plasma lipid levels is discussed in the following section (1.4).

An outline of lipid metabolism may be viewed in any biochemistry textbook. In relation to the diabetic study reported in section 2.5, it is interesting that the hypertriglyceridaemia which is characteristic of IDDM, occurs despite greatly diminished fatty acid synthesis, because a low insulin to glucagon ratio causes uncontrolled lipolysis in adipose tissue. Circulating fatty acid levels increase and there is acclerated production by the liver of ketone bodies which accumulate along with hydrogen ions resulting in ketoacidosis. Excess fatty acids which do not undergo oxidation or ketogenesis generate VLDL more rapidly than they can be cleared from the blood by lipoprotein lipase, the activity of which is also dependent upon the insulin to glucagon ratio. Diabetic rats have an increased requirement for EFA because they are more easily oxidised than saturated fatty acids (Horrobin, 1990).

Enthusiasm for the use of polyunsaturated fatty acid (PUFA) diets in the treatment of glomerular disease evolved in association with their success in ameliorating symptoms of cardiovascular disease (Kromhout *et al*, 1985, Leaf and Weber, 1988), systemic lupus erythematosus (Clark *et al*, 1989; 1993), diabetes (Horrobin, 1988; 1992) and rheumatoid arthritis (Belch, 1990, Kremer *et al*, 1987; 1990). The rationale behind the use of PUFA diets is to stimulate the production of favourable prostanoids like PGE_1 or suppress of the synthesis of eicosanoids with unfavourable effects such as thromboxane and leukotrienes (Scherhag *et al*, 1982, Lorenz *et al*, 1983, Croft *et al*, 1984). Supplying ω-6 EFAs, in the form of an evening primrose or safflower oil diet, raises the levels of monoenoic relative to dienoic prostanoids whereas ω-3 PUFA diets redirect eicosanoid synthesis in favour of 3 series prostanoids which are associated with a limited incidence of cardiovascular, atherosclerotic and inflammatory events (Saynor *et al*, 1984, Endres *et al* 1989, Laker and Alberti, 1991). Many of the complications associated with experimental and clinical diabetes have been attributed to diminished Δ-6 and Δ-5 desaturase enzyme activity in the EFA pathways which generate prostanoids: such metabolic blockades may be bypassed by dietary provision of EFA prostanoid precursors.

Much of the literature suggests that PUFA diets not only delay the onset and lessen the degree of glomerular injury but may also prevent the progression of renal disease (Barcelli and Pollack, 1985) in relation to their favourable effects on

- blood pressure and hypertension (Norris *et al*, 1986, Knapp *et al*, 1989, Bonaa *et al*, 1990)
- coagulation (Goodnight *et al*, 1981, Sanders *et al*, 1983, Knapp *et al*, 1986, von Shacky *et al*, 1985, Haines *et al*, 1986, Rogers *et al*, 1987, Hansen *et al*, 1989)
- hyperlipidaemia (Singer *et al*, 1986)
- cell proliferation (Fox *et al*, 1988)
- the immune (Endres *et al*, 1989) and inflammatory response (Meydani *et al*, 1990)
- sclerosis (Weiner *et al*, 1986)
- hypertrophy (Logan *et al*, 1990, Wight *et al*, 1990, Mene *et al*, 1990)

and

- proteinuria (see Tables 1.4.1 and 1.4.2)

To a large extent, the success of PUFA diet treatment is rated on the basis of either lowered blood pressure, lipid levels or proteinuria, improved renal function, a more favourable prostanoid balance, heightened immunosuppression or attenuated glomerulosclerosis. This section reviews the capacity of PUFA diets to lower proteinuria and retard disease progression in non-immune (see Table 1.4.1) and immune-mediated (see Table 1.4.2) glomerular diseases and diabetic renal disease.

Non-immune mediated glomerulopathies: The beneficial effects of PUFA diets appear to be dose-dependent with high linoleic acid (HLA) diets being more beneficial than low linoleic acid (LLA) diets (Barcelli *et al*, 1982, Hoffman *et al*, 1982, Izumi *et al*, 1986, Heifets *et al*, 1987) although Hirschberg *et al* (1984) reported that varying the concentrations of dietary essential fatty acids and triglycerides did not alter disease outcome either in rats with reduced renal mass or mice with experimental lupus. However, despite many favourable reports, the indiscriminate use of PUFA diets was questioned when Scharschmidt *et al* (1987) reported that fish oil diets might actually impair renal function. Deteriorating renal function and accelerated proteinuria, glomerulosclerosis and mortality in nephrectomised rats fed fish oil was attributed to the suppression of PGE_2 synthesis. Nevertheless, when the anti-proteinuric effects of fish or safflower oil diets were compared to those of a low protein diet (LPD), fish oil was seen to reduce albuminuria more effectively - but not as the result of fall in blood pressure (Clark *et al*, 1990). The study was repeated using a higher dose of fish oil and although both FO and LPD preserved renal function and lowered proteinuria, the low protein diet was more effective (Clark *et al*, 1993a). Albeit plasma lipid levels and glomerulosclerosis were reduced by both diets, the authors conceded that the lesser weight gain in the low protein diet group might explain fall in albumin excretion. In 1990, Logan *et al* claimed to have discredited the hypothetical link between hyperfiltration and hypertrophy in normal and uninephrectomised rats fed a PUFA diet. The increased kidney weights in normal rats fed fish oil were attributed to direct growth-stimulating effects of fish oil since renal function was not increased whereas in uninephrectomised rats fed fish oil, increased glomerular function was not accompanied by renal hypertrophy.

Many studies have shown that PUFA diets reduce cholesterol levels (Huang *et al*, 1986). The lipoprotein disorders common in patients with progressive renal disease are associated with lipid deposits and foam cells in the glomerulus (Diamond and Karnovsky, 1988) and hypercholesterolaemia has been implicated in the proteinuria and glomerulosclerosis of the obese Zucker rat model. The favourable effects of fish oil on histological damage, serum cholesterol and proteinuria in uninephrectomised Zucker rats were attributed to changes in plasma lipid and glomerular prostanoid

Authors	Model/Disease	Diet	Proteinuria
Barcelli *et al*, 1982	STNx (75%)	Linoleic acid (2% vs 27%)	↓
Barcelli *et al*, 1990	STZ	Evening primrose, safflower or fish oil	↓
Hoffman *et al*, 1982	STNx	Linoleic acid (H vs L)	?
Helfets *et al*, 1987	STNx	Linoleic acid	↓
Scharschmidt *et al*, 1987	STNx (85%)	Fish oil	↑
Clark *et al*, 1990	STNx	Safflower, fish oil or protein restriction (6%)	↓
Clark *et al*, 1993(a)		Fish oil (24%) or protein restriction (6%)	↓
Logan *et al*, 1990	STNx	Fish oil	?
Wheeler *et al*, 1991	Zucker rat	Fish oil (14%)	↓
Kasiske *et al*, 1991	Zucker rat	Fish, sunflower or coconut oil (20%)	↓
Jensen *et al*, 1989	IDDM	Cod liver oil (8 wks)	↔
de Caterina *et al*, 1993	GN	Fish oil (6 wks)	↓

Table 1.4.1 Effects of PUFA diets on proteinuria in non-immune mediated glomerular disease

levels since renal function was unchanged (Wheeler *et al*, 1991). However, the culpability of cholesterol was questioned when Kasiske *at al* (1991) showed that although both fish and sunflower oil diets lowered albuminuria, glomerulosclerosis and serum triglyceride levels in intact Zucker rats, cholesterol levels were reduced only in the fish oil group and that the preceding fall in albuminuria was the explanation for the favourable result. No definitive explanation was given by de Caterina *et al* (1993) for the sustained fall in proteinuria observed in patients with chronic glomerular disease, treated with high or low doses of ω-3 fatty acids. Serum cholesterol, platelet and urine prostanoid levels were all reduced and the modest reduction in blood pressure did not correlate with the fall in proteinuria.

Immune-mediated glomerulopathies: Interest in the immunosuppressive effects of PUFA diets in relation to production of E series prostaglandins, derives from reports of improved survival and decreased proteinuria in models of immune-mediated glomerular injury such as lupus nephritis (Prickett *et al*, 1981: 1983, Kelley *et al*, 1985, Robinson *et al*, 1986, Westberg *et al*, 1989) albeit Hurd *et al* (1981) showed that EFA *deficiency* improved survival and EFA-enriched and EFA-deficient diets had no effect on proteinuria or survival rate (Dubois *et al*, 1982). The anti-proteinuric effects of high linoleic acid and fish oil diets in murine apoferritin-induced ICGN were attributed to favourable effects on glomerular tissue lipids since despite differences in glomerular thromboxane levels between the diet groups, the resultant degree of protection was similar (Kher *et al*, 1985; 1986). In rats with mercuric chloride-induced ICGN, both herring and evening primrose oil diets reduced proteinuria, increased survival and suppressed immune complex deposition when compared with the thromboxane synthetase inhibitor (OKY-046) which improved survival but did not prevent glomerular IgG deposition (Papanikalou *et al*, 1987)

In contrast to a previous study, in which they had demonstrated that fish oil accelerated glomerular functional deterioration, Scharschmidt and colleagues (1990) postulated that in the NSN model of anti-GBM nephritis, fish oil preserved glomerular function and reduced proteinuria, glomerular dienoic prostanoid levels and histological damage by suppressing the immune response. Such diametrically opposite effects of fish oil diets have been attributed to suppression of lipoxygenase activity which appears to be stimulated in immune, but not non-immune mediated glomerular diseases.

A significant and sustainable reduction in proteinuria in normal rats, and lowering of established proteinuria by up to 50% by fish oil, demonstrated by Weise *et al* (1993), occurred independently of any change in blood pressure or renal function and so was attributed ω-3 PUFA incorporation into membrane phospholipids and subsequent

Authors	Model/Disease	Diet	Proteinuria
Prickett *et al*, 1981	Murine lupus	Fish oil	↓
Robinson *et al*, 1986			↓
Westberg *et al*, 1989			↔
Kher *et al*, 1985; 1986	Apoferritin ICGN	Linoleic and fish oil	↓
Papanikalou *et al*, 1987	Mercuric chloride ICGN	Herring or EPO (vs Tx inhibitor)	↓
Scharschmidt *et al*, 1990	NSN	Fish oil	↓
Thaiss *et al*, 1990			↔
Weise *et al*, 1993	Heymann nephritis	Fish or safflower oil (10%)	↓
Rahman *et al*, 1991	Experimental membranous	Fish oil	↓
Bennett *et al*, 1989	IgA nephropathy	Fish oil	?
Cheng *et al*, 1990			↑
Hamazaki *et al*, 1984			?
Donadio *et al*, 1994			?
Clark *et al*, 1989; 1993	SLE	Fish oil	↓

Table 1.4.2 Effects of PUFA diets on proteinuria in immune-mediated glomerular disease.

release of trienoic eicosanoids with attenuated inflammatory activity. However, Bennett *et al* (1989) and Cheng *et al* (1990) reported that fish oil diets did not retard the progression of IgA nephropathy despite other optimistic reports (Hamazaki *et al*, 1984, Donadio *et al*, 1990) and the results of a one year double-blind, randomised, cross-over study of fish oil diet in a group of systemic lupus erythymatosis (SLE) patients were not encouraging (Clark *et al*, 1993). Neither was there any improvement in renal function nor reduction in disease activity and only a slight, non-significant reduction in proteinuria. Nevertheless, more recent results from the Mayo Nephrology Collaborative Group on the effects of two years of fish oil treatment in patients with IgA nephropathy are favourable (Donadio *et al*, 1994).

The role of PUFA diets in the treatment of diabetic complications has been reviewed extensively by Horrobin (1988; 1990; 1992). PUFA diets may be useful in the treatment of diabetic nephropathy, in relation to the high incidence of cardiovascular, and coronary heart disease and increased mortality within the first few years following the onset of proteinuria (Jensen 1987; 1989). ω-3 fatty acids have been shown to suppress the development of diabetic cardiomyopathy in streptozotocin diabetic rats (Black *et al*, 1989). However, some of the early optimism surrounding the use of PUFA diets in the treatment of diabetic nephropathy may have been dampened by one particular study in which fasting glucose levels rose in patients with type II diabetes fed ω-3 fatty acids, returning to baseline only when the diet was discontinued (Glauber *et al*, 1988). Nevertheless, a subsequent study in diabetic rats, showed lowered proteinuria and fewer histological abnormalities in ω-6 diet groups in association with elevated PGI_2 levels and unchanged plasma lipids - whereas ω-3 diets, associated with a fall in PGI_2 and reduced plasma lipid levels, had no effect on renal disease (Barcelli *et al*, 1990). Only diets rich in ω-6 fatty acids were protective in this model of diabetic nephropathy.

The transcapillary escape rate of albumin (TER), which is a marker of systemic vascular permeability, was reduced in IDDM patients with proteinuria fed cod liver oil although urinary albumin excretion was not reduced; despite an improved lipid profile effect was attributed to modified vascular permeability and not to a fall in blood pressure (Jensen et al, 1989). Glomerular filtration rate, insulin, glycosylated haemoglobin and blood glucose levels were unaffected by cod liver oil diet.

Although at first, the indiscriminate use of PUFA diets may seem innocuous (Robinson *et al*, 1986, Scharshmidt *et al*, 1990), under certain circumstances PUFA diets can have undesirable effects (Scharshmidt *et al*, 1987, Glauber *et al*, 1988, Logan *et al*, 1990). While most PUFA diet studies show some improvements in one or more of the parameters which affect glomerular disease progression, this section has demonstrated the many inconsistencies which makes it difficult to pinpoint any single mode of action (see Table 1.4.3).

	Lowered	Raised	Unchanged/Preserved
Systemic blood pressure	Helfets *et al*, 1987 de Caterina *et al*, 1993 (Hoffman *et al*, 1982)		Barcelli *et al*, 1982 Clark *et al*, 1990 Wheeler *et al*, 1991 Weise *et al*, 1993
Plasma lipid levels	Kasiske *et al*, 1991 Wheeler *et al*, 1991 Clark *et al*, 1993 Weise *et al*, 1993* de Caterina *et al*, 1993		
Prostanoid levels	Kher *et al*, 1986* Scharschmidt *et al*, 1987; 1990* Wheeler *et al*, 1991 Weise *et al*, 1993	Barcelli *et al*, 1982*; 1990 Kher *et al*, 1986 Helfets *et al*, 1987 Kasiske *et al*, 1991*	
GFR	Scharschmidt *et al*, 1987	Scharschmidt *et al*, 1990	Jensen *et al*, 1989 Logan *et al*, 1990 Scharschmidt *et al*, 1990 Weise *et al*, 1993 Clark *et al*, 1993(a)

Table 1.4.3 Effects of PUFA diets on markers of disease activity (* = author cites more than one factor)

Section 2

EXPERIMENTAL DESIGN

MATERIALS and METHODS

RESULTS

DISCUSSION

This second section of the thesis comprises a record of the experimental work undertaken to elucidate the mechanism behind the apparent anti-proteinuric effects of PUFA diets. It is sub-divided into five sections, the first four of which attempt to relate the effects of PUFA diets to the principal factors which govern the filtration of a molecule across the glomerular capillary wall, namely:

- ***glomerular blood flow and pressure***
- ***glomerular capillary wall charge***
- ***electrostatic charge on the filtered molecule***

Sections 2.1 and 2.2 explore the interrelationships of PUFA diets and glomerular and circulating prostaglandin, thromboxane and renin levels in Lewis and DA rats.

Section 2.3 examines the possibility that, as intrinsic components of cell membrane phospholipids, PUFAs alter the permeability properties of the glomerular capillary wall with particular reference to the magnitude of the glomerular polyanion.

Section 2.4 investigates the effects of PUFA diets on the intrinsic electronegative charge (pI) on the serum albumin molecule.

The final section - 2.5 - evaluates the ability of an evening primrose oil diet to either prevent escalation of, or lower albumin excretion rate in diabetic Lewis rats.

2.1.1 Introduction

How the glomerulus responds to an antigenic or haemodynamic challenge may be determined by inherited differences in the intensity of the anionic charge on the glomerular capillary wall or the efficiency of mesangial clearance function. A diminished glomerular polyanion may facilitate deposition of macromolecules or immune complexes within the GCW and impaired mesangial function may retard the rate at which they can be cleared - such persistance can prove highly destructive. Prostanoids elicit a multitude of effects within the glomerulus (see sections 1.3 and 1.4) which might influence molecular traffic across the glomerular capillary wall and through the mesangium and thus limit the extent of proteinuria and glomerulosclerosis. Prostanoid biosynthesis can be manipulated by feeding diets rich in essential fatty acid (EFA) prostanoid precursors (see section 1.3.2).

This first study was undertaken with a view to identifying whether differences in glomerular properties between the Lewis and DA rat strains extended to differences in their prostanoid production *in vitro* and also, the feasibility of altering the glomerular prostanoid profile by feeding different PUFA diets. Diets such as evening primrose or safflower oil which contain ω-6 fatty acids are used to stimulate the synthesis of 1- and 2- series prostanoids whereas fish oil diets, rich in ω-3 fatty acids, may increase the levels of 3- series prostanoids (see section 1.3.1). Olive oil is used widely as a 'placebo' or control UFA oil supplement because it contains predominantly monounsaturated oleic acid (80%) but also a small amount of linoleic acid (7%).

2.1.2 Materials and methods

During the planning stages of this study, it became clear that because of the number of diet groups, it would be necessary to stagger the diet regimes in view of the lengthy and labour-intensive surgical and glomerular sieving techniques performed on each rat at the end of each six week diet period. Had all the diets been started simultaneously, the feeding period of some rats would have extended beyond six weeks since the glomeruli can be harvested from a maximum of only four rats on any one day. A outline of the experimental design, similar to that used in this study, is illustrated in section 2.2. (Table 2.2.2.1).

Five groups of Lewis (n = 5) and DA (n = 5) rats weighing some 200 to 250 grams at the start of the experiment, were fed *ad libitum* either standard laboratory rat chow or a fat-deficient diet supplemented with either evening primrose oil (EPO), safflower oil (SO), fish oil (FO) or olive oil (OO) diet for six weeks. Body weight was recorded weekly and at two and four weeks, the rats were put into metabolic cages to monitor both food intake and urine output and collect urine. Within any diet group, the kidneys were removed and the glomeruli recovered by a differential sieving procedure from Lewis and DA rats, alternately. Levels of PGE_2, PGI_2 (prostacyclin) and thromboxane metabolites and renin in the glomerular supernatants were measured and expressed as a function of glomerular protein content.

2.1.2.1 Control and experimental PUFA diets

The control diet was a standard laboratory rat pellet diet (Rat and Mouse No 1 Modified Maintenance diet: Special Diet Services Ltd, PO Box 705, Witham, Essex CM8 3AD). The pellets were ground up to mimic the granular consistency of the PUFA diets which prevented the rats retrieving and consuming pellets in the centre of the cage, a common behaviour pattern which can result in not only substantial amounts of food falling into the urine container below but rats in the control diet group consuming more food than those fed the powdered PUFA diets which has to be scooped up in the paws directly from the food hopper.

Experimental PUFA diets were prepared according to the method used by Chandrachud (1986). Evening primrose oil, safflower oil, fish oil and olive oil (gifted by Scotia Pharmaceuticals Ltd, Guildford, Surrey GU1 1BA) were stored under nitrogen at 4°C and added to a commercially prepared fat-deficient diet which is designed for comparative use with, and was purchased from the same source as the control diet. 43 mls of evening primrose, safflower, fish or olive oil was added to 2.7g butylated hydroxytoluene (Sigma Chemical Company: B-1378) dissolved in 150 mls of AnalaR grade diethyl ether and then stirred into 500 grams of the fat-deficient diet, giving a dietary oil content of 8.6% (v,w). Newly-prepared diets were left in a fume cupboard overnight or until the odour of ether was no longer detectable. Batches of diet were prepared as required and never stored for longer than three days because of the susceptibility of fatty acids to oxidation over time. Antioxidants such as butylated hydroxytoluene (BHT) or Vitamin E are added to PUFA diets to suppress fatty acid peroxidation and free radical generation. Constituents of both the standard and fat deficient diets are shown in Table 2.1.2.1 and Table 2.1.2.2 lists the constituent fatty acids of safflower, evening primrose, fish and olive oils.

Table 2.1.2.1 Diet components

	units	Control	Fat-deficient
Crude fibre	%	4.3	4.7
Polysaccharides	%	63	72
Digestible crude fat	%	2.4	0.07
Digestible crude protein	%	13.6	13.7
Sodium	%	0.26	0.24
Chloride	%	0.38	0.36
Magnesium	%	0.21	0.12
Potassium	%	0.65	0.52
Gross energy	MJ/kg	14.8	14.2
Digestible energy	MJ/kg	11.4	12.4
Metabolisable energy	MJ/kg	11.2	11.2

Table 2.1.2.2 Constituent fatty acids of dietary oils

		% total weight			
Fatty acid	**Chain length and double bonds**	**Evening Primrose**	**Safflower**	**Fish**	**Olive**
Myristic	C14:0	-	-	13.3	0.2
Palmitic	C16:0	5	8	0.3	10
Palmitoleic	C16:1	-	-	12.2	0.2
Hexadecadienoic	C16:2	-	-	13.3	-
Stearic	C18:0	1.7	2.6	3.8	2.6
Oleic	C18:1	9.5	13	16	**80**
Linoleic	C18:2	**77**	**76**	1.2	6.4
dihomo γ Linolenic	C20:3	4	-	1	0.5
Arachidonic	C20:4	-	-	5.6	-
Eicosapentaenoic	C20:5	-	-	**27**	-
Docosapentaenoic	C22:5	-	-	2.2	-
Docosahexaenoic	C22:6	-	-	3.7	-

2.1.2.2 Rats

Male Lewis or DA rats (Bantin and Kingman, Universal Ltd, The Field Station, Grimston, Aldbrough, Hull, U.K) were used in this and all subsequent studies. After arrival, the animals were allowed to rest for at least a week before being entered into any study and were housed in facilities with a controlled twelve hour day and night cycle and ambient temperature of 20 - 21°C. The animals remained healthy throughout the course of the studies and were inspected regularly by a vet from the Home Office. All experimental procedures were subject to the regulations of the Animals (Scientific Procedures) Act 1986.

2.1.2.3 Metabolic cage procedure

At two and four weeks, rats were put into metabolic cages for two consecutive days to monitor daily food intake and urine output and collect urine. The first day was regarded as an acclimatisation period during which measurements were recorded but not used for biochemical or statistical analyses: all metabolic cage data relates exclusively to that collected during the second day. Neither food nor water was restricted while the animals were in metabolic cages.
Urine was collected under mineral oil to minimise evaporation, absorption of carbon dioxide and food contamination. After recording the volume collected, urine was centrifuged at 1000g for 15 minutes at 4°C and kept at 4°C or frozen at -70°C.

2.1.2.4 Surgical procedures - nephrectomy

At the end of six weeks, the rats were culled and the kidneys removed. Each rat was anaesthetised with an intraperitoneal injection of Sagatal (pentobarbitone sodium BP: 60 mg in 1 ml: Rhone Merieux (Ireland), Tallaght, Dublin) at a dose of 0.1 ml per 100 g body weight. After isolating the trachea by blunt dissection, an endotracheal tube was inserted: the jugular vein was located and cannulated with polyethylene tubing (PE 50: Clay-Adams, Becton-Dickinson UK Ltd, Cowley, Oxford OX4 3LY) attached to a syringe loaded with Sagatal. (A cannula may be inserted into the carotid artery for blood pressure recording and into the femoral artery for blood sampling.) A dorsal mid-line incision was made to expose the major blood vessels and kidneys. The abdominal aorta was isolated by ligating it proximally, above both renal arteries and distally, at the level of the bifurcation: the superior mesenteric artery was tied off. A length of wide bore cannula (PE 90 tubing: Clay-Adams as above) attached to a syringe containing warm (37°C) saline (or other appropriate perfusate) was introduced into the aorta via a small incision and then tied securely. Before starting the perfusion, the vena cava and renal veins were punctured to allow the perfusate to

escape. Perfusion was continued until the kidneys turned white (blanched), at which stage they were removed and put into ice cold (0-4°C) phosphate buffered saline and kept at 4°C until they underwent the sieving procedure. There will always be an unavoidable delay of some two to three hours between removal of the kidneys and the start of the sieving procedure.

2.1.2.5 Glomerular sieving procedure

The glomeruli were recovered by sieving, after removal of the renal capsule and any extraneous fatty tissue. Renal cortical tissue was dissected and pressed through three sieves stacked in descending order of pore diameter (250 μm, 106 μm and 63 μm) (Endecotts Ltd., Lombard Road, London SW19 3BR). Fifty mls of ice-cold (0-4°C) phosphate buffered saline (PBS) in a syringe fitted with a 23 guage (blue) needle was used to force the macerated tissue with firm but gentle pressure through each successive sieve: the base of the syringe plunger was used to smear the tissue across the surface of the sieve. Finally, the 63 μm sieve was inverted within a large plastic funnel and the glomeruli collected in 50 mls of PBS in a polyethylene tube (to minimise adhesion) then centrifuged at 1000 g for 10 minutes at 4°C. The supernatant was discarded and the glomeruli resuspended in 50 mls of ice-cold PBS: this washing step was repeated twice. Finally, the glomeruli were resuspended in 10 mls of cold Hartmanns solution (Baxter Healthcare Ltd, Thetford, Norfolk) then brought to room temperature and left to stand for 30 minutes at 37°C before being rotated gently on a roller mixer for a further 30 minutes at 37°C. The tubes were plunged into ice immediately to slow down and arrest prostanoid activity and then centrifuged at 4°C for 15 minutes at 1500 g.
Aliquots of glomerular supernatants were stored at -70°C and the glomerular pellet was retained for protein estimation.

2.1.2.6 Glomerular protein content

Neither a Neubauer haemocytometer nor Coulter counter was found to be suitable for counting glomeruli because of their size and so the alternative method of determining the protein content of the glomerular pellet was used. After the supernatant had been removed, the pellets of glomeruli were hydrolysed overnight in 1 ml 1N sodium hydroxide at 37°C and the protein content of the glomerular pellet measured by the Lowry protein assay (see section 2.1.2.7). Glomerular hydrolysates may be stored frozen at -20°C.

2.1.2.7 Lowry protein assay

The Lowry protein assay has the interesting distinction of being the most widely referenced in the biochemical literature and has become the gold standard protein quantitation assay. In principle, a protein is reacted with alkaline copper sulphate in the presence of tartrate: Folin Ciocalteu's (FC) phenol reagent is then added and the copper complexes transfer electrons to the FC reagent which is reduced to a blue colour at 750 nm. Although the Lowry protein assay is some ten times more sensitive than the Biuret method, its drawbacks include the instability of the alkaline copper sulphate solution, photosensitivity of the reaction, linearity of the response and interference by lipids.

Stock reagents

Solution A: 2% $Na_2 CO_3$ in 0.1N NaOH
Solution B: 2% Na K Tartarate
Solution C: 1% Copper sulphate

Biuret reagent

Add 1 ml of solution B and 1 ml of solution C to 100 mls solution A.

Folin Ciocalteu's reagent

Folin Ciocalteu's phenol reagent (BDH Chemical Ltd, Poole, Dorset) diluted 1:1 with deionised water.

Standards

Prepared as tabulated below using rat serum albumin (Sigma Chemical Company: A-6414) at 1 mg per ml.

rat serum albumin (µls)	water de-ionised (µls)	protein concentration (µgs/400 mls)
0	400	0
25	375	25
50	350	50
75	325	75
100	300	100

Concentrated samples were diluted to a final volume of 400 µls with deionised water. Bovine serum albumin (Sigma Chemical Company: A-7906) at 0.6 mg per ml was used as a quality control (QC). 100 µls BSA was adjusted to a final volume of 400 µls with water. Standards and samples were set up in duplicate. 2 mls of Biuret reagent was added to 400 µls of standards, QC and samples. The tubes were vortex mixed and left to stand for 10 minutes. 200 µls of Folin Ciocalteu reagent was added to each tube, vortex mixed again and allowed to stand for 30 minutes. Optical density (OD) was measured at 750 nm (or 500 nm) and the values read from a standard curve.

2.1.2.8 Prostaglandin and thromboxane assays

The primary prostanoids PGE_2, PGI_2 and TxA_2, are highly potent with biological activity ranging between $10^{-13}M$ - $10^{-9}M$ and because they occur in such minute quantities and in so many structurally diverse but similar forms, assays to measure them must be highly sensitive and specific (many prostanoid assays cannot detect below $10^{-8}M$). A bioassay, for example, will measure prostanoids on the basis of their biological ability to contract vascular smooth muscle or aggregate platelets and has the advantage of detecting total biological activity, if at the expense of specificity. Radioimmunoassay, on the other hand, is highly specific with low detection limits and facilitates rapid processing of a large number of samples and although high performance liquid chromatography (HPLC) or gas chromatography - mass spectrometry (GC-MS) is highly specific and ideal for identifying metabolites of different prostanoid series (eg PGE_1, PGE_2, PGE_3), detection limits may not be sufficiently low and the rapid assay of a large number of samples is not practicable.

Over the years, refinements in prostanoid assay methodology and recognition of the importance of minimising the effects of *ex vivo* prostanoid production have improved the credibility of prostanoid measurements, but care with sample collection and processing is still essential. Some of the early prostanoid data may, unwittingly, be unreliable because the effects of surgical procedures and anaesthetics, tissue homogenisation, addition of precursor metabolites and various cofactors such as serum to culture media were not appreciated fully (Horrobin, 1990). Coagulation, for example, can raise circulating thromboxane levels several orders of magnitude higher than normal.

Highly unstable primary prostanoids circulate in the bloodstream in the low picogram range and are inactivated rapidly to be replaced by secondary metabolites, which with longer half-lives, accumulate to higher levels ($\approx$ 20- 50 picograms per ml) and so are more easily detected. Being so short-lived, PGE_2, PGI_2 and TxA_2 have to be quantified by measuring their more stable metabolites, bicyclic-PGE_2, 6-keto $PGF_{1\alpha}$ and TxB_2, respectively. Commercial radioimmunoassay kits were chosen to measure prostanoid levels, in this study, because of their speed and simplicity.

(a) 11 deoxy 13, 14 dihydro 15 keto 11β, 16ε cyclo *(bicyclic)* PGE_2 assay

In tissue, PGE_2 derived from PGH_2 by endoperoxide E isomerase may be converted to:

- 13, 14 dihydro, 15 keto- (DHK)- PGE_2 which is oxidised further to 15, ketodihydro- 2, 3, 4, 5 tetranor PGE_2, the main circulating and urinary metabolite in man.
- PGB_2 via PGA_2 and PGC_2

  or

- $PGF_{2\alpha}$ by 9 ketoreductase.

The pathways of PGE_2 and $PGF_{2\alpha}$ metabolism are very similar and there is considerable interconversion between the two. Like PGE_2, $PGF_{2\alpha}$ is converted to its DHK- derivative and oxidised further to various tetranor derivatives.

More than 90% of PGE_2 is inactivated through its conversion to 13, 14- dihydro- 15-keto- (DHK)- PGE_2 during a single passage through the lungs but DHK-PGE_2 also dehydrates spontaneously to form DHK-PGA_2, some of which binds to albumin (DHK-PGA_2-albumin) thus generating three metabolic pools of PGE_2. However, incubating samples at high pH cleaves the covalent bond between PGA_2 and albumin and converts all three (and related tetranor) metabolites to the stable bicyclic intermediate 11- deoxy- 13, 14- dihydro- 15- keto- 11β, 16ε- cyclo- *(bicyclic)* PGE_2 which can be measured by a commercial RIA system (Amersham International PLC: TRK 800).

Glomerular supernatants were assayed undiluted and in duplicate, after incubation with 1M sodium carbonate for 24 hours, according to the protocol outlined in Tables 2.1.2.3 (a) and (b). The radioimmunoassay system utilises tritiated (^3H) 11- deoxy-13, 14- dihydro- 15- keto- 11β, 16ε- cyclo- *(bicyclic)* PGE_2 as tracer and an antiserum for bicyclic PGE_2, the cross-reactivity of which is shown in Table 2.1.2.3 (c). A standard binding curve was prepared using the DHK-PGE_2 standards provided (6.9, 3.4, 1.7, 0.86, 0.43 ng per ml). The detection limits of the assay are between 43 and 690 pg of bicyclic PGE_2.

Tube identity	Control	Standards A-E	Samples
Assay buffer	50	-	-
Standard	-	50	-
Sample	-	-	250
0.9% saline	200	200	-
1M sodium carbonate	10	10	10
	Mix and incubate at 37°C for 24 hours		
1M potassium phosphate	15	15	15

All volumes are in microlitres

Table 2.1.2.3 (a) Conditions for production of bicyclic prostaglandin E_2

Tube	Total count (TC)	NSB (buffer blank)	Zero standard (B_0)	Standards A-E	Sample 1	Sample 2	Sample n
Control solution	50	50	50	-	-	-	-
Assay buffer	50	50	-	-	-	-	-
[^{3}H] Bicyclic prostaglandin E_2	50	50	50	50	50	50	
Standards or samples	-	-	-	50	50	50	50
Antiserum	-	-	50	50	50	50	50
Mix and incubate							
Charcoal suspension	500*	500	500	500	500	500	500
React, centrifuge, decant and measure ^{3}H decay							

* 500 uls of assay buffer instead of charcoal suspension

All volumes are in microlitres unless otherwise specified

Table 2.1.2.3 (b) Protocol for radioimmunoassay of bicyclic prostaglandin E_2

Prostaglandins	% Cross reactivity (50% B/B_0 replacement)
13, 14-Dihydro- 15- keto- prostaglandin E_2	100.0
13, 14-Dihydro- 15- keto- prostaglandin E_1	100.0
13, 14-Dihydro- 6, 15- diketo- prostaglandin E_1	<0.40
13, 14-Dihydro- 6, 15- diketo- prostaglandin $F_{1\alpha}$	<0.01
13, 14-Dihydro- 15- keto- prostaglandin $F_{2\alpha}$	<0.001
13, 14-Dihydro- 15- keto- thromboxane B_2	<0.001
15- keto- prostaglandin E_2	0.30
15- keto- prostaglandin E_1	0.2
15- keto- prostaglandin $F_{2\alpha}$	<0.001
15- keto- thromboxane B_2	<0.001
6, 15- Diketo-prostaglandin E_1	<0.01
6, 15- Diketo-prostaglandin $F_{1\alpha}$	<0.01
Prostaglandin E_1	<0.01
Prostaglandin D_1	<0.001
Prostaglandin $F_{1\alpha}$	<0.0001
6- Keto prostaglandin $F_{1\alpha}$	<0.0001
Prostaglandin $F_{2\beta}$	<0.0001
Prostaglandin E_2	<0.001
Prostaglandin D_2	<0.001
Prostaglandin $F_{2\alpha}$	<0.0001
Thromboxane B_2	<0.0001
2,3,4,5- Tetranor- 13, 14- dihydro- 15- keto- prostaglandin E_1	0.30
2,3,4,5- Tetranor- 13, 14- dihydro- 15- keto- prostaglandin E_1- 1,20- dicarboxylic acid	<0.001
2,3,4,5- Tetranor- 13, 14- dihydro- 15- keto- prostaglandin A_1- 1,20- dicarboxylic acid	<0.001

Table 2.1.2.3 (c) Specificity of prostaglandin E_2 antiserum

(b) 6 keto prostaglandin $F_{1\alpha}$ assay

Prostacyclin (PGI_2) is an unstable vinyl ether derived from PGH_2 by prostacyclin synthetase, with a half-life of 2 to 3 minutes which may be converted to either

- 6-keto prostaglandin $F_{1\alpha}$ by spontaneous hydrolysis and then undergo further β oxidation to 2, 3 dinor 6-keto prostaglandin $F_{1\alpha}$ (so 6-keto $PGF_{1\alpha}$ may not be the most appropriate metabolite to measure)

  or

- 6, 15 diketo- 13, 14 dihydro $PGF_{1\alpha}$ (enzymatically) and oxidised further to various 2, 3 dinor 6, 15 diketo- products (Moncada *et al*, 1976, Grygwelski *et al*, 1976).

In this study, 6-keto $PGF_{1\alpha}$ levels in glomerular supernatants were measured by a RIA system (Amersham International PLC: TRK 790). Glomerular supernatants were assayed undiluted and in duplicate, according to the assay system protocol outlined in Table 2.1.2.4. The RIA system utilises (^3H) tritiated 6-keto $PGF_{1\alpha}$ as tracer and a specific antiserum to 6-keto prostaglandin $F_{1\alpha}$, the cross-reactivity of which is displayed in Table 2.1.2.4 (a). A standard binding curve was prepared using the 6-keto $PGF_{1\alpha}$ standards provided (5, 2, 0.75, 0.3 and 0.14 ng per ml). The detection limits of the assay are between 14 and 500 pg 6-keto $PGF_{1\alpha}$ per assay tube.

Tube	Total count (TC)	NSB (buffer blank)	Zero standard (B_0)	Standards A-E	Sample 1	Sample 2	Sample n
Buffer	100	100	50	-	-	-	-
[^{3}H] keto-PGF$_{1\alpha}$	50	50	50	50	50	50	
Standards	-	-	-	50	-	-	-
Samples	-	-	-	-	50	50	50
Antiserum	-	-	50	50	50	50	50
Mix and incubate							
Charcoal suspension	500*	500	500	500	500	500	500
React, centrifuge, decant and measure H decay							

* 500 uls of assay buffer instead of charcoal suspension

All volumes are in microlitres unless otherwise specified

Table 2.1.2.4 Protocol for radioimmunoassay of 6-keto prostaglandin F$_{1\alpha}$

Prostaglandins	% Cross reactivity (50% B/B_0 replacement)
6-keto prostaglandin $F_{1\alpha}$	100.0
Prostaglandin A_1	<0.014
Prostaglandin A_2	0.014
Prostaglandin D_2	<0.014
Prostaglandin E_2	5.1
Prostaglandin $F_{2\alpha}$	0.30
Thromboxane B_2	<0.014

Table 2.1.2.4 (a) Specificity of 6-keto prostaglandin $F_{1\alpha}$ antiserum

(c) Thromboxane B_2 assay

Thromboxane A_2 is synthesised from PGH_2 by thromboxane synthetase and with a half-life of 20 to 30 seconds at 37°C is converted rapidly to its stable oxane derivative, thromboxane B_2 (Hamberg *et al*, 1975). In essence, circulating TxA_2 levels should reach little more than a few picograms per millilitre but may be raised artificially by platelet activation in association with tissue processing and coagulation.

Thromboxane B_2 can undergo either:

- oxidation to 2, 3 dinor- TxB_2 (barely detectable in blood but is the major urinary metabolite)
- dehydrogenation and further oxidation to 2, 3, dinor, 11 dehydro TxB_2 (probably the best metabolite to measure because it is not formed artefactually)

  or

- enzymatic conversion to 15, keto, 13, 14 dihydro TxB_2 followed by dehydrogenation and β and ω oxidation steps to various minor metabolites.

A RIA system (Amersham International PLC: TRK 780) was used to measure TxB_2 levels. Glomerular supernatants were assayed undiluted and in duplicate according to the assay system protocol outlined in Table 2.1.2.5. The system utilises (^3H) tritiated TxB_2 as tracer and a specific antiserum to TxB_2, the cross-reactivity of which is displayed in Table 2.1.2.5 (a). A standard binding curve was prepared using the TxB_2 standards provided (3, 1.1, 0.4, 0.13 and 0.05 ng per ml). The detection limits of the assay are between 5 and 300 pg TxB_2 per assay tube.

Tube	Total count (TC)	NSB (buffer blank)	Zero standard (B_0)	Standards A-E	Sample 1	Sample 2	Sample n
Buffer	100	100	50	-	-	-	-
[^{3}H] Thromboxane B_2	50	50	50	50	50	50	
Standards	-	-	-	50	-	-	-
Samples	-	-	-	-	50	50	50
Antiserum	-	-	50	50	50	50	50
Mix and incubate							
Charcoal suspension	500*	500	500	500	500	500	500
React, centrifuge, decant and measure ^{3}H decay							

* 500 uls of assay buffer instead of charcoal suspension

All volumes are in microlitres unless otherwise specified

Table 2.1.2.5 Protocol for radioimmunoassay of Thromboxane B_2

Prostaglandins	% Cross reactivity (50% B/B_0 replacement)
Thromboxane B_2	100.0
Prostaglandin D_2	1.2
Prostaglandin $F_{2\alpha}$	0.15
13, 14-Dihydro- 15- keto- thromboxane B_2	0.13
6β- Prostaglandin I_1	0.10
Prostaglandin E_1	0.05
Prostaglandin E_2	0.01
Prostaglandin $F_{1\alpha}$	0.01
11- epi- Prostaglandin E_1	0.01
13, 14-Dihydro- 6, 15- diketo- $PGF_{1\alpha}$	0.007
Prostaglandin $F_{1\beta}$	0.007
8- iso- Prostaglandin E_2	0.006
13, 14-Dihydro- prostaglandin E_2	0.005
13, 14-Dihydro- prostaglandin E_1	0.004
13, 14-Dihydro- prostaglandin $F_{2\alpha}$	0.004
Di- homo- 6- keto prostaglandin $F_{1\alpha}$	0.004
6- Keto- prostaglandin E_1	0.003
Prostaglandin $F_{2\beta}$	0.003
13, 14-Dihydro- 15- keto- prostaglandin E_2	0.003
15- keto- prostaglandin E_2	0.002
13, 14-Dihydro- prostaglandin $F_{1\alpha}$	0.002
6, 15- Diketo-prostaglandin $F_{1\alpha}$	0.001
Prostaglandin B_1	0.001
13, 14-Dihydro- 15- keto- prostaglandin $F_{2\alpha}$	<0.001
15- keto- prostaglandin $F_{1\alpha}$	<0.001
15- Epi- prostaglandin E_2	<0.001
13, 14-Dihydro- 15- keto- prostaglandin $F_{1\alpha}$	<0.001
15- keto- prostaglandin $F_{2\alpha}$	<0.001

Table 2.1.2.5 (a) Specificity of thromboxane B_2 antiserum

2.1.2.9 Coomassie Blue protein assay

Assays to measure urine protein may be based upon dye-binding, acid precipitation, turbidimetric or immunological principles but because urine contains varying amounts of different types of protein, it important to select the most appropriate assay. In relation to the heterogenity of the component proteins in as sample of urine, problems often arise with linearity of the response, choice of a suitable protein standard and precision and accuracy: amongst many other contaminants, high concentrations of urinary pigments interfere with many protein assays. Often, there are qualitative differences amongst assay methods in terms of detecting low molecular mass proteins and peptides as well as albumin and globulins.

The Coomassie Blue method is recommended for measuring urine total protein levels (McElderry *et al*, 1982). As the name implies, the method is based upon dye-binding and although less sensitive than the Lowry protein assay, it is more suited to measuring albumin which is usually the predominant urine protein. The shift in absorbance from 465 nm to 595 nm which occurs when Coomassie Brilliant Blue G250 binds to protein in acidic solution is reflected by a colour change from reddish-brown to blue. The sulphonic group of Coomassie Blue bind to arginine residues predominantly but also weakly with histidine, lysine, tyrosine, tryptophan and phenylalanine. Non-linearity of the colour response over a wide range of protein concentrations is a problem with this assay but can be overcome by running a standard curve each time. The number of CB reagent ligands bound to each protein molecule is thought to be proportional to the number of charges on the protein ie about 1.5 to 3 dye molecules per charge.

Preparation of CBB reagent

Commercial preparations of CBB are widely available but are expensive for large numbers of samples and so the CBB reagent was prepared in the laboratory.

200 mgs of Coomassie Brilliant Blue-G, CBB (Sigma Chemical Company: B-1131) were dissolved in 100 mls of absolute alcohol. 200 mls of concentrated phosphoric acid ($H_3 PO_4$) plus 1 litre of deionised water were added to the CBB solution and the volume made up to 2 litres with deionised water and mixed well. 16g Diatomaceous Earth: Grade II (Sigma Chemical Company: D-5509) was added to the solution which was then stirred for 6 hours and left at 4°C for 24 hours. When cold, the solution was filtered twice through Whatman No 1 filter paper. The absorbance of the CBB reagent should be 0.28 ± 0.01 when read at 595 nm against deionised water and the reagent remains stable for at least 4 months at room temperature.

A standard curve ranging from 4 to 160 mg per 100 ml was prepared by diluting mixed protein standard (Sigma Chemical Company: 540-10) to 10mls as in the table below.

Sigma standard (µls)	Protein concentration (mgs per 100mls)
5	4
10	8
20	16
50	40
100	80
125	100
150	120
200	160

Duplicate 30 µls samples of standard or urine was added to 3 mls of CBB reagent and mixed three times during colour development. The tubes were left to stand for 15 minutes before the reading the absorbance at 595 nm against CBB reagent. The standard curve of the Coomassie Blue assay is linear up to 160 mg per 100 mls with a detection limits of between 400 and 1600 µgs per ml.

2.1.2.10 Measurement of glomerular renin

Renin levels in Lewis and DA rat glomerular supernatants were measured at the Blood Pressure Unit, Western Infirmary, Glasgow, by a radioimmunoassay, the method of which is described in detail in section 2.2.2.4.

2.1.2.11 Statistical analyses

Statistical analyses were performed using Minitab version 7.2. The non-parametric Mann-Whitney U-test for unpaired data was used predominantly throughout because sample size was small and data distribution was unknown; the parametric two sample *t*-test was used for descriptive statistics of variables such as body weight or food consumption. Accordingly, median and range or mean and standard deviation are quoted in the text and 95% confidence intervals and levels of significance are tabulated in supplementary tables. A significance level (*p* value) less than or equal to 0.05 was taken to denote statistical significance (Altman *et al*, 1983, Braitman, 1991). Statistical comparisons are made only between Lewis and DA rats within a particular diet group and between the control diet and an experimental PUFA diet group, within either strain: no comparisons were made between PUFA diet groups.

2.1.3 Results

2.1.3.1 Metabolic data

Growth rate curves of normal healthy Lewis and DA rats derived from data supplied by commercial breeders (Harlan UK Ltd, Oxon, England) show that at any given age, Lewis rats are heavier than DA rats (Figure 2.1.3.1).

Although there have been reports that rats often develop an aversion to the oily PUFA diets and thus may consume significantly less food than contol rats, body weight increased steadily over the six week diet period in both strains of rat, albeit after an initial fall in all the PUFA diet groups during the first two weeks (Figure 2.1.3.1a). In both strains of rat, the percentage increase in body weight in the control group was at least double that of any of the PUFA diet groups [**Lewis**: CD: 41% vs (i) EPO: 20%, (ii) SO: 17%, (iii) FO: 13%, (iv) OO: 15%; **DA**: CD: 29% vs (i) EPO: 10%, (ii) SO: 11%, (iii) FO: 7%, (iv) OO: 11%]. The percentage weight gain was greater in Lewis rats than in all corresponding diets groups in DA rats. At six weeks, mean body weight was greater in Lewis rats than DA rats in all the diet groups and Lewis and DA rats fed PUFA diets had significantly lower body weight than their respective controls [**Lewis vs DA:** CD: mean 344 (SD 16.5) vs 262 (12.4) grams, EPO: 295 (10.45) vs 226 (15.4), SO: 281 (36) vs 228 (10), FO 281 (18.4) vs 217 (7), OO: 286 (21) vs 228 (9.12)] (see Table 2.1.3.1a for corresponding confidence intervals and significance levels).

Mean daily food consumption during time in metabolic cages is shown in Table 2.1.3.2. At both two and four weeks, Lewis and DA rats fed control diet ate more than those fed PUFA diets. However, despite differences in body weight between Lewis and DA rats, consumption of PUFA diets was remarkably similar in both strains. At four weeks, there was no significant difference in food consumption between the strains except in the control diet group [**Lewis vs DA**: mean 21 (SD 1.9) vs 17.4 (0.65) grams per day, $p = 0.014$]. Food consumption was significantly lower in the PUFA diet than control diet groups in all but DA rats fed olive oil diet [**Lewis**: CD: 21 (1.9) grams - vs (i) EPO: 11.2 (2.1), (ii) SO: 11.6 (2.3), (iii) FO: 10 (0.35), (iv) OO: 10.8 (1.8): **DA**: CD: 17.4 (0.65) grams - vs (i) EPO: 12.2 (4.01), (ii) SO: 11.2 (0.84), (iii) FO: 10.8 (1.92), (iv) OO: 13.9 (3.3)] (see Table 2.1.3.2a for corresponding confidence intervals and significance levels).

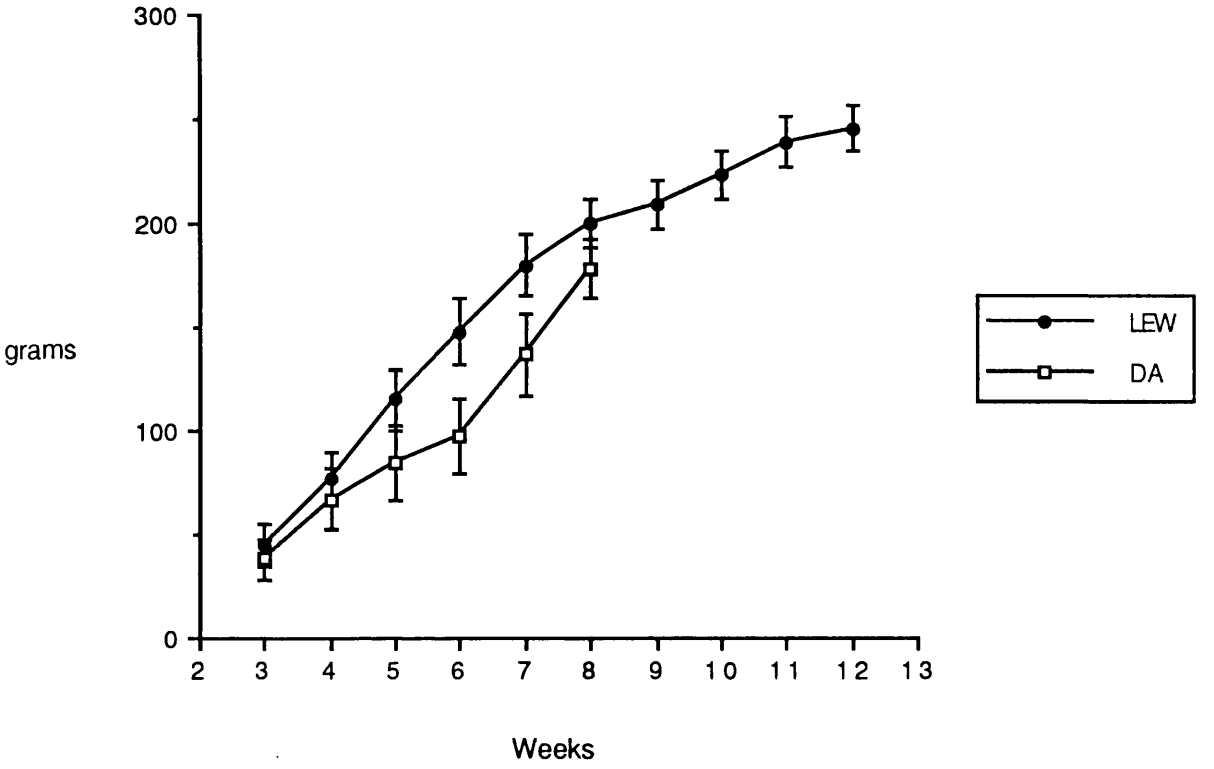


Figure 2.1.3.1 **Growth rate curves (mean and SD) of normal Lewis and DA rats (Data from Harlan UK Ltd, Oxon)**

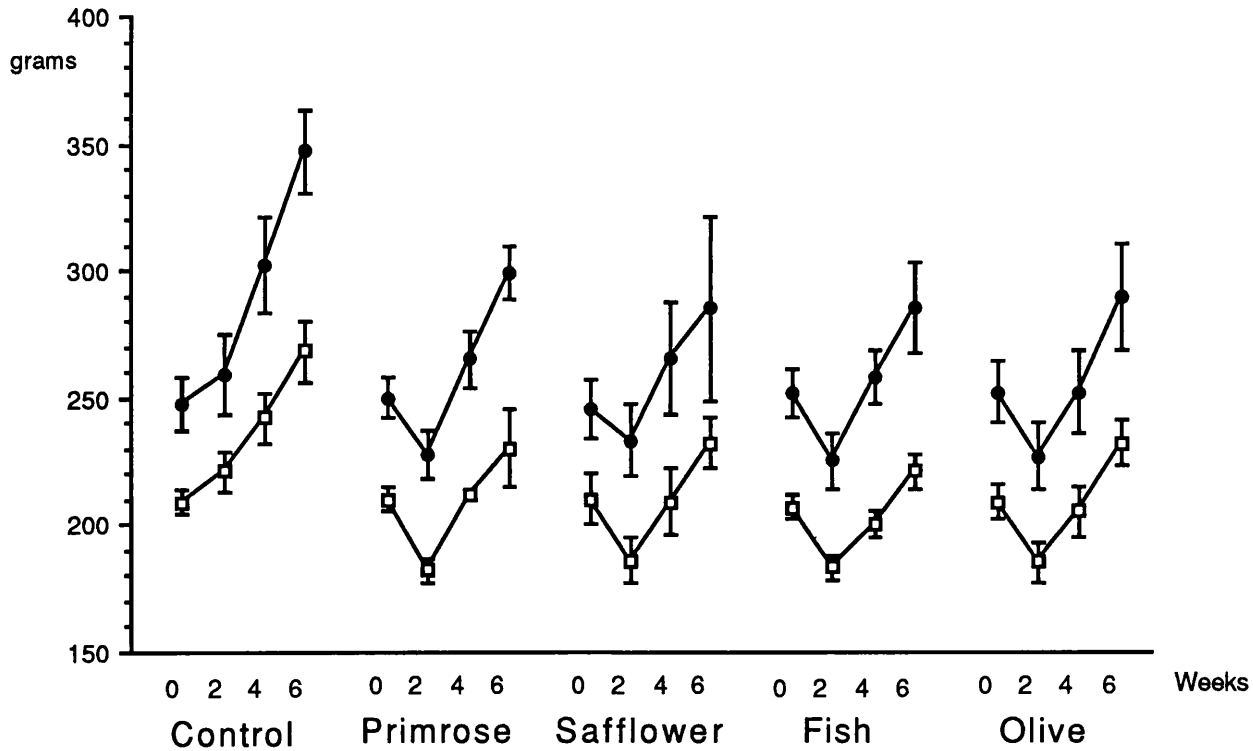


Figure 2.1.3.1(a) Growth rate curves of Lewis (●) and DA (□) rats in control and PUFA diet groups

Table 2.1.3.1(a) Body weight at six weeks - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			58 to 102	p<0.0001*
Primrose			49 to 89	p<0.0001*
Safflower			8 to 100	p= 0.03*
Fish			40 to 86	p= 0.0008*
Olive			32 to 85	p= 0.002*
Lewis rats				
Control	vs	Primrose	27 to 71	p= 0.001*
Control	vs	Safflower	17 to 108	p= 0.016*
Control	vs	Fish	36 to 89	p= 0.0008*
Control	vs	Olive	29 to 85	p= 0.002*
DA rats				
Control	vs	Primrose	17 to 59	p= 0.004*
Control	vs	Safflower	19 to 53	p= 0.0016*
Control	vs	Fish	31 to 62	p= 0.0003*
Control	vs	Olive	19 to 52	p= 0.0013*

Two Sample T Test

	Control		Primrose		Safflower		Fish		Olive	
Week	**2**	**4**	**2**	**4**	**2**	**4**	**2**	**4**	**2**	**4**
Lewis rats	15.5	21	11	14.5	14	13	12	10.5	9.5	12
	18.5	22	13.5	10.5	16	15	6	9.5	14.5	11
	19.5	24	13	10	12.5	10	10	10	13	13
	18.5	19	15	9	13.5	10	12	10	12	9
	21	20	9	12	14	10	14	10	11	9
mean (grams)	**18.5**	**21**	**12.5**	**11**	**14**	**11.5**	**11**	**10**	**12**	**11**
SD	2	1.9	2.3	2.1	1.3	2.3	3	0.35	1.9	1.8
DA rats	19	17	10.5	11	14	11	12.5	11	12.5	15
	16.5	17	20	19	11	10	12	13	11.5	13
	15.5	17.5	9.5	8.5	12	12	12	10	11	12
	16	18.5	9	10.5	13.5	11	10	12	15	19
	17	17	8.5	12	11	12	12	8	10.5	10.5
mean (grams)	**17**	**17.5**	**11.5**	**12**	**12.5**	**11**	**11.5**	**11**	**12**	**14**
SD	1.35	0.65	4.8	4	1.4	0.84	0.98	1.9	1.8	3.3

Table 2.1.3.2 Mean daily food consumption at two and four weeks

Table 2.1.3.2(a) Food consumption at four weeks - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			1.3 to 6.2	p= 0.014*
Primrose			-6 to 4	p= 0.64
Safflower			-2 to 3	p= 0.73
Fish			-3 to 1.6	p= 0.41
Olive			-7 to 1	p= 0.11
Lewis rats				
Control	vs	Primrose	7 to 13	p< 0.001*
Control	vs	Safflower	6 to 13	p= 0.0001*
Control	vs	Fish	9 to 14	p= 0.0002*
Control	vs	Olive	8 to 13	p< 0.001*
DA rats				
Control	vs	Primrose	0.15 to 10	p= 0.04*
Control	vs	Safflower	5 to 7	p< 0.001*
Control	vs	Fish	4 to 9	p= 0.002*
Control	vs	Olive	-0.66 to 8	p= 0.08

Two Sample T Test

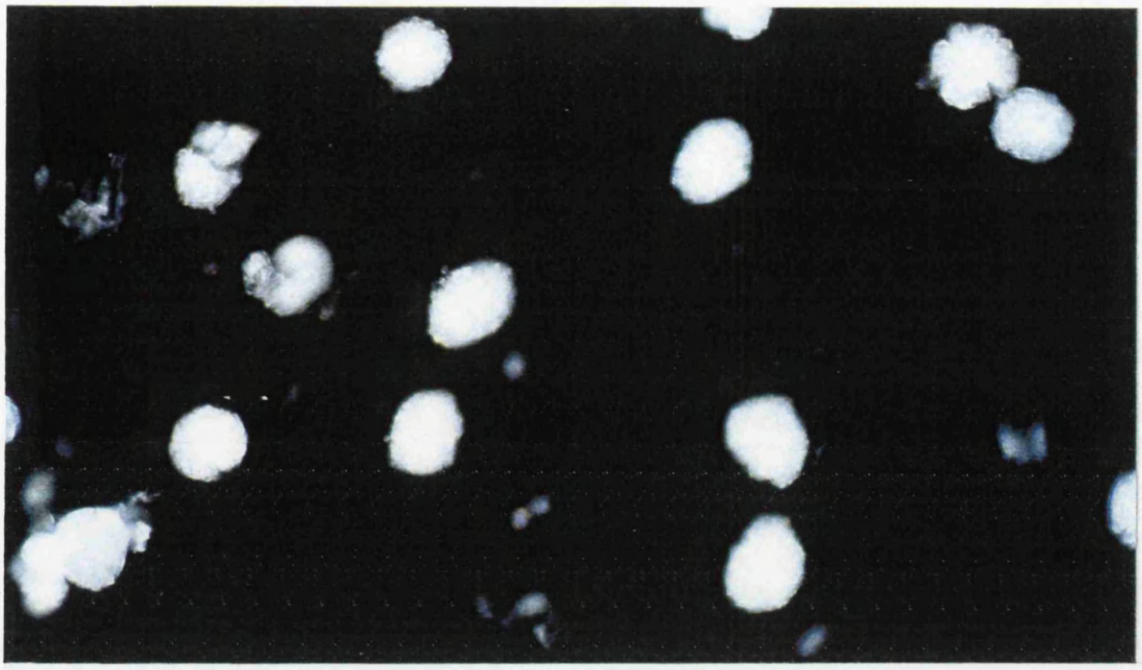
Urine volume tended to be higher in the PUFA diet groups relative to controls in DA rats. Although there was no difference in mean urine volume between control and PUFA diet groups in Lewis rats at four weeks (Table 2.1.3.3) urine volume was raised significantly in the safflower and olive oil diet groups in DA rats (Table 2.1.3.3a). Water intake was not monitored in this study.

Measuring metabolic parameters on both days on which the animals are in metabolic cages is useful for identifying 'rogue' data. Urine volume data in Table 2.1.3.3 illustrates clearly, that the first Lewis rat in the primrose oil diet group did pass more urine than others in the group.

2.1.3.2 Glomerular recovery

Viewed by light microscopy, the glomerular suspension appeared to be remarkably free of tubular contamination: isolated glomeruli viewed at x10 and x40 magnification are shown in Plate 2.1. Some glomeruli were visible with their capsule intact and others were decapsulated, with or without their associated afferent and efferent arterioles.

The protein content of the glomerular pellets from Lewis and DA rats fed control or PUFA diets are shown in Table 2.1.3.4.



x 10

Glomeruli *in vitro*, viewed by light microscopy

x 40

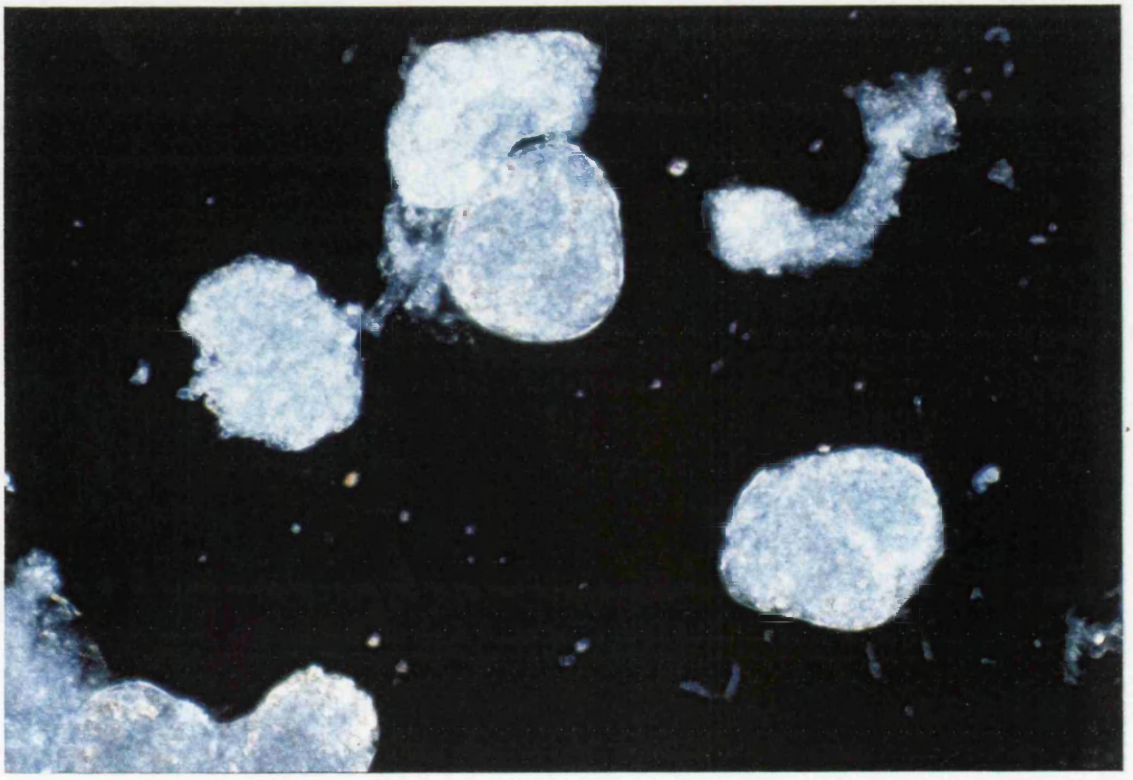


Plate 2.1

	Control		Primrose		Safflower		Fish		Olive	
Week	**2**	**4**	**2**	**4**	**2**	**4**	**2**	**4**	**2**	**4**
Lewis rats	4	6	30	18	9	6	11	8	5	7
	8	7	8	8	8	9	13	6	7	7
	7	9	8	4	8	5	6	5	5	9
	6	6	11	10	6	6	6	5	6	7
	6	8	9	6	13	9	13	7	6	5
mean (mls)	**6**	**7**	**13**	**9**	**9**	**7**	**10**	**6**	**6**	**7**
SD	1.5	1.3	9.5	5.4	2.6	1.9	3.6	1.3	0.8	1.4
DA rats	6	7	6	7	15	9	13	9	9	17
	5	5	7	6	11	8	13	17	10	11
	4	6	7	6	9	11	10	8	9	9
	7	9	13	9	8	10	8	10	6	8
	6	6	4	12	10	10	14	7	11	11
mean (mls)	**5.5**	**6.5**	**7.5**	**8**	**10.5**	**9.5**	**11.5**	**10**	**9**	**11**
SD	1.1	1.5	3.4	2.6	2.7	1.1	2.5	4	2	3.5

Table 2.1.3.3 Mean daily urine volume at two and four weeks

Table 2.1.3.3(a) Urine volume at four weeks - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-1.5 to 3	p= 0.5
Primrose			-6 to 8	p=67
Safflower			-5 to -0.2	p= 0.04*
Fish			-9.5 to 1.2	p= 0.099
Olive			-8.5 to 0.1	p= 0.055
Lewis rats				
Control	vs	Primrose	-9 to 5	p= 0.47
Control	vs	Safflower	-2.5 to 2.6	p= 0.85
Control	vs	Fish	-1 to 3	p= 0.26
Control	vs	Olive	-2 to 2	p= 0.82
DA rats				
Control	vs	Primrose	-5 to 2	p= 0.33
Control	vs	Safflower	-5 to -1	p= 0.01*
Control	vs	Fish	-8.5 to 1.3	p= 0.12
Control	vs	Olive	-9 to -0.2	p= 0.043*

Two Sample T Test

	Control		Primrose		Safflower		Fish		Olive	
	Lewis	DA	Lewis	DA	Lewis	DA	Lewis	DA	Lewis	DA
1	2.15	2.6	3.7	2.0	2.75	2.05	2.5	2.25	2.25	2.75
2	3.45	1.7	2.95	2.7	3.75	2.6	1.48	2.7	3.3	2.25
3	4.05	2.2	3.2	3.2	3.4	2.6	2.7	2.6	3.4	2.7
4	2.9	2.0	3.05	2.9	3.55	2.2	2.9	2.7	2.5	2.2
5	3.6	2.13	2.7	3.55	3.05	2.4	2.7	3.2	3.3	2.35

Results expressed in milligrams

Table 2.1.3.4 Protein content of Lewis and DA rat glomerular pellets

2.1.3.3 Prostanoid levels

Prostanoid binding curves from the assay systems used to measure bicyclic PGE_2 metabolite, 6-keto prostaglandin F_{1a} and thromboxane B_2 are shown in Figures 2.1.3.5, 2.1.3.6 and 2.1.3.7, respectively.

At six weeks, levels of bicyclic-PGE_2 in glomerular supernatants were similar between Lewis and DA rats [**Lewis vs DA:** CD: median 0.233 (range 0.048 - 0.405) vs 0.244 (0.09 - 0.69) ng per mg glomerular protein, EPO: 0.394 (0.2 - 0.46) vs 0.311 (0.2 - 0.54), SO: 0.320 (0.233 - 0.355) vs 0.455 (0.208 - 0.538), FO: 0.148 (0.007 - 0.207) vs 0.148 (0.088 - 0.284), OO: 0.303 (0.098 - 0.606) vs 0.336 (0.255 - 0.791)] (see Figure 2.1.3.5a). Although PGE_2 levels appeared to be lower in the fish oil group in both Lewis and DA rats, the results did not reach statistical significance (see Table 2.1.3.5 for corresponding confidence intervals and significance levels). None of the other PUFA diets had any effect on glomerular PGE_2 levels in either rat strain.
There were no marked differences in 6-keto $PGF_{1\alpha}$ levels in the glomerular supernatants between Lewis and DA rats (Figure 2.1.3.6a) [**Lewis vs DA:** CD: median 0.419 (range 0.398 - 0.599) vs 0.507 (0.320 - 0.583) ng per mg glomerular protein, EPO: 0.486 (0.365 - 0.926) vs 0.407 (0.379 - 0.625); SO: 0.462 (0.294 - 0.546) vs 0.439 (0.320 - 0.606), FO: 0.126 (0.088 - 0.210) vs 0.094 (0.051 - 0.209), OO: 0.459 (0.330 - 0.556) vs 0.370 (0.327 - 0.576)]. However, 6-keto $PGF_{1\alpha}$ levels were lowered to a statistically significant degree relative to controls in both Lewis (0.419 (0.398 - 0.599) vs 0.126 (0.088 - 0.210) and DA (0.507 (0.320 - 0.583) vs 0.094 (0.051 - 0.209) rats fed fish oil diet (see Table 2.1.3.6 for corresponding confidence intervals and significance levels).
Measurement of thromboxane levels in Lewis and DA rat glomerular supernatants showed that Lewis rat glomeruli generated significantly higher levels of TxB_2 *in vitro* than DA rat glomeruli, irrespective of diet [**Lewis vs DA:** CD: median 1.552 (range 1.023 - 1.646) vs 0.559 (0.333 - 0.700) ng per mg glomerular protein; EPO: 1.250 (0.875 - 1.694) vs 0.483 (0.402 - 0.791); SO: 0.978 (0.268 - 1.366) vs 0.358 (0.321 - 0.606); FO: 0.167 (0.146 - 0.241) vs 0.089 (0.075 - 0.096); OO: 1.06 (0.83 - 1.212) vs 0.543 (0.303 - 0.788)] (Figure 2.1.3.7a). Thromboxane levels were lowered to a statistically significant degree in both strains of rat in the fish oil diet group - and the significant interstrain difference was maintained within this diet group (see Table 2.1.3.7 for corresponding confidence intervals and significance levels). In Lewis rats, lowering of glomerular TxB_2 levels in safflower and olive oil diet groups did not reach statistical significance.

Table 2.1.3.7(b) shows bicyclic-PGE_2, 6-keto $PGF_{1\alpha}$ and TxB_2 levels before factoring for glomerular protein and gives some indication of the extent of *ex vivo* stimulation.

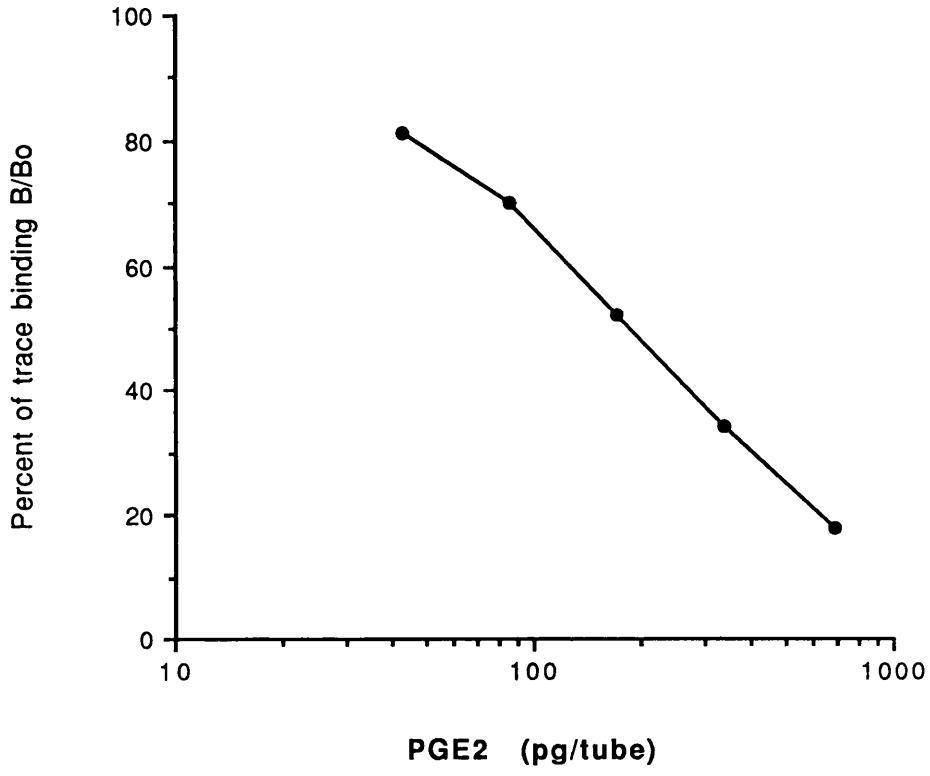


Figure 2.1.3.5 Standard bicyclic-PGE_2 binding curve

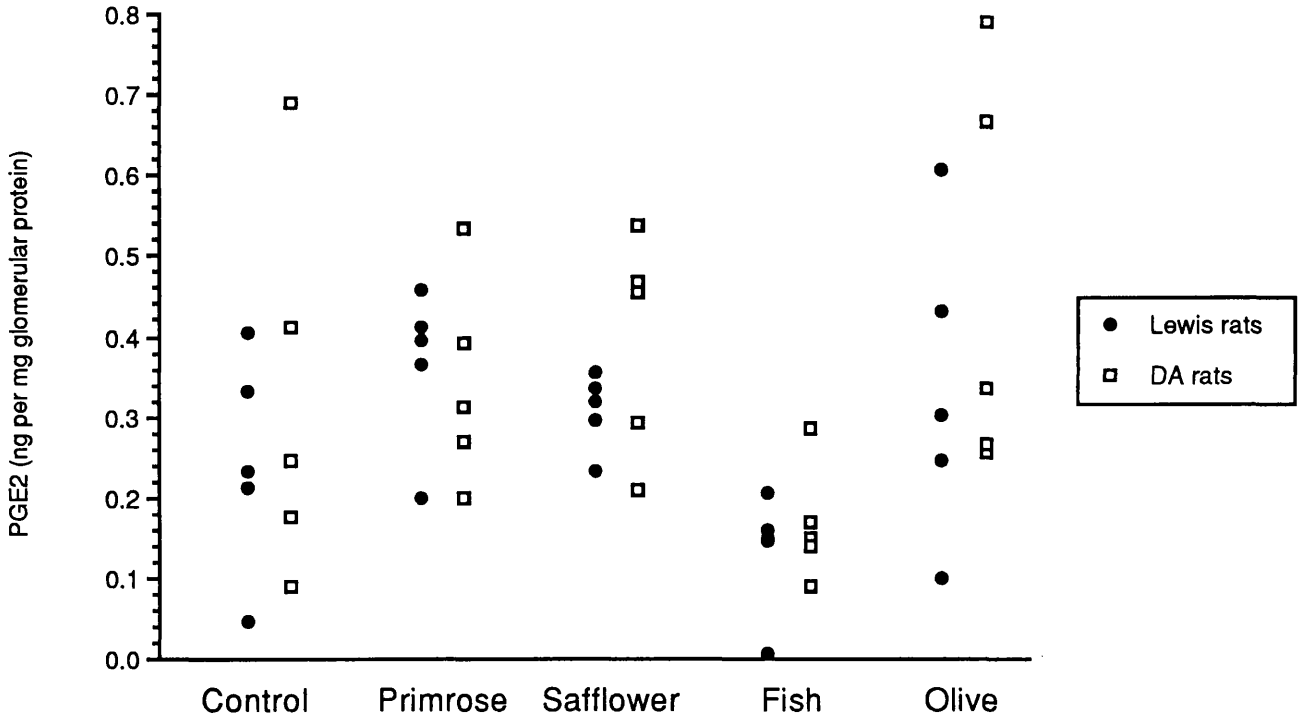


Figure 2.1.3.5 (a) Glomerular PGE_2 release *in vitro* in Lewis and DA rats after six weeks

Table 2.1.3.5 Glomerular PGE_2 release - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-0.4 to 0.23	p= 0.68
Primrose			-0.17 to 0.2	p= 0.6
Safflower			-0.23 to 0.11	p= 0.53
Fish			-0.14 to 0.07	p= 0.83
Olive			-0.5 to 0.27	p=0.4
Lewis rats				
Control	vs	Primrose	-0.3 to 0.04	p= 0.21
Control	vs	Safflower	-0.27 to 0.10	p= 0.46
Control	vs	Fish	-0.1 to 0.26	p= 0.09
Control	vs	Olive	-0.38 to 0.16	p= 0.4
DA rats				
Control	vs	Primrose	-0.3 to 0.4	p= 0.68
Control	vs	Safflower	-0.36 to 0.24	p= 0.4
Control	vs	Fish	-0.08 to 0.54	p= 0.21
Control	vs	Olive	-0.57 to 0.36	p= 0.3

Mann Whitney Confidence Interval and Test

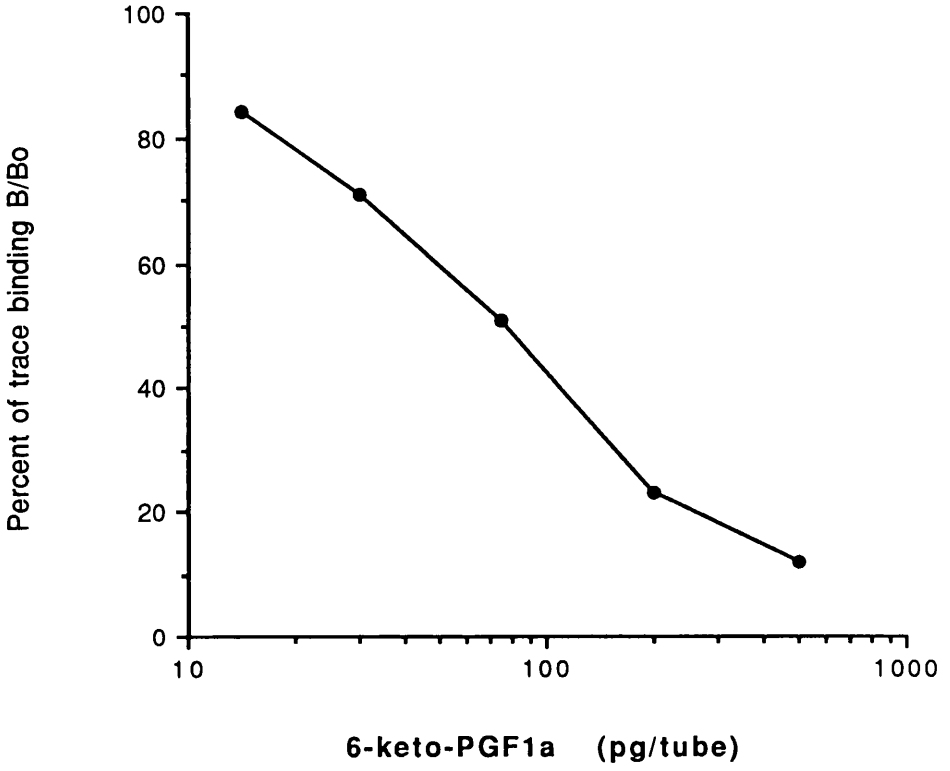


Figure 2.1.3.6 Standard 6, keto- $PGF_{1\alpha}$ binding curve

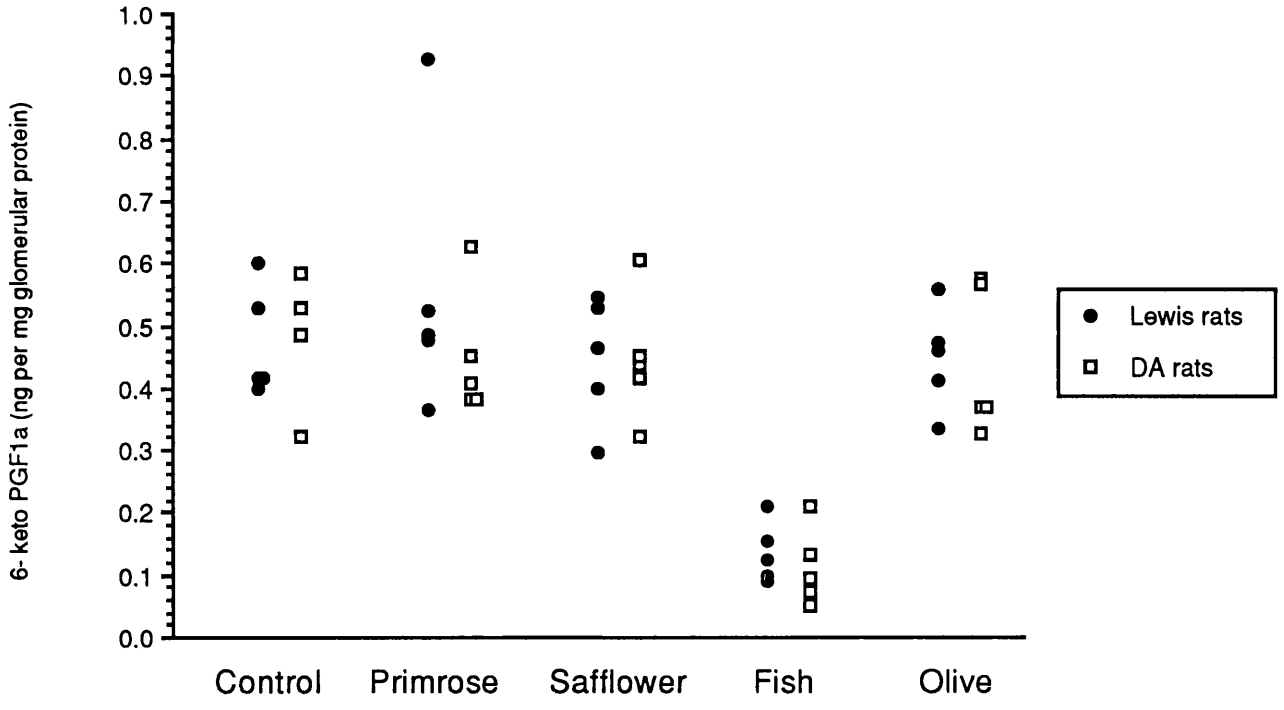


Figure 2.1.3.6 (a) **Glomerular 6, keto-$PGF_{1\alpha}$ release *in vitro* in Lewis and DA rats after six weeks**

Table 2.1.3.6 Glomerular 6-keto $PGF_{1\alpha}$ release - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-0.17 to 0.21	p= 1
Primrose			-0.13 to 0.52	p= 0.4
Safflower			-0.15 to 0.14	p= 1
Fish			-0.08 to 0.12	p= 0.4
Olive			-0.16 to 0.17	p= 1
Lewis rats				
Control	vs	Primrose	-0.5 to 0.12	p= 0.8
Control	vs	Safflower	-0.12 to 0.2	p= 0.8
Control	vs	Fish	0.2 to 0.47	p= 0.012*
Control	vs	Olive	-0.14 to 0.19	p= 1
DA rats				
Control	vs	Primrose	-0.14 to 0.20	p= 0.7
Control	vs	Safflower	-0.12 to 0.21	p= 0.6
Control	vs	Fish	0.18 to 0.51	p= 0.02*
Control	vs	Olive	-0.24 to 0.22	p= 0.9

Mann Whitney Confidence Interval and Test

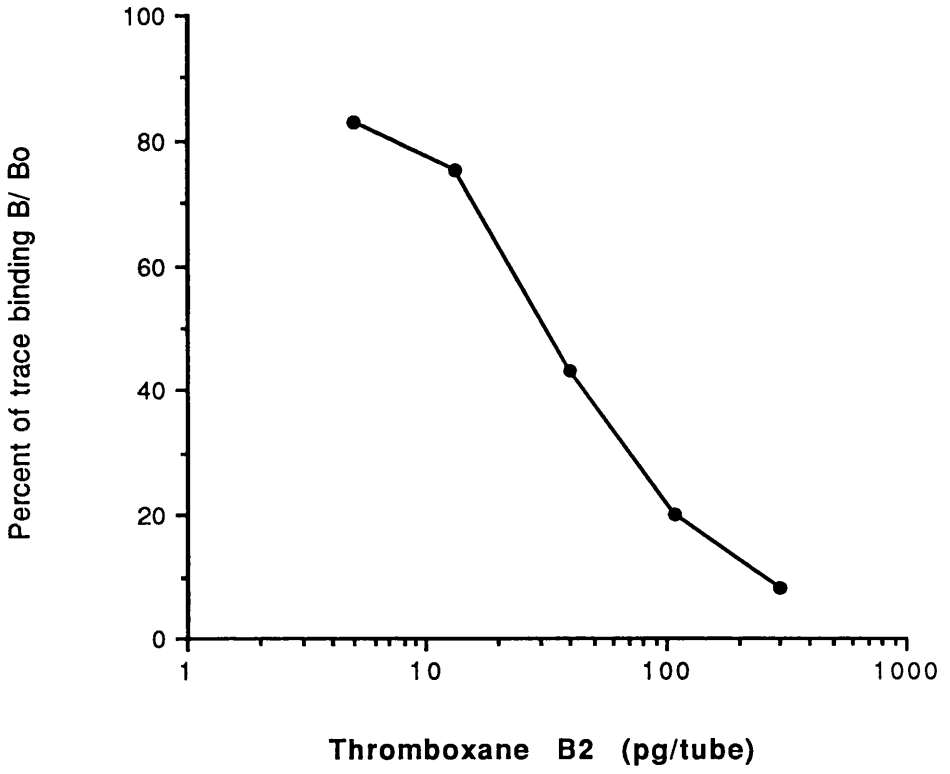


Figure 2.1.3.7 Standard thromboxane B_2 binding curve

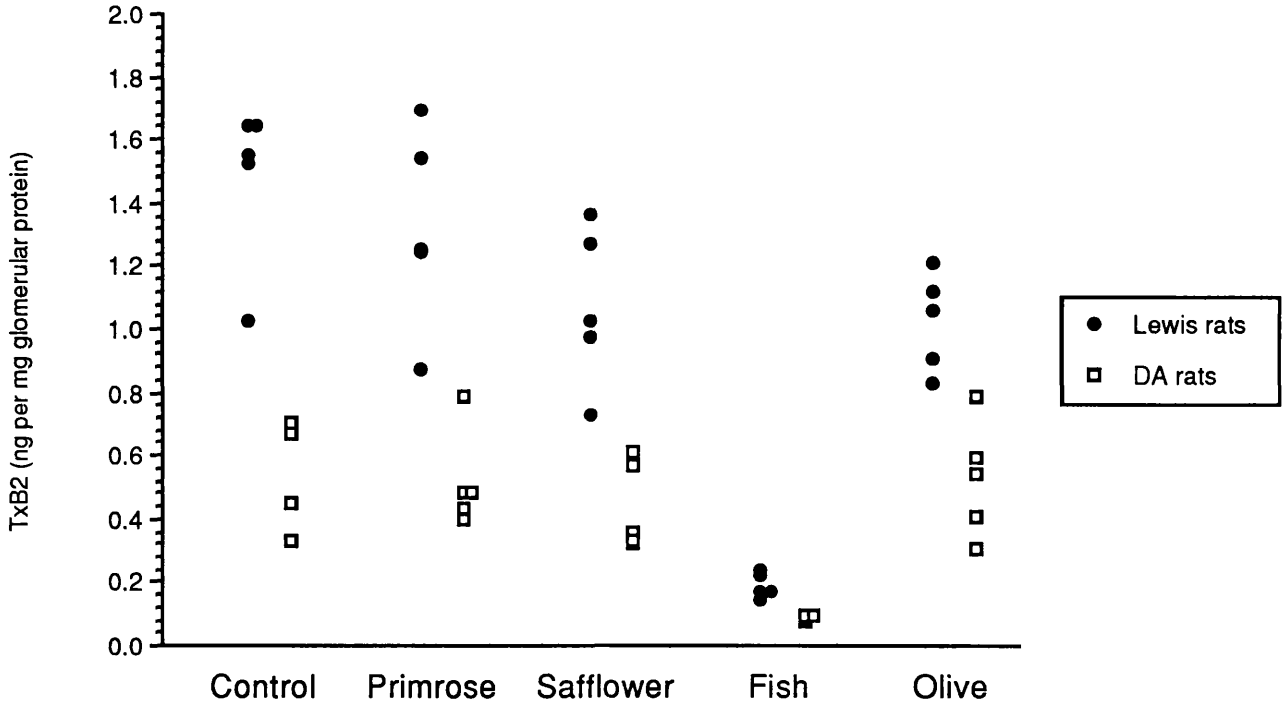


Figure 2.1.3.7(a) Glomerular thromboxane B_2 release *in vitro* in Lewis and DA rats after six weeks

Table 2.1.3.7 Glomerular thromboxane B_2 release - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			0.14 to 1.01	p= 0.037*
Primrose			0.39 to 1.2	p= 0.012*
Safflower			0.37 to 1.01	p= 0.012*
Fish			0.05 to 0.15	p= 0.012*
Olive			0.23 to 0.80	p= 0.012*
Lewis rats				
Control	vs	Primrose	-0.22 to 0.67	p= 0.53
Control	vs	Safflower	-0.006 to 0.82	p= 0.06
Control	vs	Fish	0.85 to 1.48	p= 0.012*
Control	vs	Olive	-0.03 to 0.74	p= 0.06
DA rats				
Control	vs	Primrose	0.33 to 0.27	p= 1
Control	vs	Safflower	0.23 to 0.37	p= 0.33
Control	vs	Fish	0.24 to 0.62	p= 0.02*
Control	vs	Olive	-0.33 to 0.36	p= 0.9

Mann Whitney Confidence Interval and Test

	Control			Primrose			Safflower			Fish			Olive		
	TxB_2	6, keto $PGF_{1\alpha}$	PGE_2	TxB_2	6, keto $PGF_{1\alpha}$	PGE_2	TxB_2	6, keto $PGF_{1\alpha}$	PGE_2	TxB_2	6, keto $PGF_{1\alpha}$	PGE_2	TxB_2	6, keto $PGF_{1\alpha}$	PGE_2
Lewis rats	66	27	25	84	35	32	60	33	32	11	6.6	20	56	31	11
	170	62	37	110	43	54	110	52	60	11	6.6	16	105	55	56
	200	50	82	120	46	63	105	30	50	18	12	20	93	48	42
	135	46	7	155	48	63	135	56	63	21	11	1	84	30	54
	165	43	60	125	75	62	125	50	51	16	17	28	120	33	106
DA rats	26	25	23	29	23	27	22	27	30	6	5	32	25	27	35
	23	27	35	35	33	42	25	25	27	7.3	7.6	20	40	25	30
	44	32	76	76	60	32	44	35	70	6.4	4	18	44	30	90
	42	35	9	35	33	57	40	40	50	7.8	17	23	52	38	37
	-	19	26	52	48	95	24	30	56	7.2	13	14	29	40	93

Table 2.1.3.7(a) Prostanoid levels (pg/tube) in glomerular supernatants prior to factoring for glomerular protein

2.1.3.4 Urine total protein

Only in the fish oil diet group was proteinuria significantly higher in Lewis than DA rats [**Lewis vs DA**: CD: median 13.30 (range 10.5 - 15.6) vs 11.1 (8.75 - 15.4) mgs per 24 hrs, EPO: 5.85 (4.3 - 6.6) vs 6.5 (4.9 - 8.9 mgs per 24 hrs, SO: 7.3 (4.8 - 12.3) vs 6.05 (5.2 - 7.1) mgs per 24 hrs, FO: 8.4 (8.3 - 11) vs 6 (5.6 - 7.6 mgs per 24 hrs*, OO: 7.25 (6.65 - 8.05) vs 8.3 (5.4 - 10) mgs per 24 hrs; see Figure 2.1.3.8).

Urine total protein levels were lowered in both Lewis and DA rats in all the PUFA diet groups (see Table 2.1.3.8 for corresponding confidence intervals and significance levels).
Total protein excretion at weeks 2 and 4 in all the PUFA diet groups is shown in Figure 2.1.3.8(a)

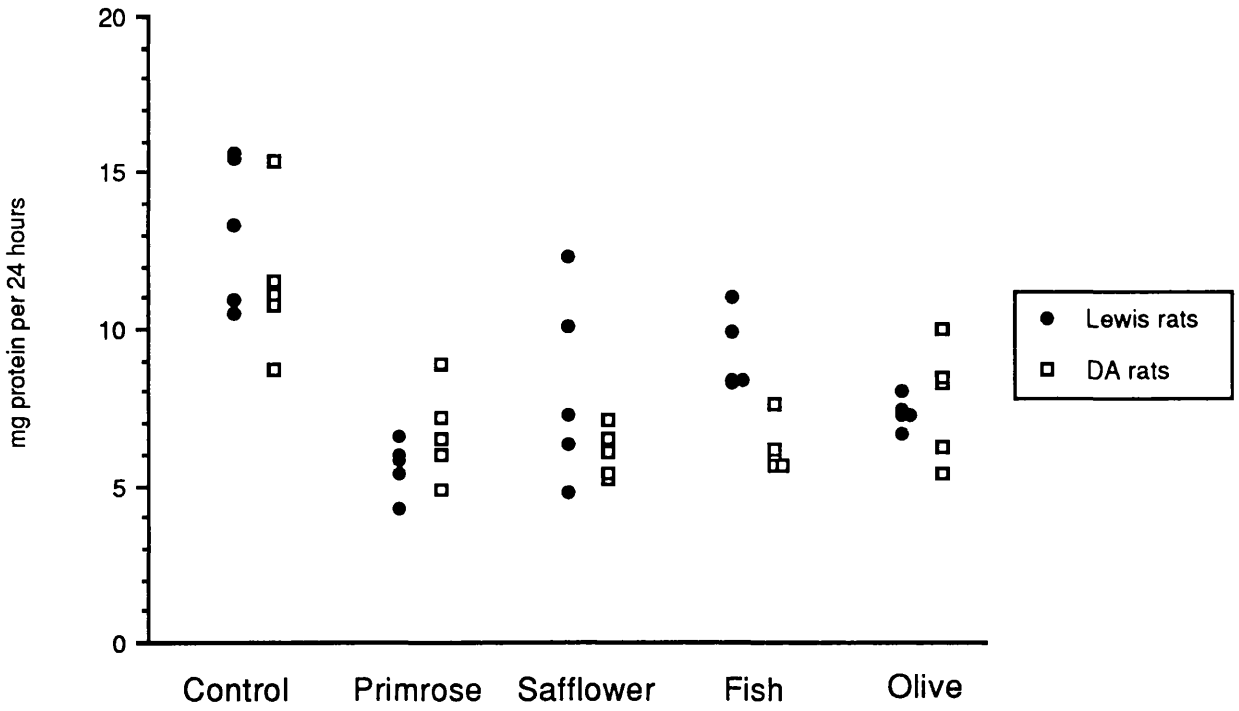


Figure 2.1.3.8 Total protein excretion in Lewis and DA rats after four weeks

Table 2.1.3.8 Total protein excretion - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-2 to 4.8	p= 0.4
Primrose			-3 to 0.95	p= 0.25
Safflower			-1 to 6.26	p= 0.29
Fish			0.8 to 5	p= 0.01*
Olive			-3 to 1.9	p= 0.67
Lewis rats				
Control	vs	Primrose	4.55 to 10	p= 0.03*
Control	vs	Safflower	0.4 to 9.3	p= 0.03*
Control	vs	Fish	0.6 to 7.2	p= 0.03*
Control	vs	Olive	3.1 to 8.4	p= 0.012*
DA rats				
Control	vs	Primrose	1.9 to 8.8	p= 0.02*
Control	vs	Safflower	2.7 to 9.4	p= 0.012*
Control	vs	Fish	2.7 to 9.4	p= 0.012*
Control	vs	Olive	0.45 to 7.1	p= 0.02*

Mann Whitney Confidence Interval and Test

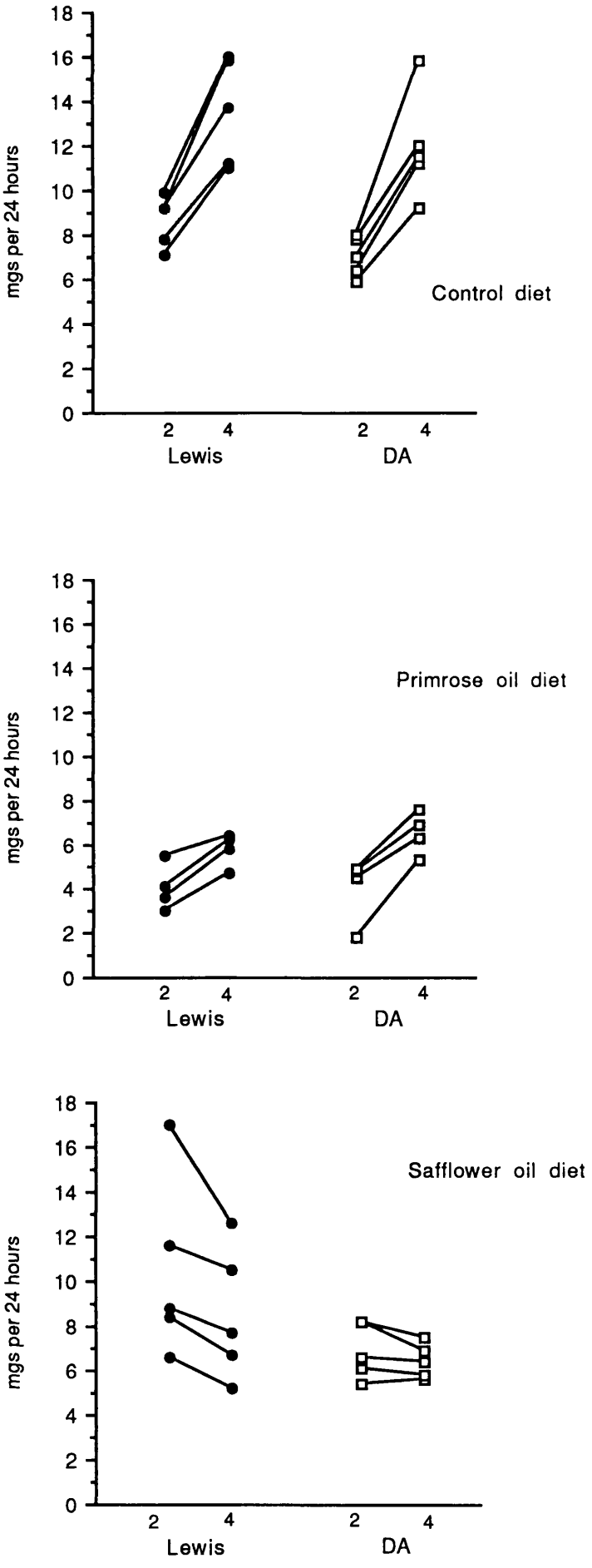
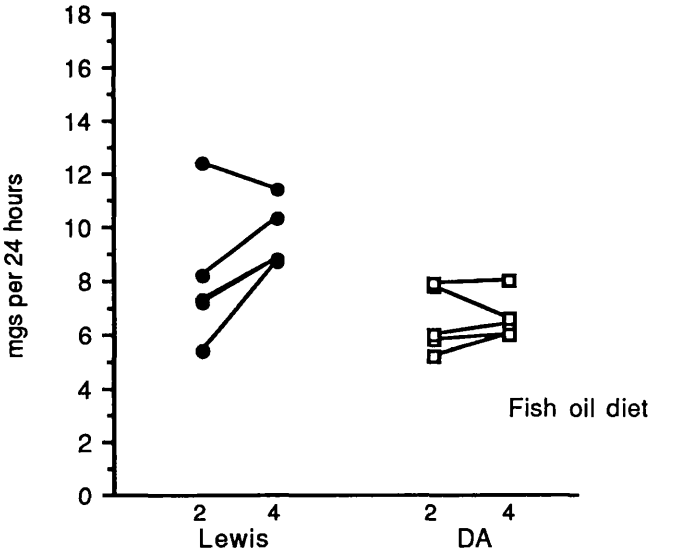


Figure 2.1.3.8(a) Total protein excretion at two and four weeks



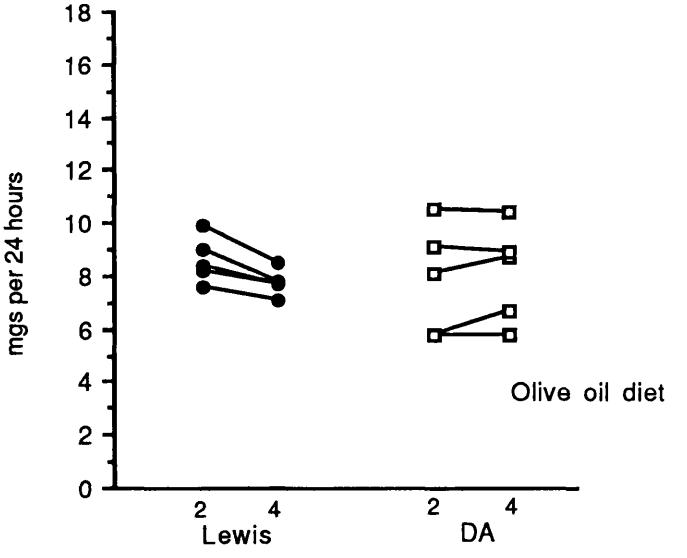


Figure 2.1.3.8(a) continued

2.1.3.5 Glomerular renin levels

Glomerular renin data shown in Figure 2.1.3.9 was derived from the difference between the standard incubation (15 minute) time value and the zero-time value (see Tables 2.1.3.9a and 2.1.3.9b) and then factored for glomerular protein content (Table 2.1.3.4).

Renin concentrations in glomerular supernatants were significantly higher in Lewis than DA rats in all diet groups [**Lewis vs DA:** CD: median 2903 (range 1311 - 105622) vs 246 (44 - 794) pg AI per hour per mg glomerular protein, EPO: 1833 (598 - 7600) vs 219 (134 - 518), SO: 3932 (1699 - 9320) vs 511 (160 - 1648), FO: 2619 (1119 - 44970) vs 446 (415 - 1724), OO: 5227 (1925 - 17493) vs 502 (336 - 640)]. However, none of the experimental PUFA diets had any effect on glomerular renin release *in vitro* in either Lewis or DA rats (Table 2.1.3.9c).

	Control		Primrose		Safflower		Fish		Olive	
	15"	0"	15"	0"	15"	0"	15"	0"	15"	0"
1	2703	314	1445	453	3064	211	1637	357	10311	471
2	92031	932	6424	819	9702	964	26011	1165	12920	1257
3	3004	604	1025	547	1992	548	2348	580	4362	834
4	1724	435	1968	470	3949	459	1161	350	1587	384
5	1692	512	3523	447	2630	605	18545	1486	2551	625

Results expressed in pg AI per unit time

Table 2.1.3.9(a) Renin concentration in Lewis rat glomerular supernatant at fifteen minutes and zero-time

	Control		Primrose		Safflower		Fish		Olive	
	15"	0"	15"	0"	15"	0"	15"	0"	15"	0"
1	568	141	451	114	162	80	445	13	396	165
2	176	69	582	440	746	229	403	111	384	52
3	118	94	267	92	481	149	280	0	376	98
4	167	47	498	131	367	88	1388	224	442	90
5	-	0	248	129	1076	87	384	27	526	231

Results expressed in pg AI per unit time

Table 2.1.3.9(b) Renin concentration in DA rat glomerular supernatant at fifteen minutes and zero-time

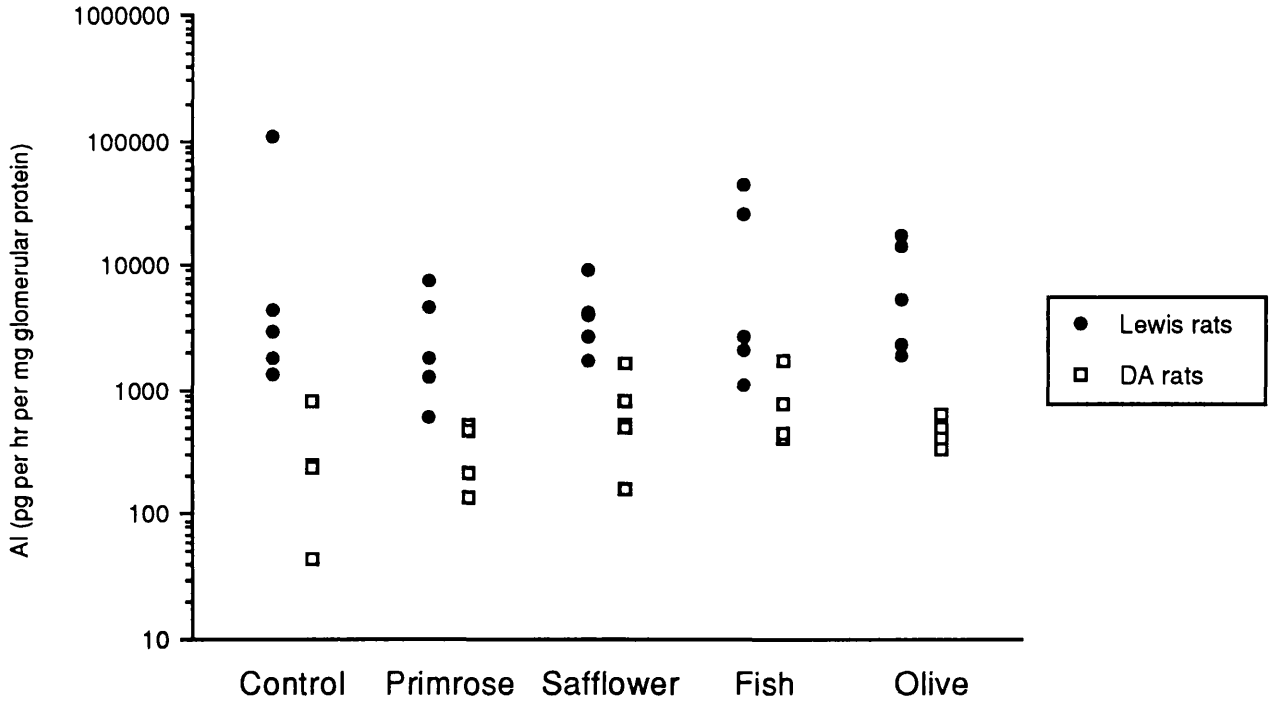


Figure 2.1.3.9 Renin concentration in glomerular supernatants from Lewis and DA rats at six weeks

Table 2.1.3.9(c) Glomerular renin concentration - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			983 to 105382	p= 0.02*
Primrose			378 to 7381	p= 0.012*
Safflower			1007 to 8809	p= 0.012*
Fish			351 to 44524	p= 0.02*
Olive			1422 to 16991	p= 0.012*
Lewis rats				
Control	vs	Primrose	-4696 to 103789	p= 0.67
Control	vs	Safflower	-6417 to 101690	p= 1
Control	vs	Fish	-42066 to 103003	p= 1
Control	vs	Olive	-14590 to 100395	p= 0.5
DA rats				
Control	vs	Primrose	-437 to 584	p= 0.9
Control	vs	Safflower	-1407 to 287	p= 0.27
Control	vs	Fish	-1483 to 361	p= 0.18
Control	vs	Olive	-546 to 382	p= 0.27

Mann Whitney Confidence Interval and Test

2.1.4 Discussion

The purpose of performimg this study was to determine whether the reported inherited differences in GCW charge, mesangial function and disease susceptibility between the Lewis and DA rat strains might relate to differences in their glomerular prostanoid profiles. The principal aims were, therefore, to establish differences in glomerular PGE_2, PGI_2 and thromboxane production *in vitro* between Lewis and DA rats and to explore the effects of PUFA diets on glomerular prostanoid levels *in vitro*.

The great bugbear of any study involving the measurement of prostanoids is that levels may be raised erroneously due to various forms of tissue stimulation outwith the body. In practice, it is impossible to avoid artefactual prostanoid production, especially with an aggressive tissue recovery technique such as the sieving procedure used in this study, but various actions can be taken to minimise the problem. Low temperatures suppress prostanoid production and so, as soon as the kidneys were removed from the rat and the glomeruli recovered, they were kept in ice-cold saline or on ice as appropriate, throughout all the stages of the procedure. Following the subsequent rest period during which the temperature of the glomerular suspensions was raised slowly to 37°C, prostanoid production was re-activated by gentle agitation on a roller mixer at 37°C and maintained thus for thirty minutes. The extent to which the glomeruli will have recovered from the various cycles of quiescence and re-initiation is unclear but the sieving and incubation technique is a common one, used widely by many workers (Stahl *et al*, 1990, Rosenberg *et al*, 1991). Although cyclooxygenase inhibitors such as aspirin or indomethacin, are often added to limit *ex vivo* prostanoid production, their use in this study would have been inappropriate because the purpose was to assess prostanoid generation rather than measure endogenous levels.

It is notable that despite the crude nature of the sieving technique, the glomerular suspensions were not contaminated, to any great extent, with tubular tissue which could alter prostanoid levels.

The problems associated with tissue stimulation can be limited by measuring prostanoid levels in urine, which is relatively free of extraneous cellular material. The advantages of measuring urine prostanoids were considered during the planning stages of this study but rejected on the basis that (i) urine contains a multitude of highly degraded tetranor metabolites originating from various tissues with as many as twenty different TxB_2 tetranor metabolites alone, (ii) the object of the study was to examine glomerular prostanoid production and the very low levels of any primary prostanoids are likely to be of tubular origin, (iii) urine collected in metabolic cages

has been at room temperature for some 24 hours, may be contaminated with food and faecal material and should, ideally, undergo some form of chemical extraction procedure which is time-consuming in a large number of experimental animals and (iv) in male rats, urine may be contaminated with seminal fluid which is a rich source of prostanoid metabolites.

Glomerular prostanoids: Interpretation of the prostanoid assay results is complicated by many factors. Although there appeared to be no differences in the glomerular levels of vasodilatory bicyclic PGE_2 or 6-keto $PGF_{1\alpha}$ between Lewis and DA rats in any of the diet groups, glomerular TxB_2 levels were significantly raised in Lewis compared to DA rats in all the diet groups and the difference was quite clear despite the small number of animals. There was no concomitant rise in glomerular PGE_2 levels in Lewis rats but rather a suggestion that PGE_2 levels might be lower in Lewis than DA rats in all the PUFA groups: however, interpretation of such data is confounded by sample size and may be subject to a type II statistical error (false-negative). In realistic terms, to detect such a difference in PGE_2 levels would require a larger number of experimental animals. A power calculation based upon the small amount of PGE_2 data gathered in this experiment, suggests that *44* Lewis or DA rats would be required in each experimental diet group to show a 20% reduction in PGE_2 levels between the strains, with 80% power at a significance level of 0.05.

In view of having fed different PUFA diets to manipulate glomerular prostanoid production, RIA was not the most appropriate method for detecting changes in the different prostanoids series. HPLC or GC-MS are the only analytical methods which can determine the levels of 1-, 2- and 3- series prostanoid metabolites simultaneously. Radioimmunoassay, as used in this study, measured specific dienoic prostanoids and so changes in biosynthesis towards 1- or 3- series prostanoids could only be insinuated from a relative fall in dienoic prostanoid levels. The fall in 6-keto $PGF_{1\alpha}$ and TxB_2 and to a lesser degree, bicyclic-PGE_2, in both strains in the fish oil diet groups, is likely to be related to preferential synthesis of 3- series prostanoids. The maintenance of a significant difference in TxB_2 levels between Lewis and DA rats in the fish oil group may reflect preferential metabolism of ω-3 fatty acids in DA rats, resulting in a higher TXA_3 : TxB_2 ratio.

That dienoic prostanoid levels were unchanged in the evening primrose oil and safflower oil diet groups in both strains was a little surprising, since the rationale behind the use of such diets is to stimulate the biosynthesis of monoenoic prostanoid derivatives. Evening primrose and safflower oils have a similar linoleic acid content but EPO also contains γ-linolenic acid (GLA) which is the immediate precursor of 1-series prostanoids; safflower oil contains no GLA and so lower dienoic prostanoid

levels might be expected in the EPO, than the SO group. It should have been possible to detect relative changes in dienoic 6-keto $PGF_{1\alpha}$ or TxB_2 levels had they occurred, since the antibodies to both metabolites do not cross react to any great extent with other prostanoid metabolites (see Tables 2.1.2.3c and 2.1.2.4a). However, it would have been impossible to distinguish between changes in PGE_1 and PGE_2 levels since the bicyclic-PGE_2 antibody cross-reacts (100%) with both PGE_1 and PGE_2.

Selection of the most suitable metabolite to measure is yet another problem of working with prostanoids. The 15 keto, 13, 14 dihydro- (or DHK-) metabolites are a major and constant index of the metabolism of the parent prostanoid *in vivo*, which are not formed by artefact and circulate at higher levels (20 to 50 pg per ml) and so are easier to detect and measure: however, the DHK- metabolites of PGE_2 and PGI_2 are metabolised further to dinor and tetranor derivatives which are the major circulating compounds. In man, there are few thromboxane metabolites other than TxB_2 in the blood, in comparison with urine which contains some twenty TxA_2 metabolites, the predominant one being 2, 3 dinor TxB_2, which is barely detectable in blood. 11, dehydro- TxB_2 is the major metabolite in both blood and urine and is not formed by artefact and so is probably the best metabolite to measure. Of the three prostanoids measured, only bicyclic PGE_2 can perhaps be regarded as a true end-product. Both 6-keto $PGF_{1\alpha}$ and TxB_2 can be metabolised further to various dinors and tetranors which might, ultimately, display a different biosynthetic pattern. Prostanoids can also interconvert or shunt between classes: in humans, for example, PGE_2 can be reduced irreversibly to a PGF_α compound - a reaction which is reversible in rats.

The purpose of this study was, however, to compare prostanoid production between the diet groups. The actual levels of prostanoids per assay tube (Table 2.1.3.7b) show that glomerular TxB_2 levels in Lewis rats are raised, relative to both DA rats and PGE_2 and 6-keto $PGF_{1\alpha}$ levels in both strains in all except the fish oil diet groups. (Prostanoid levels in glomerular supernatants relate to cumulative production over thirty minutes). PGE_2 and 6-keto $PGF_{1\alpha}$ levels did not exceed markedly, the accepted range of 20-50 pg/ml for circulating prostanoid metabolites, but TxB_2 levels were well outwith the range in Lewis compared with DA rats, in all the diet groups (see Table 2.1.3.7b).

Reasons for the raised glomerular TxB_2 levels in Lewis relative to DA rats are unclear (see Table 2.1.3.7a). Were TxB_2 levels raised due to the sieving procedure, a similar pattern would be evident in DA rats but prostanoid levels were measured in supernatants from glomeruli subjected to identical sieving and storage procedures.

Alternative explanations are that Lewis rat glomeruli are more sensitive to the mechanical stimulation of the sieving procedure or that DA rat glomeruli became exhausted more quickly *in vitro* than Lewis rat glomeruli, rather than Lewis rat glomeruli having a greater capacity to synthesise TxA_2 than DA rats. It is perhaps unusual for thromboxane levels to be raised in an apparently healthy strain of rat, since elevated thromboxane levels are associated predominantly with disease states or coagulation disorders (see section 1.3.4).

Prostaglandin and thromboxane biosynthesis is regulated enzymatically at the level of (i) lipases and phospholipases which are involved in the release of free arachidonic acid from membrane phospholipids (Zusmann and Kaiser, 1977; Benabe *et al*, 1982; Folkert *et al*, 1984), (ii) Δ- desaturases and elongases within the EFA metabolic pathways (see Figure 1.3.1), (iii) cyclooxygenase activity and (iv) specific prostanoid synthetase enzymes (see Figure 1.3.2). It is difficult therefore, to pinpoint the origin of increased thromboxane production in Lewis rats. Infiltrating neutrophils, macrophages or platelets produce TxB_2 (see section 1.3.3). Alternatively, in Lewis rats, platelets may adhere more readily to the GCW with its depleted polyanion, become activated and generate thromboxane (Pugliese *et al*, 1987). Raised glomerular thromboxane levels have also been explained as an adaptive response to an already dilated vasculature (Craven *et al*, 1987) but PGE_2 and 6-keto $PGF_{1\alpha}$ levels were not higher in Lewis compared to DA rats in this study. Agents including angiotensin II and vasopressin (Nasjletti *et al*, 1985; Ardaillou *et al*, 1987; Wilcox *et al*, 1990), complement (Couser *et al*, 1985; Stahl *et al*, 1987), endothelin (Zoja *et al*, 1990), calcium (Stork *et al*, 1985), reactive oxygen species (Baud *et al*, 1983; Adler *et al*, 1986), cytokines (Lovett *et al*, 1987a; Baud *et al*, 1989) and growth or platelet activating factors (Levine and Hassid, 1977, Schlondorff *et al*, 1984, Habenicht *et al*, 1985, Floege *et al*, 1990), hypoalbuminaemia (Schiepatti *et al*, 1984) and increased dietary protein intake (Collins *et al* 1989) can also contribute to raised thromboxane levels.

Proteinuria: Urine protein excretion appeared to be lowered in both Lewis and DA rats in all the PUFA diet groups compared with those fed standard laboratory rat chow but the finding must be viewed with caution because of the small number of rats in each diet group and the high degree of intraindividual variation inherent in urine protein measurements. However, the effect overall, was evident in a relatively large number of PUFA-fed rats and was therefore deemed worthy of further investigation (see section 2.2.1). Urine protein levels between two and four weeks are shown in Figure 2.1.3.8(a). In the control diet group, urine protein levels are rising in both Lewis and DA rats, as occurs also in the evening primrose oil group,

albeit at a lower level. In the safflower, fish and olive oil diet groups, urine protein levels are, in the main, lowered or stabilised to varying degrees. Although urine protein levels do tend to be lower in the PUFA diet groups, it is inappropriate to make any generalisation because of the small sample size and infrequent measurements.

Six weeks is a common PUFA diet feeding period and indeed, it is said that effects are evident after four weeks. The percentage increase in body weight in rats fed control diet was double that of those fed PUFA diets and could be an alternative explanation for the fall in proteinuria in the PUFA diet groups. However, the lower body weight in the PUFA groups does not appear to be related to reduced food consumption. It must also be considered that data derived from the time the animals spend in metabolic cages is only representative.

Glomerular renin: Renin levels in the glomerular supernatants of Lewis and DA rats were measured to see whether they might correlate with the fall in proteinuria in the PUFA diet groups. Generally accepted as being synthesised exclusively within the glomerulus, renin is a proteolytic enzyme involved in regulating blood pressure and fluid balance and there is a close relationship between prostanoid biosynthesis and activation of the renin-angiotensin system (see section 1.3.4). The renin assay used in this study is complicated, requiring an array of reagents which are not always readily available and so samples of Lewis and DA rat glomerular supernatants were dispatched, to be assayed using an established radioimmunoassay method. It was interesting to see that the pattern of glomerular renin was very similar to that of glomerular TxB_2, with renin levels in supernatants from Lewis rats being significantly higher than those from DA rats, irrespective of diet. However, renin levels neither correlated with urine protein levels nor the fall in protein excretion in the PUFA diet groups in either strain of rat and none of the PUFA diets had any effect on glomerular renin levels in either strain of rat.

As with the thromboxane results, it is difficult to interpret the significance of the increased glomerular renin levels in Lewis rats *in vitro*. However, unlike prostanoids, which are synthesised as required and inactivated at their site of action, renin is synthesised and stored within juxtaglomerular cells in the afferent arteriole. Raised glomerular renin levels may relate to increased synthesis and release of renin or accumulation and entrapment of renin within the glomerulus (Johnston, 1993; Lush *et al*, 1993). The release of renin from the afferent arteriole is regulated by (a) intrarenal vascular receptors and the *macula densa* which is sensitive to sodium and/or chloride load, (b) sympathetic nerve stimulation via β receptors and (c) humoral agents such as vasopressin, angiotensin II and prostaglandins (Stella and Zanchetti, 1987; Samani *et al*, 1989; Stahl *et al*, 1984a; Nath *et al*, 1986).

Whether heightened glomerular thromboxane and renin levels in Lewis rats are (i) independent or related events and (ii) mirror systemic activation of both the renin-angiotensin and cyclooxygenase systems remains unclear.

Summary

Of the three glomerular prostanoid metabolites measured - bicyclic PGE_2, 6-keto $PGF_{1\alpha}$ and TxB_2 - only thromboxane levels were raised in Lewis relative to DA rats and irrespective of diet. Proteinuria was lower in both Lewis and DA rats in all the PUFA diet groups and glomerular renin levels *in vitro* were also raised in Lewis compared to DA rats. Despite the significant differences between the strains, changes in glomerular TxB_2 and renin levels *in vitro* did not correlate with the fall in urine protein levels in the PUFA diet groups. One must remain sceptical, however, about the fall in protein excretion in the PUFA diet groups in light of the high degree of intraindividual variation in urine protein measurements, the small number of animals in each diet group and the relative infrequency of urine protein measurements.

Although the significance of raised glomerular thromboxane and renin levels in Lewis rats is unclear, such findings may relate to glomerular hypertension or heightened platelet activity which contribute to impaired glomerular permeability.

Differences in the glomerular thromboxane B_2 levels and renin activity *in vitro* between Lewis and DA rats have not been reported previously.

2.2.1 Introduction and experimental design

The previous study left many questions unanswered. Was the tendancy for protein excretion to be lower in both Lewis and DA rats fed PUFA diets a genuine anti-proteinuric effect of PUFA diets? Were the differences in glomerular thromboxane and renin levels between the two strains of rat authentic, and if they were, was the effect confined to the glomerulus or were circulating levels of these two potent vasoconstrictors also raised? Did the heightened levels of thromboxane and renin correlate with urine protein levels and were the levels of thromboxane and renin affected by the PUFA diets also? Notwithstanding the small number of rats in each diet group, it was decided that the potential protein-lowering effects of PUFA diets in the previous study could not be ignored and should be investigated further.

As discussed in section 1.1.2, a fall in urine protein levels is significant in view of the following facts:

- proteinuria is the clinical hallmark of impaired glomerular permselectivity
- very low levels of albumin excretion (microalbuminuria) is both an early predictor of the development of nephropathy in diabetic patients and an established cardiovascular risk factor reflecting a generalised increase in systemic vascular permeability (Deckert *et al*, 1989). Microalbuminuria has been associated with early age at onset and long duration of diabetes, poor metabolic control, arterial hypertension and a family history of hypertension, peripheral neuropathy, retinopathy, a high protein or fat intake and abnormal plasma lipoproteins or coagulation factor levels in IDDM patients (see section 2.5.1).
- proteinuria may reflect increased protein flux both across the glomerular capillary wall and through the mesangium which are critical factors in the initiation and persistence of sclerosis of the glomerular tuft.

In the previous study (2.1), total protein excretion was measured, but albumin excretion is a more appropriate index of glomerular permselectivity because unlike many other proteins, albumin is not secreted by the renal tubules and being negatively charged at physiological pH, it appears in the urine in increasing amounts when the glomerular polyanion is diminished. Both total protein and albumin excretion were measured in this study.

Urine albumin levels are best measured by immunological methods such as immunoturbidimetry, radioimmunoassay or ELISA which are more sensitive and specific than dye-binding and protein precipitation methods. It is important also to define the type of urine sample used for albumin estimation, such as short daytime, timed overnight or twenty four hour collection, and levels should be expressed as either a concentration, albumin excretion rate (AER) or an albumin to creatinine ratio which takes account of renal function. Each type of urine sample has inherent advantages and disadvantages: daytime samples may give higher results than an overnight sample (due to the effects of exercise) but the more accurate 24 hour urine collection is less convenient and therefore often incomplete, particularly in clinical practice. Ideally, urine should be stored refrigerated at 4°C for up to two weeks without added preservative (sodium azide at 0.03 mol/l, if necessary) or frozen at -20°C and after thawing, brought to room temperature and mixed thoroughly before assay.

The upper limits of the reference range of albumin excretion in healthy adults is some 20-30 mg per 24 hrs which is equivalent to 14-18 µgs ug per min in overnight sample or 2-3 mg per mmol creatinine. Microalbuminuria has been defined as a urinary albumin excretion rate of 20-200 µgs per minute in an overnight or 24 hour sample (equivalent to 30-300 mgs per 24 hours or 3-30 mgs per mmol creatinine) on at least two of three occasions within a period of six months (Mogensen, 1987). The predictive or threshold levels of urine albumin above which the risk of nephropathy increases can vary from 15 µgs per minute (Mogensen *et al*, 1984) to 30 µgs per minute (Viberti *et al*, 1982) to 70 µgs per minute (Mathieson *et al*, 1984) amongst different centres. Acute diuresis, sex, age and body mass can affect urine albumin excretion although the necessity for reference ranges related to such parameters remains controversial (Rowe *et al*, 1990). There are, however, substantial problems with intraindividual variation of perhaps some 40% even in normal urine albumin levels, which are related to exercise, posture and other diurnal variations (Hutchison and O'Reilly, 1985; Mogenesen, 1987; Johnston *et al*, 1993).

Because of the observed similarities between glomerular thromboxane and renin patterns, it was decided to examine glomerular and circulating renin levels. Renin converts angiotensinogen to angiotensin I (AI) which is then converted by angiotensin converting enzyme (ACE) to angiotensin II (AII) primarily in the lungs, but also in the circulation. Renin is secreted by specialised myoepithelial cells in the wall of the afferent arteriole in response to either a fall in arterial blood pressure, renal perfusion or salt-load presented to the distal tubule, the latter being detected by the macula densa (Laragh, 1981).

2.2.2 Materials and methods

The objectives of this second study were to (i) clarify whether the previously observed fall in proteinuria in Lewis and DA rats fed PUFA diets was genuine and therefore reproducible and (ii) explore the possibility of differences in glomerular and circulating thromboxane B_2 and renin levels between the two rat strains. Although the data from the previous study does suggest that PUFA diet-induced changes in TxB_2 and renin levels are not related to the fall in proteinuria, the results were inconclusive due, in part, to the small number of rats in each experimental diet group. The only feasible way to repeat the study with more rats in each diet group, was to omit one of the PUFA diets and so double the number of animals in the remaining groups. Although the fatty acid content of safflower oil and evening primrose oil are similar, EPO contains both linoleic acid and γ-linolenic acid (GLA) which are precursors of 2- and 1- series prostanoids, respectively, whereas safflower oil contains linoleic acid but no GLA. It was decided also, that since there had been no marked differences or changes in glomerular PGE_2 and PGI_2 levels in any of the PUFA diet groups (except the fish oil diet groups) that the levels of these two prostanoids would not be measured again in this study.
As outlined in Figure 2.2.2.1, the experimental design of this study is similar to that of the previous one, but with some minor refinements to address the issue of whether the heightened thromboxane and renin production is confined to the glomerulus.

Four groups of 10 Lewis rats (n = 40) and four groups of 10 DA rats (n = 40) ranging in weight between 150 and 190 grams were fed either control, evening primrose oil, fish or olive oil diet for six weeks (see section 2.1.2.1). As before, body weight was recorded weekly, but in this study, the rats were put into metabolic cages at three and six weeks to measure food consumption and collect urine. Urine total protein excretion was measured by the Coomassie Blue method (see section 2.1.2.9) and albumin excretion rate was measured in 24 hour urine volumes by immunoturbidimetry.

Week	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12
	Order	Rest	Start Control diet												
			Order	Rest	Start Primrose diet										
					Order	Rest	Start Fish diet								
						Order	Rest	Start Olive diet							
Metabolic cage						Control 3 weeks		Primrose 3 weeks	Control 6 weeks	Fish 3 weeks	Primrose 6 weeks Olive 3 weeks		Fish 6 weeks	Olive 6 weeks	
										Cull Control rats		**Cull Primrose rats**		**Cull Fish rats**	**Cull Olive rats**

Table 2.2.2.1 Outline of study design

2.2.2.1 Immunoturbidimetry

Immunoturbidimetry is based upon the principle of light absorption by antigen-antibody complexes and is suited to rapid analysis of large numbers of samples. However, the method is susceptible to non-specific light absorption or scattering from the sample but this is corrected for by blank or "initial reaction" readings. Polyethylene glycol (PEG) in the reaction mixture accelerates immunoprecipitation and the antibody must be present in excess if Beers law - which states that the amount of light absorbed or the probability of absorption is proportional to the number of molecules in the light beam - is to be obeyed. (An excess of antigen can give erroneously low results due to inadequate cross-linkage and precipitation). Turbidity is measured photometrically at 405 nm. Antibody-antigen binding may be influenced by the sample matrix which can vary markedly in urine. Highly coloured salts in the urine of normal rat are reputed to interfere with turbidimetric methods and so all urines were centrifuged at 1000 g for 10 minutes before being de-salted in a Sephadex G-25M (PD-10) ion-exchange column (Pharmacia Fine Chemicals). Samples were eluted from the column with 0.9% NaCl and thus subject to a dilution factor of 1.75. Checks were carried out to ensure that de-salting did not alter urine protein levels to any great extent.

Polyethylene glycol	5.5% polyethylene glycol 6000 (BDH Limited, Poole, England) in PBS
Rat serum albumin	Rat serum albumin (RSA) (Sigma Chemical Company: A-6416) at 5 mg per ml in PBS.
Antiserum	Goat anti-rat albumin antiserum (Cappel laboratories: Cat no 0213-0341) diluted 1: 2 with PBS. (1 ml of diluted antiserum is sufficient for 20 cuvettes)

All reagents were brought to room temperature. Duplicate standards were prepared as in the following table.

Concentration (mg/100mls)	RSA (5mg/ml) µls	Final volume mls
15	75	2.5
10	50	2.5
8	40	2.5
5	25	2.5
3	15	2.5
2	20	5.0
1	1:1 diln above	5.0
0.5	1:1 diln above	5.0
0.25	1:1 diln above	5.0

Duplicate samples of 50 µls of either RSA standard or urine were pipetted into plastic microcuvettes (1.6 mls; Sarstedt). 500 µls of PEG 6000 was added to each cuvette which was then sealed and vortex-mixed. A photometer (Clinical System 100; Beckman) fitted with a 405 nm filter was zeroed on air and an initial absorbance reading (I) was recorded. 50 µls of diluted antiserum was added to all the cuvettes which were re-sealed, vortex-mixed and incubated at 37°C for 30 minutes. After incubation, the cuvettes were vortex-mixed again and a second absorbance (II) reading was recorded. An absorbance curve was constructed by subtracting absorbance reading (I) from absorbance reading (II) and the adjusted absorbance values were read against albumin concentration.

2.2.2.2 Urine creatinine measurement

Urine creatinine was measured using a Beckman creatinine analyser.

2.2.2.3 Blood sampling procedure

At the end of six weeks, plasma and serum samples were prepared from blood withdrawn from each rat by cardiac puncture using a 25 guage needle.

(i) To measure serum thromboxane (TxB_2) levels, one ml of blood was transferred to an eppendorff tube and placed immediately in a water bath at 37°C to clot for one hour, which is an established time that dampens the effects of an initial burst of thromboxane from platelets by allowing them to equilibrate (McLaren, 1986). After one hour exactly, samples were removed from the water bath, mixed gently and placed on ice.

(ii) Plasma was prepared by withdrawing two mls of blood directly into a syringe containing 0.5 mls of prostanoid anticoagulant comprising 1 ml of 1% indomethacin (in absolute alcohol) and 1 ml of 2.7 mg per ml adenosine (Sigma Chemical

Company: A-925) added to 98 mls of 3.2% sodium citrate. Indomethacin inhibits cyclooxygenase activity and adenosine suppresses platelet activity.
Blood samples were centrifuged at 1000g at 4°C for 15 minutes and stored at -70°C, which is the optimal storage temperature for biological samples awaiting prostanoid measurements.

2.2.2.4 Glomerular sieving

The kidneys were removed and cortical tissue sieved to recover the glomeruli as detailed in section 2.1.2.5.

2.2.2.5 Thromboxane measurement

Glomerular supernatant and serum thromboxane levels were measured by radioimmunoassay as detailed in section 2.1.2.8. Serum samples were diluted 1: 4 with 0.9% sodium chloride prior to assay.

2.2.2.6 Measurement of renin

Renin assays are classified according to whether they measure plasma renin *activity* (PRA) or plasma renin *concentration* (PRC) - two distinct parameters related to the levels of circulating angiotensinogen (renin substrate) which may be rate-limiting and thus prevent attainment of maximal velocity. Plasma renin concentration is related to the generation of AI from plasma renin incubated with exogenous (or added) substrate whereas plasma renin activity is a measure of AI generated from *endogenous* substrate. Renin exists in two forms: active and inactive (prorenin). The assay method used in this study, measured the concentration of *active* renin: to measure *total* renin concentration, inactive renin is converted to its active form by acid or trypsin activation (Leckie *et al*, 1977).

In the previous study (section 2.1.3.5), renin levels were measured by staff in the laboratory of Dr B Leckie at the Blood Pressure Unit, Western Infirmary, Glasgow but in this study, their role was solely that of providing patient supervision and the reagents and buffers required for the assay. Glomerular supernatant and plasma samples from Lewis and DA rats fed control or PUFA diets were assayed.
The principles behind this renin radioimmunoassay are complex. Renin levels are measured according to a modification of the radioimmunoassay method of Miller *et al* (1980) by measuring angiotensin I generated during incubation of either rat glomerular supernatant or rat plasma (as the source of renin) with nephrectomised rat plasma (as the source of angiotensinogen). An antibody to AI is used to protect it

from hydrolysis by endogenous angiotensinases. The concentration of angiotensinogen (exogenous nephrectomised rat plasma) in the reaction mixture is sufficient to guarantee substrate excess or zero-order kinetics and thus ensures that the reaction velocity is proportional to renin concentration.

Buffers and reagents

Incubation buffer	*Tris buffer (pH 6.9): 3M Tris HCl / 0.005M EDTA* 182g Tris HCl and 37g EDTA dissolved in distilled water plus 300 mls of 5N HCl. pH adjusted to 6.9 using [90.8g Tris and 18.61g EDTA in 250 mls distilled water] to a final volume of 500 mls.
Radioimmunoassay buffer	*Tris buffer (pH 7.4): 50 mM Tris HCl / 5mM EDTA / 0.2% (w/v) neomycin sulphate / 0.5% (w/v) BSA* 24.2g Tris HCl and 7.4g disodium EDTA dissolved in 3.5 litres of distilled water and 15 mls 5N HCl, 8g neomycin sulphate and 20g bovine serum albumin added. pH adjusted to 7.4 with 5N HCl and volume made up to 4 litres with distilled water.
Modified (RIA) buffer	as above plus 7% human serum albumin
Charcoal suspension	1.25g Dextran T70 dissolved in 10 to 50 mls of Tris buffer (pH 7.4) was added to 63 g charcoal and volume made up to 1000 mls.
Dilution buffer	*Tris buffer (pH 7.5): 0.05M Tris HCl / HSA / neomycin sulphate* To dilute AI: ≈ 500 mls 0.05M HCl, 1.75 g HSA and 3.5 g neomycin dissolved in 1 litre of prepared Tris HCl (6.057 g/l) and pH adjusted to 7.5 with 0.05M HCl.

Procedures for the preparation of (i) Ile^5-angiotensin I as RIA standard and antigen for antibody production, (ii) radiolabelled ^{125}I-angiotensin I, (iii) renin standards (iv) renin substrate (angiotensinogen) derived from nephrectomised rat plasma and (v) Rat Tris-Ab-Substrate mixture are detailed in the paper of Miller *et al* (1980).

Method

All stages of the assay were performed on a refrigerated (4°C) tray. Standards, samples and pre-mixed Rat Tris-Ab-Substrate mix were brought to room temperature and placed on the cold tray as soon as thawed. Tubes (LP4: Luckham Ltd) were numbered in duplicate for both preparation of a standard curve and measurement of renin concentration in glomerular supernatant and plasma samples.
A standard curve was constructed as follows: 50 µls of modified RIA buffer (+ 7% HSA) was pipetted into the first tube and 35 µls into the remainder.

Tube no	1	2	3	4	5	6	7	8	9
pg AI	1000	500	250	125	64	32	16	8	0

20 µls of angiotensin I (AI) standard at 100 ng per ml was added to the first tube and the contents were mixed: 35 µls were then removed, added to the second tube and mixed. This procedure was repeated up to the second last tube then the 35 µls volume was discarded: the last tube containing 35 µls of buffer was used as a blank. 35 µls of Rat Tris-Ab-Substrate mix was then added to the standards, the reagents were mixed and 0.5 mls of ice-cold RIA buffer pH 7.4 added immediately. The tubes were stored at 4°C.

The next stage of the assay was to process the zero-time samples which are used to measure endogenous renin activation and the results of which will be subtracted from the test sample results in the standard incubation (15 minutes) assay. 35 µls of Rat Tris-Ab-Substrate mix was added to 5 µls of all test samples, the contents mixed and 0.5 mls of ice-cold RIA buffer, pH 7.4 added immediately to stop the reaction. The tubes were set aside at 4°C.

In the standard incubation assay, renin concentration in the test samples was measured by adding 35 µls of Rat Tris-Ab-Substrate mix to 5 µls of either glomerular supernatant or rat plasma. Glomerular supernatants from Lewis rats required dilution 1: 9 with Tris buffer (pH 6.9) but those from DA rats did not. The tubes were vortex-mixed and incubated in a water bath at 37°C for 15 minutes. The reaction was stopped by placing the tubes in crushed ice and diluting with 0.5 mls ice-cold RIA buffer (pH 7.4).

50 µls of radiolabelled (^{125}I) angiotensin I (AI) (5pg per 50 µls) was added to the standards, test sample and zero-time tubes which were then covered, shaken and stored at 4°C for 48 hours. Five empty tubes were included for normalising on counter.

The final stage of the assay is to separate the bound, from the free label (^{125}I) angiotensin I (total activity = 15.2 µCi). 200 µls of charcoal suspension was added to all but the five empty tubes and vortex-mixed. The tubes were then centrifuged at 2800 rpm at 4°C for 10 minutes, the supernatants siphoned off and the tubes counted in a gamma counter (Nuclear Enterprises NE 6162 Turbo) programmed to report the results in picograms of angiotensin I (amount bound). The final result is expressed as picograms of AI per ml of plasma per hour. Should the results fall within the lower part of the curve (ie below 25%) then the incubation time must be increased: alternatively, if they fall within the upper part (ie above 66% F) then incubation time may be decreased but not to less than 15 minutes. If the results are very high then samples should be diluted with Tris buffer (pH 6.9).

2.2.3 Results

2.2.3.1 Metabolic data

The growth rate curves shown in Figure 2.2.3.1 do not display the same initial fall as was seen in the previous study (see Figure 2.1.3.1a). At the end of six weeks, Lewis rats were significantly heavier than DA rats in all but the fish oil diet group [**Lewis vs DA:** CD: mean 235 (SD 24) vs 205 (11.5) grams, EPO: 250 (25) vs 200 (16), FO: 230 (23) vs 216 (10), OO: 274 (23) vs 206 (20): see table 2.2.3.1 for corresponding confidence intervals and significance levels]. The percentage increase in body weight in all diet groups over six weeks, was greater in this study than the previous one, presumably as a result of the lower starting body weights of the rats in this study [**Lewis**: CD: 56%, EPO: 35%, FO: 39%, OO: 58%; **DA**: CD: 37%, EPO: 20%, FO: 41%, OO: 32%] but the differences in percentage weight gain between control and PUFA diets in both strains of rat was not so marked in this study. Concern about weight loss in PUFA diet groups due to starvation appears to be unfounded: in DA rats, the increase in body weight was comparable between the fish oil (41%) and control (37%) diet groups and similarly in Lewis rats in the olive oil (58%) and control (56%) diet groups. At the end of six weeks, Lewis rats fed olive oil and DA rats fed fish oil diet were significantly heavier than their dietary controls (see Table 2.2.3.1).

As in the previous study, there was no generalised reduction in the consumption of PUFA diets compared to the control diet in either strain. Only Lewis rats fed fish oil and DA rats fed fish or olive oil diets consumed significantly less food than their respective controls [**Lewis**: CD: mean 17 (SD 3.6) grams vs (i) EPO: 17 (2.3), (ii) FO: 13.5 (2.4), (iii) OO: 16 (3); **DA**: CD: 15.2 (1.5) grams vs (i) EPO: 14 (1.6), (ii) FO: 13 (1.1), (iii) OO: 11.3 (2.1): see table 2.2.3.2(a) for corresponding confidence intervals and significance levels]. Mean food consumption differed between the strains only in the evening primrose oil diet and olive oil diet groups (Table 2.2.3.2a).

At six weeks, urine volume was significantly higher in Lewis than in DA rats in all the diet groups [**Lewis vs DA**: CD: mean 8.5 (SD 1.8) vs 6.2 (0.88) mls per day, EPO: 10.2 (4.1) vs 6.9 (2.1), FO: 9.25 (4.3) vs 6 (1.1), OO: 7.7 (1.4) vs 5.6 (1.4): see table 2.2.3.3(a) for corresponding confidence intervals and significance levels] but PUFA diets had no effect on urine volume in either strain (Table 2.2.3.3).

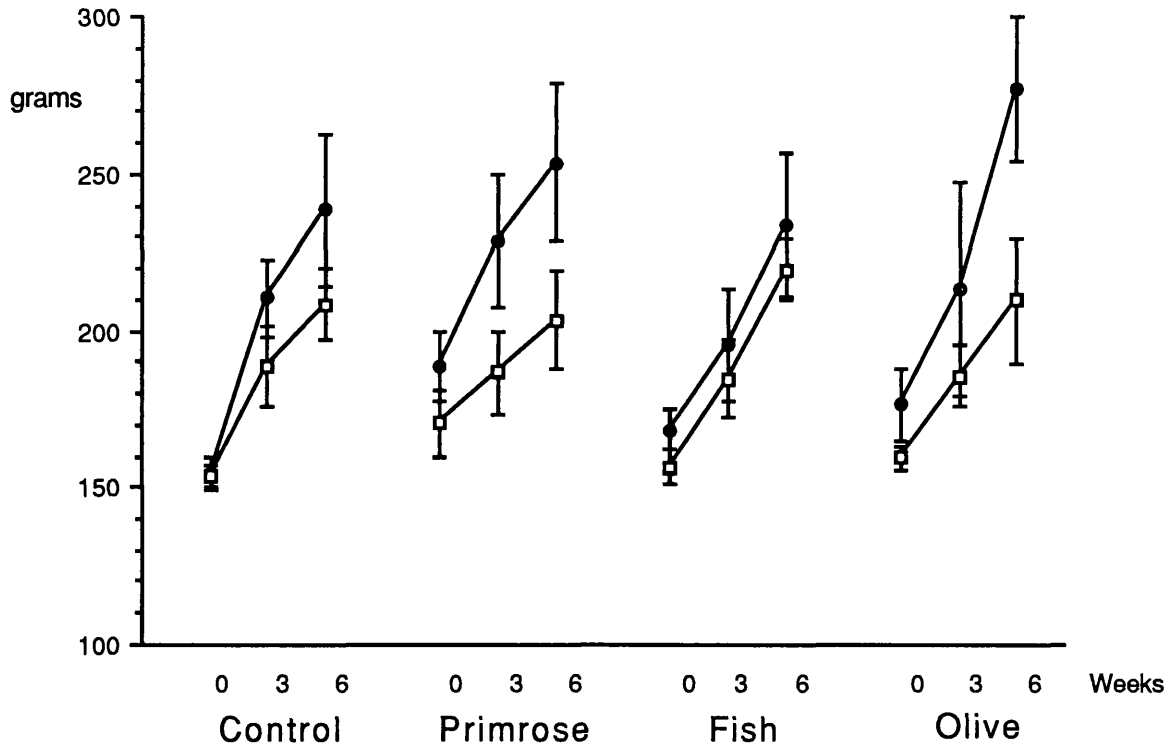


Figure 2.2.3.1 Growth rate curves of Lewis (●) and DA (❐) rats in control and PUFA diet groups

Table 2.2.3.1 Body weight at six weeks - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			11 to 48	p= 0.004*
Primrose			30 to 71	p= 0.0001*
Fish			-3 to 31	p= 0.099
Olive			48 to 89	p< 0.0001*
Lewis rats				
Control	vs	Primrose	-38 to 8	p= 0.18
Control	vs	Fish	-17 to 27	p= 0.64
Control	vs	Olive	8 to 50	p= 0.009*
DA rats				
Control	vs	Primrose	-7 to 19	p= 0.37
Control	vs	Fish	-20 to -0.4	p= 0.042*
Control	vs	Olive	-16 to 15	p= 0.94

Twosample *t* test

	Control		Primrose		Fish		Olive	
Week	**3**	**6**	**3**	**6**	**3**	**6**	**3**	**6**
Lewis rats	18	15	20	21	17	18	15	13
	20	18	16	16	16	15	13	13
	22	18	19	15	16	13	19	19
	20	20	20	20	20	11	31	17
	17	17	19	15	21	15	19	19
	20	18	20	15	16	14	25	17
	21	21	17	16	18	15	22	15
	19	8	19	15	22	10	18	15
	19	18	20	19	16	11	12	21
	22	16	17	17	16	15	29	12
mean (grams)	**20**	**17**	**19**	**17**	**18**	**13.5**	**20.5**	**16**
SD	1.6	3.6	1.5	2.3	2.3	2.4	6.45	3
DA rats	15	14	12	12	14	13	14	10
	15	14	14	16	15	14	18	8
	16	15	11	15	16	15	15	10
	17	17	13	15	19	13	16	12
	17	17	11	15	15	13	13	9
	10	13	15	14	15	13	17	14
	15	17	12	15	15	13	17	12
	19	15	12	11	15	11	21	13
	8	14	12	13	16	12	13	14
	15	16	15	14	16	12	14	11
mean (grams)	**14.5**	**15**	**12.5**	**14**	**15.5**	**13**	**16**	**11**
SD	3.3	1.5	1.5	1.6	1.35	1.1	2.5	2

Table 2.2.3.2 Mean daily food consumption at three and six weeks

Table 2.2.3.2a Daily food consumption at six weeks - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-1 to 4.4	p= 0.19
Primrose			1 to 4.8	p= 0.005*
Fish			-1 to 2.6	p= 0.42
Olive			2.3 to 7.3	p= 0.0008*
Lewis rats				
Control	vs	Primrose	-3 to 3	p= 1
Control	vs	Fish	0.4 to 6.2	p= 0.03*
Control	vs	Olive	-2 to 4	p= 0.59
DA rats				
Control	vs	Primrose	-0.2 to 2.6	p= 0.096
Control	vs	Fish	1 to 3.5	p= 0.001*
Control	vs	Olive	2.2 to 5.6	p= 0.0002*

Twosample *t* test

	Control		Primrose		Fish		Olive	
Week	**3**	**6**	**3**	**6**	**3**	**6**	**3**	**6**
Lewis rats	9	11	21	15	17	13	10	10
	9	8.5	12	10	14	14.5	8	8
	9	9	10	6	15	6.5	11	5.5
	9	9	14	9	14	4	9.5	7
	8	8.5	14	7	15	16	7.5	9
	9	8.5	20	14	16	8.5	5.5	6
	9	8	12	7	17	6	6	7.5
	10	4	19	18	10	7	9	7
	11	10	9	7	18	5	8	8
	11	8	11	8.5	22	12	10.5	9
mean (mls)	**9.5**	**8.5**	**14**	**10**	**16**	**9.5**	**8.5**	**7.5**
SD	0.9	1.8	4.3	4.1	3.1	4.3	1.8	1.4
DA rats	7	6.5	6	5	9	6	5	5
	7	5	10	9	7	4	11	3
	6	8	6	10	8	7	6	3.5
	8	6	4.5	4.5	11	7	5.5	7.5
	6	7	7	7	9	5.5	5.5	7
	3	5	6	8	10	7	6	6
	7	6	8	5	7.5	5	7.5	5
	9	6.5	6	4	10	6	7.5	6.5
	3	6	5.5	8	8	5	4	6
	7	6	7	8	10	7.5	7	6
mean (mls)	**6.5**	**6**	**6.5**	**7**	**9**	**6**	**6.5**	**5.5**
SD	1.9	0.9	1.5	2.1	1.3	1.1	1.9	1.4

Table 2.2.3.3 Mean daily urine volume at three and six weeks

Table 2.2.3.3(a) Mean daily urine volume at six weeks - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			0.8 to 3.65	p= 0.004*
Primrose			0.2 to 6.45	p= 0.04*
Fish			0.1 to 6.4	p= 0.04*
Olive			0.8 to 3.5	p= 0.0035*
Lewis rats				
Control	vs	Primrose	-4.8 to 1.4	p= 0.25
Control	vs	Fish	-4 to 2.4	p= 0.6
Control	vs	Olive	-0.8 to 2.3	p= 0.32
DA rats				
Control	vs	Primrose	-2.2 to 0.9	p= 0.38
Control	vs	Fish	-0.76 to 1.2	p= 0.67
Control	vs	Olive	-0.5 to 1.8	p=0.25

Twosample *t* test

Urine creatinine excretion was significantly higher in Lewis than DA rats only in the control and evening primrose oil diet groups [**Lewis vs DA**: CD: median 67 (range 50 - 73) vs 49.5 (41 - 59) mmols per 24 hrs, EPO: 59.5 (45 - 83) vs 47 (36 - 52) mmols per 24 hrs, FO: 50 (32 - 69) vs 48 (27 - 55) mmols per 24 hrs, OO: 65 (46 - 102) vs 55 (44 - 68) mmols per 24 hrs] (Figure 2.2.3.4). Only in the Lewis rat group fed fish oil diet was urine creatinine significantly reduced relative to the control diet group [CD: 67 (50 - 73) vs FO: 50 (32 - 69) mmols per 24 hrs: see table 2.2.3.4 for corresponding confidence intervals and significance levels].

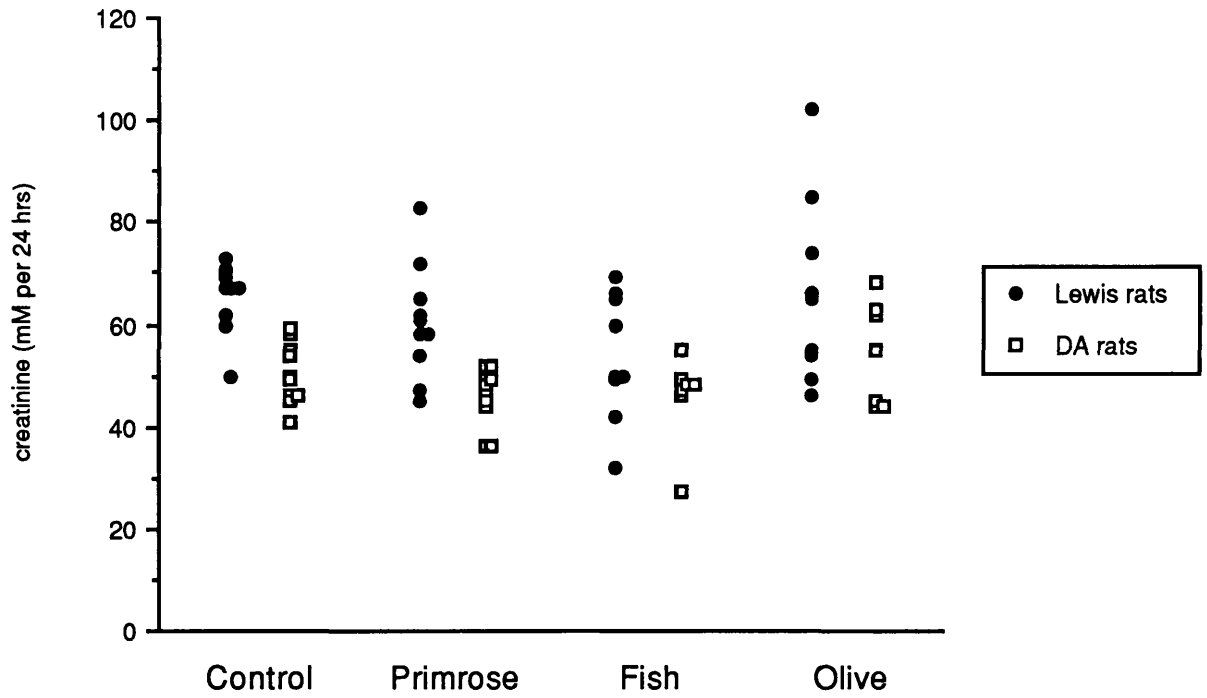


Figure 2.2.3.4 Urine creatinine levels in Lewis and DA rats at six weeks

Table 2.2.3.4 Urine creatinine levels - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			9 to 22	p= 0.0007*
Primrose			6 to 23	p= 0.004*
Fish			-5 to 19	p= 0.08
Olive			-7 to 30	p= 0.15
Lewis rats				
Control	vs	Primrose	-3 to 13	p= 0.13
Control	vs	Fish	1 to 21	p= 0.016*
Control	vs	Olive	-15 to 15	p= 0.6
DA rats				
Control	vs	Primrose	-2 to 10	p= 0.15
Control	vs	Fish	-3 to 10	p= 0.45
Control	vs	Olive	-14 to 5	p= 0.6

Mann Whitney Confidence Interval and Test

2.2.3.2 Glomerular recovery

The protein content of pellets of glomeruli recovered from Lewis and DA rat kidneys is shown in Table 2.2.3.5.

2.2.3.3 Urine total protein excretion

Proteinuria was significantly higher in Lewis than DA rats only in the control and evening primrose oil diet groups [CD: 10 (7.7 - 15.6) vs 5.95 (4.6 - 7.1) mgs per 24 hours, EPO: 10.7 (6.9 - 15) vs 7.1 (5.6 - 10.9) mgs per 24 hours, FO: 6.08 (4.8 - 11.5) vs 6.245 (3.7 to 11.4), OO: 7.6 (5.6 - 8.4) vs 6.4 (3.5 vs 7.7); see table 2.2.3.6 for corresponding confidence intervals and significance levels].

In contrast to the previous study, urine protein excretion was not reduced by all the PUFA diets in both strains of rat. Proteinuria was lowered significantly only in Lewis rats fed fish or olive oil diets [CD: 10 (7.7 - 15.6) vs (i) FO: 6.08 (4.8 - 11.5) mgs per 24 hours, (ii) OO: 7.6 (5.6 - 8.4) mgs per 24 hours; Figure 2.2.3.6). In the evening primrose oil diet group, proteinuria was not lowered in Lewis rats but there was a slight and statistically significant *increase* in proteinuria in DA rats [CD: 5.95 (4.6 - 7.1) vs EPO: 7.1 (5.6 - 10.9) mgs per 24 hours, $p = 0.015$]. Neither fish oil nor olive oil diets had any effect on protein excretion in DA rats [CD: 5.95 (4.6 - 7.1) mgs per 24 hours vs (i) FO: 6.245 (3.7 - 11.4) mgs per 24 hours, (ii) OO: 6.4 (3.5 - 7.7) mgs per 24 hours; see table 2.2.3.6 for corresponding confidence intervals and significance levels].

	Control		Primrose		Fish		Olive	
	Lewis	DA	Lewis	DA	Lewis	DA	Lewis	DA
1	1.75	0.95	4	2.1	1.9	2.4	3.3	3.7
2	2.5	2.15	3.3	3.05	1.9	3.1	3.1	2.5
3	2.25	2.0	3.0	2.6	3.05	3.25	3.33	2.5
4	2.65	1.55	3.25	3.05	3.35	3.20	3.25	3.55
5	1.95	1.55	2.9	2.25	3.2	3.75	3.05	2.6
6	2.15	1.75	2.9	1.95	2.45	3.6	3.25	2.6
7	2.9	1.7	2.9	2.45	3.1	3.6	3.4	2.4
8	2.4	1.45	2.15	1.4	2.85	3.2	2.65	3.2
9	2.15	2.15	3.55	2.15	2.55	3.6	3.05	2.65
10	2.1	1.9	2.65	2.15	3.1	2.75	2.6	2.6

Results expressed in milligrams

Table 2.2.3.5 Protein content of Lewis and DA rat glomerular pellets

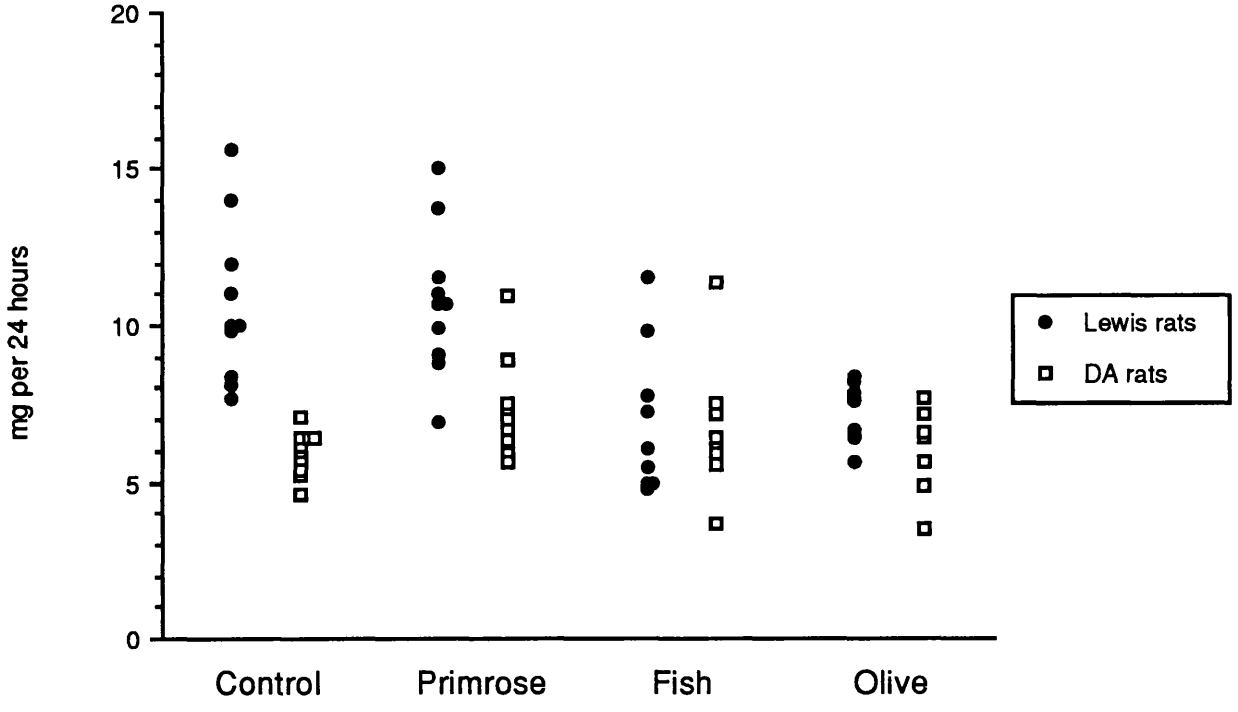


Figure 2.2.3.6 Total protein excretion in Lewis and DA rats at six weeks

Table 2.2.3.6 Total protein excretion at six weeks - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			2.7 to 6.4	p= 0.0002*
Primrose			1.3 to 4.9	p= 0.006*
Fish			-1.6 to 2.4	p= 0.96
Olive			-0.1 to 2.8	p= 0.08
Lewis rats				
Control	vs	Primrose	-2.6 to 2.2	p= 0.87
Control	vs	Fish	1.2 to 6	p= 0.007*
Control	vs	Olive	1.6 to 5.5	p= 0.002*
DA rats				
Control	vs	Primrose	-2.6 to -0.3	p= 0.015*
Control	vs	Fish	-1.8 to 0.6	p= 0.37
Control	vs	Olive	-1.3 to 1.2	p= 0.6

Mann-Whitney Confidence Interval and Test

2.2.3.4 Urine albumin excretion

Urine albumin excretion data is displayed on a logarithmic scale in Figure 2.2.3.7 and shows that at six weeks, AER was higher in Lewis than DA rats in all the PUFA diet groups [**Lewis vs DA**: CD: median 5.8 (range 0.25 - 23) vs 0.22 (0.15 - 0.46) mgs per 24 hours, EPO: 0.190 (0.160 - 4.34) vs 0.09 (0.07 - 0.13) mgs per 24 hours, FO: 0.25 (0.22 - 0.3) vs 0.145 (0.08 - 0.39) mgs per 24 hours, OO: 0.160 (0.11 - 0.21) vs 0.125 (0.09 - 0.16) mgs per 24 hours] and albumin excretion was significantly reduced in all the PUFA diet groups in both strains of rat; see table 2.2.3.7 for corresponding confidence intervals and significance levels.

Changes in AER between weeks three and six are shown only for Lewis and DA rats fed control or evening primrose oil diet (Figures 2.2.3.7a and 2.2.3.7b). In the control diet group, median AER in Lewis rats tended to rise [2.6 (0.25 - 3.62) vs 5.89 (0.25 - 23) mgs per 24 hours, CI = -4.4 to -0.02, p = 0.039] but to fall in DA rats [0.285 (0.18 - 0.91) vs 0.22 (0.14 - 0.46), CI = -0.11 to -0.03, p = 0.02] (Figure 2.2.3.7a). In the evening primrose oil diet group, median AER rose slightly but significantly in Lewis rats between weeks 3 and 6 [0.160 (0.130 - 2.4) vs 0.190 (0.160 - 4.34), CI = 0.02 to 0.23, p = 0.004] but fell in DA rats during the same period [0.160 (0.11 - 0.43) vs 0.09 (0.07 - 0.130), CI = -0.14 to -0.05, p = 0.02] (see Figure 2.2.3.7b). Statistical comparisons were made between paired samples using the Wilcoxon paired rank test.

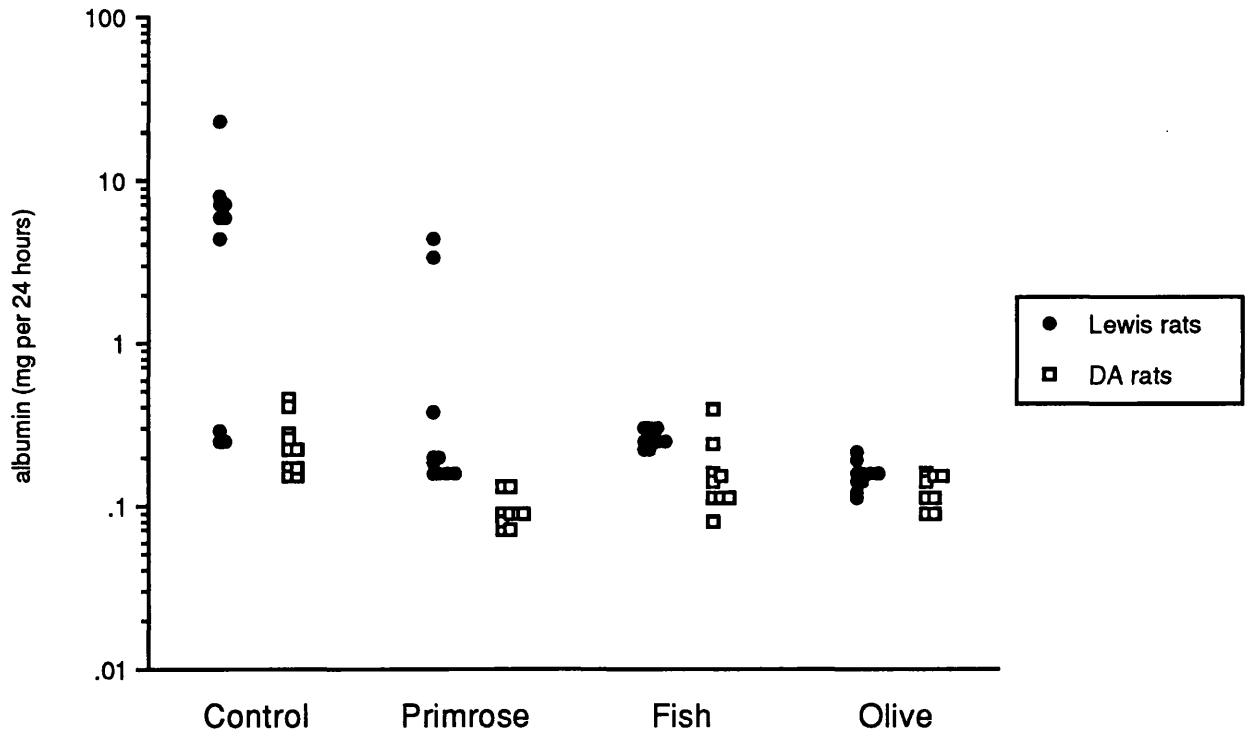


Figure 2.2.3.7 Albumin excretion rate in Lewis and DA rats at six weeks

Table 2.2.3.7 Albumin excretion rate at six weeks - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			0.1 to 6.9	p= 0.0028*
Primrose			0.07 to 0.29	p= 0.0002*
Fish			0.07 to 0.15	p= 0.006*
Olive			0.00001 to 0.06	p= 0.056
Lewis rats				
Control	vs	Primrose	0.09 to 6.9	p= 0.002*
Control	vs	Fish	0.029 to 6.8	p= 0.01*
Control	vs	Olive	0.12 to 6.9	p= 0.0002*
DA rats				
Control	vs	Primrose	0.08 to 0.2	p= 0.0002*
Control	vs	Fish	0.01 to 0.16	p= 0.017*
Control	vs	Olive	0.04 to 0.19	p= 0.0016*

Mann Whitney Confidence Interval and Test

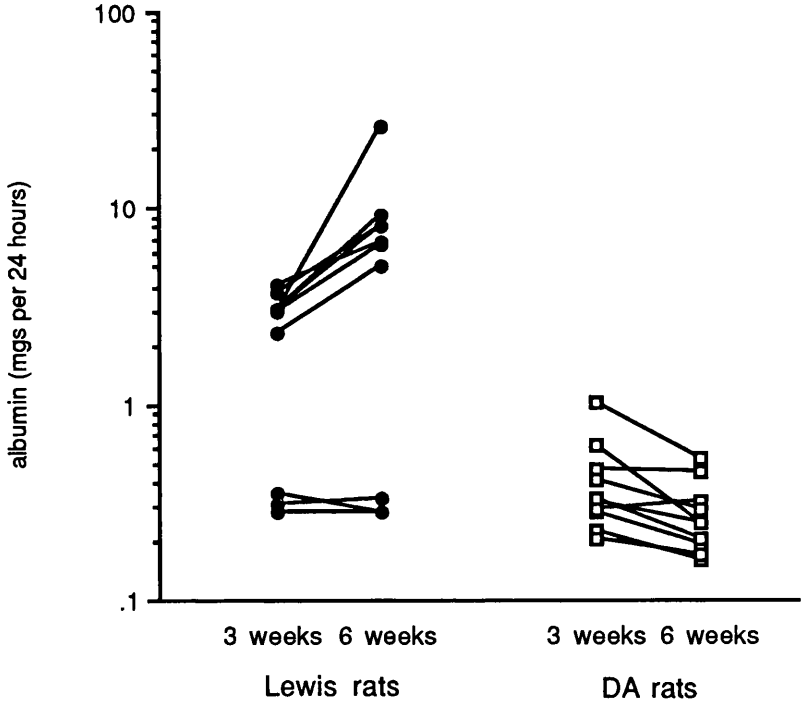


Figure 2.2.3.7(a) Albumin excretion rate after three and six weeks of control diet

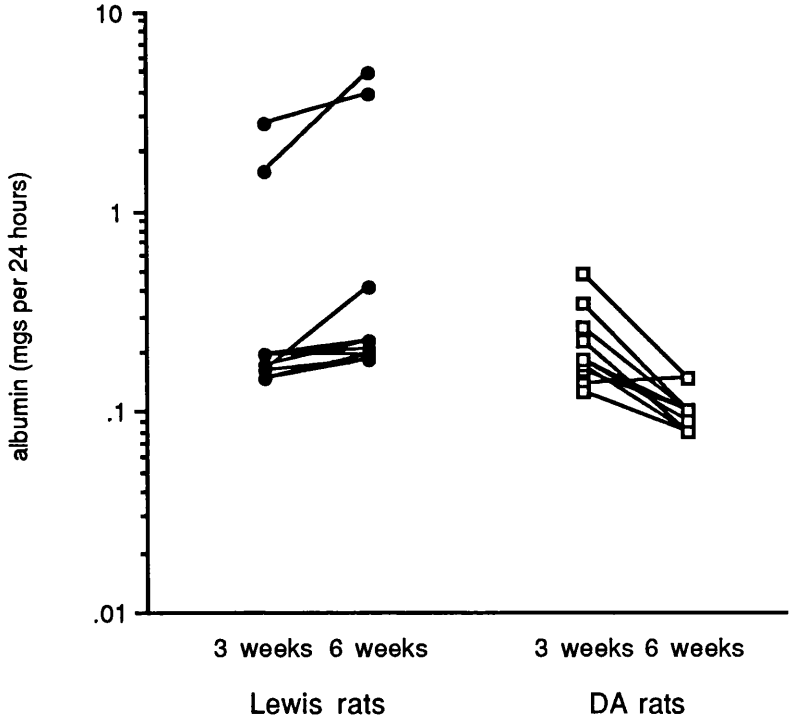


Figure 2.2.3.7(b) Albumin excretion rate after three and six weeks of evening primrose oil diet

2.2.3.5 Glomerular and serum thromboxane measurements

The standard binding curves from which glomerular supernatant and serum thromboxane B_2 levels were calculated are shown in Figures 2.2.3.8 and 2.2.3.8(a), respectively. In this study, glomerular TxB_2 levels were raised significantly in Lewis relative to DA rats only in the control and olive oil diet groups [**Lewis vs DA**: CD: median 1.063 (range 0.571 - 1.508) vs 0.223 (0.145 - 0.373) ng per mg glomerular protein, OO: 0.913 (0.485 - 1.202) vs 0.169 (0.108 - 0.616) ng per mg glomerular protein; see table 2.2.3.9 for corresponding confidence intervals and significance levels). There was no significant difference in glomerular TxB_2 levels betweenLewis and DA rats in the evening primrose oil and fish oil diet groups [**Lewis vs DA**: EPO: 0.489 (0.287 - 1.208) vs 0.549 (0.444 - 0.755) ng per mg glomerular protein, FO: 0.14 (0.108 - 0.231) vs 0.125 (0.08 - 0.200) ng per mg glomerular protein].

In the evening primrose oil diet group, glomerular TxB_2 levels were lowered in Lewis rats [CD: 1.063 (0.571 - 1.508) vs 0.489 (0.287 - 1.208) ng per mg glomerular protein] but raised in DA rats [CD: 0.223 (0.145 - 0.373) vs 0.549 (0.444 - 0.755) ng per mg glomerular protein] relative to their repective controls but in the fish oil group glomerular TxB_2 levels were significantly reduced in both Lewis [0.14 (0.108 - 0.231) ng per mg glomerular protein] and DA [0.125 (0.08 to 0.200) ng per mg glomerular protein] rats. Olive oil diet had no effect on glomerular TxB_2 levels in either Lewis or DA rats [Lew: 0.913 (0.485 - 1.202) vs DA: 0.169 (0.108 - 0.616) ng per mg glomerular protein (see Table 2.2.3.9 for corresponding confidence intervals and significance levels.

There were no marked differences in serum TxB_2 levels between the strains except in the fish oil group (Figure 2.2.3.10) [**Lewis vs DA:** CD: median 5.7 (range 4.2 - 9.3) vs 5.5 (4.8 - 6.3) ng per ml, EPO: 6.3 (6 - 7.8) vs 6.2 (5 - 9.2), FO: 3.5 (2.6 - 4.3) vs 4.1 (4 - 5), OO: 5 (4.5 - 7.7) vs 5.7 (4 - 7.3): see table 2.2.3.10 for corresponding confidence intervals and significance levels].

Serum TxB_2 levels were reduced significantly only in the fish oil group in both Lewis [5.7 (range 4.2 - 9.3) vs 3.5 (2.6 - 4.3 ng per ml); $p < 0.001$] and DA rats [5.5 (4.8 - 6.3) vs 4.1 (4 - 5) ng per ml; $p < 0.001$].

Table 2.2.3.10(a) shows actual levels of glomerular and serum TxB_2. Low serum TxB_2 values were not plotted.

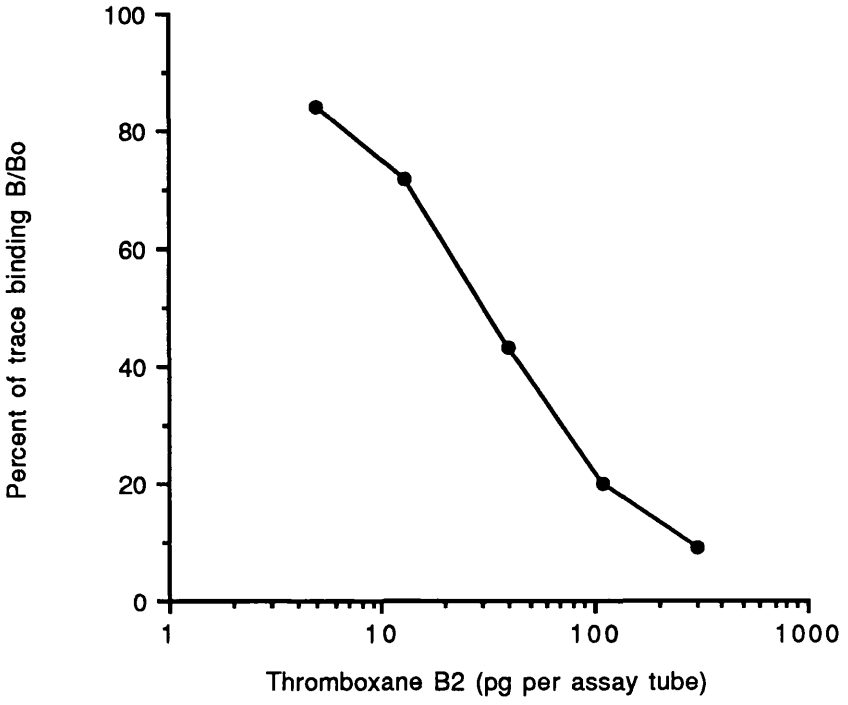


Figure 2.2.3.8 Standard TxB_2 binding curve - glomerular supernatant assay

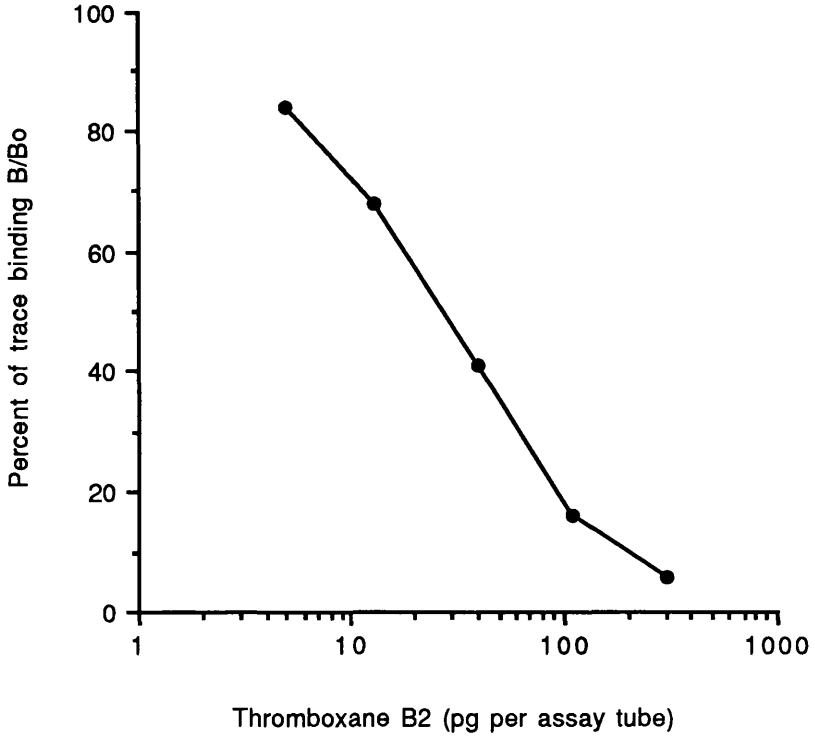


Figure 2.2.3.8a Standard TxB_2 binding curve - serum assay

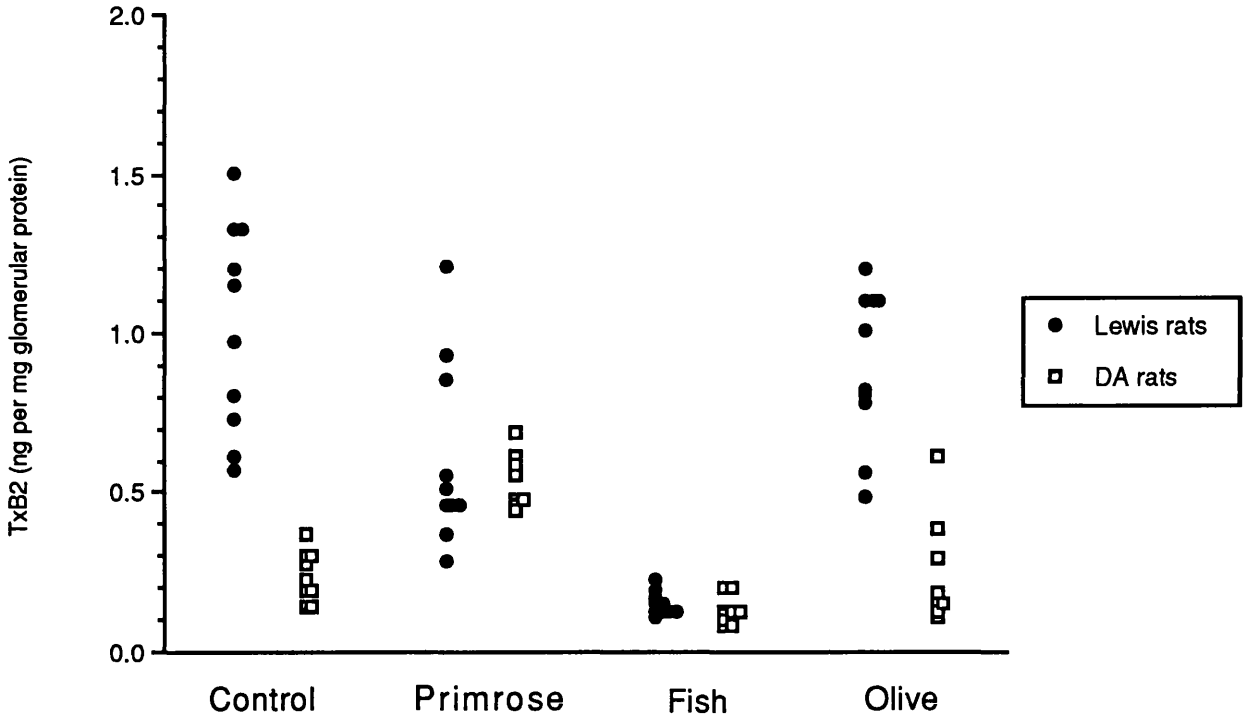


Figure 2.2.3.9 Glomerular thromboxane B_2 release *in vitro* in Lewis and DA rats at six weeks

Table 2.2.3.9 Glomerular TxB_2 release - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			0.47 to 1.05	p= 0.0002*
Primrose			-0.17 to 0.3	p= 0.76
Fish			-0.01 to 0.05	p= 0.20
Olive			0.43 to 0.9	p= 0.0003*
Lewis rats				
Control	vs	Primrose	0.11 to 0.78	p= 0.009*
Control	vs	Fish	0.57 to 1.18	p= 0.0002*
Control	vs	Olive	-0.2 to 0.40	p= 0.38
DA rats				
Control	vs	Primrose	-0.41 to -0.24	p= 0.0002*
Control	vs	Fish	0.03 to 0.17	p= 0.0028*
Control	vs	Olive	-0.14 to 0.11	p= 0.62

Mann Whitney Confidence Interval and Test

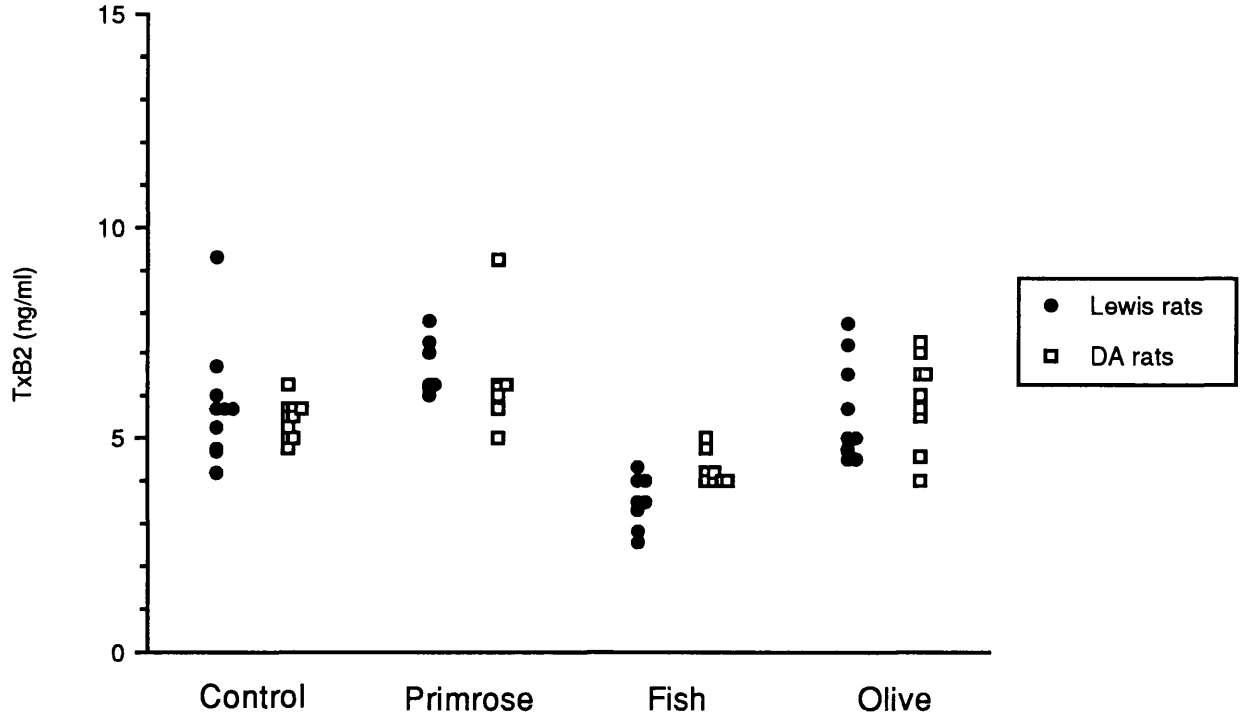


Figure 2.2.3.10 Serum thromboxane B_2 levels in Lewis and DA rats at six weeks

Table 2.2.3.10 Serum TxB_2 levels - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-0.6 to 1.4	p= 0.45
Primrose			-0.9 to 1.6	p= 0.6
Fish			-1.3 to -0.2	p= 0.008*
Olive			-1.3 to 0.9	p= 0.66
Lewis rats				
Control	vs	Primrose	-2 to 0.22	p= 0.15
Control	vs	Fish	1.2 to 3.4	p= 0.0006*
Control	vs	Olive	-0.9 to 1.5	p= 0.66
DA rats				
Control	vs	Primrose	-2.1 to 0.3	p= 0.053
Control	vs	Fish	0.75 to 1.6	p= 0.00002*
Control	vs	Olive	-1.1 to 0.5	p= 0.37

Twosample *t* test

	Control		Primrose		Fish		Olive	
	Glom	Serum	Glom	Serum	Glom	Serum	Glom	Serum
Lewis rats	30	400	145	420	7.5	5	48	290
	90	340	85	360	11	3	52	340
	78	250	42	440	15	200	80	280
	58	340	50	380	13	210	80	300
	36	290	25	370	12	210	110	300
	86	360	32	4	17	170	105	430
	70	320	40	24	10	240	80	270
	70	240	36	2.5	13	240	80	460
	86	280	50	470	11	260	100	270
	95	340	74	380	6.5	160	85	390
DA rats	4	300	34	380	9.4	1.5	12	390
	18	290	42	380	12	14	14	440
	13	340	54	13	12	300	22	240
	9	330	43	360	12	290	16	330
	7	300	30	550	9	250	30	0
	12	320	36	5	15	240	9	340
	19	340	43	4	8.4	240	30	420
	13	380	42	340	19	240	13	360
	19	330	36	370	11	250	12	275
	11	340	64	300	16	240	49	390

Table 2.2.3.10(a) Thromboxane B_2 levels (pg/tube) in glomerular supernatant and serum

2.2.3.6 Renin assay

Over the course of the study, six separate renin assays were performed: standard curve data is shown in Table 2.2.3.11 and illustrated in Figure 2.2.3.11. Renin levels in glomerular supernatants of Lewis and DA rats were derived from the difference between standard incubation (15 minutes) and zero-time values tabulated in Tables 2.2.3.11(a) and 2.2.3.11(b).

Figure 2.2.3.11(a) shows that, as in the previous study (section 2.1.3.5), glomerular renin levels in Lewis rats were significantly higher than those of DA rats in the control and all the PUFA diet groups [**Lewis vs DA:** CD: median 469 (range 145 - 911) vs 44 (12 - 80) pg AI per hour per mg glomerular protein, EPO: 1740 (327 - 3126) vs 155 (74 - 485), FO: 844 (427 - 9880) vs 265 (190 - 450), OO: 1056 (736 - 2424) vs 230 (118 - 556); see Table 2.2.3.11c for corresponding confidence intervals and significance levels]. However, in contrast to the previous study, glomerular renin levels were significantly higher in all the PUFA diet groups relative to controls (see Table 2.2.3.11c).

Plasma renin levels were derived from the difference between standard incubation (15 minutes) and zero-time values (Tables 2.2.3.11d and 2.2.3.11e). There were no statistically significant interstrain differences in plasma renin levels in any of the diet groups (Figure 2.2.3.11b) [**Lewis vs DA:** CD: median 149 (range 55 - 213) vs 230 (25 - 589) pg AI per hour, EPO: 102 (36 - 152) vs 125.5 (29 - 207), FO: 89.5 (62 - 252) vs 123.5 (30 - 220), OO: 134.5 (48 - 232) vs 135.5 (94 - 233); see Table 2.2.3.11f for corresponding confidence intervals and significance levels].

None of the PUFA diets had any marked effect on plasma renin levels in Lewis rats but in DA rats, plasma renin levels were lowered significantly in the evening primrose and fish oil diet groups (see Table 2.2.3.11f).

	1	2	3	4	5	6	CV%
pg AI							
8	74.93	75.37	74.84	81.71	69.95	75.53	4.96
16	73.87	75.49	73.70	80.73	67.79	74.95	5.6
32	72.17	72.16	70.50	78.73	62.93	72.30	7.08
64	68.07	68.58	64.79	76.30	61.69	67.69	7.2
125	58.76	58.16	54.47	70.49	55.13	58.70	9.76
250	46.52	46.55	41.39	58.82	45.16	46.23	12.45
500	32.49	31.57	27.53	42.66	27.02	33.73	17
1000	20.49	20.17	17.07	26.29	20.88	18.88	15

Results expressed as B/F%

Table 2.2.3.11 Binding curve data from six renin assays

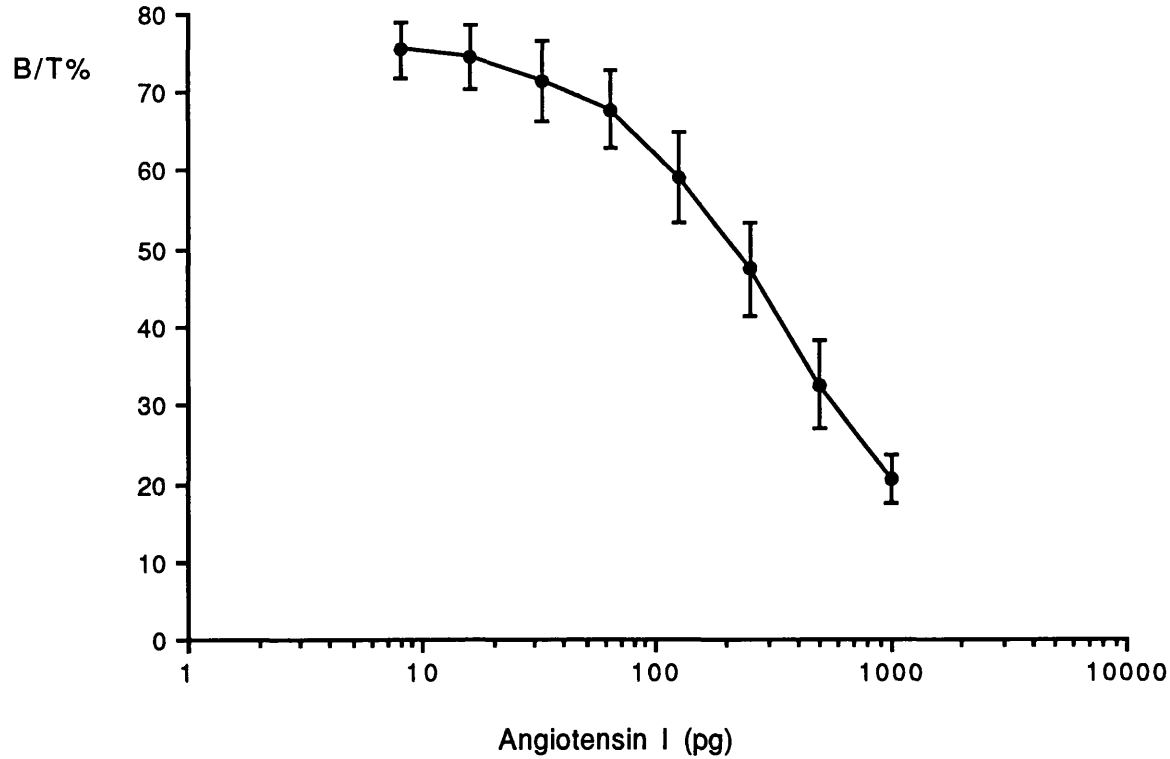


Figure 2.2.3.11 Renin standard binding curves - mean and SD of six assays.

	Control		Primrose		Fish		Olive	
	15"	0"	15"	0"	15"	0"	15"	0"
1	-	-	1691	11	435	0	742	6
2	145	0	2185	19	1021	2	1181	3
3	243	0	2183	12	1312	3	2443	19
4	492	23	1406	39	1915	3	1930	18
5	520	35	1156	2	533	0	1318	2
6	513	0	1968	169	11625	1745	1167	191
7	800	162	3661	535	872	-	905	18
8	264	35	3281	553	999	182	954	-
9	193	-	822	495	427	-	1225	257
10	917	8	880	106	600	27	1136	-

Results expressed in pg AI per unit time

Table 2.2.3.11a Renin concentration in Lewis rat glomerular supernatants at fifteen minutes and zero time.

	Control		Primrose		Fish		Olive	
	15"	0"	15"	0"	15"	0"	15"	0"
1	-	-	100	0	190	0	370	30
2	42	0	160	35	490	62	230	0
3	56	0	300	50	520	70	190	0
4	56	20	210	30	470	30	620	64
5	80	0	130	0	250	30	0	0
6	60	16	267	32	399	65	129	11
7	47	19	607	122	242	27	320	38
8	30	18	107	15	357	47	253	36
9	78	14	90	16	220	29	227	-
10	68	12	211	21	214	24	481	-

Results expressed in pg AI per unit time

Table 2.2.3.11b Renin concentration in DA rat glomerular supernatants at fifteen minutes and zero time.

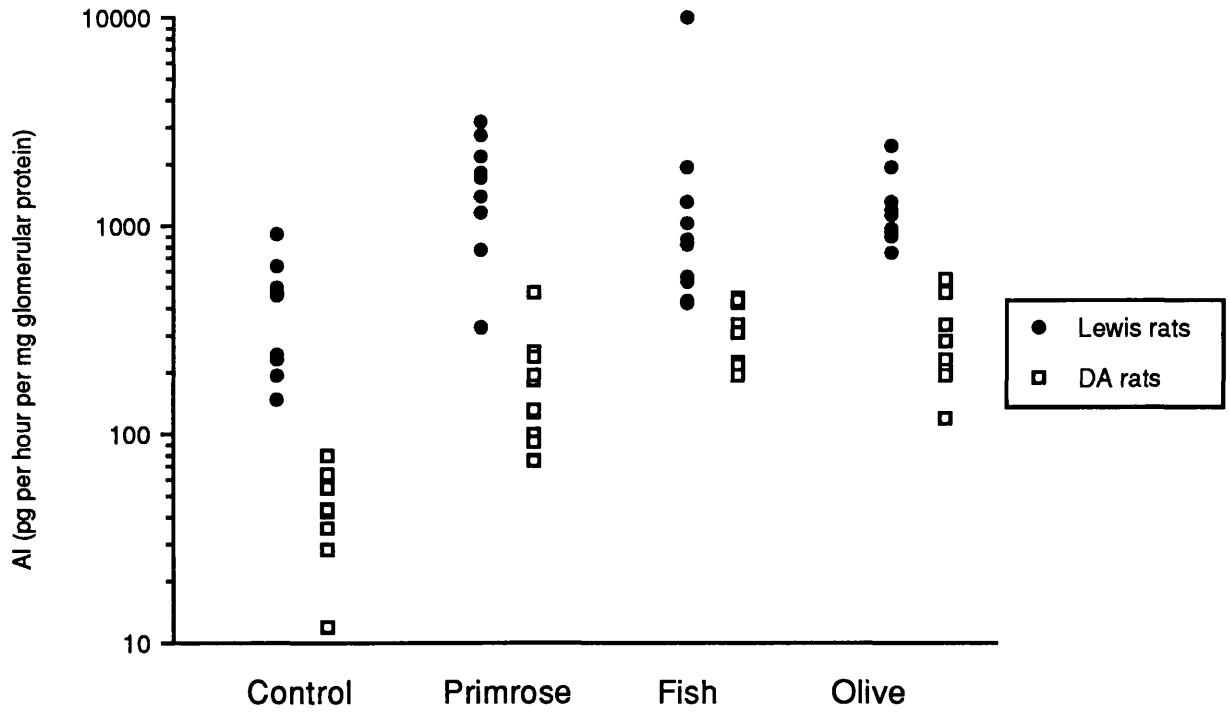


Figure 2.2.3.11a Renin concentration in glomerular supernatants of Lewis and DA rats at six weeks

Table 2.2.3.11c Glomerular renin concentration - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			163 to 558	p= 0.0004*
Primrose			919 to 2074	p= 0.0002*
Fish			237 to 1094	p= 0.0008*
Olive			618 to 1099	p= 0.0003*
Lewis rats				
Control	vs	Primrose	-1973 to -629	p= 0.0017*
Control	vs	Fish	-1066 to -88	p= 0.02*
Control	vs	Olive	-1073 to -455	p= 0.0005*
DA rats				
Control	vs	Primrose	-191 to -56	p= 0.0004*
Control	vs	Fish	-376 to -151	p= 0.0003*
Control	vs	Olive	-401 to -150	p= 0.0004*

Mann-Whitney Confidence Interval and Test

	Control		Primrose		Fish		Olive	
	15"	0"	15"	0"	15"	0"	15"	0"
1	129	38	85	29	84	22	275	53
2	290	81	54	18	165	59	169	32
3	91	14	144	32	127	25	285	53
4	62	7	81	8	126	28	183	36
5	108	29	213	61	130	45	276	65
6	231	18	115	17	106	12	93	45
7	163	16	140	23	79	4	190	59
8	168	17	129	23	280	28	177	66
9	207	30	139	24	117	42	127	52
10	215	21	104	21	132	64	169	37

Results expressed in pg AI per unit time

Table 2.2.3.11d Lewis rat plasma renin concentration at fifteen minutes and zero-time.

	Control		Primrose		Fish		Olive	
	15"	0"	15"	0"	15"	0"	15"	0"
1	161	20	161	36	30	0	173	79
2	244	30	213	6	50	11	199	79
3	211	19	169	43	190	68	237	118
4	279	22	71	42	122	63	196	82
5	381	38	186	33	152	27	197	70
6	45	20	139	50	209	82	249	51
7	240	60	139	66	38	-	194	50
8	652	63	200	47	242	43	233	49
9	294	63	124	32	324	104	294	70
10	216	75	189	47	171	46	306	73

Results expressed in pg AI per unit time

Table 2.2.3.11e DA rat plasma renin concentration at fifteen minutes and zero-time.

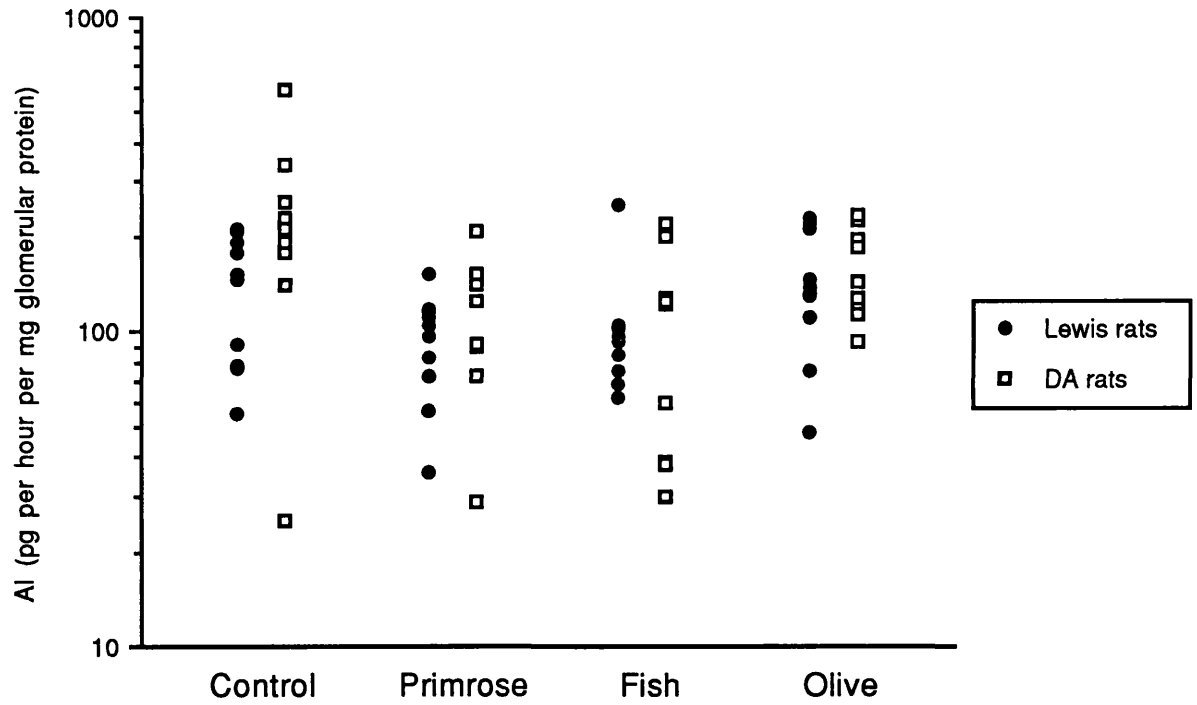


Figure 2.2.3.11b Plasma renin concentration in Lewis and DA rats at six weeks

Table 2.2.3.11f Plasma renin concentration - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-154 to 10	p= 0.10
Primrose			-69 to 23	p= 0.19
Fish			-57 to 47	p= 0.79
Olive			-71 to 37	p= 0.85
Lewis rats				
Control	vs	Primrose	-18 to 97	p= 0.16
Control	vs	Fish	-17 to 107	p= 0.21
Control	vs	Olive	-60 to 63	p= 1
DA rats				
Control	vs	Primrose	16 to 168	p= 0.026*
Control	vs	Fish	16 to 193	p= 0.02*
Control	vs	Olive	-18 to 130	p= 0.14

Mann-Whitney Confidence Interval and Test

2.2.4 Discussion

It was disappointing - but not surprising - that the fall in proteinuria in the PUFA diet groups was not so marked and did not attain the same degree of statistical significance in this, as in the previous study, being lowered significantly only in the fish and olive oil diet groups in Lewis rats and in the evening primrose oil group in DA rats. Single urine protein measurements are notoriously unreliable because of a high degree of intra-individual variability (Feldt-Rasmussen and Mathiesen, 1984, Hutchison and O'Reilly, 1985; Johnston *et al*, 1993). Ideally, urine protein levels would have been monitored more frequently to establish both a normal range and the extent of intraindividual variation but this was neither practicable nor possible due to number of experimental animals, utilisation of metabolic cages and manpower limitations.

Figures 2.2.3.7(a) and 2.2.3.7(b) show the pattern of albumin excretion in Lewis and DA rats between weeks three and six in the control and evening primrose oil diet groups. In Lewis rats, urine albumin levels were higher and a larger proportion were rising more steeply in the control than evening primrose oil diet group; in DA rats, the levels of albumin excretion were lower than in Lewis rats and the fall was more pronounced in the evening primrose oil diet group than control diet group. Despite the initial intention, it became difficult to accommodate measurement of both urine protein and albumin levels at three and six weeks, within the study protocol because the urine samples had to be de-salted - a procedure which could take up to thirty minutes per sample - prior to immunoturbidimetry: time constraints prevented measurement of the three week fish and olive oil samples. Because of the staggered diet regimes, each experimental group of twenty rats (10 Lewis and 10 DA) is a at different stage and in any one week, one group may have to be put into metabolic cages and monitored while another has to be culled, their kidneys removed and glomeruli harvested by sieving (see Table 2.2.2.1). In addition, blood and tissue samples must be collected, labelled, stored and/or processed and control and PUFA diets prepared as required. For a solitary worker, the practical problems associated with studies of this kind are substantial.

Despite the inability to reproduce the fall in total protein excretion in all the PUFA diet groups, albumin excretion rates were lowered in both Lewis and DA rats in all the PUFA diet groups and while it is acknowledged as unwise to measure urine albumin following strenuous exercise or an acute fluid overload, all the rats were of the same sex (male), of comparable body weight and relatively inactive within the confines of the metabolic cages when urine was collected.

Again, as was seen in the preceding study, glomerular TxB_2 levels were raised in Lewis compared to DA rats - but this time, only to a statistically significant extent in the control and olive oil diet groups. Glomerular TxB_2 levels were reduced significantly in both rat strains in the fish oil diet group and were lowered in Lewis, but raised in DA rats in the evening primrose oil group. Olive oil had no effect on glomerular TxB_2 levels in either strain of rat, as before. These findings raise the question of whether the slightly larger sample size makes these thromboxane results more accurate than those from the previous study.

A major reason for, in effect, repeating the previous study was to establish whether circulating, as well as glomerular levels of thromboxane (and renin) were raised. Although measurement of circulating thromboxane levels is not advocated because of artefactual synthesis related to blood sampling and coagulation, it has been customary to estimate serum thromboxane levels by measuring the capacity of platelets to generate TxB_2 in blood allowed to clot for an hour at 37°C (McLaren, 1986). There appeared to be no difference in serum TxB_2 levels between Lewis and DA rats in any of the diet groups and levels were lowered only in the fish oil diet group relative to controls: fish oil was the only diet to have any effect on both glomerular and serum thromboxane levels in both strains of rat. It is very likely, however, that the inability to detect any difference in serum TxB_2 levels between the groups is because the levels are close to the upper detection limit of the assay: serum samples could perhaps have been diluted a further ten, or even hundred fold. It had been anticipated that thromboxane levels would be high after the 30 minute incubation period but one drawback of using commercial assay kits is that they do not afford the luxury of protracted testing to establish the optimal dilution factor. Measurement of alternative TxB_2 metabolites such as 11 dehydro- TxB_2 found in both blood and urine, or 2, 3 dinor TxB_2 which predominates in urine and is highly reflective of platelet TxB_2 might have been more appropriate but the facilities to measure these metabolites were not readily available. Although blood was withdrawn into prostaglandin anti-coagulant (section 2.2.2.3), plasma prostanoid levels were never measured. Few problems were encountered with the cardiac puncture procedure but any samples in which clotting was suspected, were discarded.

Renin levels too, were again raised in glomerular supernatants of Lewis rats compared to DA rats, but in contrast to the previous results, in which PUFA diets had no effect on renin levels in either rat strain, glomerular renin levels were raised in *all* the PUFA diet groups in *both* strains of rat. It was notable that Lewis rat glomerular supernatants required dilution prior to assay whereas those from DA rats did not.

Raised glomerular renin levels in association with apparently lowered rates of albumin excretion in the PUFA diet groups was unexpected.
Renin levels were measured in plasma containing prostaglandin anticoagulant and while median plasma renin levels are higher in DA than Lewis rats, the differences do not reach statistical significance. The suggestion that plasma renin levels are raised in DA rats compared to Lewis rats with raised glomerular renin levels, may indicate that renin is trapped within the Lewis rat glomerulus. Renin zero-time values too, which relate to endogenous activation, appear to be higher in the glomerular supernatants of Lewis rats compared to DA rats (Table 2.2.3.11b), particularly in the evening primrose oil diet group (Table 2.2.3.11a) but the amount and distribution of the data makes it difficult to analyse and interpret. There were no obvious differences in plasma renin zero-time values between Lewis (Table 2.2.3.11d) and DA (Table 2.2.3.11e) rats. That no significant differences in plasma renin activity were observed either between Lewis and DA rats within any diet group or within either strain in any of the PUFA diets groups suggests that the increased glomerular renin activity in Lewis rats is indeed localised.

Renin results are most meaningful when related to sodium balance and blood pressure but neither parameter was measured in this or the previous study. Renin production is inversely proportional to sodium intake and affected by dietary protein (Paller and Hostetter, 1986; Rosenberg *et al*, 1987). Although dietary protein and sodium intake were not measured directly, overall, there was no difference in food consumption between Lewis and DA rats (see Table 2.2.3.2).

Even within the normal human kidney, there is a large degree of nephron heterogeneity with respect to renin synthesis and storage. Histological studies have shown that not all nephrons produce renin and that superficial nephrons may store more than deeper nephrons (Taugner *et al*, 1981; Gomez *et al*, 1988). Although renin is synthesised and stored in the JGA, the raised glomerular renin levels in Lewis rat glomerular supernatants may be explained by (i) an increased number of renin-secreting glomeruli, (ii) increased rates of renin synthesis and secretion or (iii) the glomerulus itself becoming an alternative site of renin synthesis either in relation to renin-containing cells migrating from the afferent arteriole into glomerular tuft or native glomerular cells such as mesangial cells starting to produce renin. Using the same glomerular sieving procedure as in this study, Rosenberg *et al* (1991) showed that compared to adriamycin nephrosis (ADR) rats, in which neither glomerular nor circulating renin levels were raised, in rats with reduced renal mass, glomerular renin levels were raised while circulating levels were unchanged. Evidence of increased glomerular renin was reinforced by immunofluorescence staining and increased

transcription of messenger RNA although neither parameter was heightened in ADR rats (Rosenberg *et al*, 1993). The explanation for the increased renin levels in Lewis rat glomeruli might be that they store more renin which is released by the sieving procedure. It should be noted that there was no difference between Lewis and DA rats, in the number of glomeruli with attached or detached afferent arterioles (section 2.1.3.2).

Other issues to consider are that renin levels are not a good marker of the biological effects of AII since angiotensinogen can be cleaved by other means. Also, there is a storage pool of both active and inactive renin in the kidney and as much as 50% may be inactive. Not all procedures to measure renin necessarily reflect true amounts of active renin - although the method used in this study measured active renin: to have measured total renin would have involved trypsin activation of the samples.

It is unclear whether the heightened levels of both glomerular TxB_2 and renin in Lewis rats are independent or related events but concomitant rises in thromboxane and renin levels are common (Shibouta *et al*, 1979; Podjarny *et al*, 1986; Lefkowith *et al*, 1987). The two kidney-one clip rat model of renovascular hypertension (Stahl *et al*, 1984a) and the hydronephrotic, ureter-obstructed kidney (Purkerson *et al*, 1986; Yanagasawa *et al*, 1990) are characterised by increased glomerular levels and urinary excretion of TxB_2 and renin. It has been shown that AII releases thromboxane within the kidney and that thromboxane may mediate the vascular effects of AII within the normal rat glomerulus (Welch and Wilcox, 1990, Wilcox *et al*, 1991).

The nature of the link between elevated thromboxane and renin levels in the glomeruli of Lewis rats remains unclear. A possible explanation is that either a fall in glomerular blood flow, or platelet aggregation and a subsequent increase in plasma viscosity induced by thromboxane, may be detected by cells in the JGA and renin released in response. Alternatively, renin may elevate thromboxane levels through angiotensin II-mediated hydrolysis of membrane phospholipids (Luft *et al*, 1989; Welch *et al*, 1990; Mistry *et al*, 1990). Other explanations for raised thromboxane levels are discussed in section 2.1.4.

Studies centred around dietary manipulations are often difficult to interpret because of problems in regulating and standardising food intake. Glomerular filtration can be influenced by both protein and calorie intake (Klahr and Alleyne, 1973; Ichikawa *et al*, 1980; 1985, Tapp *et al*, 1989; Woods, 1993) and so a reduction in either dietary parameter could contribute to the fall in AER in experimental PUFA-fed rats. However, it has been reported that changes in dietary protein intake have no effect on urine albumin levels (Solling *et al*, 1986). The fall in albumin excretion in this and

the previous study, is unlikely to be linked to a reduced dietary protein or calorie intake or a resultant fall in body weight since the protein content of the control and PUFA diets was similar (see Table 2.1.2.1) and rats fed PUFA diets did not consume less than rats fed control diet. Although the initial calorie content of both the standard pellet and fat-deficient diets were similar, it is inevitable that the addition of the oils to the fat-deficient diet would raise the calorie content of the PUFA diets relative to the control diet; a reduced calorie intake is therefore unlikely to explain the fall in albumin excretion in PUFA-fed rats. Even had the PUFA-fed rats consumed significantly less food, the higher calorie content of the PUFA diets should compensate for any calorie deficit. Furthermore, if there is a link between increased calorie intake and proteinuria it might have been evident in the experimental PUFA diet groups. Dietary components other than the oils themselves are unlikely to have accounted for the fall in AER. Neither does the fall in AER correlate with changes in body weight: the absence of a fall in body weight, similar to that observed at two weeks in all the diet groups in previous study, and the greater increase in body weight may be related to the rats in this study having a lower body weight at the outset. Urine creatinine levels in Lewis rats reflected their greater body weight relative to DA rats but PUFA diets had no effect on urine creatinine levels in either strain of rat.

It is interesting that olive oil, as a UFA control with attenuated prostanoid activity, was as effective as the other oils in lowering urine albumin levels despite no change in glomerular prostanoids and renin levels.

Summary

It is impossible to state with conviction, at the end of this study, that PUFA diets lower albumin excretion rate although there is certainly little evidence of urine albumin levels being raised in the PUFA diet groups. There is some suggestion that the anti-proteinuric effects of PUFA diets are more pronounced in Lewis rats than DA rats.

Despite some discrepancies between this and the previous study, glomerular TxB_2 and renin levels *in vitro* appear to be raised in Lewis compared to DA rats. Notwithstanding some doubts about the accuracy of the serum thromboxane results, elevated TxB_2 and renin levels seem to be confined to the glomerulus since their circulating levels were not raised. The functional significance of raised glomerular TxB_2 and renin levels in Lewis rats is unclear but may be linked to their reportedly higher levels of proteinuria and heightened susceptibility to various forms of glomerular injury.

PUFA diet-induced changes in glomerular or systemic thromboxane or renin levels did not correlate with the fall in albumin excretion rate in Lewis and DA rats. That a PUFA diet-induced fall in intraglomerular pressure (P_{gc}) was responsible for the fall in AER is not compatible with the pattern of glomerular thromboxane or renin production.

It is unlikely that factors such as restricted calorie or protein intake or differences in body weight account for the fall in albumin excretion rate in both rat strains.

The possibility that glomerular thromboxane and renin levels are depressed in DA rats, rather than raised in Lewis rats, should not be overlooked .

2.3.1 Introduction and experimental design

Experimental data from the two previous studies suggests that changes in glomerular or circulating prostanoid or renin levels do not contribute to the apparent fall in the albumin excretion in healthy Lewis and DA rats fed PUFA diets. It was decided therefore, that other aspects of PUFA function such as their incorporation into the lipid bilayer of glomerular cell membranes should be explored (see section 1.3.6). Manipulation of the fatty acid content of glomerular membrane phospholipids by PUFA diets might alter the magnitude of the intrinsic negative charge on the glomerular capillary wall by altering the linkage to, and expression of glycoproteins on the cell surface.

The glomerular capillary wall controls the passage of albumin from the plasma into the glomerular ultrafiltrate in relation to its ability to discriminate amongst molecules on the basis of their size, electrostatic charge and shape (see section 1.2). Within the three-layered sandwich structure of the GCW, the endothelial and epithelial cell layers are separated by the non-cellular glycoprotein gel matrix of the glomerular basement membrane (GBM). The effective negative charge (or polyanion) on the GCW which repels anionic circulating proteins such as albumin, is derived from charged moieties in all three layers of the glomerular capillary wall.

Experimental design

The hypothesis underlying this study was that the trend towards lowered rates of urinary albumin excretion observed in Lewis and DA rats fed PUFA diets in the previous studies was the result of heightened glomerular capillary wall charge in rats fed PUFA diets. Quantifying the charge on the glomerular capillary wall is made difficult by the heterogeneous nature of the charged molecules which comprise the polyanion and their ubiquity in all three layers of the GCW. Using the same dietary regime and experimental design as in the two preceding studies, the effects of PUFA diets on the glomerular polyanion were assessed by measuring the uptake of radiolabelled cationic (+) human IgG by the glomeruli of Lewis and DA rats. Uptake of cationic IgG by whole spleen was also measured.

2.3.2 Animals, materials and methods

It is important to establish a dose of labelled cationic protein (^{125}I-IgG^{+}) that is high enough to detect a difference in GCW charge but not so high as to overwhelm the glomerulus. Before beginning the study proper, therefore, a small preliminary study was performed to determine the dose of ^{125}I-IgG^{+} necessary for optimal detection of glomerular uptake.

Two groups of 5 Lewis and 5 DA rats fed evening primrose oil diet were given either 1 or 5 mg of ^{125}I-IgG^{+} and similarly, one group of 5 Lewis and 5 DA rats fed control diet were given 5 mg of ^{125}I-IgG^{+}. The methods used to cationise and radiolabel IgG are described in sections 2.3.2.1 and 2.3.2.2.
Incorporation of the results from this pilot study explains the larger number of Lewis and DA rats in the control diet groups in the main study.

Main study

Four groups of 10 Lewis rats and four groups of 10 DA rats (n = 80) ranging between 200 and 225 grams in weight at the start of the study, were fed either standard laboratory diet, evening primrose oil diet, fish oil diet or olive oil diet for six weeks (see section 2.1.2.1). At three and six weeks, the rats were put into metabolic cages and at the end of six weeks, seven or eight rats in each diet group were given an intravenous bolus injection of 5 mgs of ^{125}I-IgG^{+} and the remainder were given an equivalent dose of radiolabelled native IgG (^{125}I-IgG^{N}) to assess the extent of non-specific binding to the glomerular capillary wall. Each animal was killed fifteen minutes after injection of label, the kidneys were removed (without saline perfusion because of the high levels of radioactivity) and the glomeruli were recovered by sequential sieving as described in section 2.1.2.5. The protein content of the glomerular pellets was measured by the Lowry protein assay (see section 2.1.2.7) and the amount of radioactivity accumulated by isolated glomeruli and whole spleen was measured in a gamma counter. A small sample of renal cortex was removed for immunofluorescence.

2.3.2.1 Preparation of native and cationic IgG

Native IgG Human immunoglobulin G (Sigma Chemical Company: I-4506) reconstituted to 50 mg per ml in PBS.

Cationic IgG

IgG was cationised by activation of the carboxyl groups with carbodiimide (EDC) followed by nucleophilic attack with the base 3-dimethylaminopropylamine (DMPA). Cationisation takes place in a protein modification buffer (Buffer A, pH 5.5) prepared by dissolving 3.28 g *di*-sodium hydrogen phosphate and 8.77 g sodium chloride in just under 1 litre distilled deionised water then lowering the pH to 5.5 with HCl. The same buffer was used to prepare the following solutions.

Human IgG 50 mgs of human IgG (as above) were dissolved in 0.5 mls of Buffer A and heated in a warm oven (≈ 37°C) to dissolve, if necessary.

EDC 30 mgs 1-ethyl-3-(3-dimethyl-amino-propryl)-carbodiimide hydrochloride (EDC: Sigma Chemical Company: E-7750) in 500 µls of Buffer A.

IgG and EDC solution were mixed together in a small tube and then 20 µls of the free base, DMPA added. DMPA must be removed from the preparation by five one hour dialyses against two litres of cold (4°C) PBS (pH 7.2-7.4). [Ten litres of PBS were prepared using sodium chloride (8 g/l), potassium dihydrogen phosphate (0.34 g/l) and *di*-potassium hydrogen phosphate (1.2 g/l)].

The initial pH of the IgG^+ solution when tested using a pH strip (Sigma Chemical Company: P-4536) was greater than 10 but had fallen to 7 (in PBS) by the end of dialysis.

2.3.2.2 Radioiodination of native and cationic IgG

The following stock solutions were prepared for iodination of IgG (native and cationic):

IgG (native or cationic)	50 mgs per ml in PBS
Iodine monochloride	ICl at 25 nmols per µl in 1N NaCl. Molar ratio of iodine monochloride to protein = 2.5 : 1
Glycine buffer	250 µls of 1M Glycine buffer, pH 10, per ml of protein. (7.51 g glycine in 100 mls deionised water: adjust pH to 10.
Iodine-125	^{125}-Iodine - 10 µls ≡ 37 MBq ≡ 1 mCi (Amersham International plc: IMS 30)

250 µls of glycine buffer was added to 1 ml of cationic or native IgG in a 5 ml tube with snap-on cap (Sarstedt) and 31.25 µls of ICl pipetted into the cap. 10 µls of ^{125}-Iodine was added to the contents of the tube before replacing the cap (containing the iodine monochloride) carefully and mixing the contents by inversion. Free ^{125}-Iodine was removed from the preparation by dialysis for 1 hour, five times, against two litres of cold (4°C) PBS (prepared as in the previous section).

2.3.2.3 Isoelectric focusing

Isoelectric focusing (IEF) was used to confirm the cationisation of IgG. 15 µls of native and cationic human IgG and 20 µls of IEF reference markers (Isoelectric focusing calibration kit from Pharmacia Biotech: Code no 17-0471-01) were run in an Ampholine^{R} PAGplate, pH 3.5 - 9.5 polyacrylamide gel plate (Pharmacia Biotech: Code no 80-1124-80) on an Multiphor 2117 (LKB) electrophoresis unit. 1M H_3PO_4 was used as the anolyte and 0.1 M sodium hydroxide as the catholyte solution. Samples were placed 6.67 cm from the cathode and power was supplied at 750V and current at 25mA for 1 hour from a power pack (LKB 2301 Macrodrive 1).

The gel was stained using the following solutions for the stated times:

Fixing solution	29 g trichloroacetic acid and 8.5 g sulphosalicylic acid in 250 mls distilled water - 30 minutes.
Destaining solution	500 mls ethanol and 160 mls acetic acid made up to 2 litres with distilled water - 30 minutes.
Staining solution	0.1% Coomassie Blue G-250 in aqueous solution of 25% methanol, 5% acetic acid (w,v,v,v) - 10 - 20 minutes.
Preserving solution	25 mls glycerol made up to 250 mls with destaining solution. Change solution as required and leave until gel is clear.

The gel was dried overnight at room temperature.

2.3.2.4 Surgical procedure: administration of IgG

Three syringes (1 ml) were labelled and primed with anaesthetic, saline or cationic or native ^{125}I-IgG at 5 mgs per 100 µls. Each rat was anaesthetised by an intraperitoneal injection of Sagatal and an endotracheal tube inserted. The jugular vein was exposed by blunt dissection and cannulated first, with the anaesthetic line and then the saline line. Each cannula was constructed from syringes fitted with a 23 guage needle attached to a length of PE 50 polyethylene tubing (see section 2.1.2.4).

To introduce ^{125}I-IgG^{+}, the saline line was clamped and the syringe removed and exchanged for one containing radiolabelled native or cationic IgG. After the contents of the syringe had been expelled, the saline syringe was re-attached and 100 µls of saline flushed through slowly to wash out the line. After fifteen minutes, the animal was killed by an overdose of Sagatal via the anaesthetic line. The kidneys and spleen were removed and placed in ice cold PBS; the weight of both kidneys and the spleen were recorded and a sample of cortical tissue was snap-frozen immediately for immunofluorescence (see following section).

The glomeruli were recovered from both kidneys of each rat by sieving as described in section 2.1.2.5 and resuspended in a final volume of 2 mls of Hartmanns solution (Baxter Healthcare). The quantity of radioactivity in the glomerular suspension and intact spleens was measured in a gamma counter (Packard). A ^{125}I-IgG reference standard equivalent to 500 µgs of IgG was prepared by adding 10 µls of radiolabelled native IgG (50 mg per ml) to 990 µls of saline and counted with glomeruli and spleen each day to compensate for radioactive decay. The amount of ^{125}I-IgG taken up by the glomeruli and spleen was expressed per mg of glomerular protein or wet spleen weight, respectively. The protein content of the glomerular pellet was measured by the Lowry method (see section 2.1.2.7).

2.3.2.5 Immunofluorescence staining and microscopy

To prepare the antiserum, fluorescein isothiocyanate (FITC) conjugated sheep anti-human IgG (Scottish Antibody Production Unit, Law Hospital, Carluke, ML8 5ES) was reconstituted as directed. 200 mgs of mouse liver powder (Cappel Laboratories: Cat no 20311) was washed with 2 mls of PBS, centrifuged at 1000 g for 5 minutes and the supernatant discarded. To minimise non-specific tissue binding, the antiserum was mixed with the washed liver powder for 1 hour at room temperature and then centrifuged twice at 1000g for 5 minutes and dispensed into 10 µls volumes and stored at -20°C.
Antiserum was titrated and checked for specificity and the optimal conjugate dilution was determined by the immunofluorescence chessboard titration method and diluted 1:160, 1:320 and 1:640 with saline. Serial dilutions of a positive control serum with an identified pattern and end-point were tested with a series of conjugate dilutions. A negative serum and buffer control were tested simultaneously.

Tissue was prepared for immunofluorescence by immersing 3-5 mm pieces of fresh rat kidney cortex in gelatine capsules, filled with embedding medium for frozen tissue sections (Tissue-Tek; Miles Scientific). The capsules were snap frozen immediately by floating them in a mixture of methanol and dry ice (solid CO_2) and then stored in a sealed container at -70°C.

Frozen sections (4 - 6 µm) were cut on a cryostat (Slee) and air-dried for 30 minutes at room temperature. The slides were washed twice in PBS for 10 minutes, on a shaker, prior to fixing in an ice-cold mixture of ether and alcohol (50: 50) for 10 minutes, to precipitate proteins. With the apparatus on ice, the tissue was fixed in 95% alcohol for 20 minutes and then washed three times in PBS, for 5 minutes each time. After drying, the sections were kept in a moist chamber. Antiserum diluted 1: 320 was dropped on to the sections which were then incubated for 30 minutes at room temperature. Sections from control kidneys were stained at the same time. The slides were washed three times in PBS each time for 7 minutes, mounted in Citifluor and then stored in the dark at 4°C. The sections were examined under a fluorescence microscope (Leitz) and scored subjectively on a scale of + / ++++.

2.3.3 Results

2.3.3.1 Isoelectric focusing

An isoelectric focusing gel illustrating the cationisation of IgG is shown in Plate 2.3.1. Lanes 1, 6 and 11 represent the IEF marker proteins [trypsinogen (pI 9.3), lentil lectin - basic band (pI = 8.65), lentil lectin - middle band (pI = 8.45), lentil lectin - acidic band (pI = 8.15), myoglobin - basic band (pI = 7.35), myoglobin - acidic band (pI = 6.85), human carbonic anhydrase β, pI = 6.55), bovine carbonic anhydrase β (pI = 5.85), β lactoglobulin A (pI = 5.2), soybean trypsin inhibitor (pI - 4.55) and amyloglucosidase (pI = 3.5). Lanes 2, 4, 8, and 9 contain cationic (+) IgG and lanes 3, 5, 7 and 10 contain native IgG.

Cationic (+) IgG exhibited bands ranging between ≈ 8.45 and 9 whereas native IgG displayed bands between ≈ 6.85 and 8.55.

2.3.3.2 Immunofluorescence microscopy

Immunofluorescence was used to illustrate the distribution of ^{125}I-IgG^{+} deposited within the glomerular capillary loops of Lewis and DA rats. There was a perceptible, but not remarkable difference in glomerular uptake of ^{125} I-IgG^{+} between the two strains in the control and PUFA diet groups but differences in glomerular uptake between the diet groups within either strain were not discernible. Differences in glomerular uptake of ^{125}I-IgG^{+} between Lewis and DA rats fed control diet are shown in Plate 2.3.2.

2.3.3.3 Dose-dependancy study

The results showed a difference in glomerular uptake of ^{125}I-IgG^{+} between Lewis and DA rats with DA rats taking up more than Lewis rats, irrespective of diet or IgG dose [**CD**: Lewis (5 mg): median 22 (range 18 - 24) vs DA: 18 (14 - 21) μgs ^{125}I-IgG^{+} per mg glomerular protein, p = 0.056, EPO (5 mg): Lewis: 30 (28 - 33) vs DA: 20 (19 - 22), p = 0.02, EPO (1 mg): Lewis: 13 (11 - 19) vs DA: 8.5 (8 - 11), p = 0.016: see Figure 2.2.3.1]. The lack of a statistically significant difference between the strains in the control diet group given 5 mg of ^{125}I-IgG^{+} may relate to sample size but the decision to use ^{125}I-IgG^{+} at a dose of 5 mg the main study was based upon the more marked distinction between the strains in the evening primrose oil diet group. Measurement of the glomerular uptake of 1 mg IgG^{+} in the control diet group was omitted because of time and numbers.

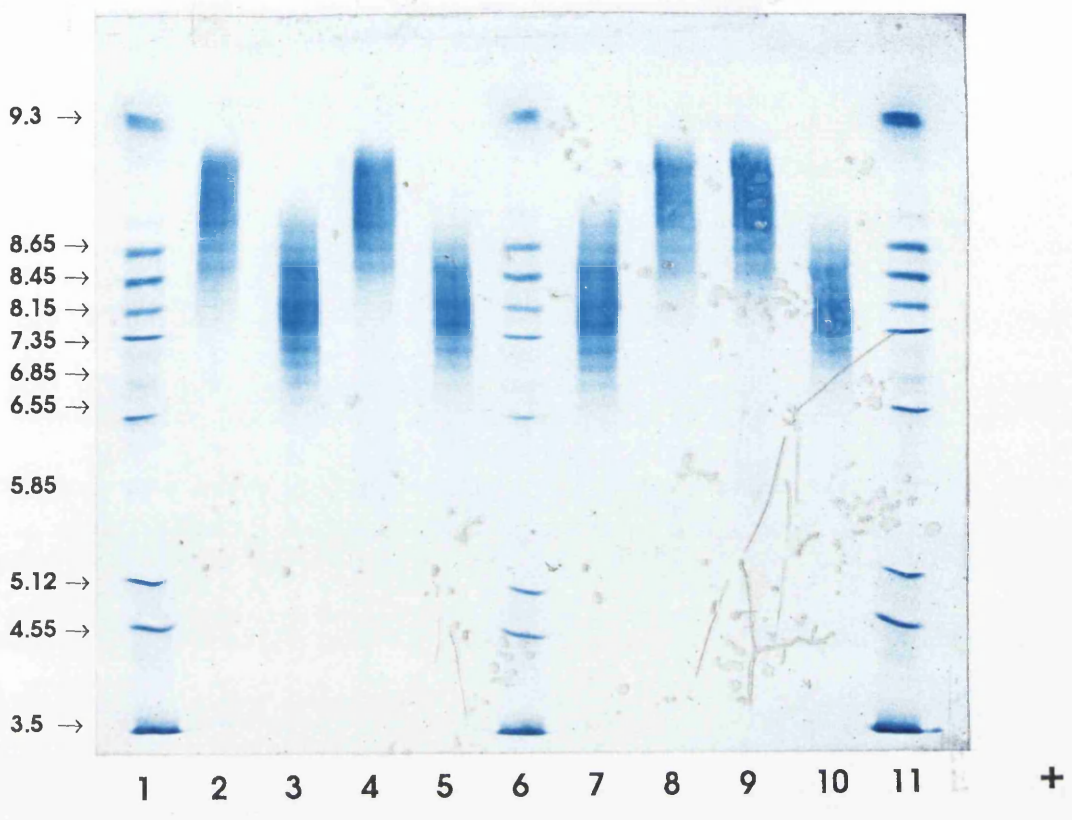
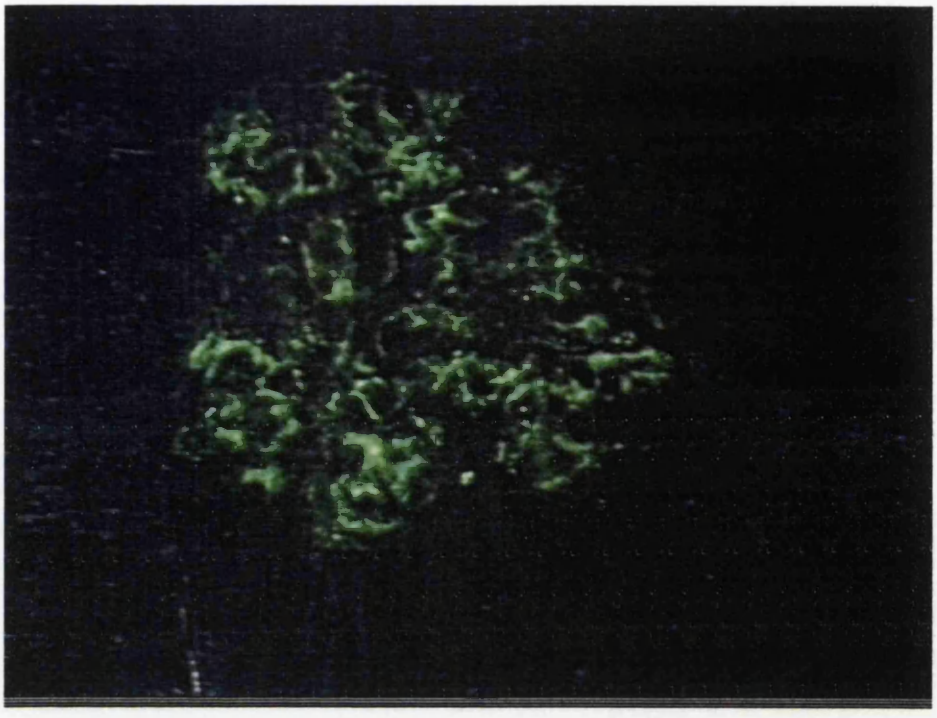
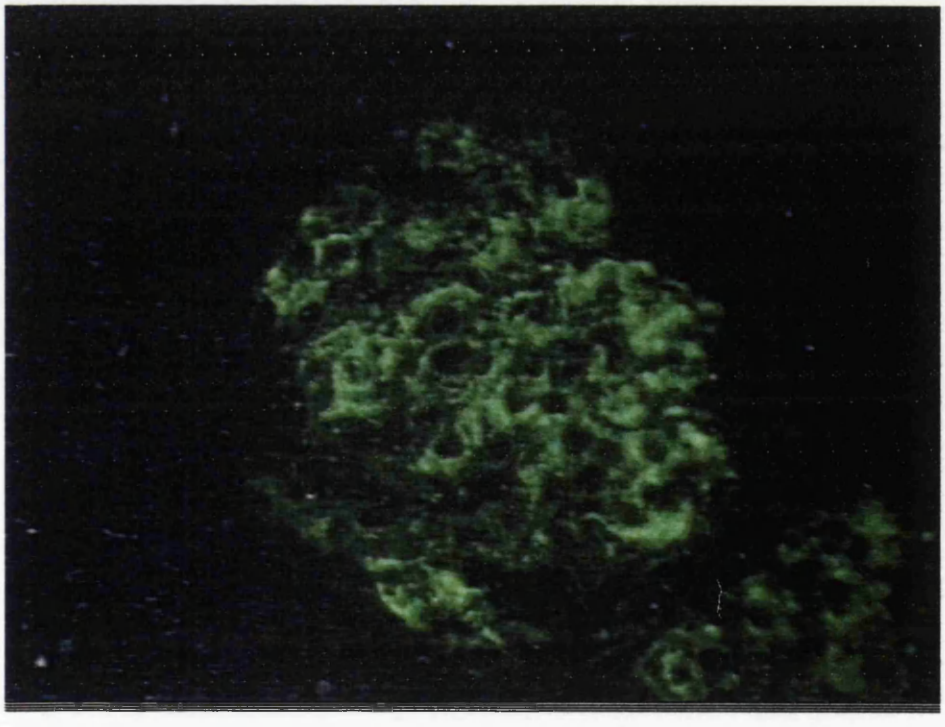


Plate 2.3.1 Isoelectric focusing of cationic and native human IgG (see section 2.3.3.1 for details)



Lewis rat glomerulus - Control diet



DA rat glomerulus - Control diet

Plate 2.3.2

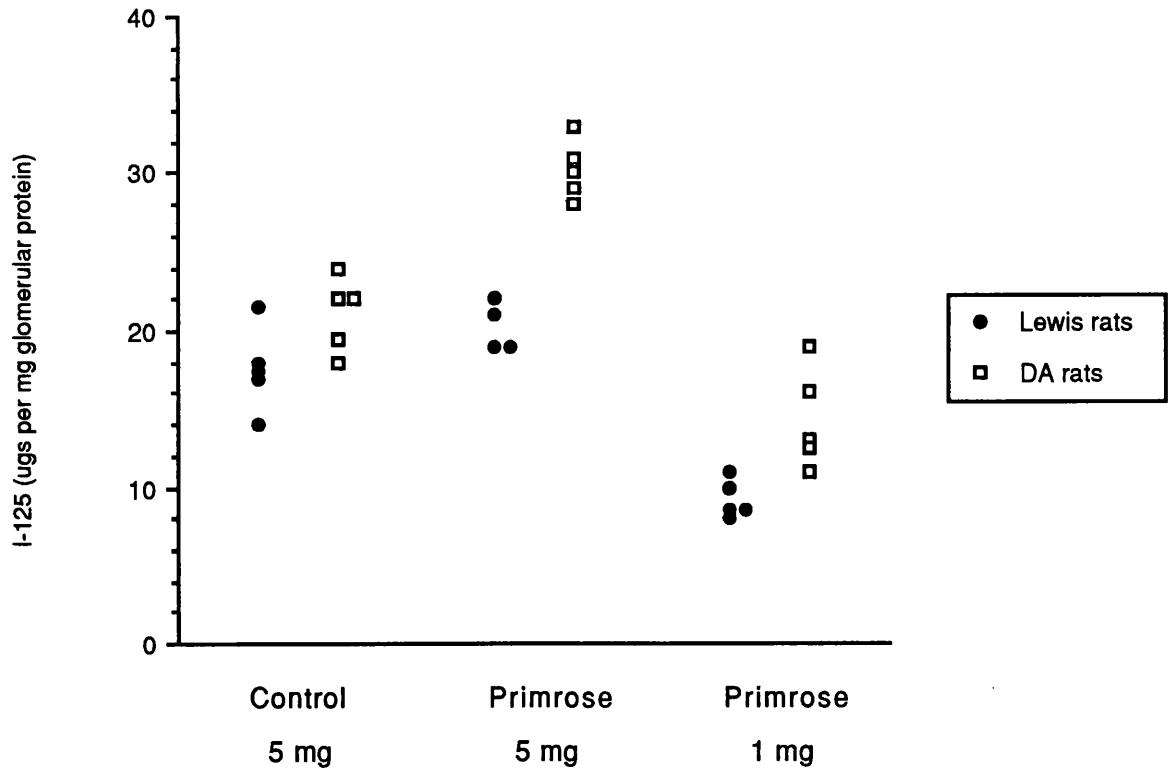


Figure 2.3.3.1 Dose dependent differences in glomerular uptake of ^{125} IgG+

	Control		Primrose		Fish		Olive	
	Lewis	DA	Lewis	DA	Lewis	DA	Lewis	DA
1	3.20	1.50	3.25	2.70	2.80	2.35	2.86	2.90
2	2.30	2.55	2.95	2.80	2.75	2.85	3.30	2.60
3	2.95	1.70	2.50	2.45	2.7	2.25	3.90	1.95
4	2.45	2.35			3.55	1.35	2.85	2.45
5	3.30	1.60				2.70	2.75	1.85
6	2.65	2.50	3.70	3.45	3.60	2.40	2.45	2.40
7	2.45	2.45	3.55	2.06	3.10	3.30	3.85	3.20
8	1.10	2.10	3.05	2.95	2.50	2.60	2.55	2.15
9	2.50	2.35			2.80	2.65	3.75	2.15
10	2.45	2.45			3.65	2.20	2.15	3.00

Results expressed in milligrams

Table 2.3.3.1 Protein content of Lewis and DA rat glomerular pellets

2.3.3.4 Albumin excretion rate

Figure 2.3.3.2 shows that albumin excretion rate was significantly higher in Lewis than DA rats only in the evening primrose oil diet group [**Lewis vs DA:** CD: 3.54 (range 0.13 - 11.34) vs 0.392 (0.103 - 1.7) mg per 24 hrs, EPO: 0.156 (0.102 - 0.752) vs 0.1 (0.057 - 0.15), FO: 0.206 (0.13 - 0.32) vs 0.232 (0.18 - 0.64), OO: 0.165 (0.058 - 1.26) vs 0.102 (0.05 - 0.238); see Table 2.3.3.2 for corresponding confidence intervals and significance levels]

In both strains of rat, AER was lowered significantly in both the primrose and olive oil diet groups [**Lewis**: CD: 3.54 vs (i) EPO: 0.155 mg per 24 hrs, (ii) OO: 0.165 mg per 24 hrs; **DA**: CD 0.392 vs (i) EPO: 0.098 mg per 24 hours, (ii) OO: 0.102 mg per 24 hours; see Table 2.3.3.2 for corresponding confidence intervals and significance levels]. AER was unchanged in both Lewis and DA rats in the fish oil diet group.

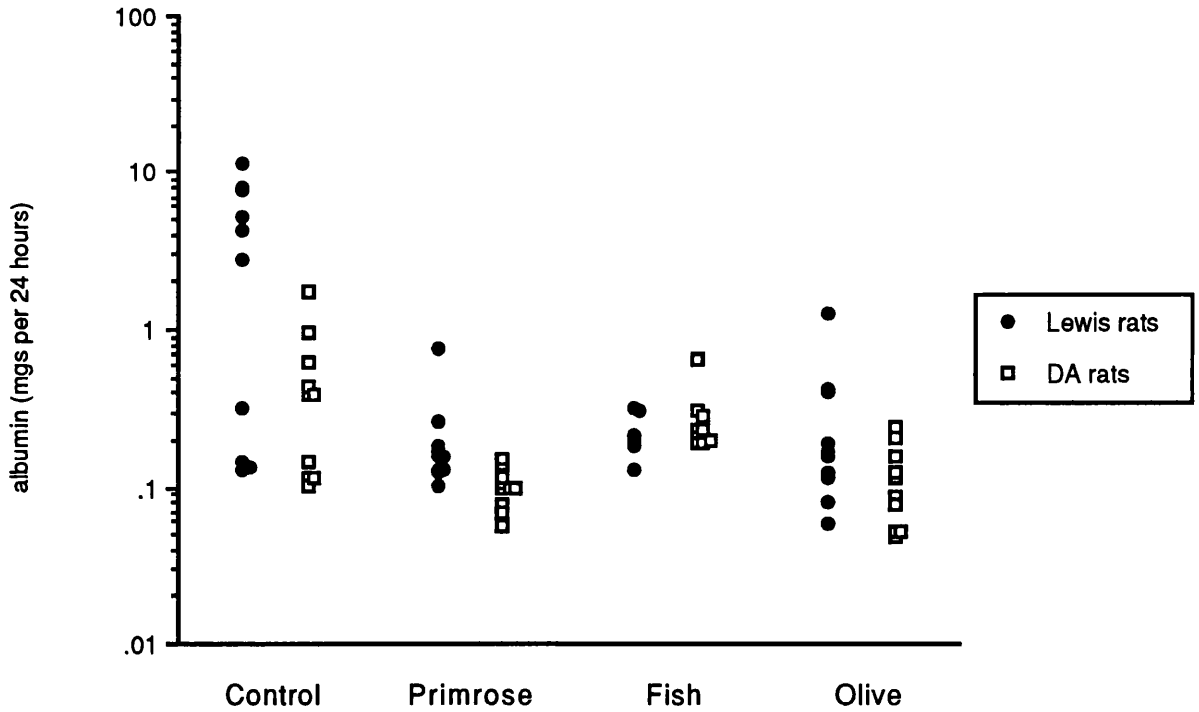


Figure 2.3.3.2 Albumin excretion rate in Lewis and DA rats at six weeks

Table 2.3.3.2 Albumin excretion rate at six weeks - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-0.07 to 7.2	p= 0.09
Primrose			0.02 to 0.11	p= 0.0028*
Fish			-0.1 to 0.07	p= 0.51
Olive			-0.01 to 0.3	p= 0.29
Lewis rats				
Control	vs	Primrose	0.004 to 7.5	p= 0.025*
Control	vs	Fish	-0.05 to 7.8	p= 0.13
Control	vs	Olive	0.017 to 7.5	p= 0.03*
DA rats				
Control	vs	Primrose	0.02 to 0.5	p= 0.0025*
Control	vs	Fish	-0.11 to 0.39	p= 0.67
Control	vs	Olive	0.03 to 0.55	p= 0.013*

Mann Whitney Confidence Interval and Test

2.3.3.5 Glomerular uptake of ^{125}I-IgG^{+}

The protein content of the glomerular pellets are shown in Table 2.2.3.1. (The group mean was used to replace any missing data points).

Significantly more ^{125}I-IgG^{+} was taken up by the glomeruli of DA rats than Lewis rats in the control and all the experimental PUFA diet groups (Figure 2.3.3.3); **[Lewis vs DA:** CD: median 18.5 (14 - 27) vs 22 (range 17 - 30) μgs ^{125}I-IgG^{+} per mg of glomerular protein, EPO: 18.5 (15 - 22) vs 28 (19 - 33), FO: 16 (14 - 17) vs 18.5 (16 - 21), OO: 16 (13 - 19) vs 20 (19 - 23); see Table 2.2.3.2 for corresponding confidence intervals and significance levels].
In Lewis rats, glomerular uptake of ^{125}I-IgG^{+} was unaffected in the evening primrose oil group but was significantly reduced in the fish and olive oil diet groups **[Lewis** CD: 18.5 (14 - 27) vs (i) FO: 16 (14 - 17) μgs ^{125}I-IgG^{+} per mg of glomerular protein, (ii) OO: 16 (13 - 19) μgs ^{125}I-IgG^{+} per mg of glomerular protein]. In DA rats, neither evening primrose oil nor olive oil diet had any effect on glomerular ^{125}I-IgG^{+} uptake but glomerular uptake was reduced significantly in the fish oil group **[DA:** CD: 22 (range 17 - 30) vs FO: 18.5 (16 - 21) μgs ^{125}I-IgG^{+} per mg of glomerular protein; see Table 2.2.3.2 for corresponding confidence intervals and significance levels].
Uptake of native human IgG was regarded as being low enough to be discounted.

2.3.3.6 Splenic uptake of ^{125}I-IgG^{+}

Splenic uptake of ^{125}I-IgG^{+} was also significantly greater in DA rats than Lewis rats in all the diet groups **[Lewis vs DA:** CD: median 190 (132 - 260) vs 304.5 (18. - 494) μgs per gram of spleen, EPO: 260 (246 - 295) vs 409 (311 - 456), FO: 299 (263 - 330) vs 408 (269 - 503), OO: 205 (151 - 277) vs 360 (178 - 456); see Figure 2.3.3.4 and Table 2.3.3.4 for corresponding confidence intervals and significance levels].
In Lewis rats, splenic uptake of ^{125}I-IgG^{+} was significantly higher in the evening primrose and fish oil diet groups than control diet group **[Lewis:** CD: 190 (132 - 260) vs (i) EPO: 260 (246 - 295) μgs per gram of spleen, (ii) FO: 299 (263 - 330) μgs per gram of spleen]. There is a suggestion that splenic uptake of ^{125}I-IgG^{+} may be raised in the evening primrose and fish oil diet groups which did not reach statistical significance; see Table 2.3.3.4 for corresponding confidence intervals and significance levels.
Splenic uptake of native IgG also was low enough to be discounted.

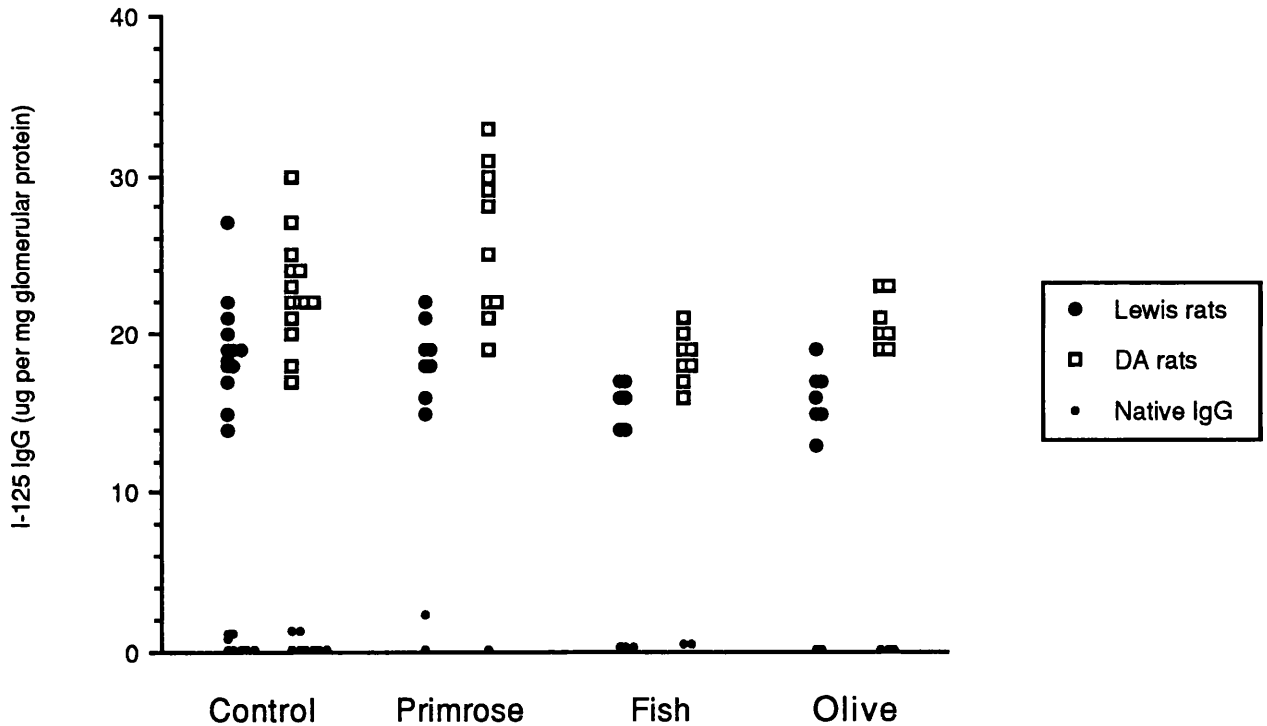


Figure 2.3.3.3 Glomerular uptake of IgG(+) in Lewis and DA rats at six weeks

Table 2.3.3.3 Glomerular uptake of ^{125}I-IgG^{+} - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-6 to -2	p= 0.0035*
Primrose			-11 to -3	p= 0.0037*
Fish			-5 to -1	p= 0.01*
Olive			-7 to -2	p= 0.003*
Lewis rats				
Control	vs	Primrose	-2 to 3	p= 1
Control	vs	Fish	0.9 to 5	p= 0.009*
Control	vs	Olive	1 to 5	p= 0.02*
DA rats				
Control	vs	Primrose	-8 to 1	p= 0.13
Control	vs	Fish	2 to 6	p= 0.004*
Control	vs	Olive	-1 to 4	p= 0.19

Mann Whitney Confidence Interval and Test

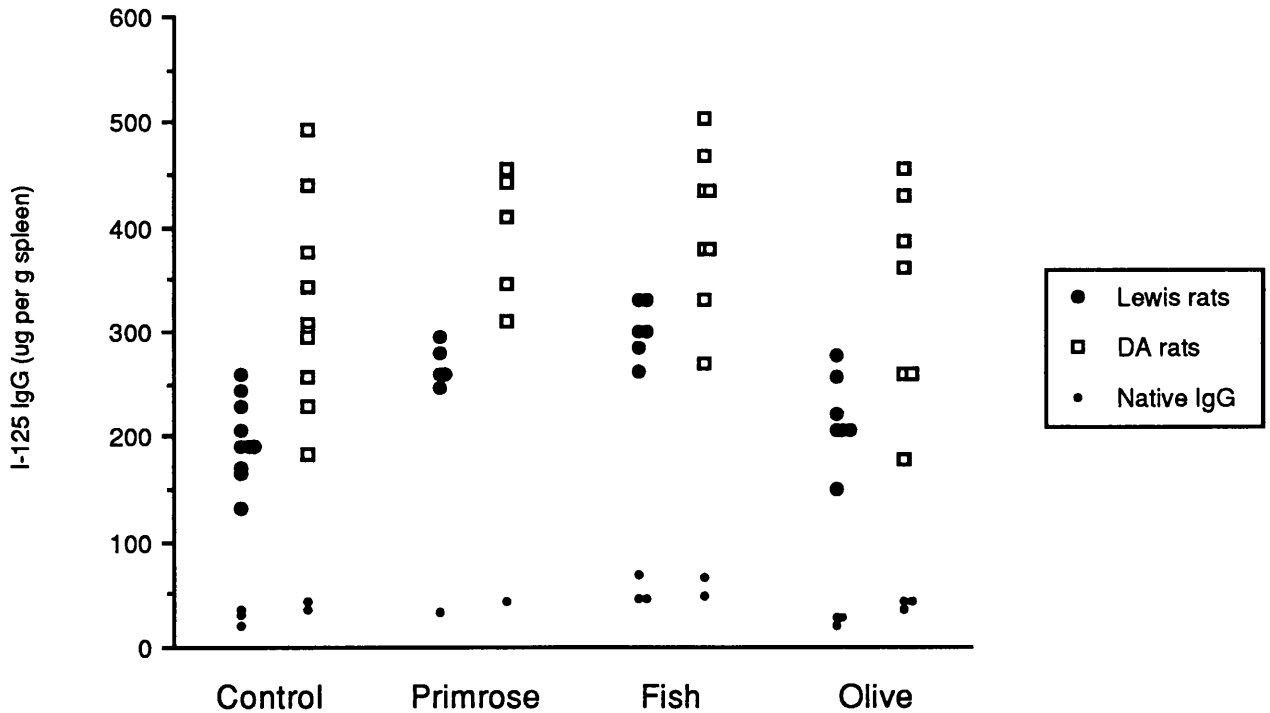


Figure 2.3.3.4 Splenic uptake of IgG(+) in Lewis and DA rats at six weeks

Table 2.3.3.4 Splenic uptake of ^{125}I-IgG^{+} - statistical analyses

			95% confidence interval	significance level
Lewis vs DA rats				
Control			-186 to -51	p= 0.003*
Primrose			-196 to -51	p= 0.012*
Fish			-173 to -32	p= 0.016*
Olive			-224 to -2	p= 0.04*
Lewis rats				
Control	vs	Primrose	-109 to -31	p= 0.005*
Control	vs	Fish	-140 to -70	p= 0.0013*
Control	vs	Olive	-68 to 24	p= 0.35
DA rats				
Control	vs	Primrose	-163 to 31	p= 0.09
Control	vs	Fish	-173 to 11	p= 0.08
Control	vs	Olive	-130 to 107	p= 0.8

Mann Whitney Confidence Interval and Test

2.3.4 Discussion

There are countless reports of diminished GCW charge in various glomerulopathies (see sections 1.2.1 and 1.2.6) and it has been suggested that the inheritance of a diminished glomerular polyanion heightens susceptibility of animals and humans to progressive forms of renal disease (Boulton-Jones *et al*, 1986, Deckert *et al*, 1989). The extent to which the filtration of an anionic protein like albumin is retarded while the clearance of cationic molecules is facilitated, is regarded as being proportional to the intensity of the negative charge on the glomerular capillary wall. The glomerular polyanion comprises negatively charged groups located in all three layers of the glomerular capillary wall (Szarfman *et al*, 1982, Timpl, 1986, Smith *et al*, 1989, Kobayashi *et al*, 1989, Wada *et al*, 1990). Through their incorporation into the membrane lipid bilayer, PUFAs may affect membrane fluidity and thus alter the topography of endogenous lipid domains and micro-domains and so influence the orientation and expression of anionic glycoproteins within glomerular cell membranes (see section 1.3.6). At the most fundamental level, PUFAs may intensify GCW charge through the introduction of additional carboxylate (COO-) groups on to the cell surface.

To date, there is no ideal technique to evaluate the magnitude of the negative charge on the glomerular capillary wall, principally, because the inherent heterogeneity of the component sialoglycoproteins and glycosaminoglycans makes it difficult to develop sensitive and specific immunological methods. Measuring the glomerular uptake of a radiolabelled cationic (+) protein such as IgG is the best method available currently, to reflect the intensity of the glomerular polyanion and should therefore correlate inversely with the rate of albumin excretion (Border *et al*, 1981, Oite *et al*, 1982; 1985). It is imperative, when employing this sequestration method, to measure the uptake of an equivalent amount of the same radiolabelled protein in its native, unmodified form to establish the extent of its non-specific binding to the glomerular capillary wall (and to give some indication of glomerular blood flow). That the uptake of native ^{125}I-IgG^{N} was negligible in both strains of rat in this study, strengthens the hypothesis that the differences in uptake are due to differences in GCW charge.

The cationisation procedure resulted in not one, but several IEF bands ranging between $\approx$ 8.45 and 9 which was higher than those of native IgG ($\approx$ 6.85 - 8.55). Whether it is possible to attain electrophoretic homogeneity is unclear.

A sample of renal cortex was taken from each rat, for IF microscopy but it would have been impossible to cut sections from all eighty rats and so only three or four frozen sections from each strain within each diet group were processed. The

differences in glomerular uptake of ^{125}I-IgG^{+} between the strains as assessed by immunofluorescence, were neither outstanding nor very enlightening and so did not warrant displaying photographs of all the data. Immunofluorescence could have been utilised to greater effect had time allowed.

Although the fall in albumin excretion in the PUFA diet groups did not reach the same degree of statistical significance as in the previous study (section 2.2), AER tended to be lower in both strains of rat in all the experimental PUFA diet groups. Figure 2.3.3.3 shows that DA rat glomeruli take up significantly more ^{125}I-IgG^{+} than Lewis rat glomeruli in the control and all the experimental PUFA diet groups, but there is little evidence of a correlation between a PUFA diet-induced increase in GCW charge and fall in albumin excretion rate. The link between GCW charge and albumin excretion in this study may be tenuous when one considers that the significantly diminished glomerular uptake of ^{125}I-IgG^{+} in the fish oil diet groups in both Lewis and DA rats was not accompanied by any rise in albumin excretion. In general, PUFA diets tended to reduce glomerular uptake of cationic IgG (ie lower GCW charge) which one would expect to accompany a *rise* in the excretion rate of albumin.

It was interesting that the differences in glomerular uptake of ^{125}I-IgG^{+} between Lewis and DA rats were mirrored in the spleen. Both evening primrose and fish oil diets increased splenic uptake of ^{125}I-IgG^{+} to a statistically significant extent in Lewis rats and there is a suggestion that splenic uptake also, was raised in the same two diet groups in DA rats although but the results did not reach statistical significance. That the increased splenic uptake of ^{125}I-IgG^{+} occurred in the EPO and FO diet groups suggests that the effect might be prostanoid-related and could be linked to changes in blood flow.

It would be encouraging if the differences in glomerular uptake of ^{125}I-IgG^{+} between Lewis and DA rats were related to GCW charge, but if the lower splenic uptake of ^{125}I-IgG^{+} in Lewis rats is also charge-mediated, it suggests a diminished charge on all tissue surfaces in Lewis rats relative to DA rats (Deckert *et al*, 1989). Such a global diminution of cell surface charge would surely have significant implications for a variety of intercellular interactions including those between platelets and the endothelium (Rogers *et al*, 1992).

Alternative explanations for the differences in uptake of ^{125}I-IgG^{+} between the strains include:

(i) Differences in reticuloendothelial uptake. Increased glomerular and splenic uptake in DA, compared to Lewis rats suggests diminished mesangial or splenic

function in Lewis rats although the perfusion of native IgG (and IF) does preclude this to some extent. The effects of PUFA diets on glomerular or splenic blood flow are unknown. If the differences in glomerular uptake are related to mesangial function, DA rats may have an increased rate of mesangial uptake, but a slower rate of egress. Mesangial function can be assessed by measuring the uptake of chemical or heat aggregated IgG.

(ii) The delivery of ^{125}I-IgG^{+} to the glomerulus and spleen. The reduced glomerular uptake of ^{125}I-IgG^{+} in Lewis rats could be linked to vasoconstriction, coagulation and increased plasma viscosity and impaired mesangial function as a result of raised glomerular thromboxane and renin levels.

(iii) Differences in glomerular uptake of IgG (+) between Lewis and DA rats might be related to differences in the magnitude of the non-selective shunt pathway through which IgG can filter unimpeded (Tomlanovich *et al*, 1987). Were the GCW of Lewis rats to have a greater proportion of enlarged pores, more ^{125}I-IgG^{+} might pass into the glomerular ultrafiltrate: DA rats, on the other hand, may have no such increase in pore size or number and so much of the ^{125}I-IgG^{+} remains trapped within the GCW. Withdrawal of urine directly from the bladder during surgery and subsequent measurement of ^{125}I-IgG^{+} levels might help to clarify this point. Such a hypothesis does not, however, help to explain differences in splenic uptake.

Further doubts about whether this technique measures the net charge on the glomerular capillary wall relate to:

(i) The size of the IgG molecule (M_r = 55Å) in relation to the size of the GCW pores. IgG is of similar dimensions to the effective GCW pore diameter and so although IgG will pass through the endothelial fenestrae (100Å) with relative ease, it should be impeded increasingly by the GBM and epithelial slit diaphragms - although cationisation will facilitate its passage. Whether IgG is distributed evenly throughout all three layers of the GCW is unclear and only more sophisticated microscopy might elucidate this issue. Table 1.2.1 shows that both cationic ferritin (61Å, pI >8) and catalase (52Å, pI 5.7) are impeded at the level of the GBM and slit diaphragm. Perhaps the use of a smaller cationic molecule such as lactoperoxidase (38Å, pI 8) or cationised albumin, might pinpoint anionic charges within the GCW more accurately.

(ii) The use of a cationic molecule to locate anionic sites within the glomerular capillary wall may neutralise the glomerular polyanion to some extent and thus impair the normal permselective properties of the GCW (Purtell *et al*, 1979).

(iii) The effects of cationisation and radioiodination on the physico-chemical characteristics of the IgG molecule are not entirely clear.

It has been reported that four weeks of PUFA diet supplementation is sufficient to induce near-maximal fatty acid substitution of glomerular membrane phospholipids

(Marshall *et al*, 1983, Huang *et al*, 1986, Anderson *et al*, 1989) and so if the fall in albumin excretion rate was indeed mediated by the effects of PUFAs on the glomerular capillary wall, it should be apparent by this stage.

The importance of PUFA incorporation into membrane phospholipids has been acclaimed by many groups but none has attempted to assess GCW charge (Weise *et al*, 1993, Heifets *et al*, 1987, Kasiske *et al*, 1991, Clark *et al*, 1990, Clark *et al*, 1993: see section 2.3). The extent of PUFA incorporation and the effects of PUFAs on membrane fluidity (as defined by *anisotropy*) was not investigated in this study but can be studied using spin labelled probes (Dominiczak *et al*, 1991, Le Grimellec *et al*, 1992).

Summary

Despite a reduced glomerular uptake of cationic IgG (representing a diminished glomerular polyanion) in Lewis relative to DA rats, a change in the intensity or magnitude of the negative charge on the glomerular capillary wall is unlikely to be the mechanism behind the anti-proteinuric effects of PUFA diets. No evidence of any parallel increase in the uptake of ^{125}I-IgG^{+} which could be related to a fall in albumin excretion rate in the PUFA diet groups was observed in either strain of rat. In anything, PUFA diets tended to reduce capillary wall charge which would be associated with an increase, rather than a decrease in albumin filtration across the glomerular capillary wall.

The method of measuring GCW charge used in this study, may be criticised on the basis that it is neither sufficiently specific nor sensitive. The optimal dose of IgG to detect differences in GCW charge should be titrated more thoroughly in a large number of animals.

2.4.1 Introduction and experimental design

Characteristics such as the size, shape and electrostatic charge of the albumin molecule contrain its passage across the GCW (Brenner *et al*, 1978, Rennke *et al*, 1978; 1979, Purtell *et al*, 1979). The human serum albumin molecule is oval-shaped (≈ 38Å x 150Å) with a mass of ≈ 69,000 kD, effective radius of 36Å and isoelectric point of 4.7 - 4.9 (Kuntz *et al*, 1974). It is a single polypeptide chain comprising 585 amino acids folded into three semi-autonomous but homologous domains and has a half-life ($t_{1/2}$) of 19 days. Compared to other proteins, albumin contains very few tryptophan and methionine residues but an abundance of cysteine and other charged amino acids such as aspartic and glutamic acid, lysine and arginine which endow human and rat albumin with an net charge of -15 and -12 mEq/L, respectively, at physiological pH. Rat serum albumin has a molecular mass of 64, 000 kD and a half-life of 2.2 days (Peters and Peters, 1972).

Because of its inherent anionic charge, circulating serum albumin is repelled, to a large extent, by the complement of fixed negative charges on the glomerular capillary wall. The tubular reabsorption of albumin is also dependent upon its charge and the effects of tubular fluid pH (Christensen *et al*, 1983, Christensen and Bjerke, 1986). Cationic albumin is not only filtered more readily but is also reabsorbed preferentially by the proximal tubule and when perfused, increases the permeability of the rat glomerulus to both modified and endogenous albumin (Baldamus *et al*, 1975, Purtell *et al*, 1979). In contrast, less of the anionic forms of ferritin, albumin, lysozyme and cytochrome C were reabsorbed by the proximal tubules relative to their cationic isoforms, relative to their cationic isoforms (Christensen *et al*, 1983). In addition to maintaining serum oncotic pressure, albumin transports and distributes calcium, fatty acids, bilirubin, hormones and drugs and it is this capacity to bind ligands which gives albumin its structural diversity and heterogeneity and makes its troublesome to study (Spector, 1975, Folco *et al*, 1979, Naval *et al*, 1982). Changing pH conditions can also generate isomers of albumin with altered molecular characteristics, known as N-F transitions (Foster *et al*, 1977).

Glucose binds covalently to albumin at lysine-525 and it is estimated that 6-15% of serum albumin is glycosylated but the effects of glycosylation on the charge on the albumin molecule are unclear (Day *et al*, 1979, Dolhofer and Weiland, 1980, Shaklai *et al*, 1984). In theory, glycosylation should render albumin more anionic but more often, cationic isoforms are observed (Candiano *et al*, 1983 and Ghiggeri *et al*, 1984). Glycosylated albumin is also filtered more readily and reabsorbed more avidly by endothelial cells than native albumin (Williams *et al*, 1981, Sampietro *et al*, 1987).

The binding of ligands can elicit both competitive and cooperative effects within the albumin molecule. For instance, aspirin inhibits glucose binding to albumin by acetylating lysine-199 (Walker *et al*, 1976, Garlick and Mazer, 1983) and fatty acid binding can induce conformational changes within the albumin molecule which heighten its affinity for calcium (Aguanno *et al*, 1982). Reduced calcium excretion in rats (Wortsman and Traycoff, 1980) and humans (Ladenson and Shyong, 1977, Whitsett and Tsang, 1977) has been attributed to sequestration of free calcium by circulating fatty acids. Physiological levels of fatty acids do not affect calcium-to-albumin binding but dietary manipulation can raise plasma fatty acid levels high enough to alter calcium levels. Evening primrose oil, rich in γ-linolenic acid, reduced urine calcium excretion in both normal and hypercalciuric diabetic rats (Tulloch *et al*, 1994)

The rate of, and strength with which ligands bind to albumin can vary greatly. Not only does the glycosylation of albumin *in vivo* occur some ten times more rapidly than that of haemoglobin but it reduces the affinity of albumin for bilirubin by 50% and that for long chain fatty acids some twenty-fold (Brown *et al*, 1982, Shaklai *et al*, 1984, Kragh-Hansen *et al*, 1990). Calcium ions bind to albumin more weakly than organic anions like bilirubin, or the long chain fatty acids, oleate, stearate, linoleate and palmitate which, with binding affinities in the region of of pKa = 10^{-8} M, are amongst the most tightly bound ligands transported by albumin. Acylated albumin is the predominant form circulating in the bloodstream and is more stable and resistant to hydrolysis and heat denaturation than native de-fatted albumin (Klapper *et al*, 1964, Yu *et al*, 1984). In analbuminaemic subjects with a high fatty acid to albumin ratio exogenous albumin is catabolised more slowly than native albumin (Dammacco *et al*, 1980, Mego *et al*, 1984).

Serum albumin has six high affinity, and several more (20 to 60) lower affinity binding sites for fatty acids (Brown and Shockley, 1982) and under normal physiological conditions, these fatty acid binding sites are not fully occupied. The strength of binding between albumin and fatty acids is derived principally from hydrophobic interactions between the acyl tail of the fatty acid and the hydrophobic interior of the binding site(s). The tail of the fatty acid inserts into the binding site, leaving carboxyl (COO-) groups in the head of molecule free to introduce additional anionic charges on the surface of the albumin molecule (Brown, 1977, Peters and Sjoholm, 1977, Peters, 1985, Kragh-Hansen, 1990). The carboxyl (COO^-) groups in the head of the fatty acid molecule do not play a major role in its binding to albumin (Peters, 1985).

The binding of fatty acids induces conformational changes within the albumin molecule which affect its filtration (Soetewey *et al*, 1972). Not only is the charge on the albumin molecule modified, but it becomes more spherical, compact and rigid, all of which are characteristics that retard its passage through the glomerular capillary wall. Removal of the fatty acids bound to human serum albumin converted it to a electrophoretically heterogeneous material but their replacement restored its electrophoretic homogeneity (Evenson and Deutsch, 1978). In relation to the degree of unsaturation and double bond configuration *(cis* or *trans),* fatty acids may alter the conformation of albumin to liberate additional COO- groups to bind calcium. Low levels (2 moles) of *cis* unsaturated fatty acids can influence calcium - albumin binding markedly (Aguanno and Ladenson, 1982).

Experimental design

Although initial speculation that the anti-proteinuric effects of PUFA diets were related to changes in either glomerular vasoactive mediators or the magnitude of the glomerular polyanion in the preceding studies (2.1-2.3), seems to have been groundless, the effects of PUFA diets on the electrostatic charge on the albumin molecule remained to be explored. The purpose of this study was to establish whether feeding PUFA diets altered the charge or isoelectric point (pI) of the serum albumin molecule.

The isoelectric point of a molecule is often measured by isoelectric focusing (IEF) but in this study, was measured by chromatofocusing (CF). The isoelectric point (pI) of a molecule is that pH at which it is electrically neutral. Proteins with a low pI value are negatively charged (anionic) whereas those with a high pI value are positively charged (cationic). The principal behind chromatofocusing is that at a pH below its isoelectric point, a protein will become positively charged and pass straight through an anion (-) exchange column, emerging in the eluting buffer. However, if as it migrates down the column, it encounters buffer of increasing pH, the charge on the protein reverses and when the pH of the buffer is higher than the pI of the protein, it will bind to the anion exchanger and remain bound until the developing pH gradient again drops below the pI of the protein, when it is released - until the pH rises above the pI and it binds again. This process is repeated until the protein emerges from the column at its isoelectric point.

The pI of albumin was measured by chromatofocusing in a selection of serum samples collected in study 2.2. Only Lewis rats fed control diet (n = 7), evening primrose oil diet (n = 4), fish oil diet (n = 3) and olive oil diet (n = 2) were studied.

2.4.2 Materials and methods

2.4.2.1 Chromatofocusing

All solutions used for chromatofocusing were prepared with deionised water and degassed using a water vacuum pump for 30 minutes before use.

Start buffer (pH 6.2) 0.025M Histidine buffer. 1.19 g l-histidine (Sigma Chemical Company: H-8000) dissolved in 500 mls of deionised water and pH adjusted to 6.2 after further degassing.

Eluate buffer (pH 4) 62.5 mls of Polybuffer™ 74 (Pharmacia Biotech) was reconstituted to a final volume of 500 mls with deionised water and the pH adjusted to 4 with HCl. The solution was degassed again and the pH adjusted if necessary.

Sephadex G25 5g of Coarse Sephadex G25 (Pharmacia Fine Chemicals) was swollen in 50 mls of *start* buffer in a boiling water bath for one hour and then allowed to cool.

To generate the *anion exchange column*, a slurry was prepared by mixing 40 mls of Polybuffer exchanger, PBE 94™ (Pharmacia Biotech) with 20 mls of start buffer. The slurry was degassed for 30 minutes and then poured into a C 10/40 glass chromatography column (Pharmacia Biotech). Sephadex G25 was layered on top to a depth of about 2 cm to facilitate even mixing of sample and buffer. With a top adapter in position, the column was equilibrated with at least four column volumes of start buffer.

Attempts were made to maintain the volumetric flow rate at around 28 -30 cm per hr for optimal resolution. The volumetric flow rate through the packing (in mls per hour) is equal to the cross sectional area (mm) of the column multiplied by the linear flow rate (in cm per hour). Column packing and flow were checked using cytochrome C which, with a pI of 10.5, should pass straight through the column.

Control samples

One ml sample volumes of human serum albumin (Pentex® Fraction V, Miles Laboratories; 10 mg per ml^{-1}), human serum diluted tenfold and rat serum albumin (Sigma Chemical Company: A-6414) diluted to 2 mg per ml in start buffer (pH 6.2) were run as control samples.

Test samples

One ml of rat serum diluted 1 : 9 with start buffer (pH 6.2) was introduced on to the column with *eluate* buffer (pH 4) using a pump (LKB Varioperpex® II) at a flow rate of 0.35 mls per minute which was equivalent to an effective linear flow rate of 27 mls cm^{-1} hr^{-1} under these conditions. Eluate from the column was collected in 1.5 - 2 mls fractions by a fraction collector (LKB 2070 Ultrorac® II) and the tubes containing albumin were located using Labstix. In each consecutive fraction, pH was measured to two decimal places using a digital read-out pH meter (Horiba F.8L) and albumin levels were measured by immunoturbidimetry (see section 2.2.2.1). At the end of each run, the column was regenerated using several column volumes of 1M NaCl before it was re-equilibrated with start buffer (pH 6.2).

2.4.3 Results

During the initial chromatofocusing runs, every fraction was collected and the pH of every fifth fraction measured to monitor the development of the pH gradient (see Figures 2.4.3.1 - 2.4.3.7). Each fraction volume was between 1.5 and 2 mls.

The precision of the IT standard curve is shown in the following table

Standard mg/100 mls	n	OD (405 nm) mean	SD	CV (%)
1	22	0.0645	0.005	7.65
2	22	0.0965	0.006	6.55
3	22	0.1280	0.006	4.60
5	22	0.1805	0.008	4.50
8	22	0.2520	0.012	4.80
10	22	0.2870	0.012	4.40
15	22	0.3705	0.012	3.40

The chromatofocusing profiles of various control and test serum samples are shown as follows:

Control samples

- human serum albumin - 10 mg (Figures 2.4.3.1a and 2.4.3.2a).
- human serum (tenfold dilution) (Figures 2.4.3.3a and 2.4.3.4a)
- rat serum albumin - 2 mg (Figure 2.4.3.5a)

Test serum samples

• Control diet	Rat 7	Figure 2.4.3.6(a)
	Rat 9	Figure 2.4.3.6(b)
	Rat 9	Figure 2.4.3.6(c) rpt
	Rat 10	Figure 2.4.3.6(d)
	Rat 1	Figure 2.4.3.6(e)
	Rat 3	Figure 2.4.3.6(f)
	Rat 6	Figure 2.4.3.6(g)
	Rat 2	Figure 2.4.3.6(h)

• Primrose oil diet	Rat 7	Figure 2.4.3.7(a)
	Rat 6	Figure 2.4.3.7(b)
	Rat 9	Figure 2.4.3.7(c)
	Rat 4	Figure 2.4.3.7(d)
• Fish oil diet	Rat 6	Figure 2.4.3.8(a)
	Rat 10	Figure 2.4.3.8(b)
	Rat 4	Figure 2.4.3.8(c)
• Olive oil diet	Rat 6	Figure 2.4.3.9(a)
	Rat 9	Figure 2.4.3.9(b)

The total amount of albumin recovered was calculated and displayed in the box under the curve.

Chromatofocusing profiles illustrate that serum albumin eluted from the anion exchange column as an unresolved double peak or with a distinct shoulder. Only the first few control samples (human serum albumin (Figures 2.4.3.1a and 2.4.3.2a) and tenfold dilutions of human serum (Figures 2.4.3.3.a and 2.4.3.4a) were run in duplicate.

Because pH is measured on a logarithmic scale, data were log-transformed prior to calculating the mean value and a virtual mid-point value between the two peaks was recorded as the pI (see Table 2.4.3.1). For example, serum albumin from Lewis rat (Figure 2.4.3.6g) fed control diet eluted with one peak at pH 4.98 and another at pH 4.91 and so a pI value of 4.944 was assigned.
The pI of serum albumin from rats fed evening primrose oil, fish oil or olive oil diet was significantly lower than that from rats fed a standard laboratory rat diet. There was a significant difference in the median pI values of serum albumin between the control and collective PUFA (EPO, FO and OO) diet groups [**Control vs PUFA:** 4.944 vs 4.835, CI = 0.064 to 0.170, p = 0.001] (see Figure 2.4.3.10).

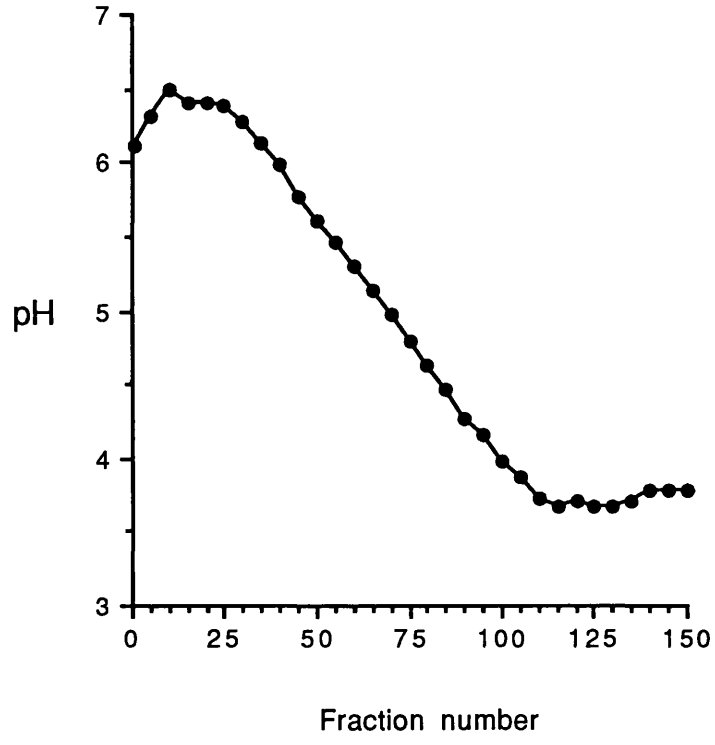


Figure 2.4.3.1 Development of pH gradient (flow rate 38 cm hr $^{-1}$)

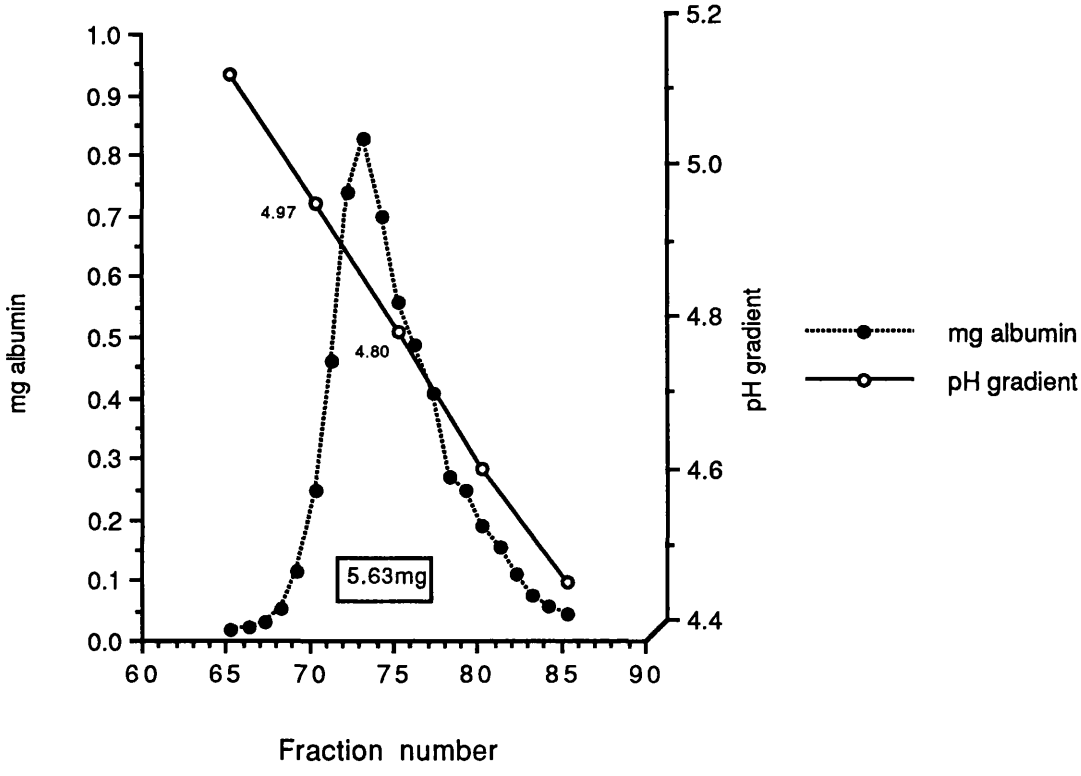


Figure 2.4.3.1(a) Chromatofocusing of human serum albumin (10 mgs)

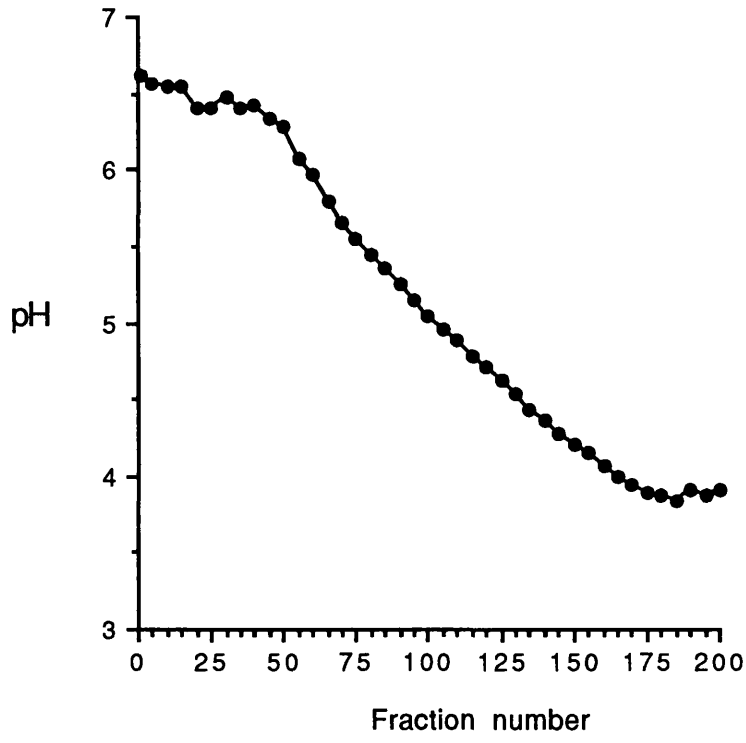


Figure 2.4.3.2 Development of pH gradient (flow rate = 30 cm hr^{-1})

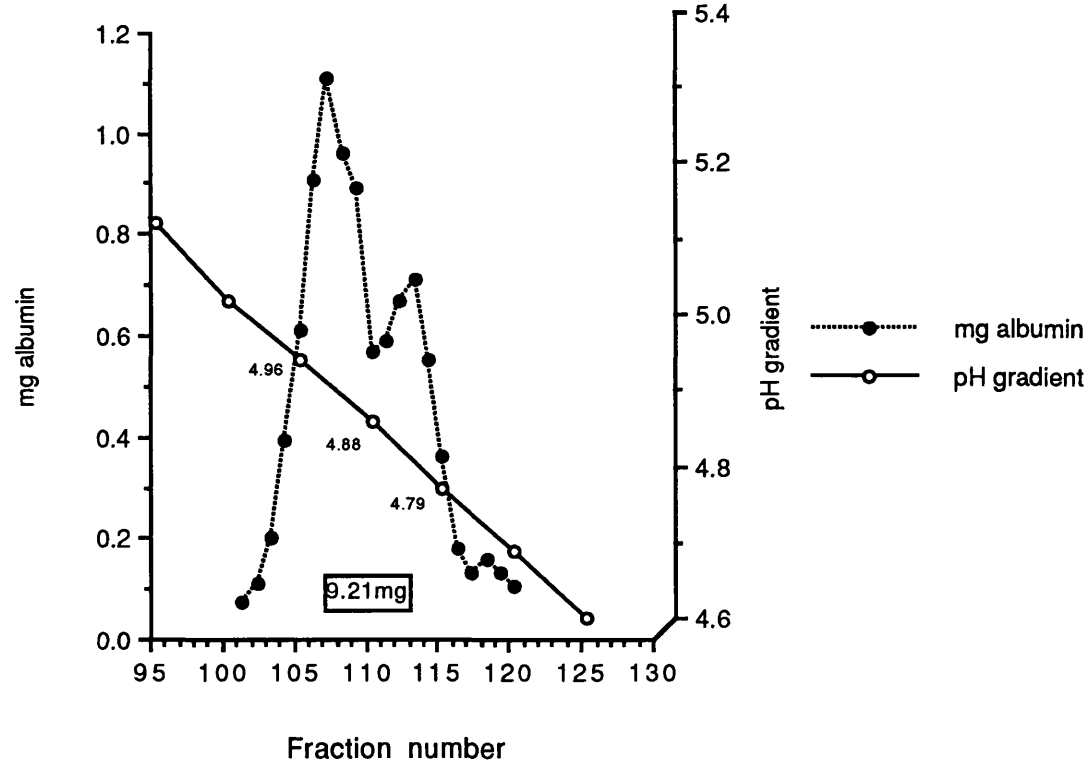


Figure 2.4.3.2(a) Chromatofocusing of human serum albumin (10 mgs)

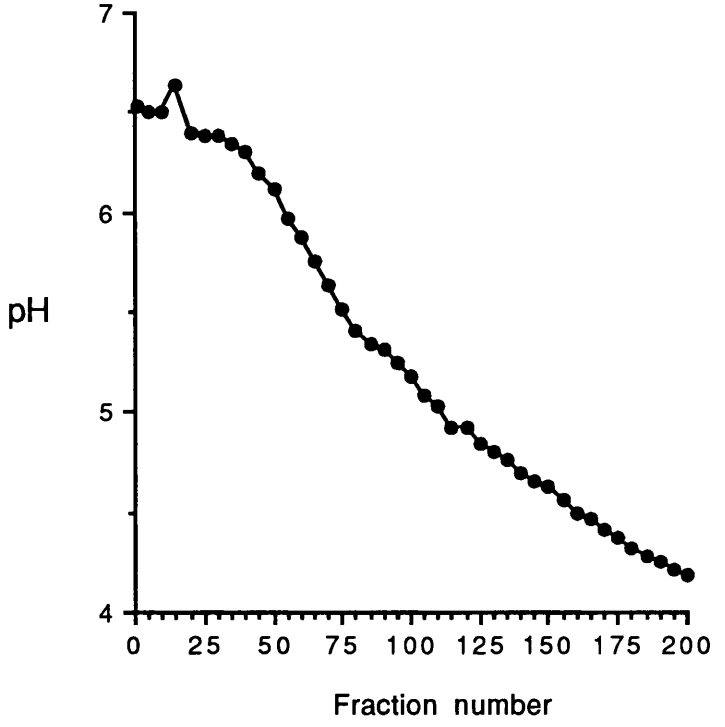


Figure 2.4.3.3 Development of pH gradient (flow rate = 25 cm hr^{-1})

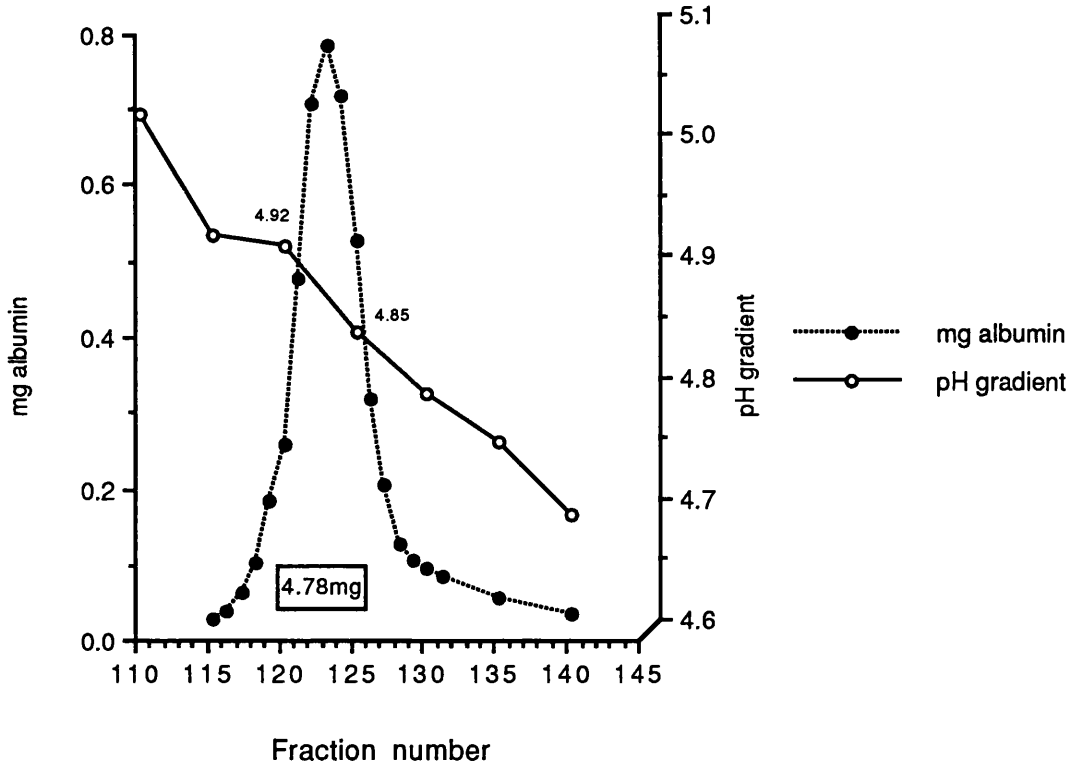


Figure 2.4.3.3(a) Chromatofocusing of human serum (1:9)

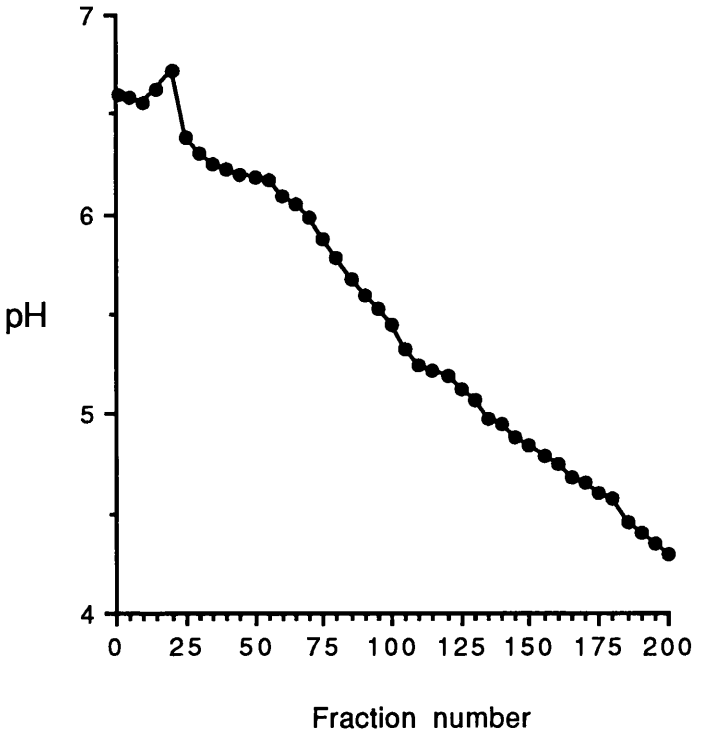


Figure 2.4.3.4 Development of pH gradient (flow rate = 25 cm hr^{-1})

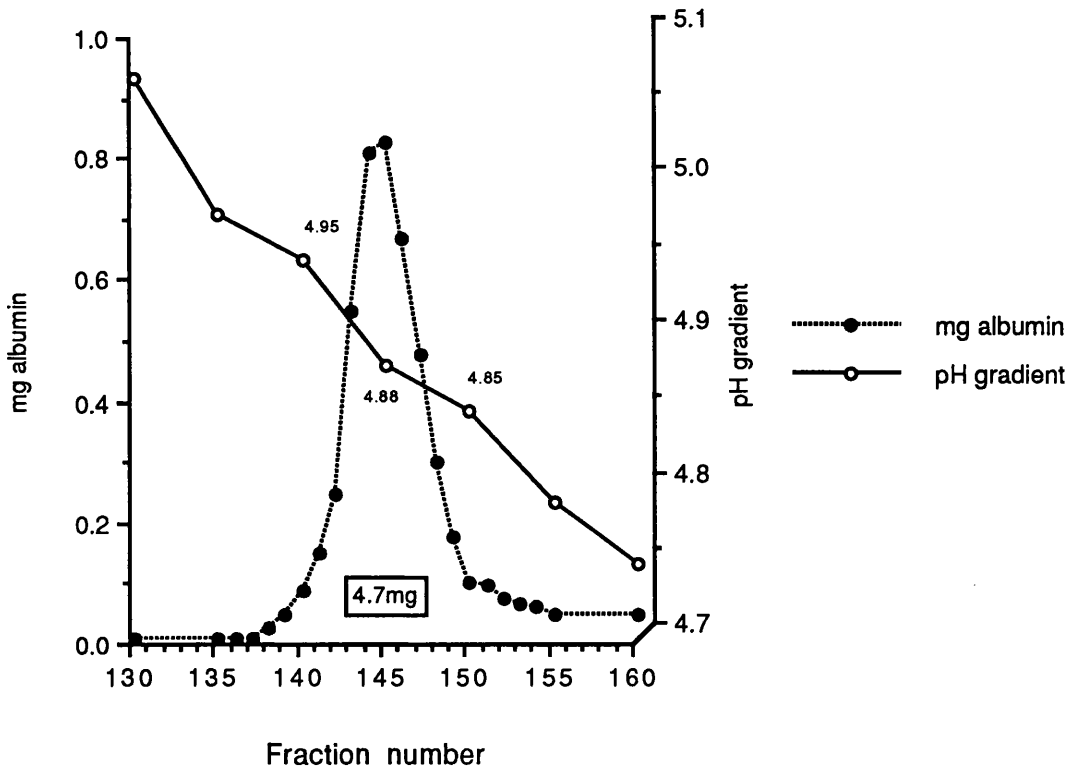


Figure 2.4.3.4(a) Chromatofocusing of human serum (1:9)

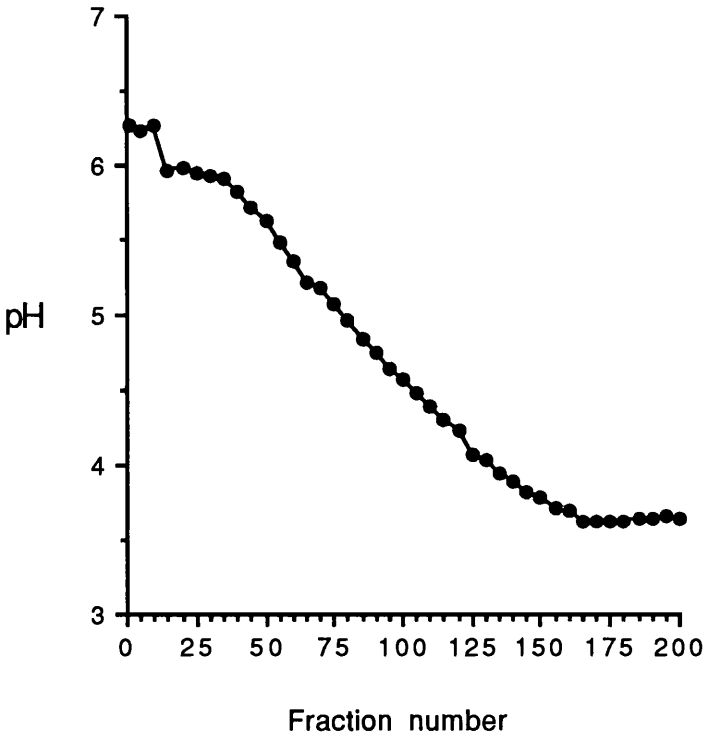


Figure 2.4.3.5 Development of pH gradient (flow rate = 30 cm hr^{-1})

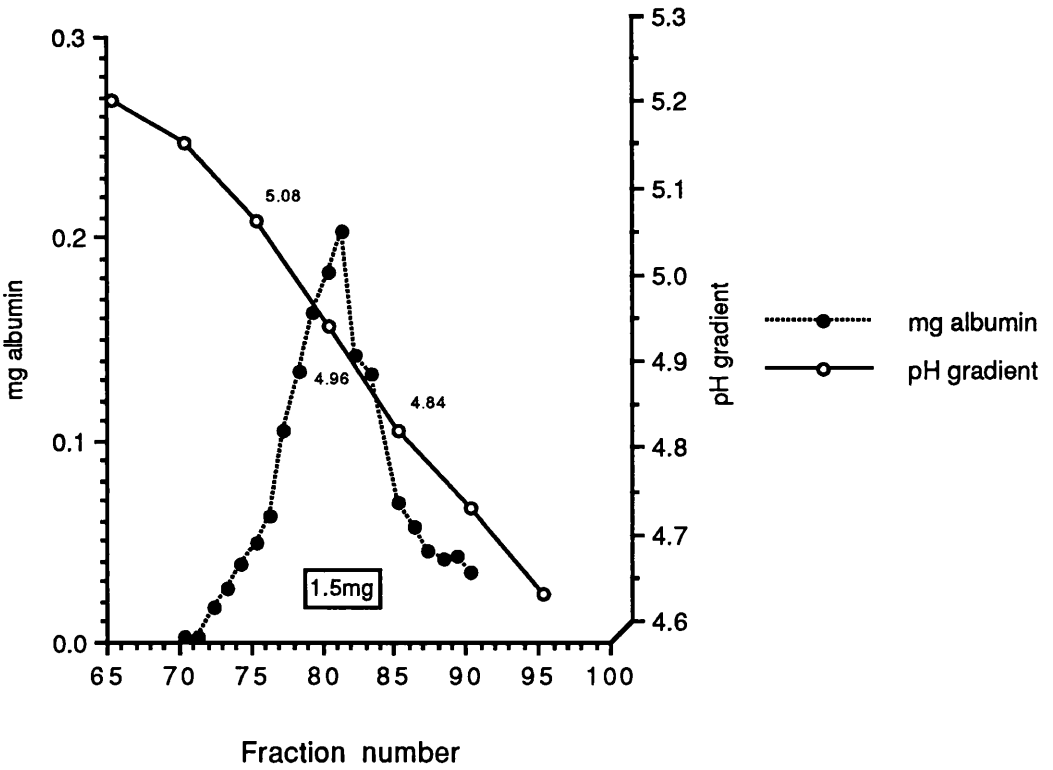


Figure 2.4.3.5(a) Chromatofocusing of rat serum albumin (2 mgs)

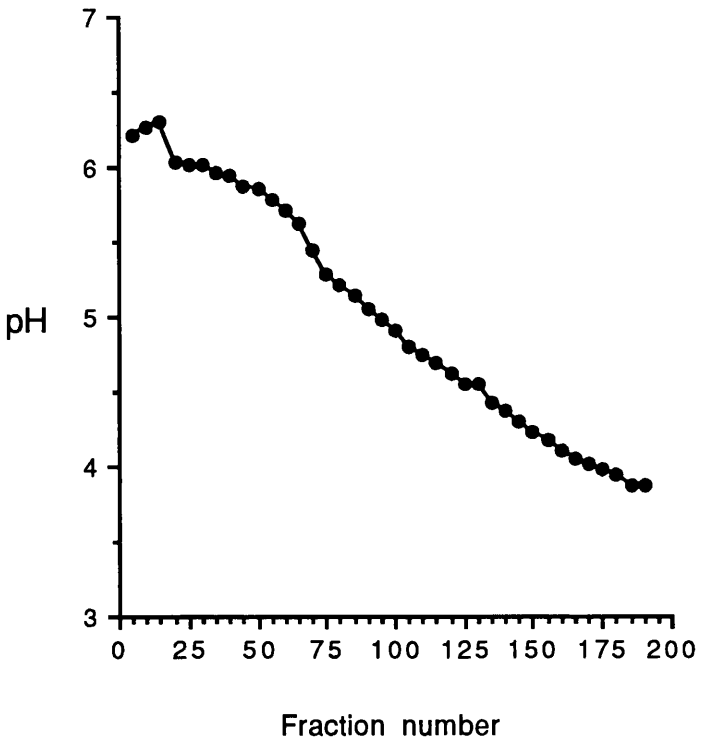


Figure 2.4.3.6 Development of pH gradient (flow rate = 27 cm hr^{-1})

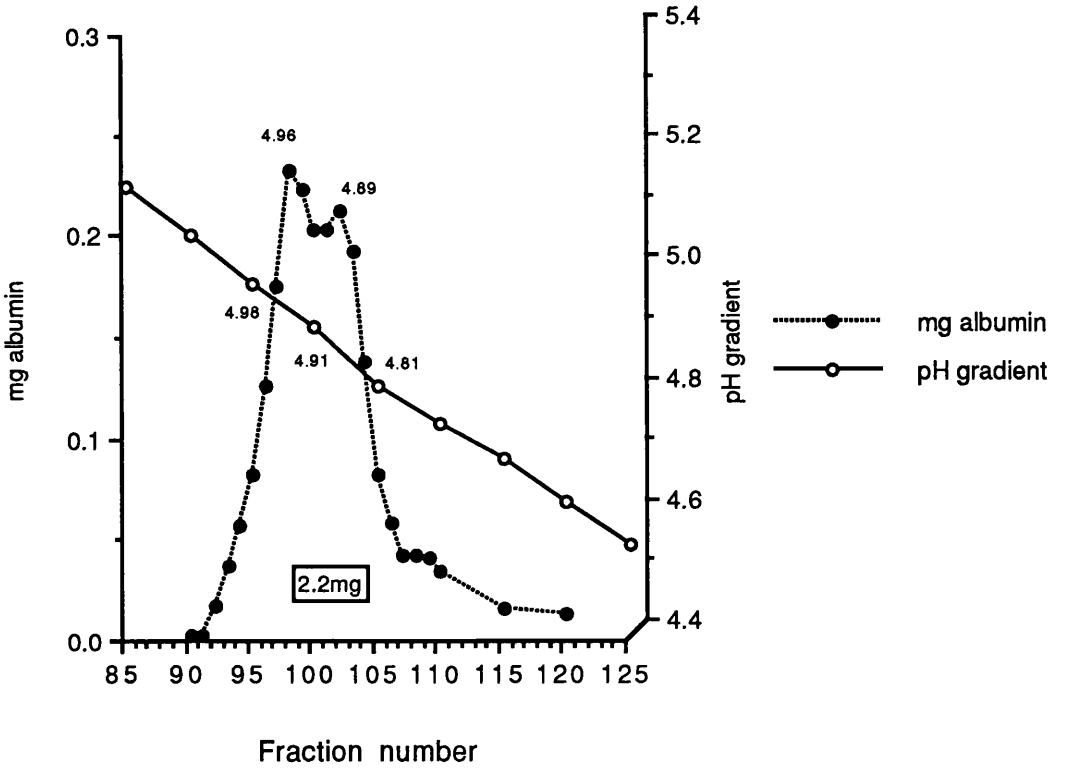


Figure 2.4.3.6(a) Chromatofocusing of Lewis rat (7) serum - control diet

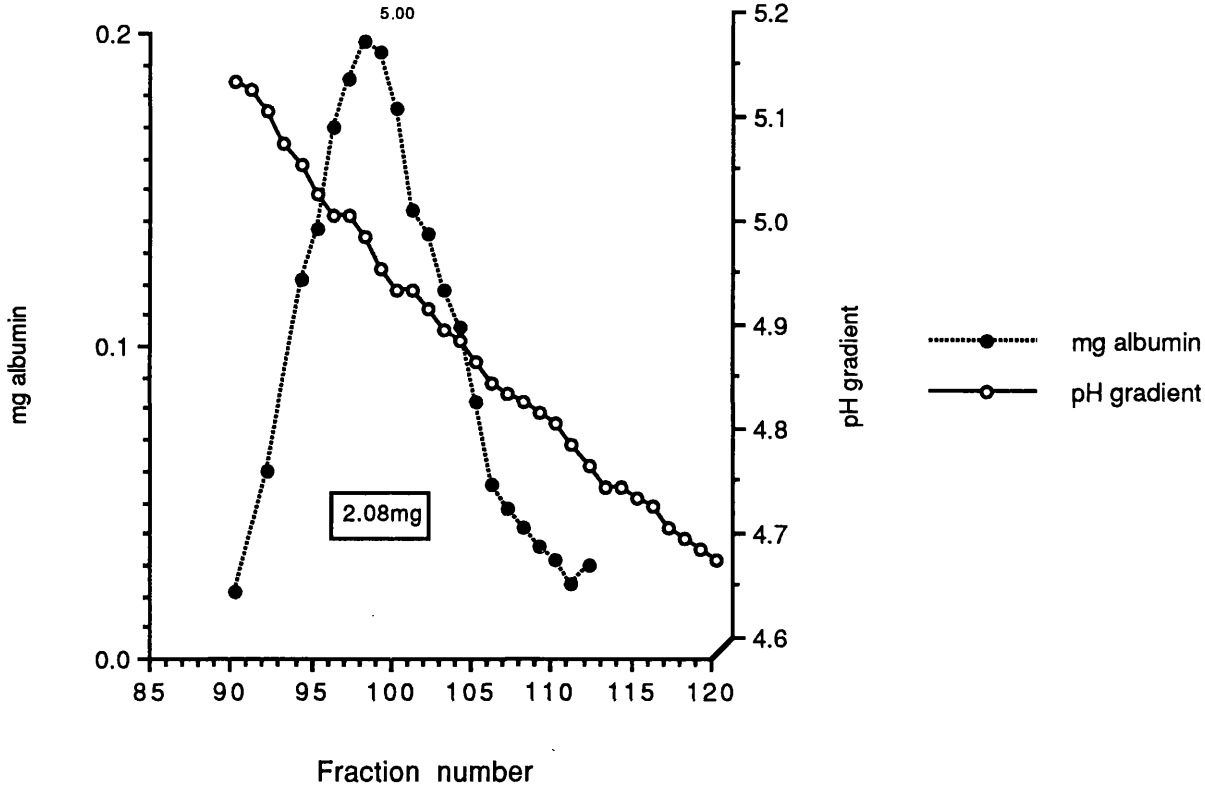


Figure 2.4.3.6(b) Chromatofocusing of Lewis rat (9) serum - control diet

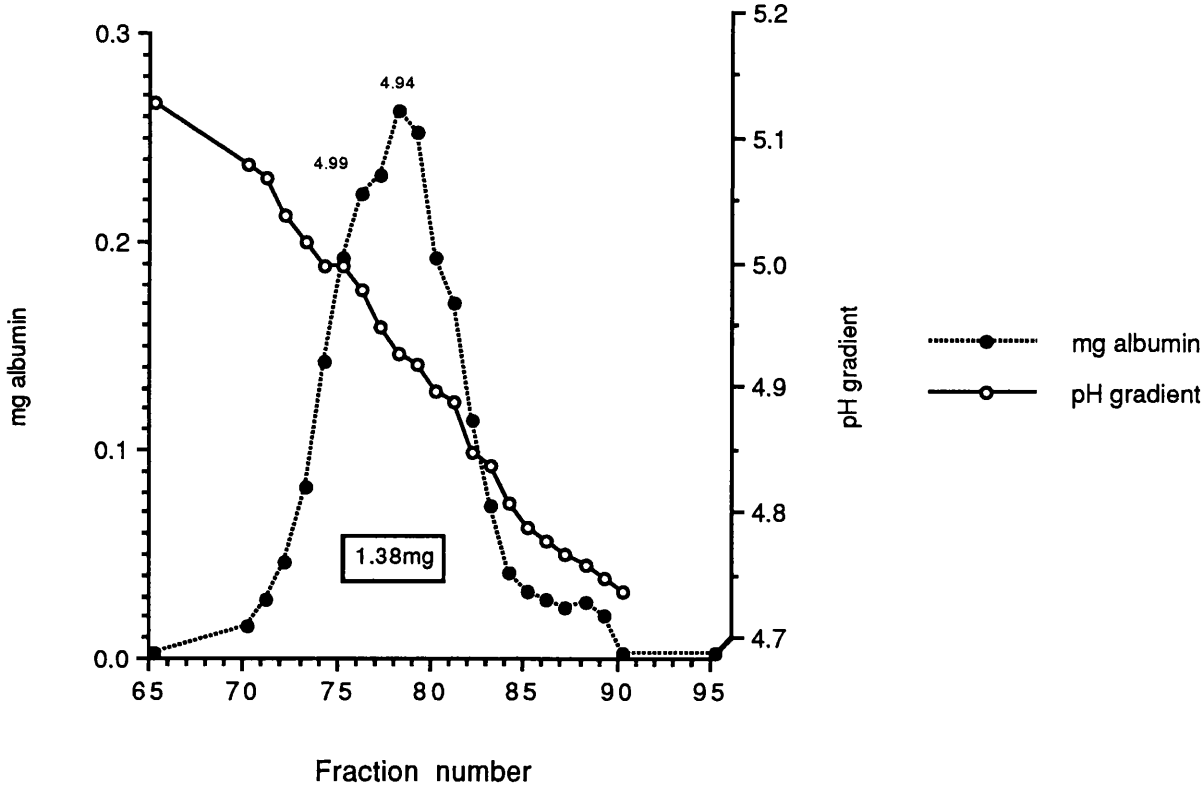


Figure 2.4.3.6(c) Chromatofocusing of Lewis rat (9) serum - control diet (repeat)

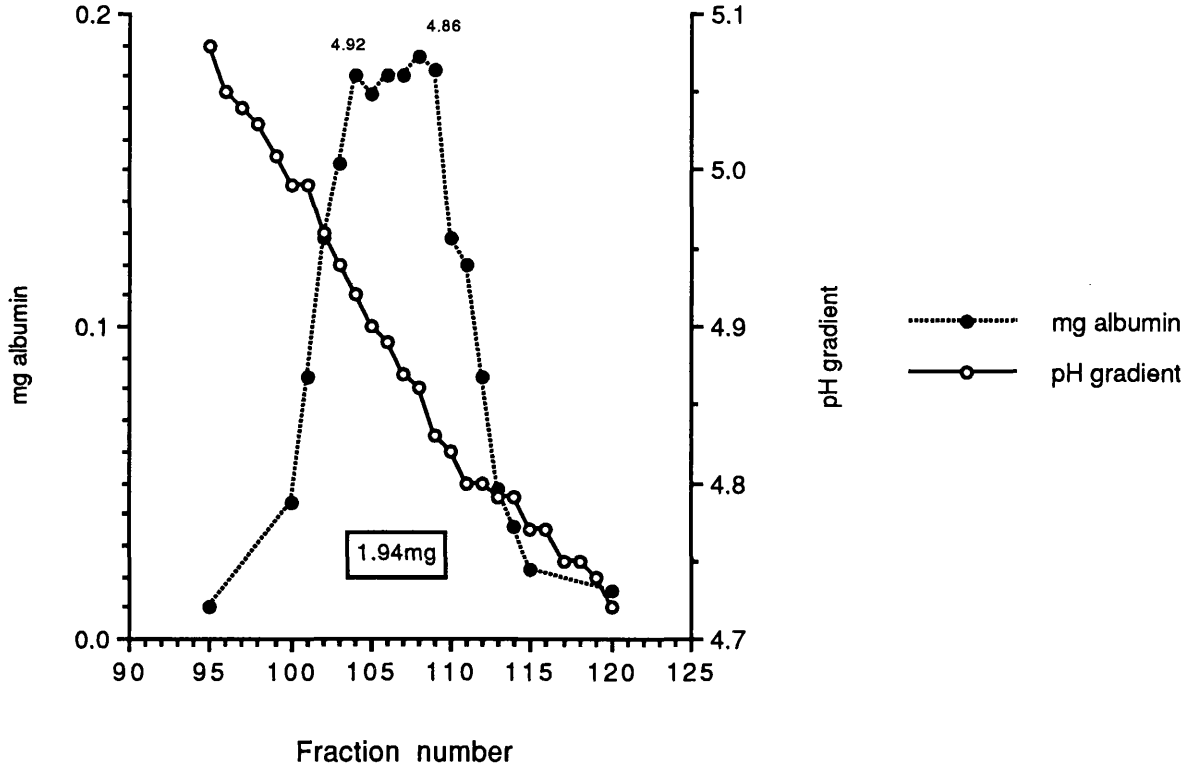


Figure 2.4.3.6(d) Chromatofocusing of Lewis rat (10) serum- control diet

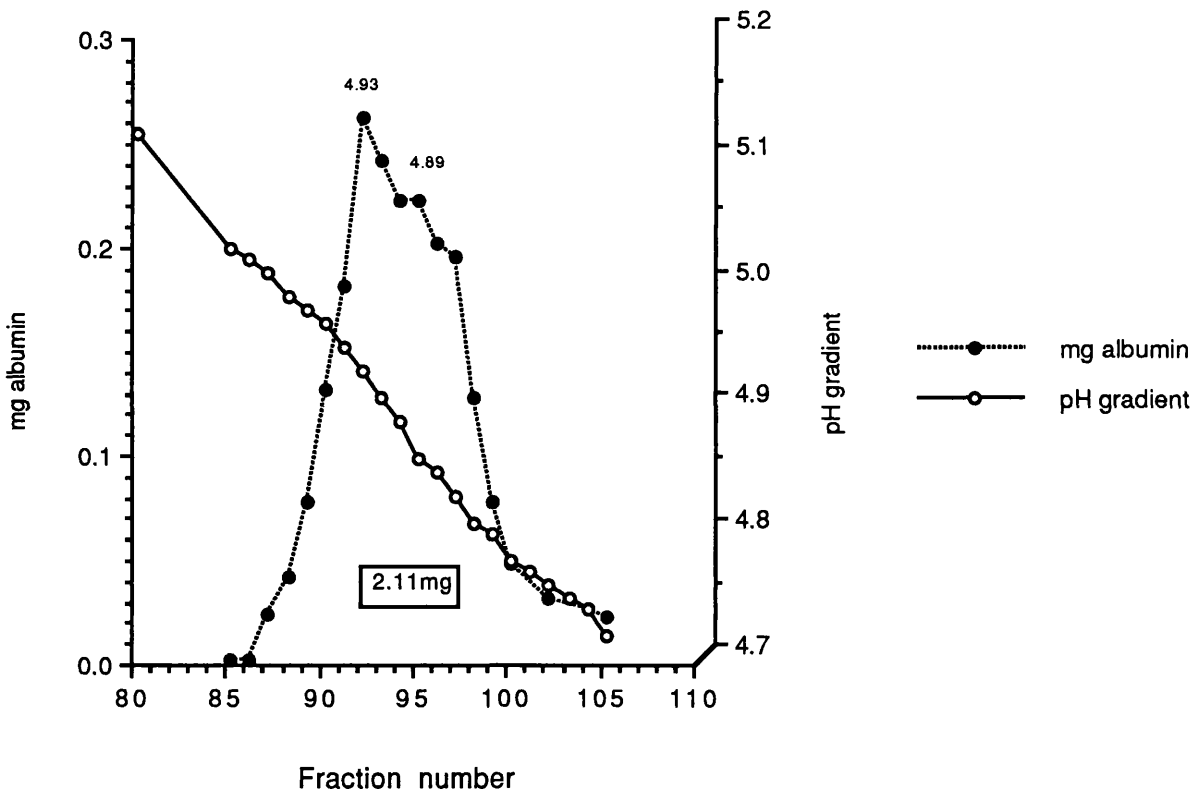


Figure 2.4.3.6(e) Chromatofocusing of Lewis rat (1) serum - control diet

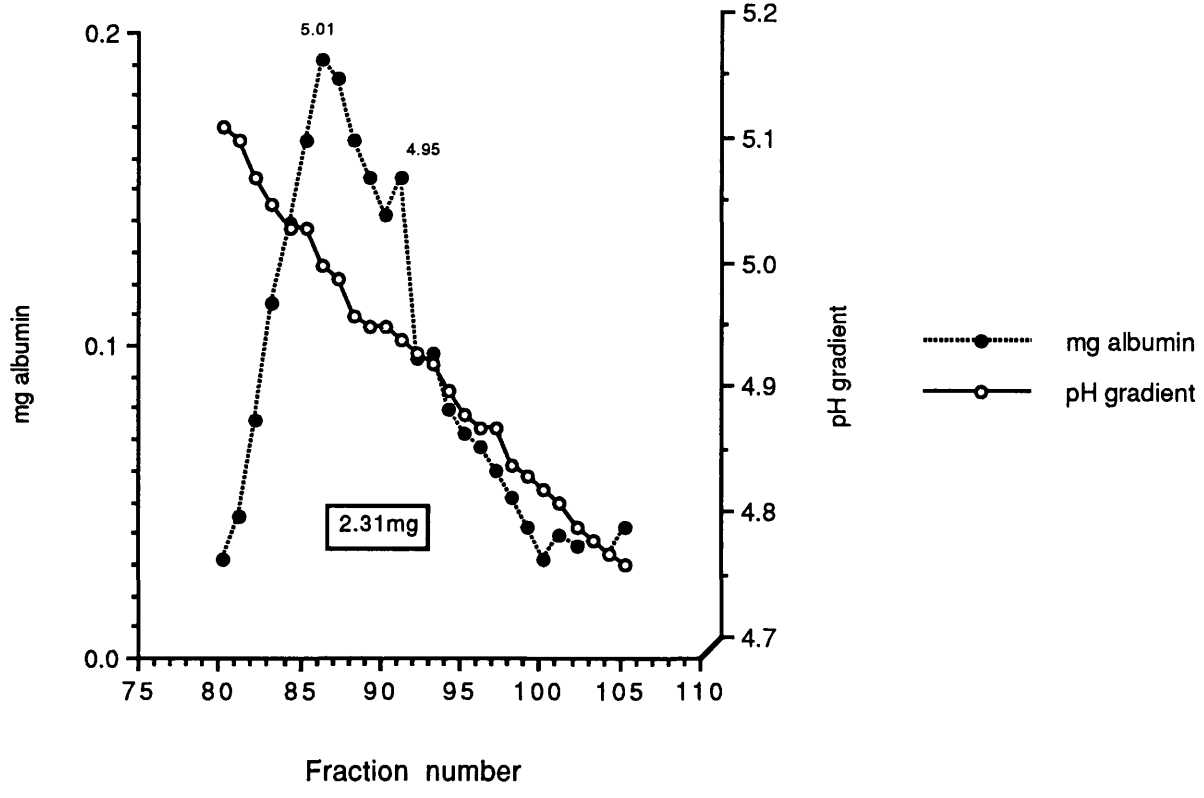


Figure 2.4.3.6(f) Chromatofocusing of Lewis rat (3) serum - control diet

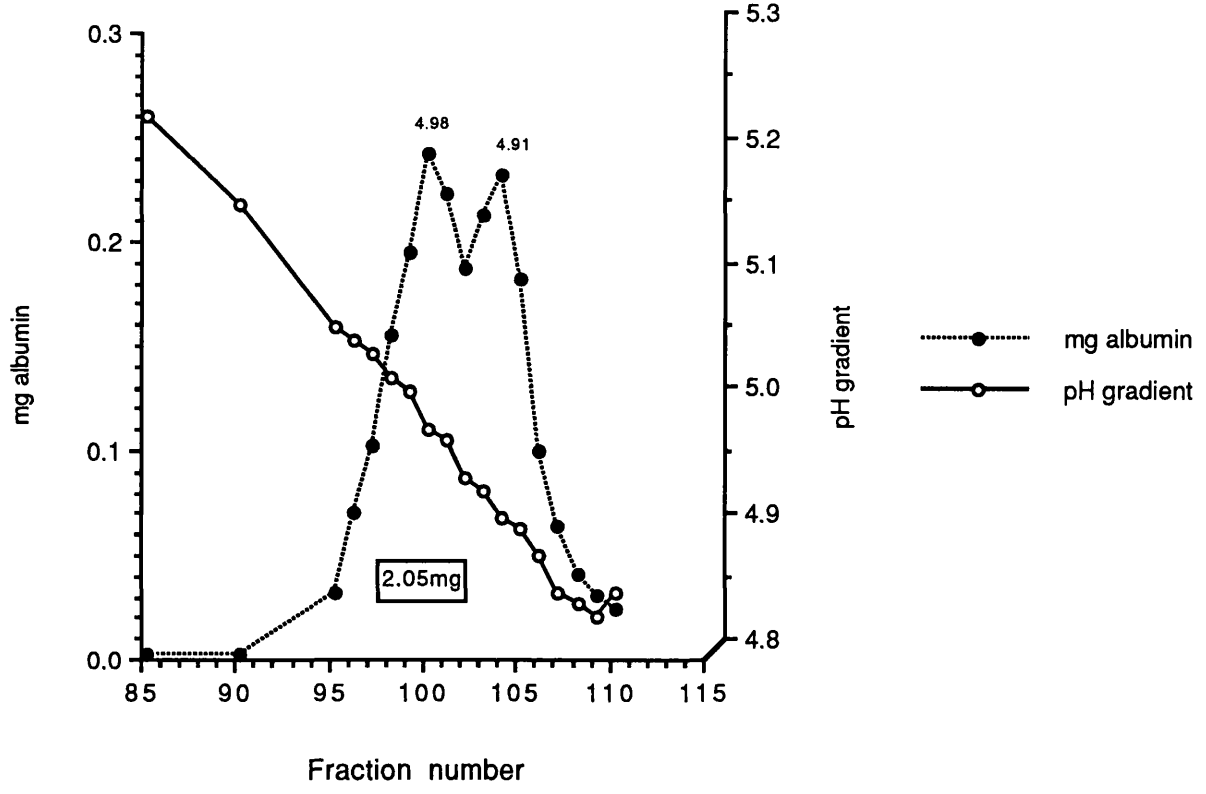


Figure 2.4.3.6(g) Chromatofocusing of Lewis rat (6) serum - control diet

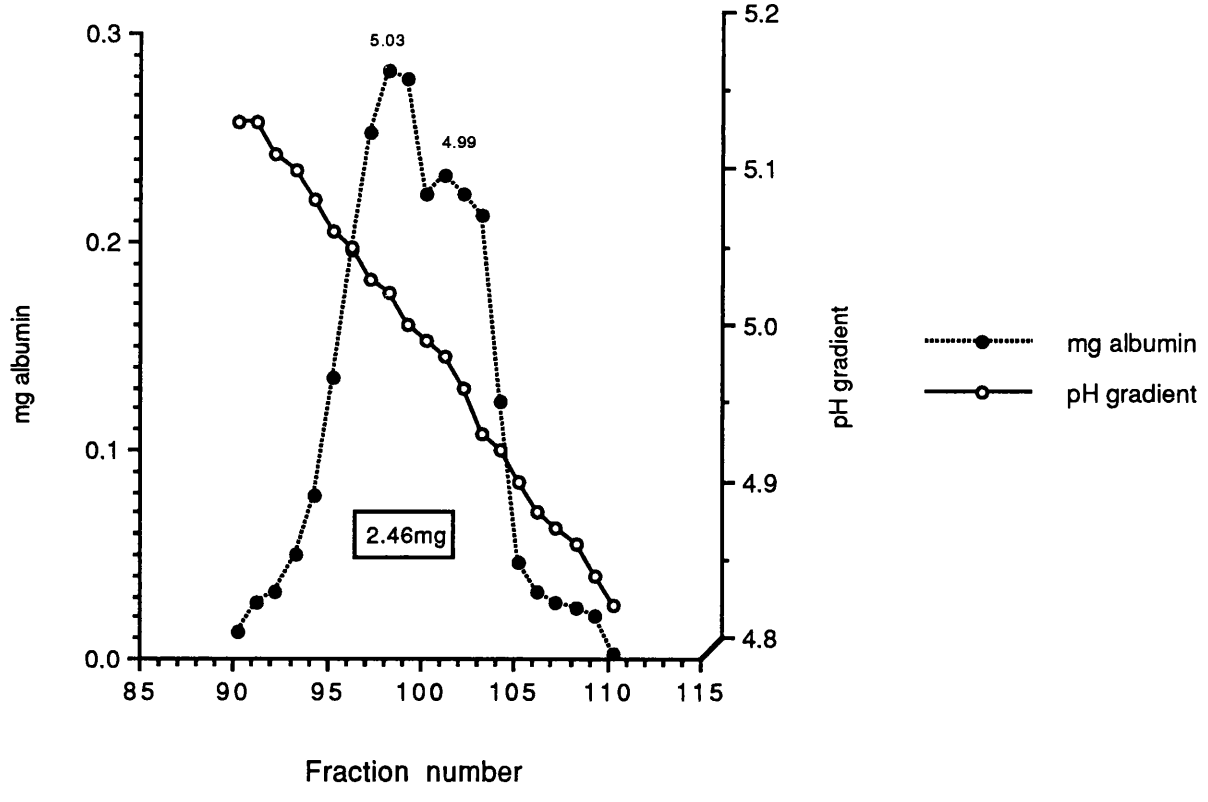


Figure 2.4.3.6(h) Chromatofocusing of Lewis rat (2) serum - control diet

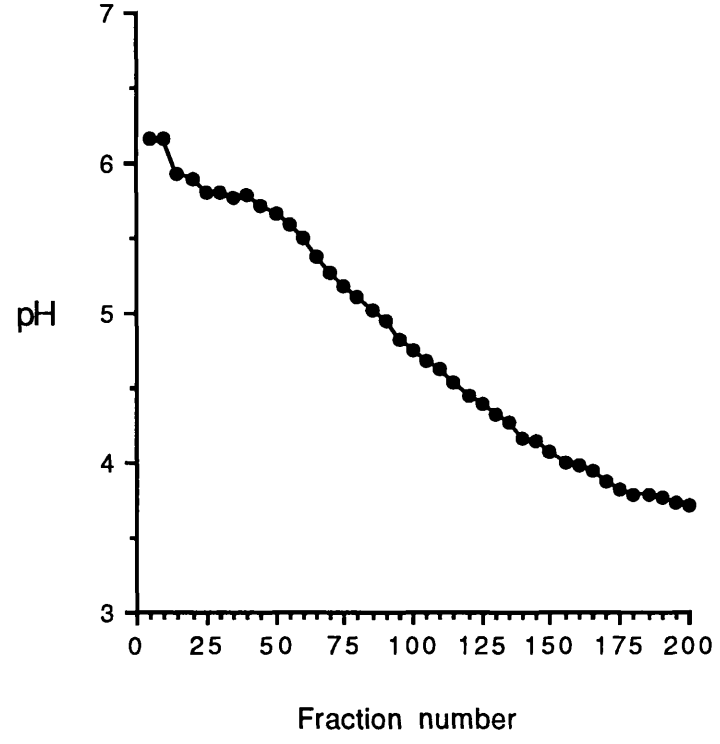


Figure 2.4.3.7 Development of pH gradient (flow rate = 27 cm hr^{-1})

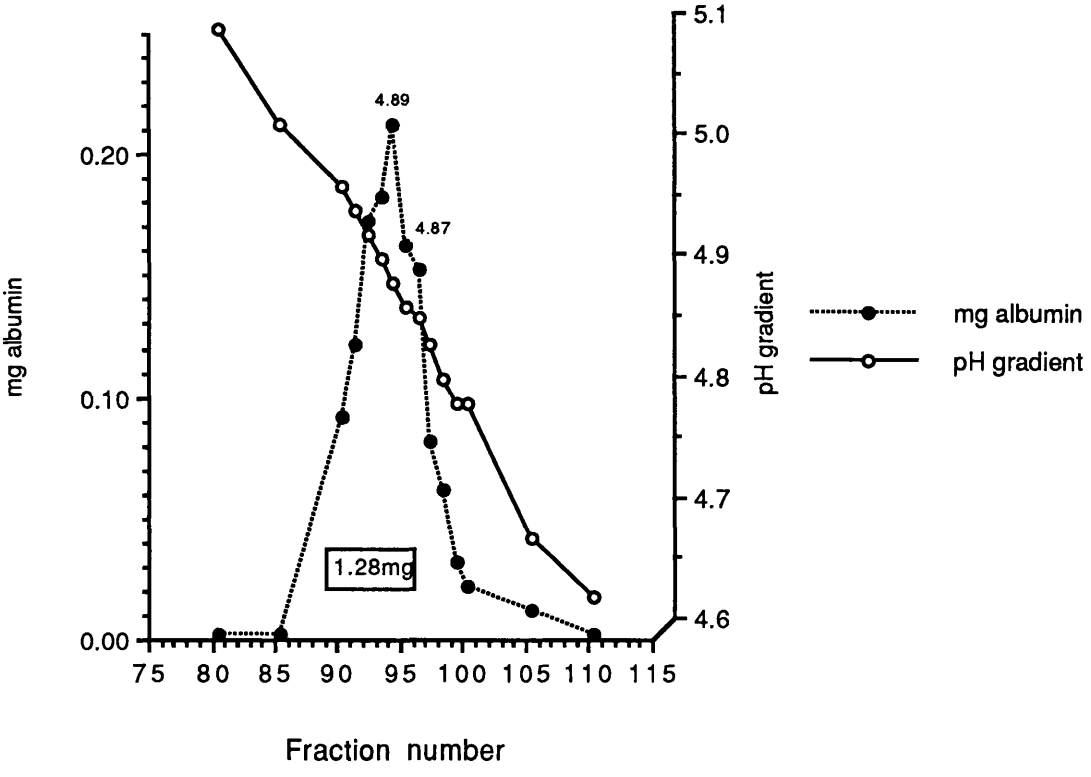


Figure 2.4.3.7(a) Chromatofocusing of Lewis rat (7) serum - Primrose diet

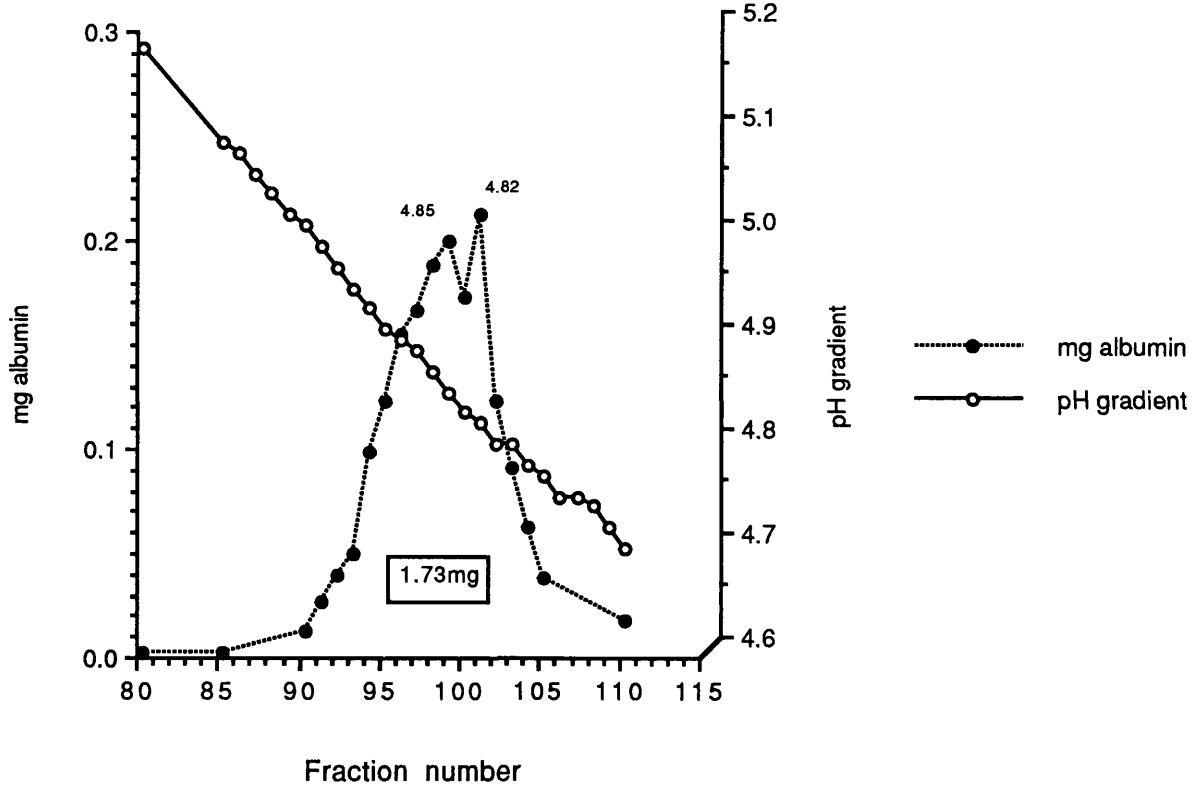


Figure 2.4.3.7(b) Chromatofocusing of Lewis rat (6) serum - primrose oil diet

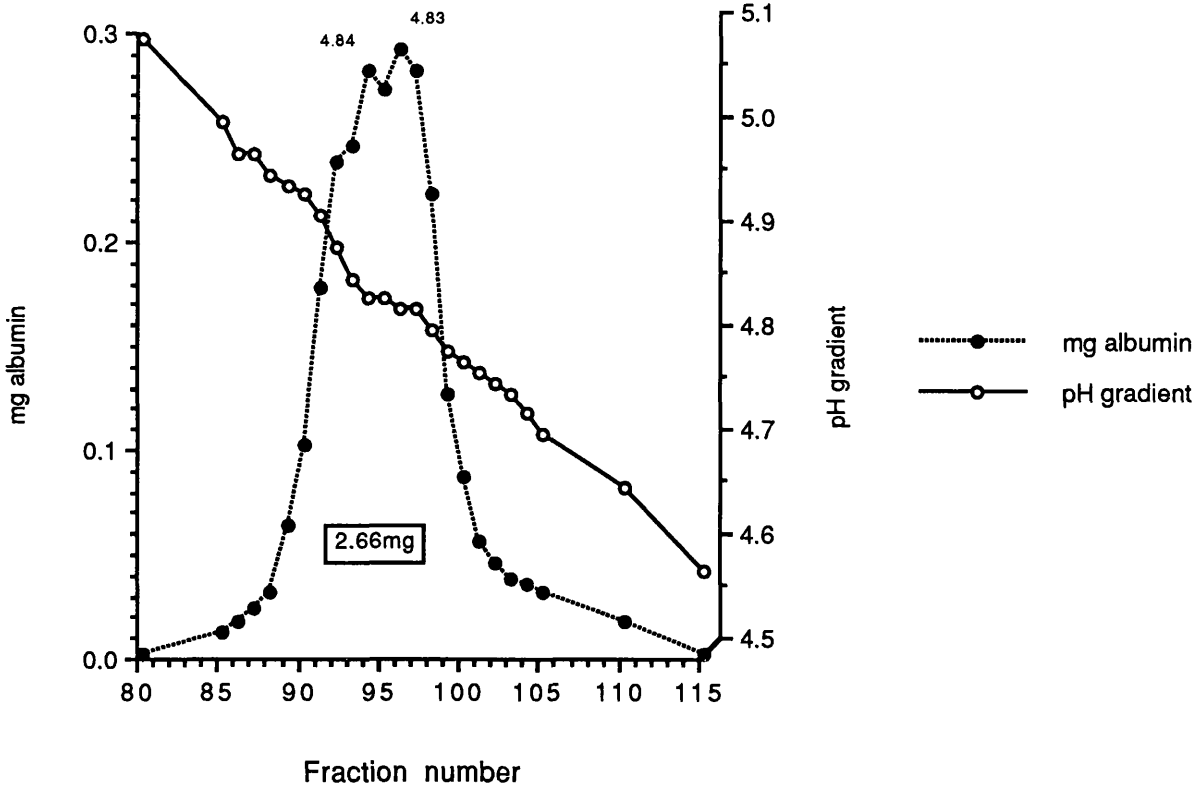


Figure 2.4.3.7(c) Chromatofocusing of Lewis rat (9) serum - primrose oil diet

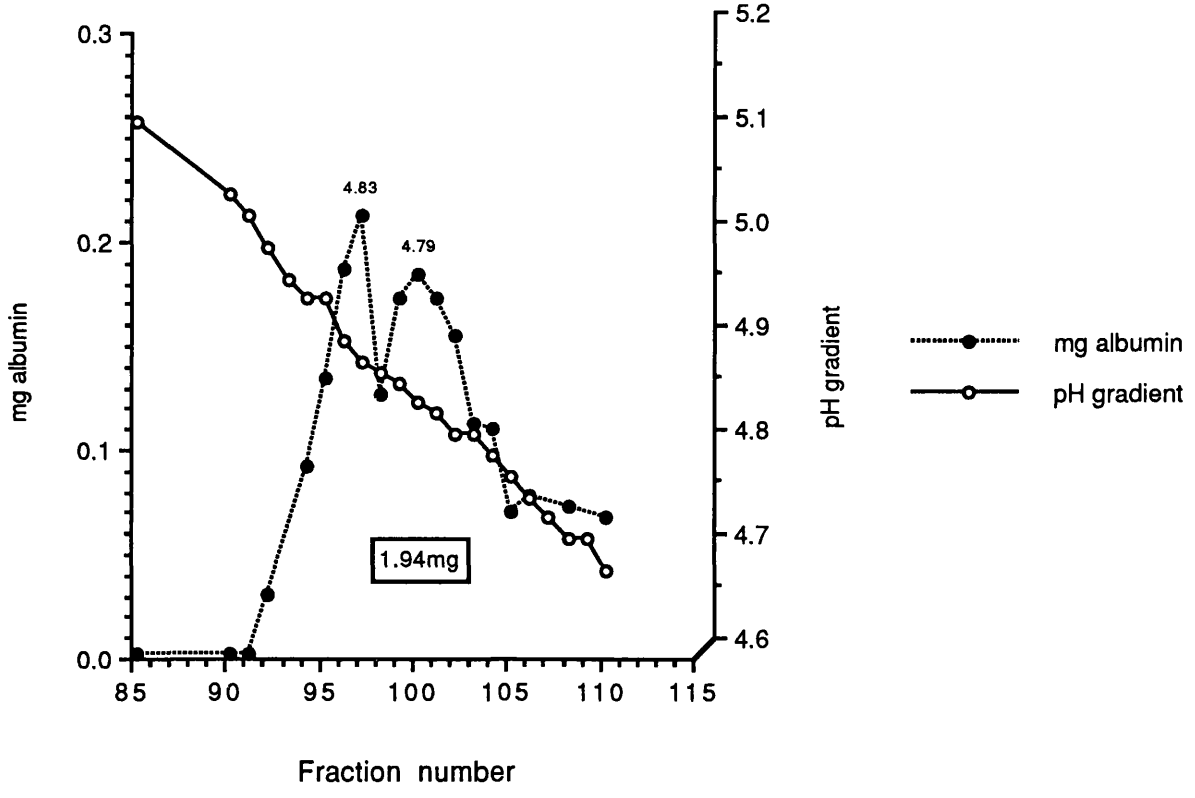


Figure 2.4.3.7(d) Chromatofocusing of Lewis rat (4) serum - primrose oil diet

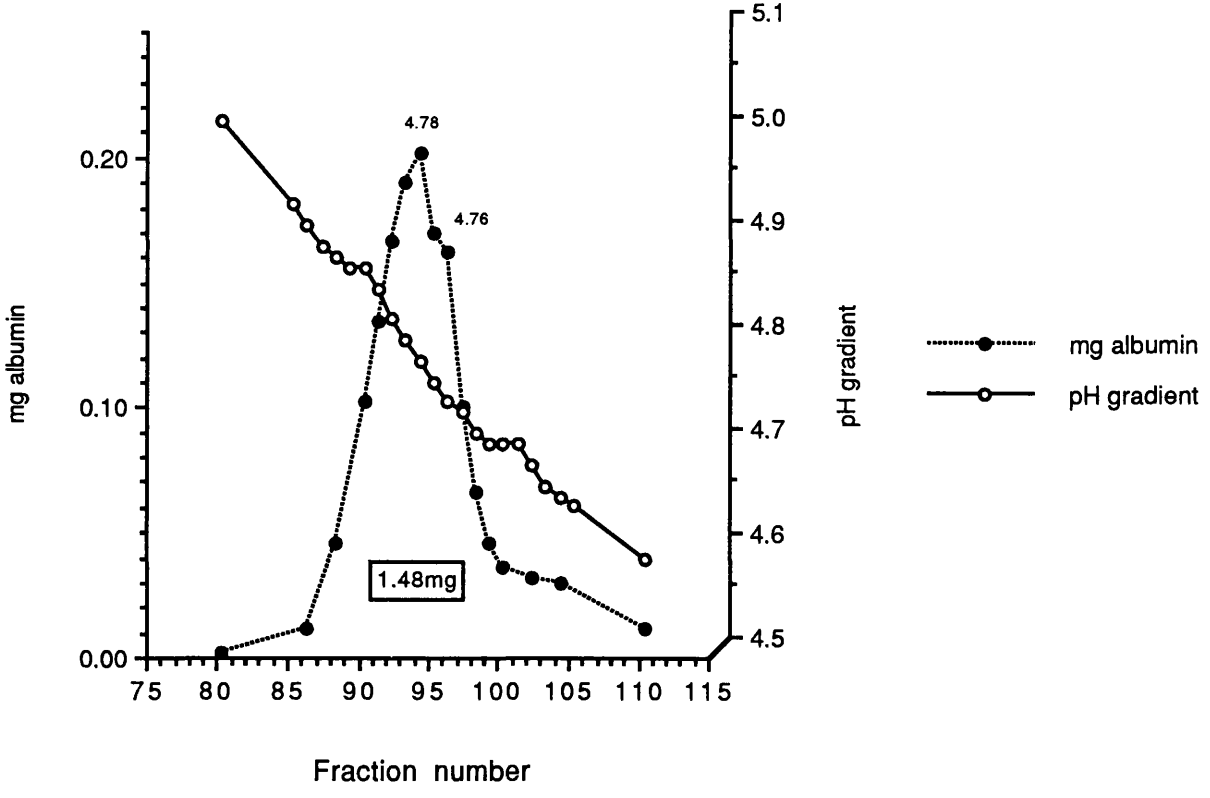


Figure 2.4.3.8(a) Chromatofocusing of Lewis rat (6) serum - fish oil diet

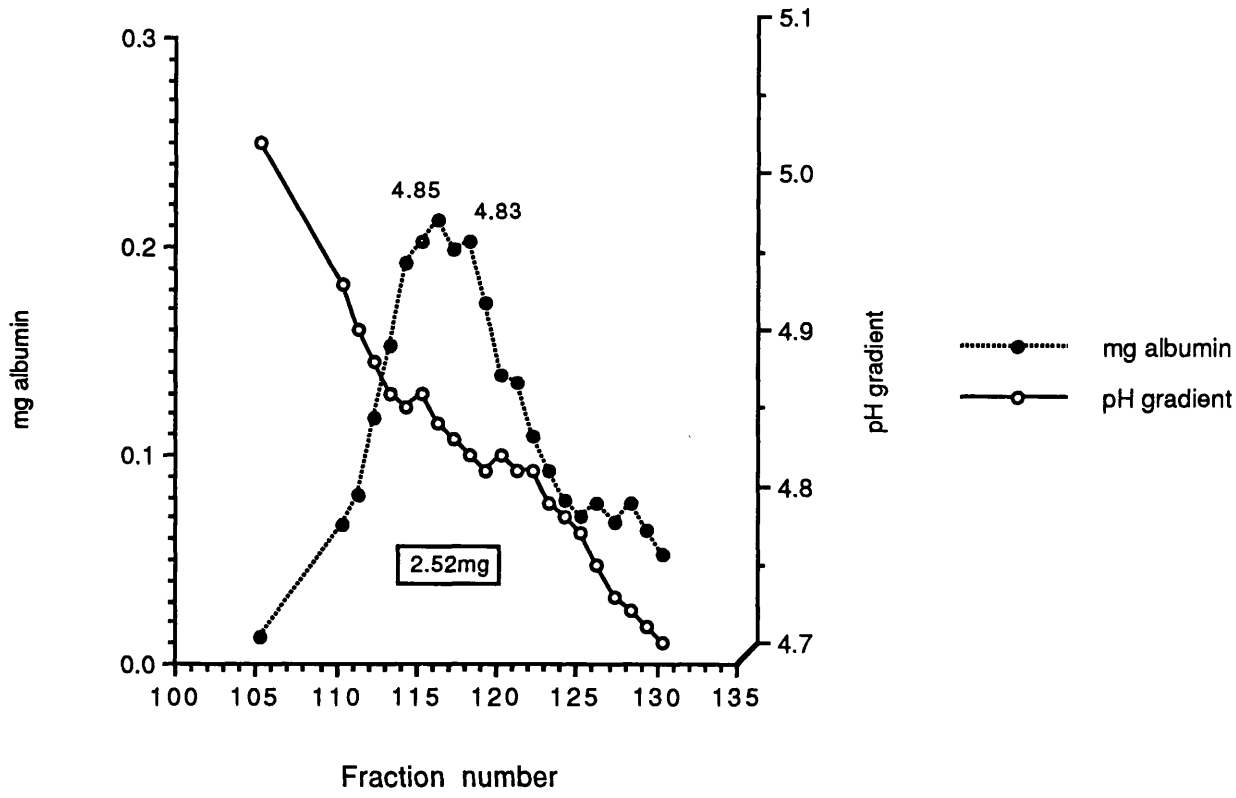


Figure 2.4.3.8(b) Chromatofocusing of Lewis rat (10) serum - fish oil diet

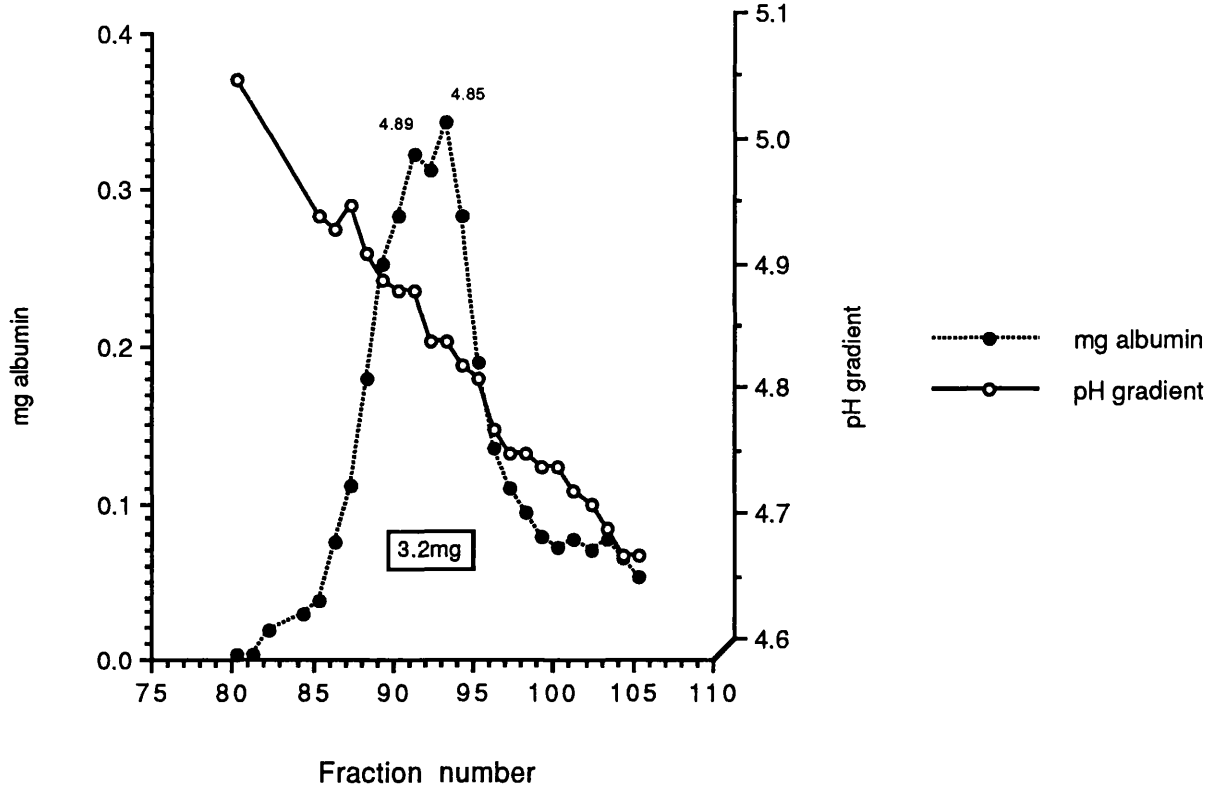


Figure 2.4.3.8(c) Chromatofocusing of Lewis rat (4) serum - fish oil diet

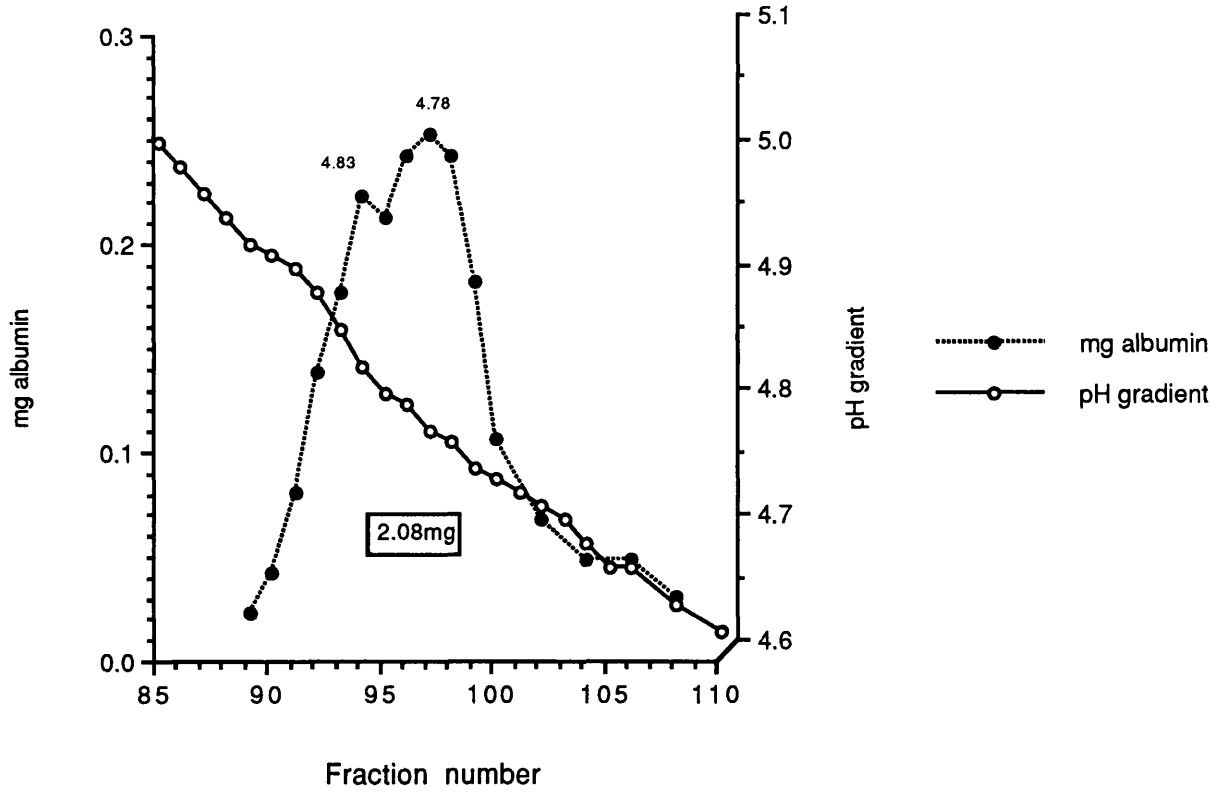


Figure 2.4.3.9(a) Chromatofocusing of Lewis rat (6) serum - olive oil diet

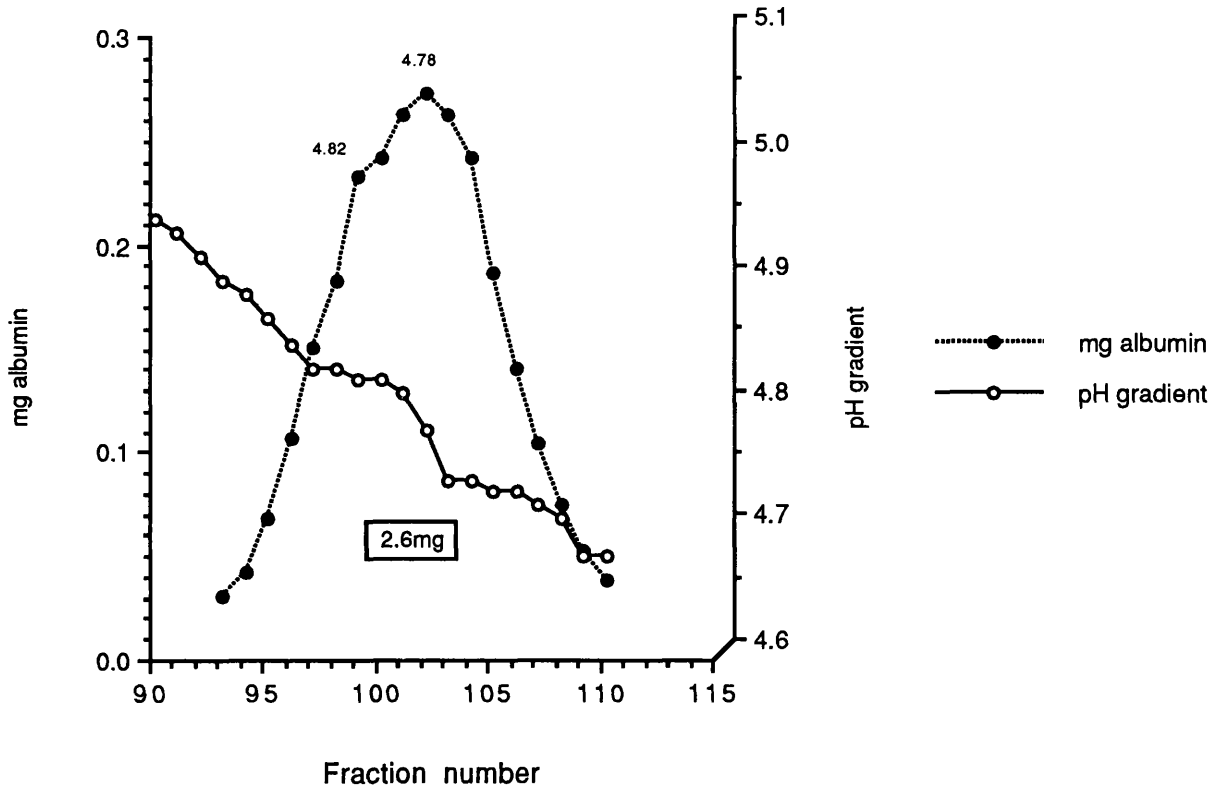


Figure 2.4.3.9(b) Chromatofocusing of Lewis rat (9) serum - olive oil diet

pH	5.03	5.02	5.01	5.00	4.99	4.98	4.97	4.96	4.95	4.94	4.93	4.92	4.91	4.90	4.89	4.88	4.87	4.86	4.85	4.84	4.83	4.82	4.81	4.80	4.79	4.78	4.77	4.76
Control 7								X							X													
Control 9					X					X																		
Control 10												X						X										
Control 1											X				X													
Control 3			X						X																			
Control 6						X							X															
Control 2	X				X																							
Primrose 7															X		X											
Primrose 6																			X			X						
Primrose 9																				X	X							
Primrose 4																					X				X			
Fish 6																										X		X
Fish 10																			X		X							
Fish 4															X				X									
Olive 6																					X					X		
Olive 9																						X				X		

Table 2.4.3.1 CHROMATOFOCUSING PEAK MAXIMA

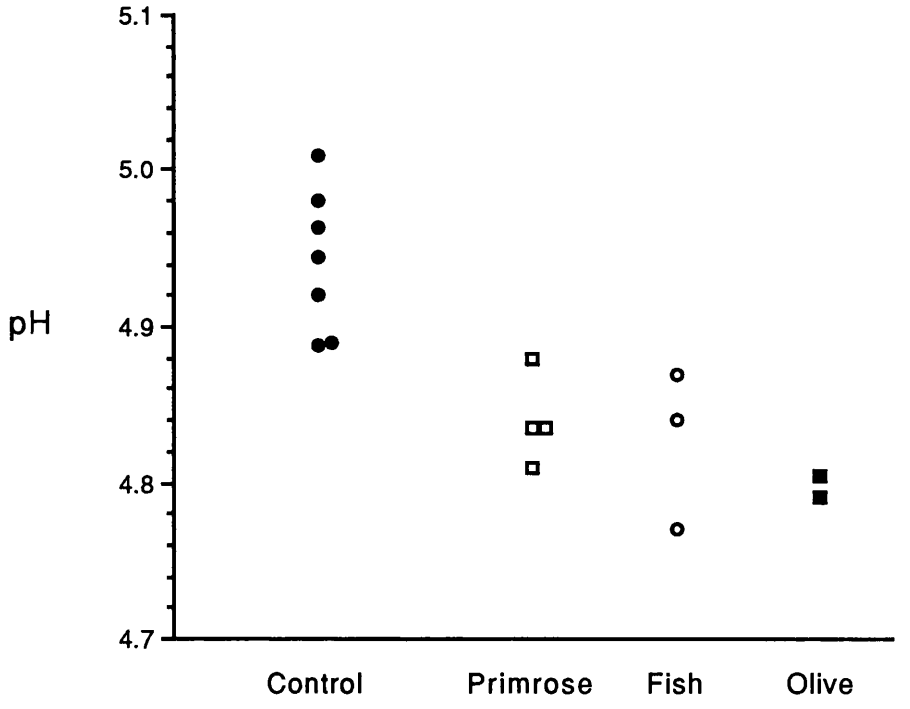


Figure 2.4.3.10 Isoelectric point (pI) of Lewis rat serum albumin in control or PUFA diet groups.

Median pI of control vs amalgamated PUFA diet groups = 4.942 vs 4.826, p = 0.001.

2.4.4 Discussion

Although isoelectric focusing is a more traditional method of establishing the electrical charge on a molecule, it was decided that the novel chromatofocusing technique might discriminate more accurately between small differences in pH because of its superior resolving power and focussing effects. It soon became clear however, that chromatofocusing is heavily dependent upon the sensitivity, accuracy and precision of the pH meter. In this study, a digital pH meter was used and calibrated with pH 4 and pH 7 standards at 20°C before each run, but like all equipment, it is subject to minor fluctuations which may introduce a certain degree of inaccuracy at the level of 0.01 pH units. In general, no major problems were encountered with development of the pH gradient although often it did become a little more 'bumpy' when consecutive fractions were measured. Fractions were collected overnight, usually, and although the tubes were covered, it is conceivable that some degree of evaporation or adsorption of carbon dioxide might cause minute changes in either fraction volume or pH. Also, measuring pH in small fraction volumes with a standard pH probe is not ideal.

More often than not, albumin eluted from the column either as a double peak or with distinct shoulders and this made it difficult to collate and interpret the data. Peak shape could be due entirely to procedural artefacts such as an inaccurate pH reading but this is unlikely. Several isoforms of albumin exist; for example, that albumin expands and expresses some forty carboxyl groups at around pH 4 to 4.5 (N-F transitions: Foster, 1977, Peters, 1985) might also affect the chromatofocusing profile. As in any chromatographic procedure, peak resolution may be improved through various technical manipulations including (i) increasing the length of the column, (ii) lowering the buffer concentration to expand the pH gradient, (iii) slowing the flow rate through the column and (iv) ensuring the uniformity of the column packing. Ultimately, the limits of resolution may have been reached in this instance. It is unclear whether the elution of albumin as a double peak in the control and PUFA diet groups is genuine or artefactual. One of the peaks could be glycosylated albumin (Ghiggeri *et al*, 1985) or some other albumin-ligand moiety which elutes close by. A paper by Evenson and Deutsch (1978) shows similar profiles of native serum albumin eluting in two peaks which are attributed to the quantity of fatty acids bound: one albumin peak, at pH 5.6, was de-fatted and another at pH 4.8 had some 2-3 moles of bound fatty acids. The pH 5.6 isoform of serum albumin could be generated by removing fatty acids and re-converted to the pH 4.8 isomer by the addition of long chain fatty acids. The authors attributed the isoelectric heterogeneity of HSA to variable losses of fatty acids during isolation and storage. The chromatofocusing profile of HSA in Figure 2.4.3.2(a) shows two similarly distinct peaks.

The recovery of albumin was poor and might be due, in part, to small amounts of albumin in both tails of the elution profile which were not measured: alternative explanations include loss of albumin through surface adsorption or the capacity of the anion exchanger. Recovery can be improved by lowering the pH, or increasing the ionic strength of the eluting buffer. The accuracy of the immunoturbidimetry method used is also unknown.

Ideally, a larger number of serum samples would have been processed in duplicate at least, but chromatofocusing is an expensive technique. The bias in sample type and number is related to over-enthusiastic assay of control and evening primrose oil samples in the early stages, which limited the availability of chromatofocusing reagents for processing fish and olive oil samples. Chromatofocusing is not only expensive but is also very slow, requiring no fewer than two days to process a single serum sample. The most efficient arrangement was to collect fractions overnight and measure albumin levels the following day while regenerating the column for the next run. Chromatofocusing is very much slower than IEF in which several samples can be analysed simultaneously.

Although sample number is small and only Lewis rats were studied, the results of this study provided a persuasive explanation for the tendancy for albumin excretion to fall in all the PUFA diet groups. A fall in the isoelectric point of serum albumin in the PUFA diet groups implies increased anionic charge which heightens the degree to which it is repelled by the glomerular polyanion. That carboxyl groups in the polar head of the fatty acid molecule augment the anionic charge on the surface of the albumin molecule is the most likely mode of action: under normal physiological conditions, many of the fatty acid binding sites within the albumin molecule are unfilled.

The median pI of Lewis rat serum albumin was calculated as being around 4.95 which might be a little higher than that normally quoted for rat serum albumin ($\approx$ 4.7 - 4.9) but often proteins do not elute at their exact pI as measured by IEF, when measured by chromatofocusing. Polymorphisms within the albumin gene are said to uncommon and are unlikely, therefore, to explain the elution pattern of serum albumin in Lewis rats (Kragh-Hansen, 1990). However, Lewis rat serum albumin could be depleted of fatty acid binding sites and thus more cationic and filtered more readily than that of DA rats. PUFA diets may restore or increase the anionic charge on the albumin molecule.

Few studies have given much consideration to the effects of fatty acid binding on the physico-chemical properties of albumin but Jensen *et al* (1989) did suggest that the

fall in the transcapillary escape rate (TER) of albumin in IDDM patients with albuminuria was due to conformational changes in the albumin molecule induced by its binding fatty acids derived from cod liver oil. On the other hand, it is suggested that, more menacingly, fatty acids bound to albumin may generate damaging toxic lipid hydrolysis products following their uptake by renal tubular cells (Thomas and Schriener, 1993).

The functional significance of physical modification of the serum albumin molecule in glomerulopathies such as diabetic and minimal change nephropathy has been explored. In diabetic patients, the pI of serum albumin ranged from 3.5 to 7 and there was a preponderance of heavily glycosylated, more anionic isomers of albumin in the urine, perhaps as a result of increased reabsorption of cationic albumin (Ghiggeri *et al*, 1985). In diabetics with normal albumin excretion, the pI of urine albumin was more anionic (4.1 - 4.7) than those with microalbuminuria, in which albumin was present as a single band at pI 4.7 (Candiano *et al*, 1990). In children with minimal change nephropathy, regarded as being related to a fundamental defect GCW charge, greater quantities of the more cationic (pI 4.8 - 5.5) isoforms of albumin were found in the urine (Ghiggeri *et al*, 1987) and following steroid therapy, proteinuria fell in association with disappearance of the more cationic bands and appearance of numerous more anionic pI (< 4.7) isoforms. Ghiggeri *et al* (1987a) suggested that in MCN patients, the pI of albumin is a function of its fatty acid content and indeed, analysis of the fatty acids bound to albumin showed that, relative to its serum homologue, de-fatted albumin was not only more cationic, and thereby filtered in excess of native acylated albumin but was deficient in linoleic acid, in particular. It was speculated that in MCN, changes in the pI of albumin from 4 to 6.1 reflect the fatty acid content of the albumin molecule (ranging from 20-30 to 2-3 moles of fatty acid per mole of albumin for the most, and least anionic albumin isoforms, respectively). The amount of fatty acids bound to albumin fell further during the proteinuric phase of MCN. The heightened filtration of de-fatted (cationic) albumin in MCN suggests that some degree of glomerular charge selectivity was maintained and that the fall in albumin excretion was due to a fundamental change in the structure of the albumin molecule itself.

It would be intriguing if binding fatty acids, of whatever degree of double bond unsaturation or configuration or acyl chain length, reduced the clearance of albumin, but the results must be viewed with caution (Aguanno and Ladenson, 1982). Two major flaws associated with the hypothesis of increased acylation of albumin lowering its excretion rate are that (i) hyperlipidaemic individuals would be expected to have low levels of albumin excretion albeit the anti-proteinuric effects of PUFA binding

may be dependent upon the type of fatty acid bound and (ii) the pI of serum albumin might fluctuate considerably in response to circulating fatty acid levels with marked effects on protein excretion. It must also be said that there can be a high degree of subjectivity in determining the pI from chromatofocusing profiles

Unfortunately, this study did not establish if the pI of DA rat serum albumin was also lowered in the PUFA diet groups. The issues of whether (i) the pI of DA rat serum albumin is lower than that of Lewis rats under normal conditions and (ii) if a simultaneous change in the size or conformation of the albumin molecule also occurred in the PUFA diet groups could be addressed in the future. It is unclear whether a difference of 0.12 pH units (4.95 - 4.83) in pI between control and PUFA diet groups would be sufficient to elicit a fall in the clearance rate of albumin and designing an experiment to answer this question could be a difficult. The binding of PUFAs to albumin can also increase its size and shape or configuration (see section 2.4.1). That olive oil had the same anti-proteinuric effect as evening primrose and fish oil diets, tends to preclude the role of prostanoids while strengthening the hypothesis that some structural modification of the serum albumin molecule, induced by fatty acid binding, altered its pI and reduced its clearance.

Summary

A change in the charge on the serum albumin molecule induced by binding polyunsaturated fatty acids (PUFAs) may explain the reductions in albumin excretion rate observed in the studies reported in this thesis.

Chromatofocusing was a satisfactory, but perhaps not ideal, method of measuring the pI of serum albumin because contrary to reports, it is expensive for assaying a large number of samples and is both time- and labour-intensive. IEF might have distinguished these differences just as well and lessened the problems of inter-assay variation.

2.5.1 Natural history of diabetic nephropathy

Renal disease is still the leading cause of death amongst patients with type I insulin-dependent diabetes (IDDM) who present before the age of twenty but clearly, there are differing susceptibilities to nephropathy since only around 35 to 40% develop this complication (Andersen *et al*, 1983). The prognosis of established diabetic nephropathy is poor but strategies for preventing or arresting the progression of renal damage in diabetes are being pursued with some success (Viberti *et al*, 1987, The Diabetes Control and Complications Trial (DCCT) Research Group, 1993).
Diabetic renal disease progresses through five well-defined stages (Viberti *et al*, 1982, Mogensen, 1987).

Stage I: Glomerular hyperfiltration, defined as a GFR in excess of 150 mls per min per 1.73 m^2, is common in diabetic patients at diagnosis although albumin excretion rate (AER) may be normal (Mogensen, 1986). Kidney size increases but no other symptoms can be attributed to changes in renal function (Mogensen and Andersen, 1973).

Stage II: A silent period during which lesions develop mainly in the glomerulus but AER remains normal.

Stage III: The early, incipient phase starts some five years after presentation. Symptoms include an albumin excretion rate of 20-200 µgs per minute (30-300 mgs per day) termed microalbuminuria (Albustix-negative) and which, although potentially reversible, is associated with a high risk of developing nephropathy. AER remains stable except perhaps during periods of poor diabetic control, severe exercise or infection (Viberti *et al*, 1982a, Christensen, 1984, Feldt-Rasmussen *et al*, 1985, Jensen *et al*, 1988a). The duration of this phase is unpredictable but in most patients it progresses to stage IV.

Stage IV: The overt or clinical phase is much less amenable to treatment than the incipient phase and is characterised by a rise in AER to levels in excess of 200 µgs per minute (300 mgs per day: Albustix-positive). It may last for three to five years and is marked by a progressive decline in GFR of about 1 ml per minute per 1.73 m^2 per month and an increase in AER of some 20 to 40% per year. Hypertension becomes progressively more severe, the patient may become nephrotic and other complications of diabetes such as retinopathy are common.

Stage V: End-stage renal failure with uraemia.

Research into the treatment of diabetic nephropathy has developed rapidly and may already have reduced the proportion of patients with IDDM who develop renal complications. During the *incipient phase*, the efficacy of treatments is often assessed by their ability to reduce albumin excretion and both strict glycaemic control (Viberti *et al*, 1979; 1983, Feldt-Rasmussen *et al*, 1986) and antihypertensive therapy (Christensen and Mogensen, 1985, Parving *et al*, 1987; 1988, Anderson *et al*, 1989) may help accomplish this objective. Compared to those with good glycaemic control, diabetic patients with inadequate glycaemic control are more likely to display a sustained rise in GFR associated with a rise in intraglomerular blood pressure and progressive glomerular sclerosis (Mogensen, 1976, Wiseman *et al*, 1985, Sabbatini *et al*, 1992). Those patients with the highest early increase in GFR and AER are more likely to develop progressive disease (Mogensen, 1987). For any given degree of glycaemic control, a predisposition to hypertension may aggravate the resultant glomerular injury. Throughout the *clinical phase*, a reduction in proteinuria and retarded disease progression has been achieved by controlling blood pressure (especially intraglomerular hypertension) with ACE inhibitors (Taguma *et al*, 1985, Bjorck *et al*, 1986, Hommel *et al*, 1986a, Parving *et al*, 1988, Valvo *et al*, 1988, Marre *et al*, 1987; 1988) or low protein diets (Wiseman *et al*, 1987, Zeller *et al*, 1987; 1987a).

Various hypotheses to account for a predisposition to diabetic nephropathy have been advanced but it is still unclear whether diabetic renal disease is precipitated by haemodynamic or metabolic derangements (Zatz *et al*, 1985, The DCCT Research Group, 1988). Elevated rates of sodium-lithium countertransport (SLC) across the erythrocyte membrane are associated with an inherited predisposition to essential hypertension and have been observed in both patients with diabetic nephropathy and their relatives (Mangili *et al*, 1988; 1993, Krolewski *et al*, 1988, Carr *et al*, 1990a, Walker *et al*, 1990).

The primary cause of glomerular hyperfiltration in diabetic renal disease remains unresolved but has been linked to disturbances in the renin-angiotensin system (Christlieb *et al*, 1978, Dworkin *et al*, 1983, Ballerman *et al*, 1984, Fioretto *et al*, 1991, Beretta-Piccoli and Weidmann, 1981, Christiansen *et al*, 1988), growth factors (Christiansen *et al*, 1982, Flyvbjerg *et al*, 1990), glucagon (Parving *et al*, 1980), insulinopenia (Harrison *et al*, 1980, Pfaffman *et al*, 1982, Kreisberg *et al*, 1983, Schambelan *et al*, 1985) and heightened prostaglandin levels (Johnson *et al*, 1979, Silberbauer *et al*, 1979, Gerrard *et al*, 1980, Axelrod 1982, Rogers *et al*, 1982, Funakawa *et al*, 1983, Kirschenbaum *et al*, 1981; 1985, Craven *et al*, 1987, Jensen *et al*, 1986, Gambardella *et al*, 1988, Green *et al*, 1988).

Prostanoid levels are altered in diabetes and the vasodilatory actions of prostaglandins may contribute to glomerular hyperfiltration (Zatz *et al*, 1985, Quilley *et al*, 1985, Schambelan *et al*, 1985; Wilson *et al*, 1985, Benigni *et al*, 1986, Bunke et al, 1986). It has been suggested that prostaglandins sustain only the early stages of glomerular hyperfiltration and that the fall in GFR observed in the latter stages of diabetes is due to increased thromboxane synthesis (Craven *et al*, 1987). The protective effects of aspirin and indomethacin in diabetic renal disease may be related to their ability to reduce or prevent glomerular hyperfiltration (Jensen *et al*, 1986, Craven and DeRubertis, 1989). Aspirin prevented hyperfiltration, GBM thickening and a fall in GFR in STZ diabetic rats (Moel *et al*, 1987) and indomethacin lowered both glomerular filtration and albumin excretion rate in patients with type I diabetes (Hommel *et al*, 1987). However, experimental evidence implicating prostaglandins in diabetic hyperfiltration is weak. Despite raised glomerular PGE_2 levels in STZ rats, compared to genetically diabetic (BB) and non-diabetic control rats, the response of isolated glomeruli to AII and mesangial contractility were similar in all the groups (Barnett *et al*, 1987). Christiansen *et al* (1985) and Stirati *et al* (1986) showed that cyclooxygenase inhibitors did not reduction hyperfiltration in diabetic patients.

2.5.2 Experimental design, animals, materials and methods

This study was undertaken to establish whether feeding an evening primrose oil diet could attenuate or prevent increased rates of albumin excretion in rats made diabetic by an injection of streptozotocin (Carney *et al*, 1979, Jensen *et al*, 1987). Proteinuria had been shown to be higher in diabetic Lewis rats than diabetic DA rats and the difference became more marked over time. Mesangial expansion too, was greater in Lewis rats than DA rats after six months of diabetes (Payton and Boulton-Jones, 1989). For practical and procedural reasons only one strain of rat and one PUFA diet could be studied. Lewis rats were selected because of their propensity to raised levels of proteinuria and heightened susceptibility to diabetic nephropathy compared to DA rats. It was more difficult to decide upon which diet to test, but evening primrose oil diet was chosen because of some adverse reports on the use of fish oil in diabetes (Glauber *et al*, 1988) and a strong endorsement of the use of linoleic acid diets in experimental diabetic nephropathy (Barcelli *et al*, 1989).

2.5.2.1 STZ ranging study

Compared to most other studies, the dose of streptozotocin to be used in this study (80 mg per kg) is rather high but Payton *(personal communication)* had found that a lower dose often did not induce diabetes. A small ranging study was performed in a group of ten Lewis rats to check the optimal dose of STZ required to induce diabetes. Rats were anaesthetised by ether inhalation and injected via the tail vein with streptozotocin prepared in citrate buffer, pH 4.5 at doses of 40, 50, 60, or 70 mg per kg (see method in section 2.5.2.3); two rats were given citrate buffer only and the progress of the animals was followed for three to four days. Body weight was recorded and urine glucose and protein levels were monitored using Labstix (Miles Laboratories, Slough SL2 4LY). Blood was withdrawn from the tail vein under ether anaesthesia for measurement of plasma glucose levels (see Table 2.5.3.1).

2.5.2.2 Animals

The main study was started after the dose of streptozotocin (80 mg per kg) had been confirmed by the ranging study. Two groups of 10 Lewis rats (total n = 20), of mean body weight around 100 grams at the start of the study, were fed either standard laboratory rat diet or evening primrose oil diet (section 2.1.2.2) for three weeks before STZ and throughout a subsequent six week period of diabetes. A six week diet period was selected because (i) the protein lowering effects of PUFA diets had been observed within this time period in the preceding studies and (ii) the Home Office stipulated that the study should be kept as brief as possible.

Both groups of rats were made diabetic by receiving an injection of the cytotoxic drug, streptozotocin (STZ) which destroys insulin-producing β cells in the pancreas. As there was to be no non-diabetic control group, data from normal Lewis rats fed standard laboratory rat chow in earlier studies was used for comparison, as required. Rats were put into metabolic cages one and three weeks before, and one, three and six weeks after STZ injection and food and water intake and urine output was measured. Urine urea levels were measured by a Hitachi 704 discrete autoanalyser in urine diluted 1:49 as an assessment of dietary protein intake.

2.5.2.3 Induction of diabetes

After anaesthesia, induced by ether inhalation, a single injection (80 mg per kg body weight) of streptozotocin (Zanosar, The Upjohn Company, Kalamazoo, Michigan 49001, USA) reconstituted to 100 mg per ml in 0.1M citrate buffer, pH 4.5 [8 mls of 0.1M citric acid (21.1 g/l) and 12 mls of trisodium citrate (29.4 g/l)] was administered to each rat. 200-500 µls of blood were withdrawn from the tail vein using a 25 guage needle into a fluoride tube (Teklab) for measurement of baseline blood glucose levels. After recovering from the anaesthetic, the animals were returned to communal cages but as soon as diabetes was confirmed (within two to three days) by the development of polyuria and glycosuria, the rats were placed in individual gridded cages. Each morning throughout the six week diabetic period, body weight was recorded and urine was tested by Labstix (Ames) for glucose, ketones, protein and pH. Food and water intake were not restricted during the study.

Another pre-condition of performing the study, set down by the Home Office, was that insulin be given to control the severity of diabetes. The criteria for insulin administration were either or both a urine glucose concentration of 3+ to 4+ (by Labstix) or any decrease in body weight to less than 80% of the body weight of a

non-diabetic control rat. In the early stages, a daily subcutaneous injection of 1 unit of insulin (Human Ultratard insulin: Novo) was given to each rat but this dose proved insufficient to control and the dose was increased gradually to 4 units per day. Although urine glucose levels may not accurately reflect plasma levels, measuring blood glucose involves tail venepuncture under anaesthesia and so was measured only at week three in the pre-STZ phase and at one, three and six weeks during the diabetic phase, using a Beckman glucose analyser.

2.5.2.4 Blood pressure measurement

The equipment for non-invasive blood pressure monitoring became available for this study. Arterial blood pressure was measured using a Buffington Cuff (Small Animal Indirect Blood Pressure Plethysmograph. Buffington Clinical Systems: PO Box 24241 / Cleveland, Ohio 44124 / Area 216/442-3850) at one week before, and five weeks after injection of STZ.

The Cuff records blood pressure in the tail of the rat by differential pulse volume plethysmography (Pfeffer *et al*, 1971). Arterial blood flow to the tail is blocked by inflating an occluding cuff placed proximally to a detecting transducer cuff. Each rat was placed in an incubator at 37°C for 15 minutes to dilate the tail veins and subdue the animal and then restrained gently in a cloth and kept as still and calm as possible. The occluding cuff placed high on the tail with the transducing cuff about 1 cm below, was inflated until the trace from the pulse transducer was steady and noise-free and then released gradually until the first low amplitude pulsation - which represents systolic pressure - appeared on the trace. Each blood pressure measurement was the mean of five separate readings (see Plate 2.5.1).

2.5.2.5 Urine protein measurement

Urine total protein was measured by the Coomassie Blue method (see section 2.1.2.9). In all the previous studies and during the pre-diabetic phase of this one, urine *albumin* was measured by immunoturbidimetry (see section 2.2.2.1) but it had not been anticipated that, with detection limits of 2.5-150 µgs per ml, the method would not be sensitive enough to measure the low levels of albumin in the dilute urine of diabetic rats, even after further dilution. The options were to measure urine albumin either by radioimmunoassay (RIA) or ELISA (Enzyme-Linked ImmunoSorbent Assay): because a specific RIA method was not readily available, would be difficult to develop and with detection limits of ≈ 0.5-20 µgs per ml was perhaps not much more sensitive than immunoturbidimetry, it was decided to use an ELISA to detect albumin within the nanogram range (7.5-150 ng per ml).

Procedures from published rat serum albumin ELISA methods were followed (Mohamed *et al*, 1984, Torffvit and Weislander, 1986) and antibodies were titrated to establish optimal working dilutions. Sheep anti-rat albumin (The Binding Site, PO Box 4073, Birmingham B29 6AT: PC341) was used as the coating (or capture) antibody and peroxidase-labelled sheep anti-rat albumin (The Binding Site, as above: PP341) was used as the secondary (or conjugate) antibody. Immediately, problems were encountered with both a lack of sensitivity at the lower end of the standard binding curve and a poor absorbance range from the bottom to top of the binding curve (see Figure 2.5.3.12).

The sensitivity of an ELISA may be improved by manipulating various procedural parameters - but only one at a time - since changing several at once can have devastating consequences: such parameters include

- the source and dilutions of the primary and secondary antibodies
- quality of the microtitre plates
- ambient temperature and duration of the different reaction steps
- number of washes
- use of agents such as gelatin, casein or serum which block non-specific binding sites.

After several months of attempting to improve the sensitivity of the method, the best results could only be obtained by using the coating and conjugate at unacceptably low dilutions of between 1:50 to 1:80 and 1:75 to 1:125, respectively. Microtitre plates were coated for 1 hour at room temperature (or overnight at 4°C) and blocked for 2 hours at room temperature with a 1% casein solution. Rat serum albumin standards ranged from 500 to 4 ng per ml by doubling dilutions (Figure 2.5.3.12).
Ultimately, it was acknowledged that an alternative method would have to be found and two fundamental changes were implemented: a double antibody labelling procedure using the highly sensitive and specific biotin-streptavidin system was introduced and both the commercial and biological source of the antibodies were changed. The two-step streptavidin-biotin labelling procedure amplifies the detection stage of the assay and the sensitivity of the ELISA improved immediately (see Figure 2.5.3.12a). The principle behind double-labelling is that instead of the secondary antibody being labelled directly with peroxidase, it is instead conjugated with biotin which has a very high affinity for avidin. The final reagent layer of horseradish peroxidase (HRP)-labelled Avidin D binds with high avidity to the biotin moiety of second antibody and thus heightens the sensitivity of the ELISA.

The new rat serum albumin ELISA method evolved through the use of:

- goat anti-rat albumin antiserum (Cappel Laboratories: 0213-0341) as coating antibody
- a greater number of washing steps
- rat serum albumin standards ranging from 50 to 7.8 ng per ml
- 0.1% gelatin as blocking solution
- biotin-conjugated goat anti-rat albumin (see method) and
- horseradish peroxidase (HRP)-labelled Avidin D (Vector laboratories, Peterborough PE3 8RF: Cat no A2004, 5mg).

The optimal dilutions of coating and conjugate antibodies and avidin-HRP were determined by titration.

2.5.2.6 Rat serum albumin ELISA

The first stage of the ELISA involves biotinylation of the antiserum. Goat anti-rat albumin serum was dialysed extensively against PBS and then diluted with PBS to an IgG concentration of 5 mg per ml. 2.13 mg of N-hydroxy-succimidobiotin (Sigma Chemical Company: H-1759) and 100 µls of dimethylformamide (DMF: Sigma Chemical Company: D-8654) were mixed and added immediately to 1 ml of antiserum (IgG = 5 mg per ml) and mixed again. (This gives a biotin to protein ratio of 200:1 which is equivalent to binding 5 to 150 molecules of biotin per molecule of protein.) The mixture was left at room temperature for two hours, dialysed against one litre of PBS five times and then stored at 4°C.

The following reagents and buffers were prepared:

Washing buffer — *15M PBS-Tween, pH 7.2*: Sodium chloride (40 g/l); potassium chloride (1 g/l); *di*-sodium hydrogen phosphate (5.75 g/l); potassium *di*-hydrogen phosphate (1 g/l): Tween 20 (1 ml/l). Prepare 5 litres with distilled water - add Tween last.

Coating buffer — *1.5 M carbonate-bicarbonate, pH 9.6:* Sodium carbonate, pH 11 (3.8 g/l); sodium hydrogen carbonate, pH 8.3 (5.86 g/l). Mix equal volumes (approximately) of the two solutions until the correct pH is reached.

Blocking solution — 0.1% gelatine in phosphate buffered saline (PBS)

Substrate buffer — *0.15M citrate-phosphate, pH 5*: Citric acid (21 g/l); *di*-sodium hydrogen phosphate (35.6 g/l). Mix equal volumes (approximately) of the two solutions until correct pH is reached.

Albumin standards — Stock rat serum albumin (RSA: Sigma Chemical Company: A-6414) prepared at 5 mgs per ml diluted to 1 mg per ml (10 µls + 40 µls) then according to the following table:

Dilution	RSA (ng/ml)
1:5T	200
1:10T	100
1:20T	50
1:40T	25
1:80T	12.5
1:160T	6.25
1:320T	3.13
1:640T	1.56
1:1280T	0.78

(T = x 10^3)

Method

Ninety four wells of a 96 well microtitre plate (Maxisorp; Nunc-Immuno Plate) were coated with 100 µls of goat anti-rat serum albumin diluted 1 in 2600 with coating buffer. The plate was covered and left overnight in a moist chamber at 4°C and then washed five times with washing buffer. 200 µls of 0.1% gelatine solution as blocking agents was pipetted into each well. The plate was left covered for one hour at room temperature and then washed five times with washing buffer. 100 µls of either rat serum albumin standards (see table) or urine diluted 1:800 (see validation) was added to the wells. The plate was covered and left at room temperature for two hours then washed five times with washing buffer. 100 µls of biotin-labelled goat anti-rat serum albumin (see above) diluted 1:4000 (5 µls in 20 mls of PBS) was added to each well and the plate was left covered at room temperature for one hour and then washed five times with washing buffer. Finally, 100 µls of horseradish peroxidase (HRP)-labelled Avidin D diluted 1:5000 (2 µls in 10 mls of PBS) was added to each well. The plate was left covered at room temperature for one hour and then washed five times with washing buffer. Substrate buffer was prepared for immediate use. One tablet (15 mg) of o-phenylenediamine dihydrochloride (OPD) (Sigma Chemical Company: P-8787) was dissolved in 44 mls of citrate buffer, pH 5 and then 18 µls of 30 % hydrogen peroxide was added: 100 µls of this substrate solution was added to each well. The plate was left in the dark, at room temperature, until sufficient colour had developed to read the lowest standard. The reaction was stopped by adding 50 µls of 4N sulphuric acid to each well and the absorbance was read at 490 nm on a Microplate^R reader (Dynatech).

The precision and accuracy of the ELISA were assessed. Precision is a measure of the reproducibility of an assay both within and between batches and is defined as the coefficient of variation CV(%) = SD/mean x 100. A urine sample (C2-5 diluted 1: 3200) was used as quality control (QC) during optimisation of the standard curves. The accuracy of the assay relates to its ability to determine the true value of analyte present in the sample and can be calculated from recovery experiments. Recovery was assessed by "spiking" ultrafiltered rat urine (UFU) with rat serum albumin (Sigma Chemical Company: A-6414) at 6, 4, 2, 1 and 0.5 ng per ml. Urine was ultrafiltered by centrifugation in a disposable Centriflo ultrafiltration membrane cone (Amicon Ltd. Gloucester GL10 2BJ) which, with a molecular weight cut-off of 25,000 kD, claims to removes in excess of 95% of endogenous albumin. A stock preparation of UFU was spiked with 3.2 μgs per ml (≡ 3200 ng per ml) of rat serum albumin which, following 800- fold dilution, results in a level of ≈ 4 ng per ml which falls in the middle of the binding curve (previously determined). Ideally, ultrafiltered rat urine should be used as the diluent as PBS may alter the sample matrix.

Parallelism is another index of assay validation which determines whether albumin in urine behaves in an immunochemically similar manner to the albumin standard used. A urine sample (C2-5) diluted x200, x400, x800, x1600, x3200, x6400 was measured to establish the appropriate dilution factor and the parallelism of the response.

2.5.3 Results

Results from the ranging study (Table 2.5.3.1) show that doses of 40 or 50 mg per kg of streptozotocin were insufficient to induce diabetes in Lewis rats. By day three, only rats given 60 or 70 mg per kg of STZ showed signs of glycosuria (+, ++ and +++ equivalent to 14, 28 and 55 mmol/l glucose, respectively). Protein levels measured by Labstix were lower in the dilute urine of diabetic rats. Urine pH ranged between 8 and 8.5 in most rats.

Many of the graphs in the following sections include both non-diabetic (*pre-STZ)* and diabetic (*post-STZ*) data. It should be noted that week zero represents a baseline period during which both groups of rats were fed standard laboratory rat diet albeit the legends on the graphs depicts evening primrose oil (EPO) diet. When it is necessary to make comparisons between *pre- and post- STZ*, they are made relative to the week 1 *pre-STZ* group since it was deemed that (i) comparisons made between, for example, 3 weeks *pre-* and 1 week *post-* STZ were too close and (ii) the effects of EPO diet are often apparent after one week (see urine pH results).

2.5.3.1 Body weight

Pre-STZ: Mean body weight increased by 80% in the control group and by 63% in the EPO diet group (Figure 2.5.3.1). Only at week three were control rats significantly heavier than EPO rats [CD vs EPO: 248 (24) vs 218 (22) grams, CI = 9 to 52, p = 0.009] (Table 2.5.3.1a).

Post-STZ: Growth rate slowed down during the diabetic period; only at week one were control rats significantly heavier than at week one in the non-diabetic phase [Table 2.5.3.1a]. Despite a fall in body weight following streptozotocin, the animals continued to gain weight, albeit at a slower rate than during the non-diabetic phase. Growth rate curves of rats in both the Control and EPO diets groups are shown in Figures 2.5.3.1a and 2.5.3.1b. There was no significant difference in mean body weight between diet groups during the diabetic phase of the study.

RAT	BWt Day 1	BWt Day 2	STZ mg/kg	INJ VOL mls	BGl mM/L	BWt Day 3	UGl	UpH	UPROT	BWt Day 4	UGl	UPROT	UpH	BWt Day 5	UGl	UPROT	UpH
1	209	213	40	0.08	5.4	220	0	8		224	0	+/++	8.5	224	0	+++	8
1A	207	210	40	0.08	4.1	213	0	8.5		214	0	++/+++	8.5	215	0	+++	8.5
2	208	215	50	0.1	4.2	215	0	8.5		216	0	+/++	8.5	215	0	+++	8.5
2A	212	215	50	0.1	4.9	217	0	8.5	++	222	0	++/+++	8.5	224	0	++/+++	8.5
3	201	203	60	0.12	5.2	207	+/++	8	++	202	++/+++	Tr	8.5	204	++/+++	Tr	8
3A	206	207	60	012	4.7	210	0	8.5	++	210	+/++	+	8.5	210	Tr/+	+/++	8.5
4	194	199	70	0.14	5.6	203	+	8	++/+++	197	+++	0	8.5	197	+++	Tr	8
4A	195	199	70	0.14	5.1	199	+++	8.5	0	197	+++	Tr	8.5	190	+++	Tr	6.5
5	222	226	Buffer	0.1	DEAD												
5A	232	218	Buffer	0.1	5.2	224	0	8	++/+++	224	0	+	8.5	223	0	+	8.5

(**BWt**: Body weight; **BGl**: Blood glucose; **UGl**: Urine glucose; **UpH**: Urine pH; **UPROT**: Urine protein; **Inj Vol**: Injection volume)

Table 2.5.3.1 Streptozotocin ranging study

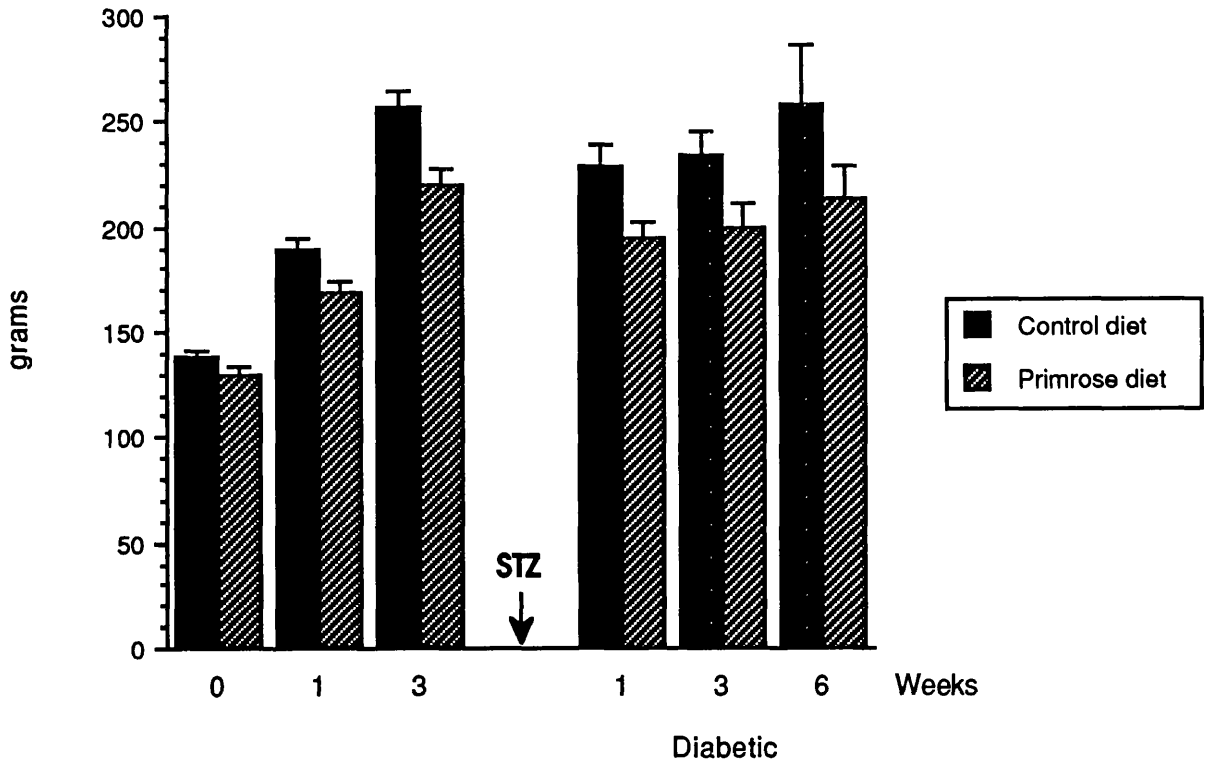


Figure 2.5.3.1 **Body weight (mean and SD) before and after streptozotocin (STZ).** At week 0 (baseline) both groups were fed control diet

Table 2.5.3.1(a) Body weight before and after STZ - statistical analyses

	95% confidence interval	significance level
Control vs Primrose		
0 weeks - pre	-6 to 14	p= 0.45
1 weeks - pre	-1 to 35	p= 0.06
3 weeks - pre	9 to 52	p= 0.009*
1 week - post	-8 to 75	p= 0.10
3 weeks - post	-5 to 79	p= 0.10
6 weeks - post	-35 to 71	p= 0.4
Control		
1 week pre vs 1 week post	3 to 56	p= 0.03*
1 week pre vs 3 week post	-33 to 55	p= 0.6
1 week pre vs 6 week post	-40 to 66	p= 0.5
Primrose		
1 week pre vs 1 week post	-7 to 73	p= 0.09
1 week pre vs 3 week post	-9 to 39	p= 0.2
1 week pre vs 6 week post	-23 to 23	p= 0.97

Two Sample *t* Test

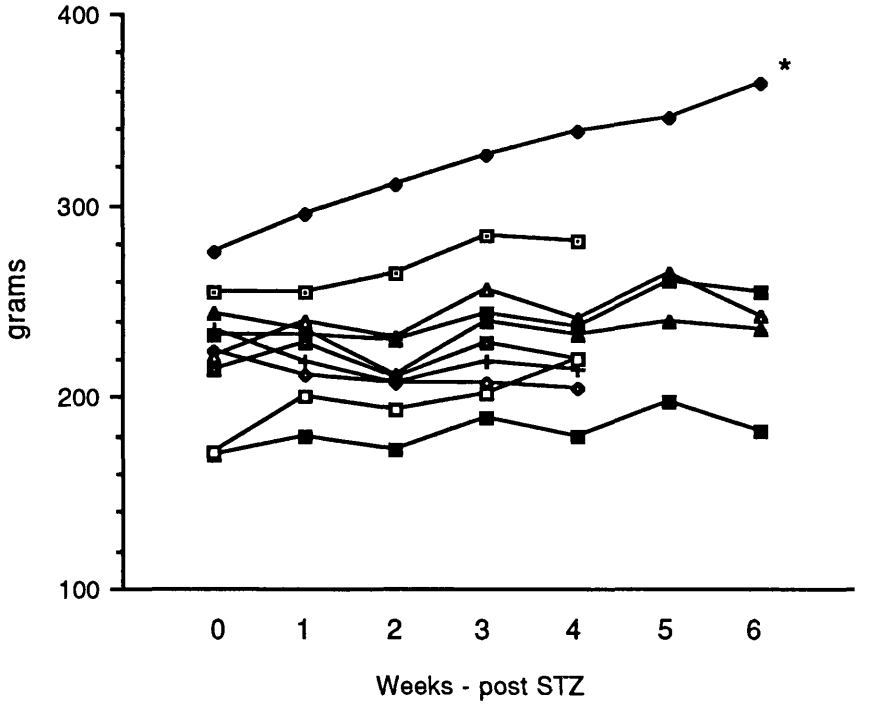


Figure 2.5.3.1(a) Growth rate curves of diabetic rats fed control diet (* denotes non-diabetic rat)

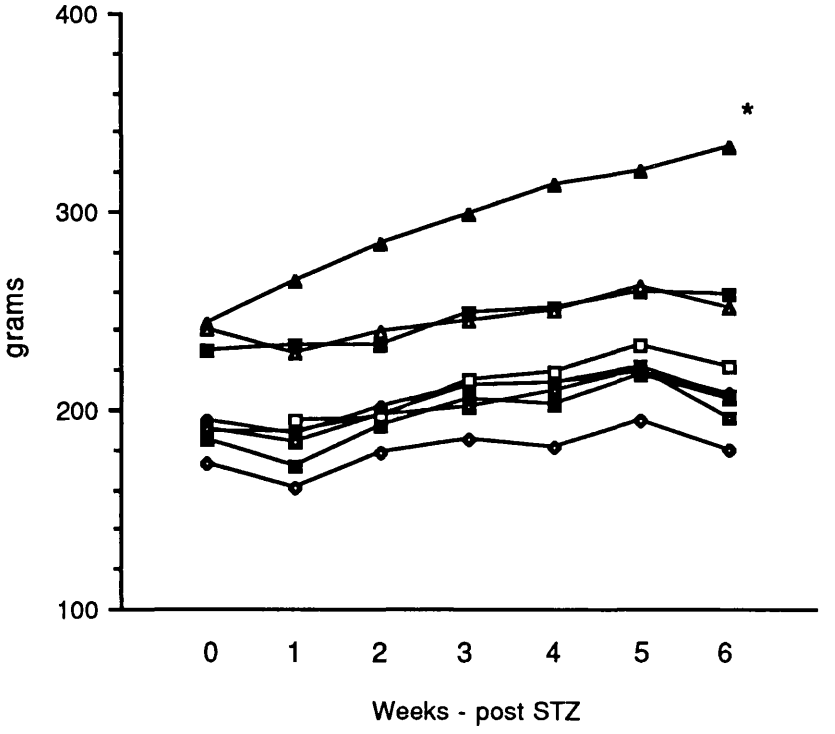


Figure 2.5.3.1(b) Growth rate curves of diabetic rats fed primrose oil diet (* denotes non-diabetic rat)

2.5.3.2 Food consumption

Pre-STZ: Mean food consumption was similar between the groups at baseline (week 0) when both were fed control diet [19.5 (4.5) vs 21.6 (4.12) grams, $p = 0.3$] but was significantly lower in the evening primrose oil diet group at weeks one [CD vs EPO: 20.8 (3.15) vs 15 (4.48), $p = 0.0046$] and three [CD vs EPO: 21 (2.36) vs 15.7 (2.36) grams, $p < 0.001$]. The fall in food consumption is likely to be related to the change from control to evening primrose oil diet (Figure 2.5.3.2).
Post-STZ phase: Mean food consumption increased in both diet groups during this period but there were no significant differences between the groups (Table 2.5.3.2).

2.5.3.3 Water intake

Pre-STZ: At week one, water intake was significantly lower in the evening primrose oil group [CD vs EPO: 19 mls (2.2) vs 14 mls (2.3), $p = 0.0002$] (Figure 2.5.3.3 and Table 2.5.3.3)
Post-STZ phase: Water intake increased markedly in both groups of rat after streptozotocin (Figure 2.5.3.3). Only at week one was there a significant difference in mean water intake between the diet groups [CD vs EPO: 113 mls (50) vs 180 mls (43), $p = 0.008$].

2.5.3.4 Urine volume

Pre-STZ: Urine volume was not affected by diet during this period (Figure 2.5.3.4).
Post-STZ phase: There was a significant increase in mean urine volume at week one in the evening primrose oil diet group [CD vs EPO: 104 mls (42) vs 169 mls (44) mls, $p = 0.006$] which reflected the significant increase in water intake in Figure 2.5.3.3 (see Table 2.5.3.4).

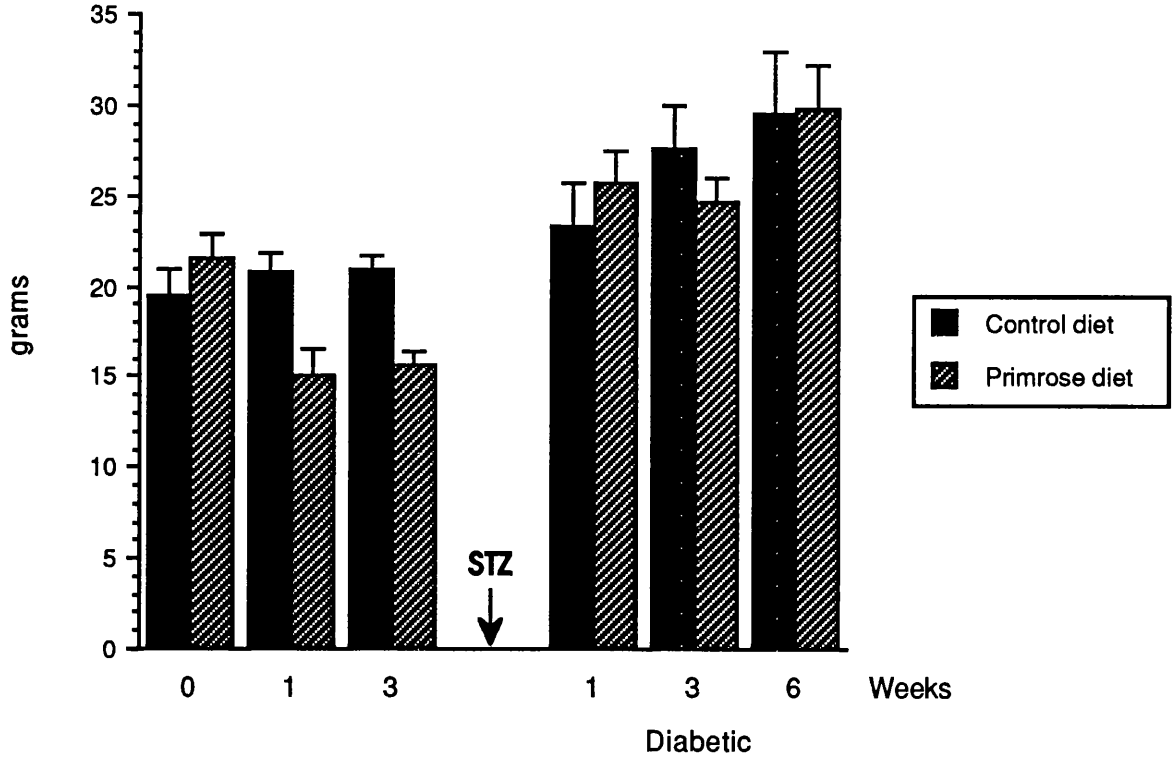


Figure 2.5.3.2 **Food consumption (mean and SD) before and after streptozotocin (STZ).** At week 0 (baseline) both groups were fed control diet

Table 2.5.3.2 Food consumption before and after STZ - statistical analyses

	95% confidence interval	significance level
Control vs Primrose		
0 weeks - pre	-6 to 2	p= 0.3
1 weeks - pre	2 to 9.5	p= 0.0046*
3 weeks - pre	3 to 7.5	p= 0.0001*
1 week - post	-8 to 3	p= 0.37
3 weeks - post	-4 to 9	p= 0.37
6 weeks - post	-11 to 14	p= 0.71
Control		
1 week pre vs 1 week post	-9 to 1.5	p= 0.14
1 week pre vs 3 week post	-14 to -1	p= 0.03*
1 week pre vs 6 week post	-22 to 2	p= 0.075
Primrose		
1 week pre vs 1 week post	-15 to -8	p<0.0001*
1 week pre vs 3 week post	-13 to -7	p<0.0001*
1 week pre vs 6 week post	-18 to -10	p<0.0001*

Two Sample *t* Test

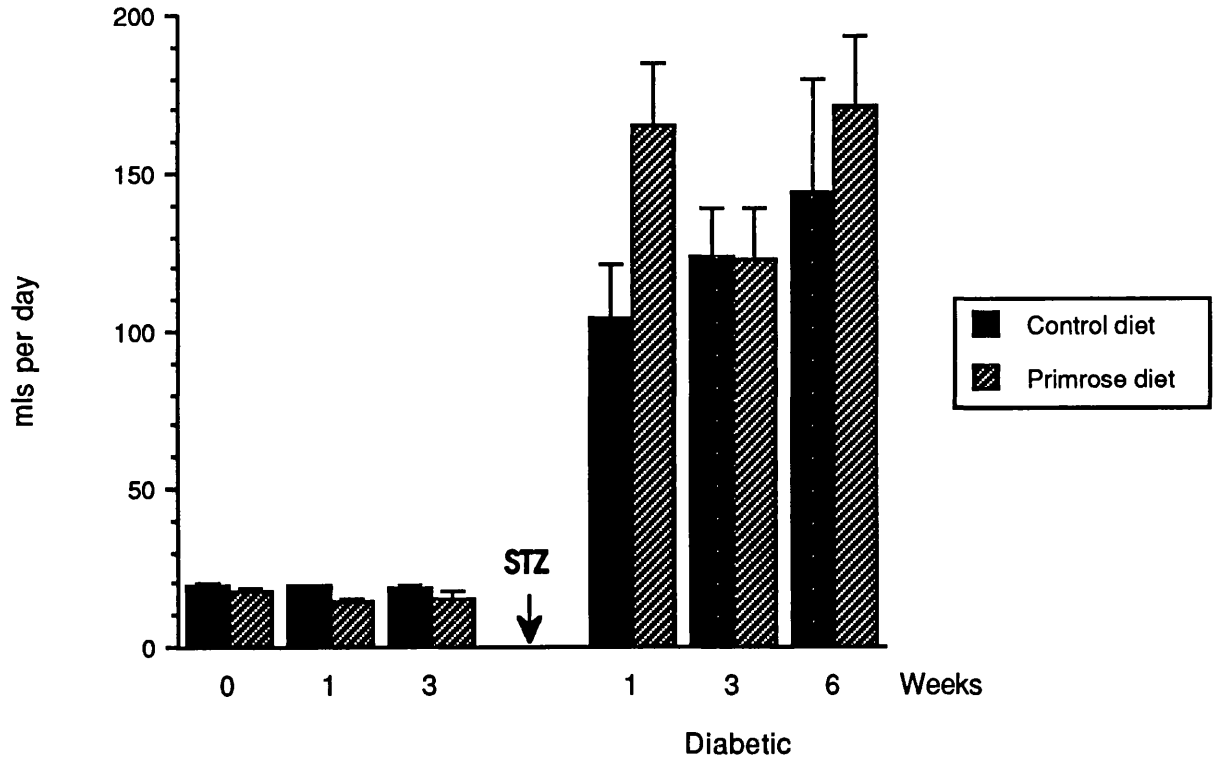


Figure 2.5.3.3 **Water intake (mean and SD) before and after streptozotocin (STZ).** At week 0 (baseline) both groups were fed control diet

Table 2.5.3.3 Water intake before and after STZ - statistical analyses

	95% confidence interval	significance level
Control vs Primrose		
0 weeks - pre	-2 to 5	p= 0.36
1 weeks - pre	3 to 7	p= 0.0002*
3 weeks - pre	-3 to 8.5	p= 0.31
1 week - post	-114 to -21	p= 0.008*
3 weeks - post	-41 to 38	p= 0.9
6 weeks - post	-77 to 47	p= 0.56
Control		
1 week pre vs 1 week post	-133 to -56	p= 0.0004*
1 week pre vs 3 week post	-145 to -85	p<0.0001*
1 week pre vs 6 week post	-224 to -91	p= 0.005*
Primrose		
1 week pre vs 1 week post	-198 to -131	p<0.0001*
1 week pre vs 3 week post	-151 to -88	p<0.0001*
1 week pre vs 6 week post	-204 to -146	p<0.0001*

Two Sample *t* Test

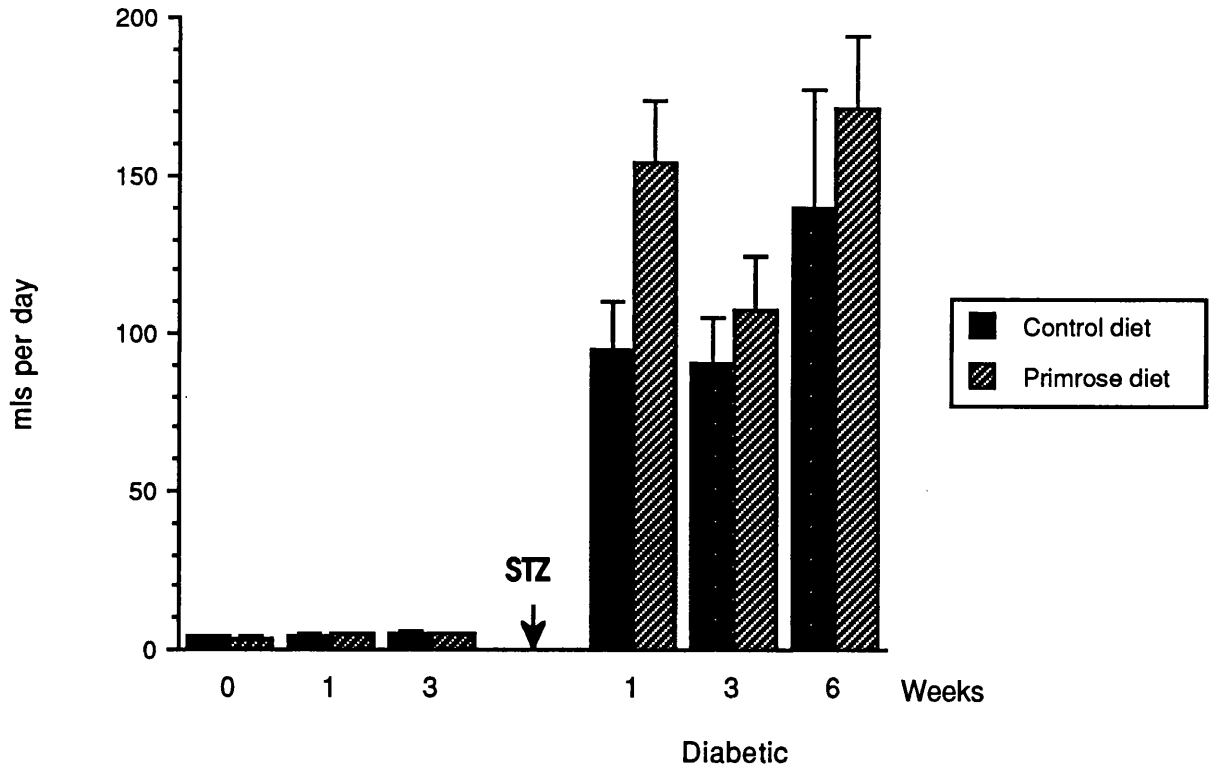


Figure 2.5.3.4 Urine volume (mean and SD) before and after streptozotocin (STZ). At week 0 (baseline) both groups were fed control diet

Table 2.5.3.4 Urine volume before and after STZ - statistical analyses

	95% confidence interval	significance level
Control vs Primrose		
0 weeks - pre	-1 to 1.5	p= 0.67
1 weeks - pre	-1.3 to 0.6	p= 0.4
3 weeks - pre	-0.4 to 1.6	p= 0.24
1 week - post	-107 to -21	p= 0.006*
3 weeks - post	-58 to 17	p =0.26
6 weeks - post	-87 to 50	p= 0.49
Control		
1 week pre vs 1 week post	-131 to -67	p=0.0001*
1 week pre vs 3 weeks post	-123 to -67	p<0.0001*
1 week pre vs 6 weeks post	-237 to -97	p= 0.005*
Primrose		
1 week pre vs 1 week post	-198 to -130	p<0.0001*
1 week pre vs 3 weeks post	-146 to -86	p<0.0001*
1 week pre vs 6 weeks post	-213 to -160	p<0.0001*

Two Sample *t*- Test

2.5.3.5 Urine pH

Pre-STZ: There was no difference in urine pH at baseline (week 0) when both groups were consuming control diet [**CD vs EPO**: 7.45 (0.685) vs 7.25 (0.589)] (Figure 2.5.3.5). However, in the control diet group, urine pH rose significantly at weeks one and three [Week 0: 7.45 (0.685) vs (i) week 1: 8.2 (0.632), CI = -1.4 to -0.1, p = 0.002, (ii) week 3: 8.35 (0.530), CI = -1.5 to -0.32, p = 0.005] and similarly, in the evening primrose oil group, urine pH fell significantly at weeks one and three [Week 0: 7.25 (0.589) vs (i) week 1: 6.2 (0.258), CI = 0.61 to 1.49, p = 0.0002, (ii) week 3: 6.1 (0.211), CI = 0.71 to 1.59, p = 0.0001] (see Table 2.5.3.5).

Post-STZ phase: The difference in urine pH between the diet groups was maintained at weeks one [**CD vs EPO:** 6.5 (0.35) vs 6 (0)] and three [8.4 (0.2) vs 6.1 (0.5)] but at six weeks the difference did not reach statistical significance due perhaps to sample size [**CD vs EPO:** 7.3 (0.7) vs 6.6 (0.2): p = 0.07].

In the control diet group, despite a significant fall at week 1, pH remained raised at weeks 3 and fell to around the starting level at week 6 relative to week 1 in the pre-STZ phase [*Pre-STZ* - week 1: 8.2 (0.632) vs *Post-STZ* (i) week 1: 6.5 (0.354), CI = 1.2 to 2.2, p <0.0001, (ii) week 3: 8.4 (0.221), CI = -0.66 to 0.280, p = 0.39, (iii) week 6: 7.3 (0.671), CI = 0.05 to 1.75, p = 0.041]. In the evening primrose oil diet group, urine pH remained low at weeks 1, 3 and may be starting to rise again at week 6. [*Pre-STZ* - week 1: 6.2 (0.258) vs *Post-STZ* (i) week 1: 6 (0), (ii) week 3: 6.1 (0.486), CI = -0.31 to 0.49, p = 0.63, (iii) week 6: 6.6 (0.177), CI = -0.582 to -0.143, p = 0.003].

2.5.3.6 Systolic blood pressure

There was no significant difference in systolic blood pressure between the control diet and evening primrose oil diet groups either in the pre-STZ [CD vs EPO: 138 (16) vs 144 (17) mm Hg, CI = -24 to 11, p = 0.4] or diabetic [CD vs EPO: 136 (19) vs 139 (10) mm Hg, CI = -25 to 21, p = 0.8] periods (Figure 2.5.3.6).

Neither was there any difference in the control diet or evening primrose oil diet groups between non-diabetic and diabetic phases [*Pre-STZ* vs *post-STZ*: 138 (16) vs 136 (19), CI = -21 to 24, p = 0.9] or EPO [*Pre-STZ* vs *post-STZ*: 144 (17) vs 139 (10), CI = -10 to 22, p = 0.5] diet groups.

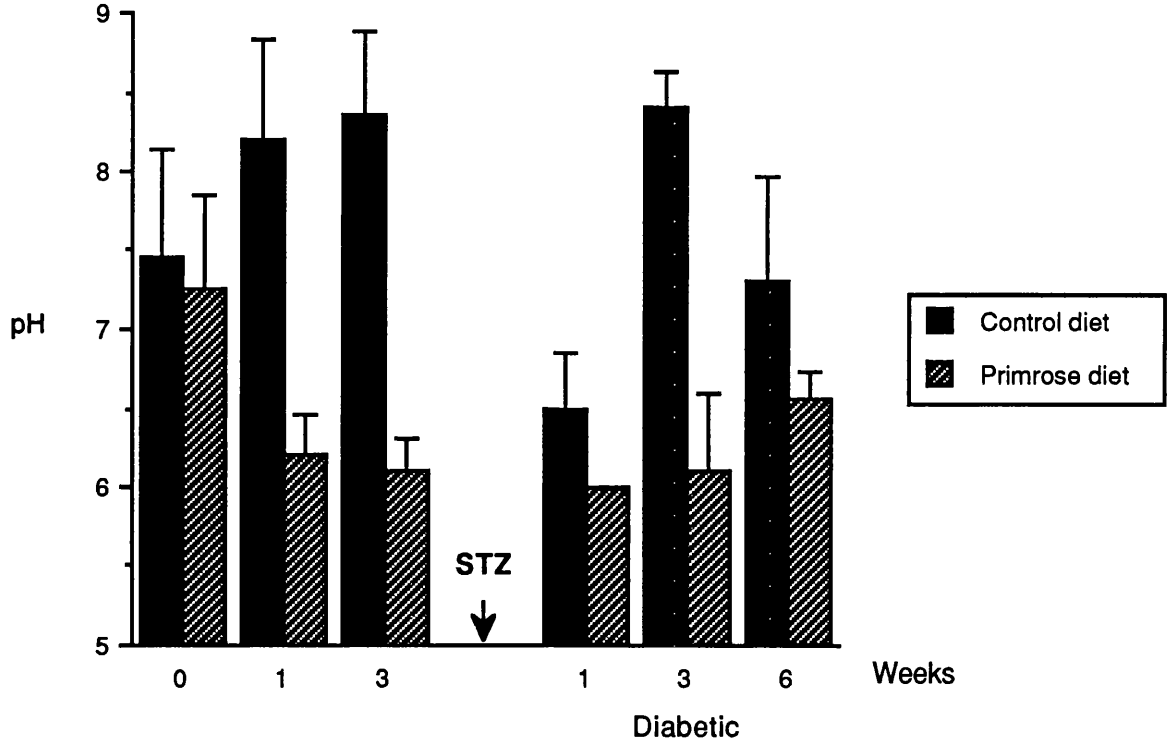


Figure 2.5.3.5 **Urine pH before and after streptozotocin (STZ).** At week 0 (baseline) both groups were fed control diet

Table 2.5.3.5 Urine pH before and after STZ - statistical analyses

	95% confidence interval	significance level
Control vs Primrose		
0 weeks - pre	-0.4 to 0.8	p= 0.5
1 weeks - pre	1.5 to 2.5	p<0.0001*
3 weeks - pre	2 to 2.7	p<0.0001*
1 week - post		
3 weeks - post	1.8 to 2.7	p<0.0001*
6 weeks - post	-0.1 to 1.6	p= 0.07
Control		
1 week pre vs 1 week post	1.4 to 2.3	p<0.0001*
1 week pre vs 3 week post	-0.4 to 0.36	p= 0.84
1 week pre vs 6 week post	0.2 to 1.9	p= 0.022*
Primrose		
1 week pre vs 1 week post		
1 week pre vs 3 week post	-0.4 to -0.38	p= 0.95
1 week pre vs 6 week post	-0.6 to -0.3	p= 0.0001*

Two Sample *t* Test

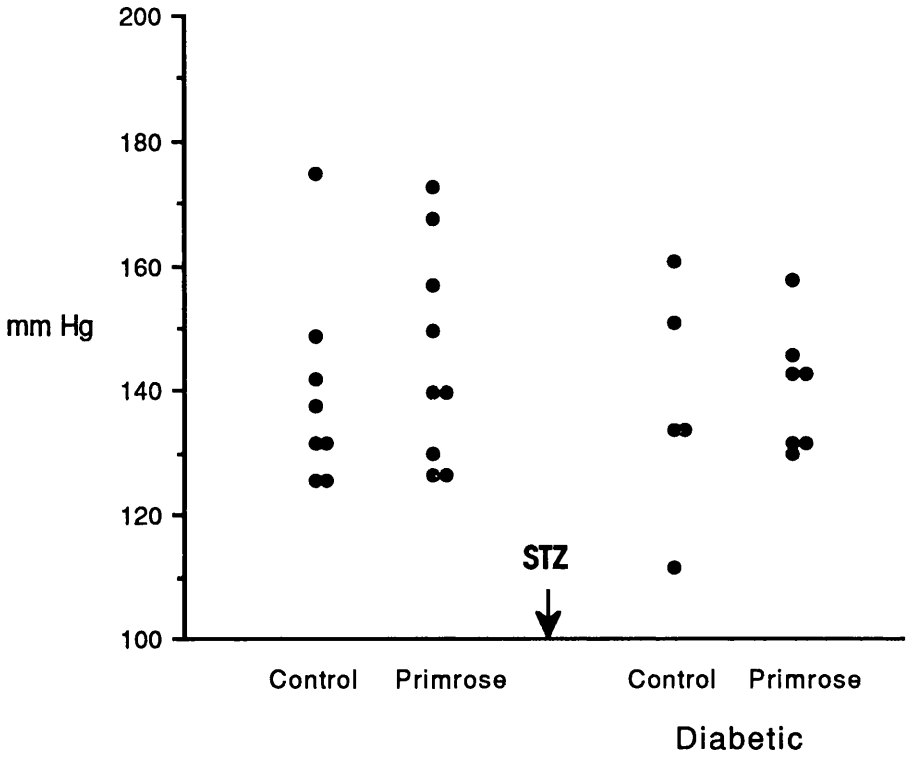


Figure 2.5.3.6 Systolic blood pressure one week before and five weeks after STZ injection. (Each point represents the mean of five readings)

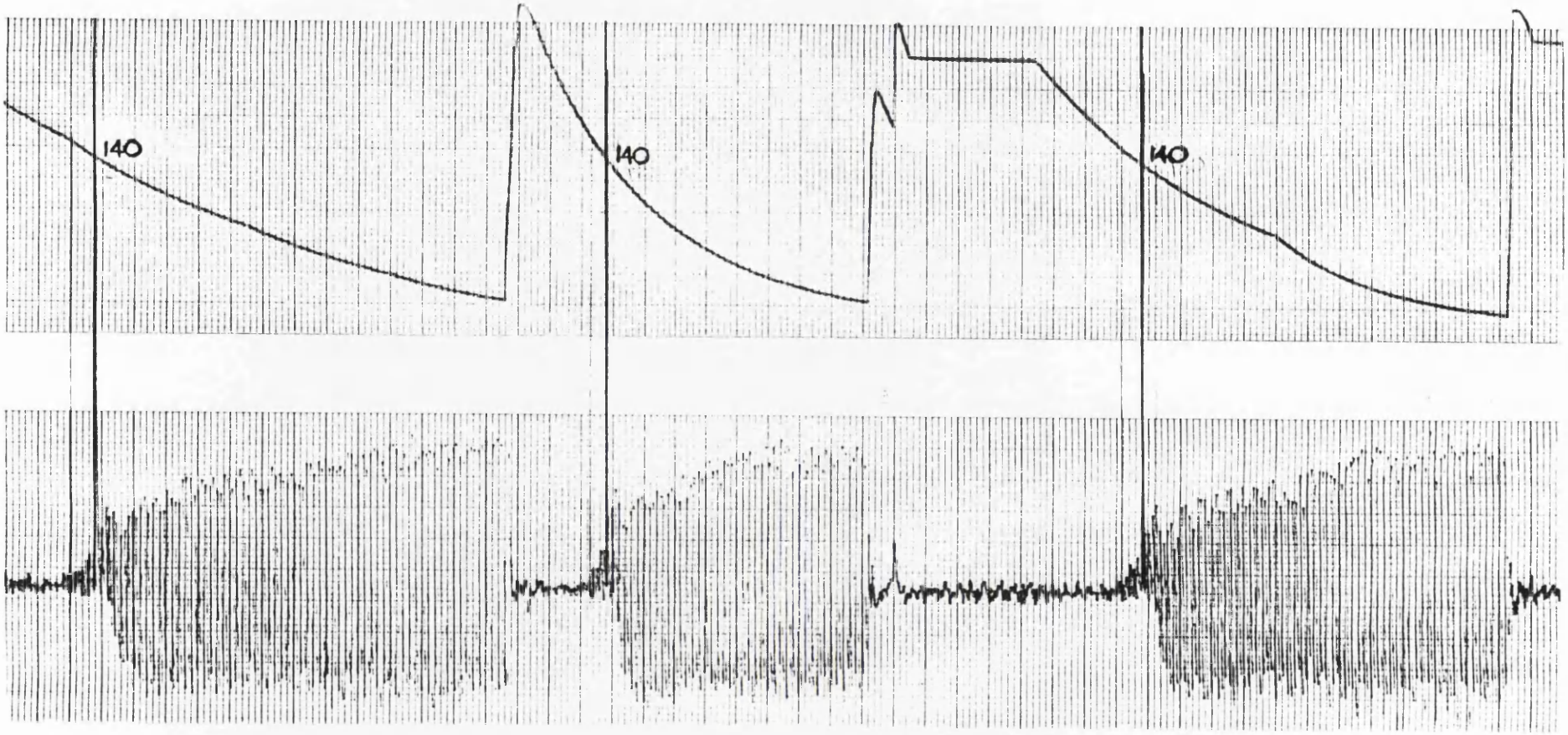


Plate 2.5.1 Systolic blood pressure measurement (in mm Hg) using the Buffington Cuff

2.5.3.7 Kidney weight

At the end of six weeks kidneys from diabetic rats were significantly heavier than those of normal Lewis rats fed standard laboratory rat diet, irrespective of diet [*Pre-STZ* CD: median 0.67 (range 0.54 - 0.76) grams vs: (i) *post-STZ* CD: 1.425 (1.3 - 1.5) grams, CI = -0.85 to -0.65, p = 0.0019, (ii) *post-STZ* EPOD: 1.59 (1.4 - 1.8) grams, CI = -0.99 to -0.84, p < 0.0001] (Figure 2.5.3.7)]. The results suggest that kidneys from diabetic rats fed evening primrose oil diet may be heavier than those fed control diet [*Post-STZ* CD: 1.425 (1.3 - 1.5) vs EPO: 1.59 (1.4 - 1.8), 95% CI = -0.3 to 0, p = 0.055].

2.5.3.8 Blood glucose

Pre-STZ: At three weeks, plasma glucose levels were significantly higher in the EPO diet group [CD vs EPO: 4.9 (1.35) vs 6.5 (1.1) mmol/l, CI = -2.8 to -0.4, p = 0.011] (Figure 2.5.3.8).

Post-STZ phase: Mean plasma glucose levels were higher than in the non-diabetic phase in both diet groups at weeks one but there were no statistically significant differences between the diet groups [**CD vs EPO**: week 1: 32.75 (2.7) vs 33 (3.9) mmol/l], week 3: 34 (2.8) vs 33 (3.0) mmol/l], week 6: 47.5 (2.4) vs 41.6 (8.2) mmol/l].

2.5.3.9 Urine urea

Urea excretion was similar in both diet groups at weeks one and three during the non-diabetic phase of the study (Figure 2.5.3.9) [**CD vs EPO**: week 1: median 5.005 (range 3.74 - 6) vs 4.3 (3.54 - 5.76) mmol/l, 95% CI = -0.02 to 1.44, p = 0.038), week 3: 5.4 (4.86 - 6.51) vs 5.63 (4.58 - 6.18) mmol/l: 95% CI = -0.6 to 0.6, p = 0.94]. Urine urea was not measured in diabetic rats.

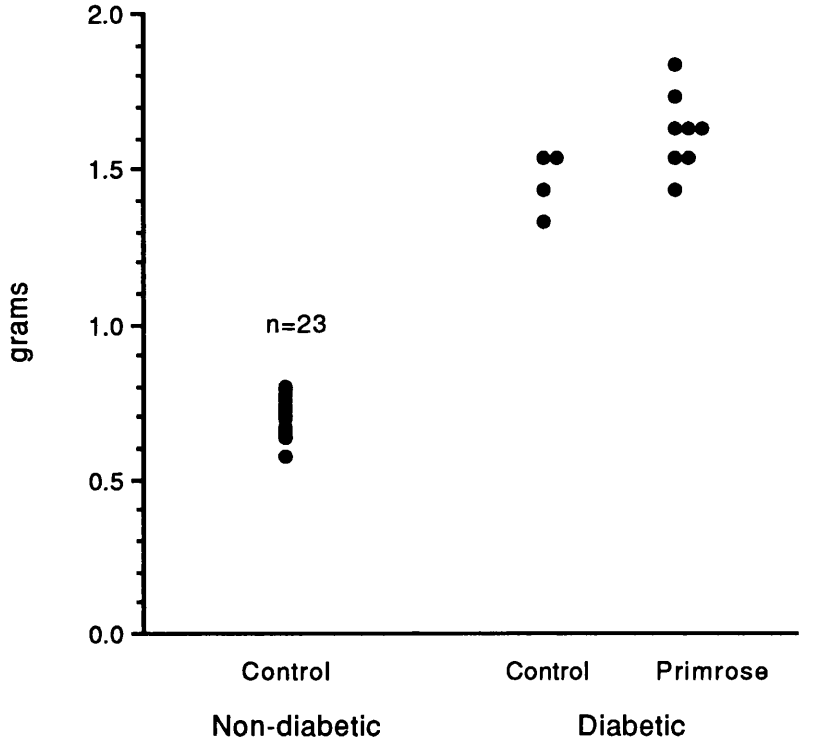


Figure 2.5.3.7 **Kidney weights of (i) non-diabetic Lewis rats fed control diet for six weeks and (ii) diabetic rats after six weeks of either control or evening primrose oil diet**

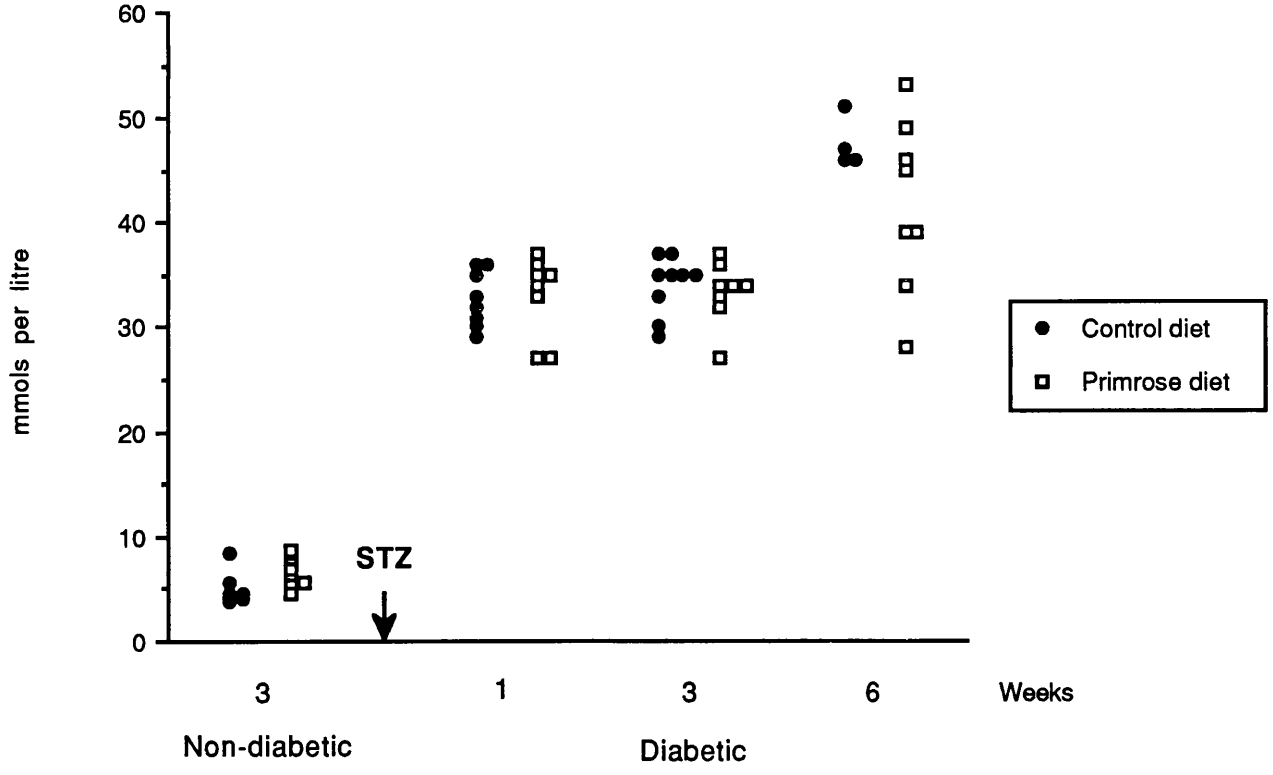


Figure 2.5.3.8 Plasma glucose levels before (after 3 weeks) and after streptozotocin

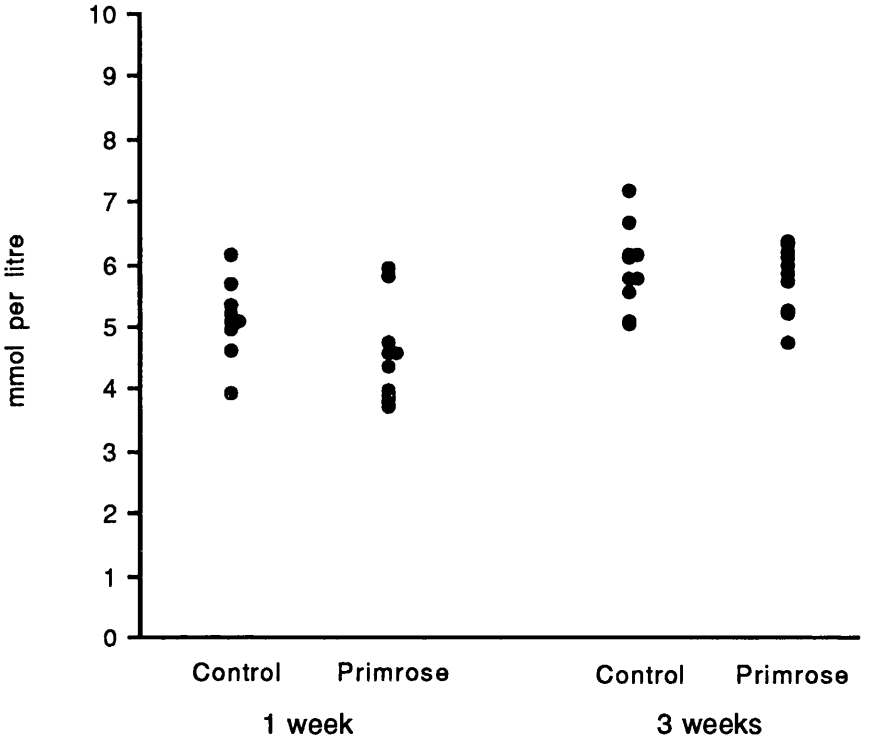


Figure 2.5.3.9 Urine urea levels at one and three weeks in non-diabetic (before STZ) rats

2.5.3.10 Total protein and albumin excretion

Pre-STZ: Urine total protein excretion was significantly lower in the evening primrose oil diet group after three weeks [CD vs EPO: median 12.05 (range 9 - 14.5) vs 5.6 mg per 24 hours (3.7 - 6.7), CI = 5.4 to 8.4, p = 0.0002] (Figure 2.5.3.10).

Albumin excretion rate was significantly lower in the evening primrose oil diet group after three weeks [CD vs EPO: median 0.625 (range 0.221 - 0.875) vs 0.249 (0.137 - 0.403) mg per 24 hours, CI = 0.206 to 0.546, p < 0.0017] (Figure 2.5.3.11).

2.5.3.11 Validation of ELISA method

The poor results obtained from the first sandwich ELISA method (Figure 2.5.3.12) may be compared to the immediate improvement in sensitivity using the streptavidin-biotin method (Figure 2.5.3.12a).

Optimal dilutions of coating and capture antibodies and avidin-HRP were derived by titration. Coating antibody was diluted 1: 2600, biotinylated antibody at 1:4000 and avidin-HRP at 1: 5000 dilution (Figure 2.5.3.12b) which are much higher dilutions than those used in the first method (see section 2.5.2.5). The standard curve now ranged over nearly two units of optical density.

Standard curve data of doubling dilutions from 25 to 0.2 ng per ml are shown in Figure 2.5.3.13. Not surprisingly, the precision of the assay (25%) was poor in the early stages of the assay (QC mean = 1.169 and standard deviation = 0.288).
The results of diluting a urine sample x200, x400, x800, x1600, x3200 or x6400 is shown in Table 2.5.3.14. Actual levels at dilutions of 200- and 400- fold were too high but higher dilution factors gave more linear results and the intra-assay imprecision at all dilutions was less than 10%. Using this data, a dilution factor of 800 was selected for assay of the test urine samples.

The results of spiking ultrafiltered urine (UFU) with rat serum albumin at 6, 4, 2, 1, 0.5 ng per ml are shown in Table 2.5.3.15. Recovery and precision (CV%) are shown in table 2.5.3.15(a).

Assay imprecision may be estimated from the measurements shown in Tables 2.5.3.16, 2.5.3.16a and 2.5.3.16b but by omitting results of Assay E which tended to read high.

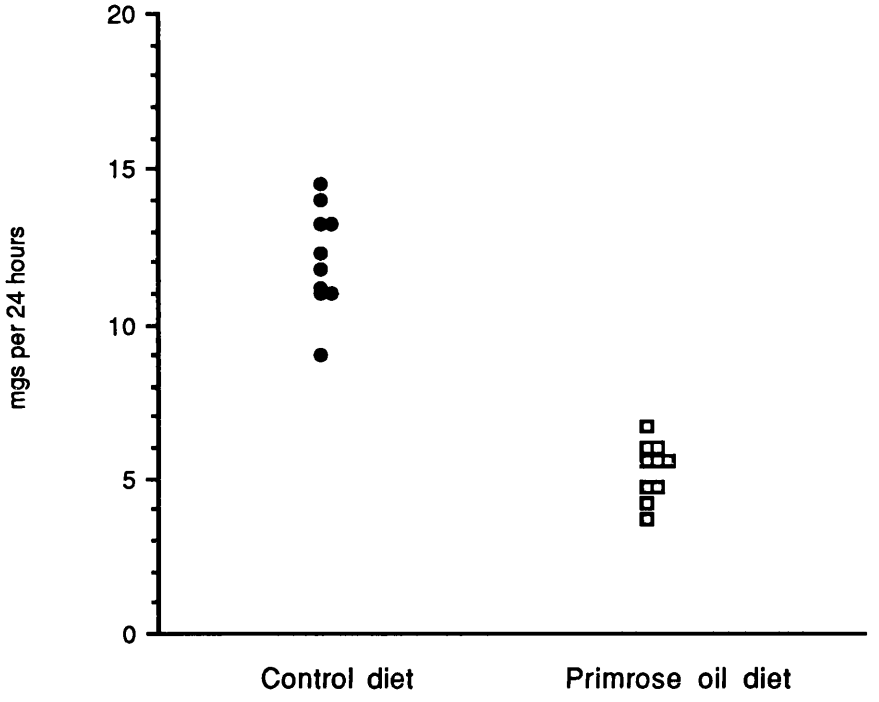


Figure 2.5.3.10 Total protein excretion before STZ - (at three weeks)

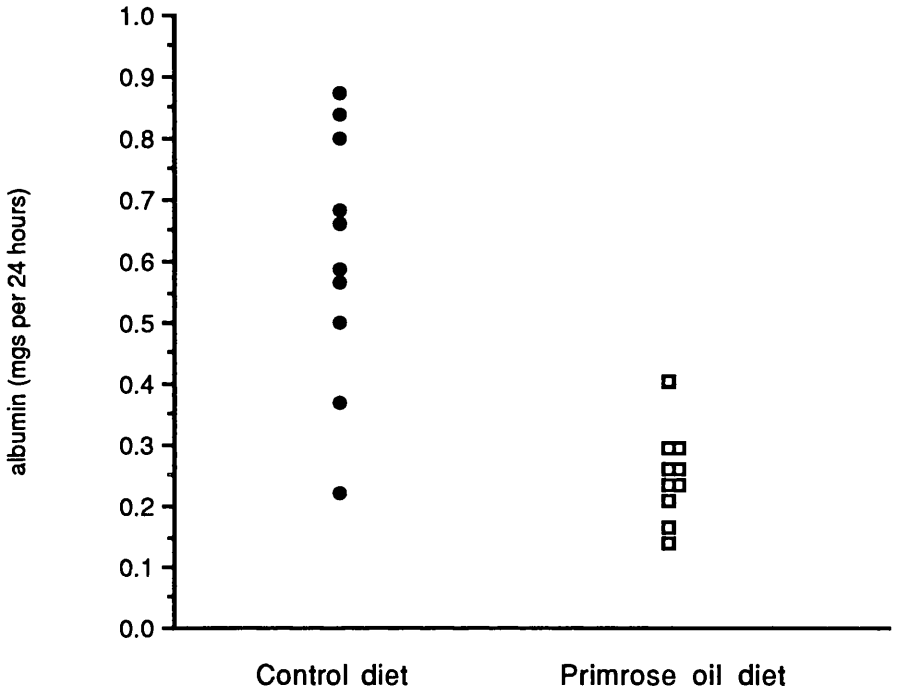


Figure 2.5.3.11 Albumin excretion rate before STZ - (at three weeks)

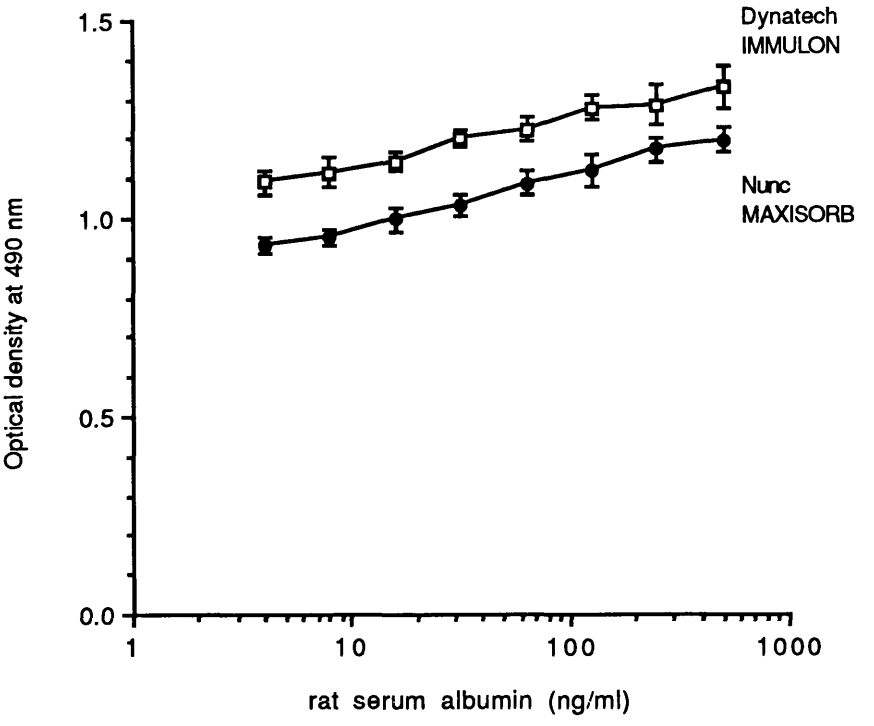


Figure 2.5.3.12 **Best obtainable binding curve using double sandwich ELISA method**

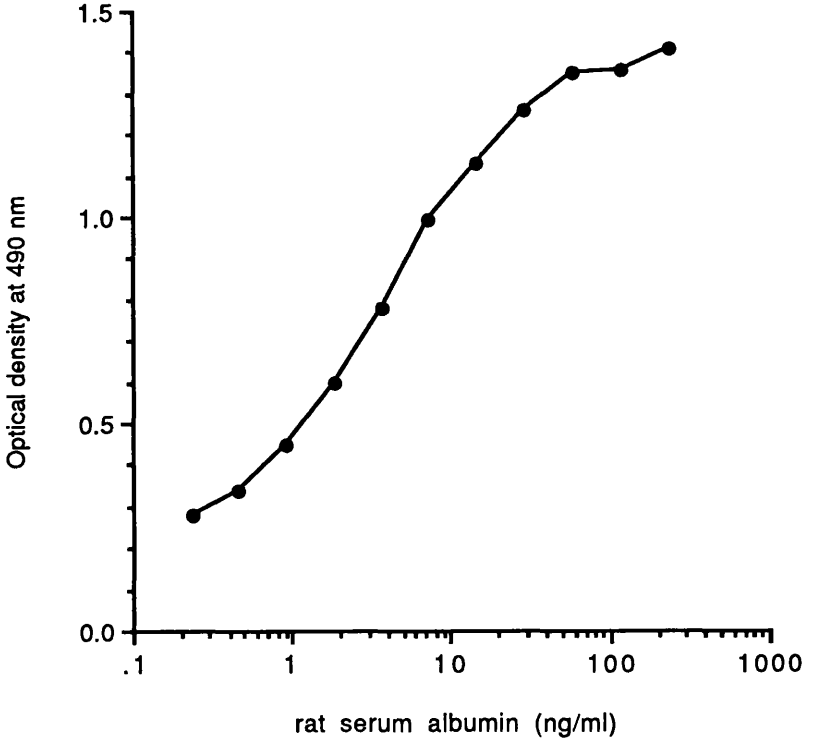


Figure 2.5.3.12(a) **First standard binding curve using the biotin-avidin detection system**

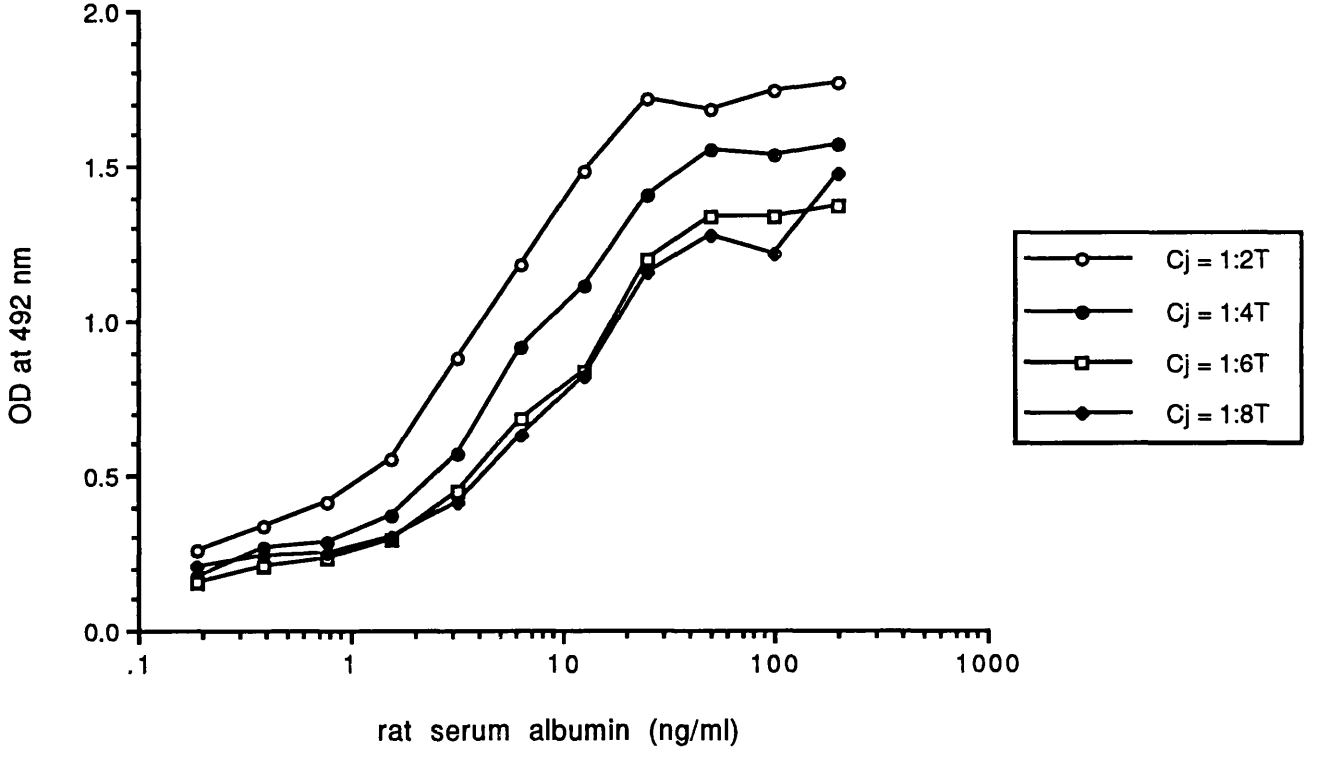


Figure 2.5.3.12(b) Optimisation of standard binding curves. Biotinylated α–RSA at 1: 2000, 1: 4000, 1: 6000 and 1: 8000 and avidin - HRP at 1: 5000

	200	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0	QC (ng/ml)
1	1.380	1.332	1.326	1.235	1.102	0.965	0.749	0.574	0.420	0.311	0.252	0.227	
2	1.206	1.162	1.049	0.887	0.720	0.704	0.567	0.420	0.371	0.337	0.324	0.321	
3	1.284	1.311	1.140	1.153	1.029	0.806	0.661	0.443	0.399	0.364	0.349	0.357	
4	1.427	1.505	1.492	1.485	1.429	1.312	1.086	0.852	0.609	0.454	0.333	0.241	
5	1.817	1.826	1.793	1.751	1.704	1.676	1.417	1.126	0.784	0.550	0.400	0.205	
6			1.498	1.441	1.312	1.225	0.832	0.588	0.386	0.283	0.194	0.126	
7			1.726	1.655	1.580	1.501	1.387	1.092	0.830	0.568	0.268	0.129	
8				1.653	1.596	1.364	1.112	0.786	0.523	0.340	0.220	0.231	1.748
9				1.522	1.316	1.115	0.842	0.586	0.406	0.274	0.199	0.123	1.342
10				2.038	1.900	1.679	1.263	0.807	0.541	0.346	0.245	0.214	1.269
11				1.862	1.748	1.563	1.245	0.879	0.566	0.346	0.236	0.087	1.064
12				1.796	1.705	1.517	1.223	0.874	0.566	0.354	0.235	0.090	1.060
13				2.097	2.031	1.967	1.638	1.196	0.774	0.495	0.324	0.134	0.999
14				OVER	2.063	1.931	1.637	1.212	0.790	0.479	0.309	0.110	0.787
15				2.031	1.987	1.832	1.575	1.174	0.772	0.506	0.345	0.138	1.085
16				2.064	2.010	1.925	1.708	1.413	1.059	0.722	0.478		
17				2.066	2.043	1.916	1.670	1.320	0.947	0.614	0.401		
18				1.932	1.922	1.811	1.630	1.349	0.965	0.577	0.394		

Table 2.5.3.13 ELISA standard curve data

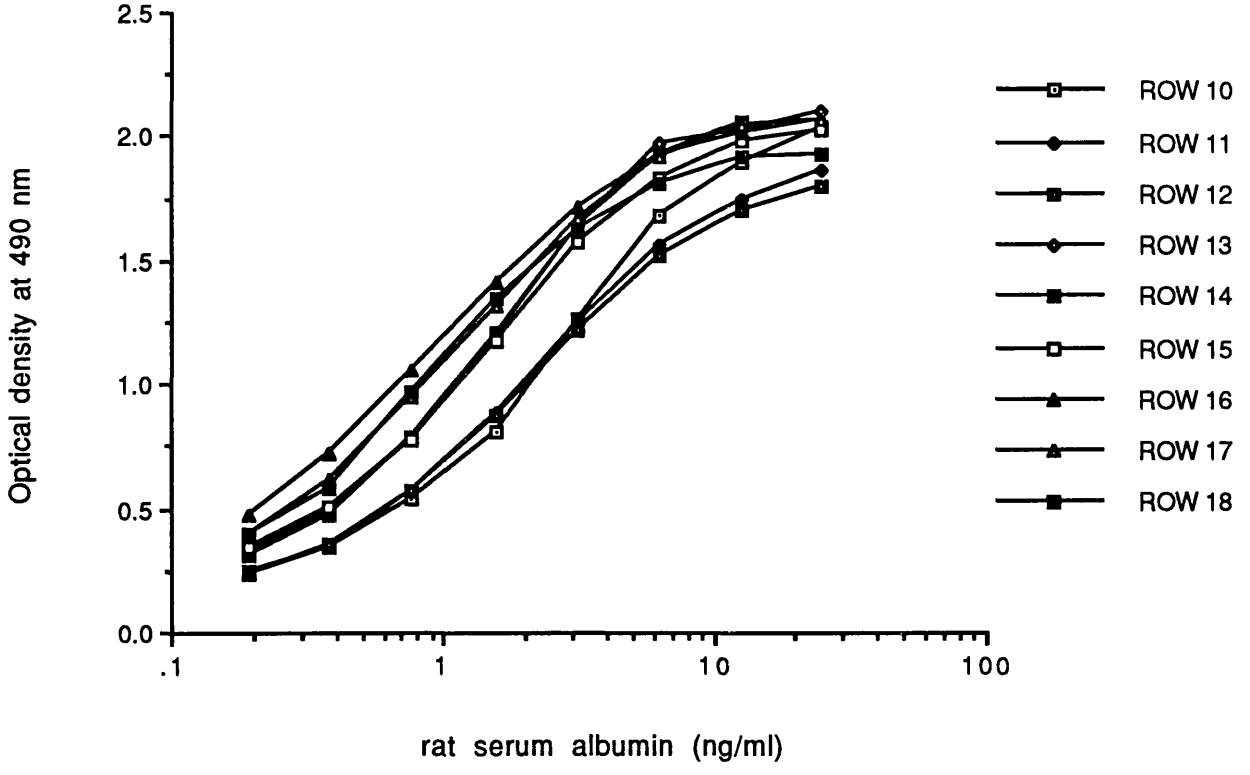


Figure 2.5.3.13 ELISA binding curves from data in Table 2.5.3.13

		50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0	0	0	CV(%)
A		1.580	1.467	1.360	1.161	0.899	0.600	0.398	0.292	0.184	0.128	0.109	0.122	
B		1.585	1.463	1.360	1.123	0.860	0.587	0.378	0.255	0.184	0.112	0.098	0.100	
C	x200	1.522	1.522	1.527	1.489	1.517	1.517	1.503	1.494	1.480	1.480	1.485	1.480	
		18.478	**18.478**	**18.736**	**16.860**	**18.223**	**18.223**	**17.528**	**17.096**	**16.444**	**16.444**	**16.674**	**16.444**	**5.2**
D	x400	1.446	1.403	1.463	1.403	1.418	1.414	1.418	1.392	1.385	1.357	1.370	1.364	
		14.963	**13.279**	**15.686**	**13.279**	**13.844**	**13.691**	**13.844**	**12.879**	**12.632**	**11.687**	**12.116**	**11.916**	**9**
E	x800	1.306	1.297	1.327	1.294	1.300	1.297	1.300	1.280	1.280	1.263	1.280	1.260	
		10.144	**9.893**	**10.753**	**9.811**	**9.976**	**9.893**	**9.976**	**9.437**	**9.437**	**9.002**	**9.437**	**8.928**	**5.2**
F	x1600	1.093	1.098	1.115	1.074	1.071	1.089	1.074	1.069	1.069	1.017	1.059	1.048	
		5.615	**5.694**	**5.969**	**5.327**	**5.282**	**5.553**	**5.327**	**5.253**	**5.253**	**4.547**	**5.109**	**4.956**	**6.9**
G	x3200	0.834	0.831	0.840	0.795	0.826	0.816	0.820	0.803	0.797	0.776	0.806	0.774	
		2.736	**2.713**	**2.781**	**2.455**	**2.675**	**2.602**	**2.631**	**2.510**	**2.468**	**2.329**	**2.531**	**2.316**	**6**
H	x6400	0.594	0.573	0.596	0.566	0.570	0.566	0.574	0.571	0.566	0.557	0.569	0.555	
		1.405	**1.325**	**1.413**	**1.300**	**1.314**	**1.300**	**1.329**	**1.318**	**1.300**	**1.268**	**1.311**	**1.261**	**3.5**

Upper value = Optical density at 490 nm
Lower value = RSA (ng/ml)

Table 2.5.3.14 Urine sample dilutions measured by ELISA

Standards	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0	0	QC	QC
A	OVER	2.036	1.890	1.571	1.190	0.784	0.512	0.341	0.214	0.220	1.241	1.017
B	2.031	1.938	1.774	1.579	1.158	0.760	0.501	0.348	0.215	0.217	1.080	1.001
ng/ml	6	6	4	4	2	2	1	1	0.5	0.5	UFU	UFU
C	5.835	5.580	3.632	3.257	2.002	1.561	0.711	0.738	0.454	0.482	0.260	0.247
D	5.521	5.519	3.068	3.154	1.581	1.445	0.743	0.741	0.460	0.463	0.252	0.248
E	5.898	5.812	3.580	3.469	1.519	1.537	0.721	0.785	0.461	0.460	0.251	0.251
F	5.515	5.440	3.752	3.304	1.631	1.362	0.703	0.780	0.446	0.450	0.249	0.250
G	5.730	6.141	3.411	3.095	1.653	1.512	0.727	0.802	0.450	0.436	0.248	0.245
H	5.187	5.646	4.000	3.243	1.722	1.436	0.699	0.753	0.437	0.425	0.247	0.255

Results expressed as concentration (ng/ml)

Table 2.5.3.15 Recovery of albumin from 'spiked' ultrafiltered urine

ng/ml	6	4	2	1	0.5	UFU
n	12	12	12	12	12	12
mean	5.652	3.414	1.580	0.742	0.452	0.250
SD	0.25	0.28	0.17	0.033	0.015	0.004
CV (%)	4.4	8.2	10.75	4.4	3.3	1.6
Recovery (%)	94.2	85.4	79	74.2	90.5	

Table 2.5.3.15(a) Statistical analysis of ELISA recovery data

2.5.3.12 Albumin excretion - post STZ

Tables 2.5.3.16, 2.5.3.16(a) and 2.5.3.16(b) depict mean albumin concentration in urine diluted 800- fold (2.5 µls to 2 mls). Urine samples were assayed several times in different assays from separate frozen aliquots and it is from this data that Figure 2.5.3.17 is derived. There was little difference in albumin excretion rate between the diet groups at weeks one and three but at six weeks, albumin excretion started to rise in the EPO diet group [**CD vs EPO**: (i) week 1: median 311 (range 126 - 429) vs 299 (185 - 793) µgs per 24 hours, CI = -198 to 56, p = 0.8, (ii) week 3: 208 (152 - 341) vs 229 (171 - 309) µgs per 24 hours, CI = -78 to 46, p = 0.46, (iii) 230 (215 - 725) vs 821 (522 - 937) µgs per 24 hours, CI = -701 to -51, p = 0.034].

Within the control diet group, there was no change in AER at weeks three and six compared with week one [Week 1: 311 (126 - 429) µgs per 24 hours vs (i) week 3: 208 (152 – 341), CI = -30 to 134, p = 0.22, (ii) week 6: 230 (215 - 725) µgs per 24 hours, CI = -413 to 104, p = 0.7].

In the evening primrose oil group, compared with week one, median AER may be slightly lower at week three but was raised significantly at six weeks [Week 1: 299 (185 - 793) mgs per 24 hours vs (i) week 3: 229 (171 - 309), CI = -9 to 236, p = 0.07, (ii) week 6: 821 (522 - 937), CI = -613 to -241, p = 0.003].

Statistical comparisons made using six week data must be viewed with caution since there are so few surviving rats in the control group.

Only one rat in each diet group did not become diabetic following injection of streptozotocin. At the end of six weeks, four rats remained in the control diet group and eight in the evening primrose oil diet group (Figure 2.5.3.18).

Rat	Urine volume	Assay A (ng/ml)	Assay B (ng/ml)	Assay C (ng/ml)	Assay D (ng/ml)	Assay E (ng/ml)	Assay F (ng/ml)	mean (µgs/24 hrs)
C1	104	3.19 2.64	3.51 3.77	4.93	3.147	5.780	3.328	3.84 (319)
C2	6							
C3	110	3.76 3.33	5.36 4.42	4.86	3.596	8.145	4.194	4.87 (429)
C4	104	3.05 2.95	3.72 3.1	3.54	2.873	6.145	3.425	3.73 (310)
C5	76	2.100 2.003	2.94 1.45	2.08	1.282	2.290	2.514	2.07 (126)
C6	15	8.88 9.62		10.92	10.869	17.946	6.769	11.15 (134)
C7	148	1.307 1.358		3.115	2.536	2.4	1.870	2.25 (266)
C8	98	2.654 2.955		4.078	4.159	6.059	2.734	3.97 (311)
C9	130	2.22 1.88		2.54	2.522	3.694	2.037	2.57 (267)
C10	154	2.115 1.950		2.389	2.342 2.178	3.901	2.386	2.59 (320)
P1	208	2.52 2.39	3.45 3.65	3.02	2.948	4.916	2.088	3.16 (526)
P2	170	1.48 1.40	2.04 3.86	1.997	1.918	2.540	1.234	2.01 (274)
P3	194	3.47 4.16	5.4 7.56	4.63	5.502	6.496	3.745	5.11 (793)
P4	167	0.99 1.055	1.93 2.75	2.29	1.744	2.536	1.422	1.89 (253)
P5	65	4.09 4.57	7.9 9.82	6.06	5.259	7.601	5.281	6.23 (324)
P6	190	1.299 1.195		2.112	1.714	2.476	2.300	1.97 (299)
P7	192	1.516 1.422		1.64 1.79	1.962	2.497	2.116	1.95 (300)
P8	18							
P9	193	1.019 0.99		1.357	0.822	1.658	1.143	1.20 (185)
P10	140	1.793 1.642		2.77	2.454 2.222	2.836	2.104	2.35 (264)

Table 2.5.3.16 **Urine albumin excretion in rats fed control (C) or evening primrose oil (P) diet - 1 week post STZ**

Rat	Urine volume	Assay C (ng/ml)	Assay D (ng/ml)	Assay E (ng/ml)	Assay F (ng/ml)	mean (µgs/24 hrs)
C1	110	2.44	1.790	3.107	2.278	2.40 (212)
C2	7					
C3	135	1.954	1.645 1.429	2.529 1.815	2.006	1.92 (207)
C4	135	1.817	1.220	2.179	1.632	1.71 (185)
C5	72	4.683	4.324	6.684	3.682	4.84 (279)
C6	85	2.026	2.019	2.647	2.397	2.27 (155)
C7	75	6.185	4.571	7.250	4.716	5.68 (341)
C8	130	2.311	1.918	2.628	2.385	2.31 (240)
C9	130	1.505	1.177 1.090	2.178 1.478	1.397	1.47 (152)
C10	32	8.263	7.677	10.150	6.373	8.12 (208)
P1	150	3.044	1.929	2.709	2.629	2.58 (309)
P2	90	4.432	2.730	3.466	2.322	3.24 (233)
P3	140	2.717	1.728	6.602	1.694	2.05 (229)
P4	110		2.091	1.866	2.798	1.94 (171)
P5						
P6	150					
P7	120	4.256 4.090	3.056	2.498	2.362	3.02 (290)
P8	8					
P9	155	1.986	1.381 1.330	2.086	1.943	1.84 (229)
P10	52	6.273	3.508	3.594	5.066	4.61 (192)

Table 2.5.3.16(a) Urine albumin excretion in rats fed control (C) or evening primrose oil (P) diet - 3 weeks post STZ

Rat	Urine volume	Assay D (ng/ml)	Assay E (ng/ml)	Assay F (ng/ml)	mean (μgs/24 hrs)
C1					
C2	8				
C3					
C4					
C5	125	6.882 6.476	6.682 9.926	7.052 6.495	7.25 (725)
C6					
C7	190	1.323 1.324 1.290	1.477 1.905 1.483	1.393 1.355	1.44 (218)
C8	150	1.599 1.598	1.966 2.124	1.774 1.752 1.700	1.80 (215)
C9	225	1.112 1.033	1.587 1.642	1.405 1.334	1.35 (243)
C10					
P1	215	2.119 2.141	2.667 3.623	4.200 4.138	3.15 (541)
P2	160	6.335 6.295	6.596 8.815	6.510 6.493 6.326	6.82 (873)
P3	155	5.945 6.089	6.961 9.715	7.135 6.030	6.98 (865)
P4	195	5.584 5.341	5.327 7.089	6.240 5.766	5.89 (919)
P5					
P6	225	3.347 3.094	3.664 3.928	4.476 4.599	3.85 (693)
P7	220	5.222 4.607	5.424 5.703 5.138	5.691 5.577	5.32 (937)
P8	7				
P9	210	4.213 3.754	4.155 4.450	6.130 5.015	4.62 (776)
P10	150	4.144 3.849	3.636 4.504 4.369	5.056 4.712	4.35 (522)

Table 2.5.3.16(b) Urine albumin excretion in rats fed control (C) or evening primrose oil (P) diet - 6 weeks post STZ

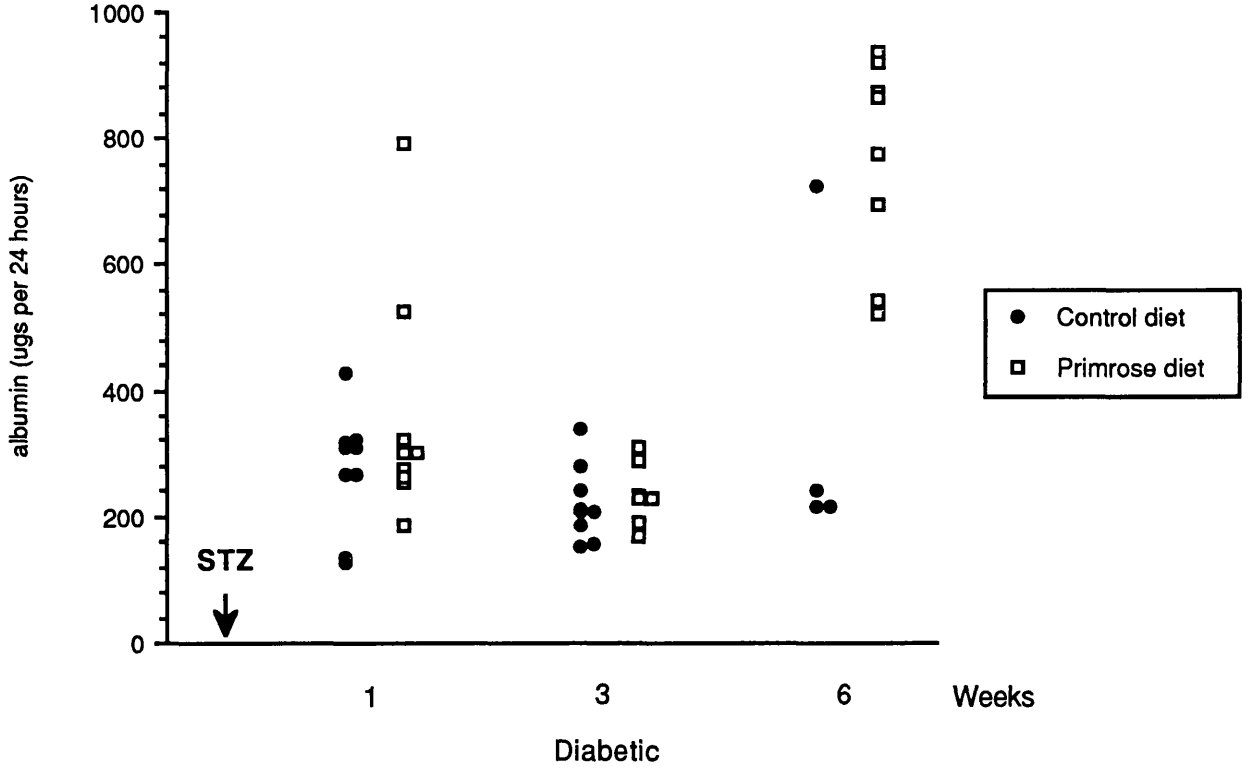


Figure 2.5.3.17 Albumin excretion rate (AER) in diabetic rats fed control or primrose oil diet

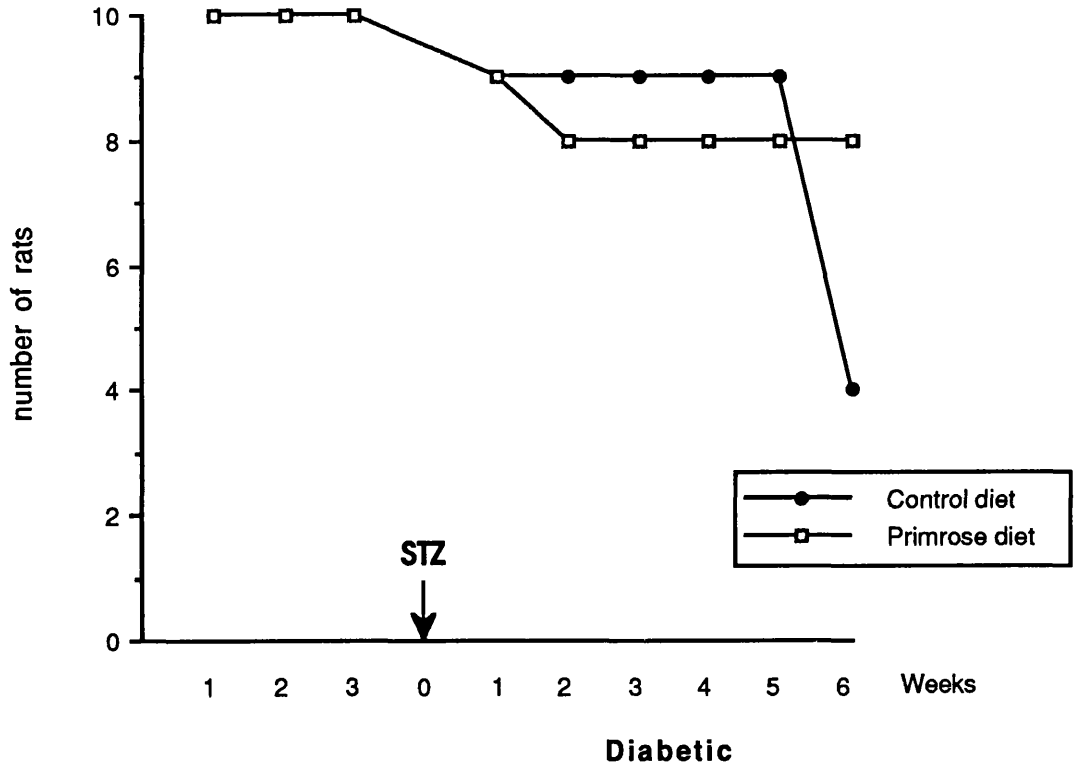


Figure 2.5.3.18 Survival of Lewis rats in control and evening primrose oil groups

2.5.4 Discussion

The propensity of PUFA diets to reduce proteinuria and albuminuria in healthy Lewis and DA rats had raised hopes that an evening primrose oil diet might moderate or prevent an increase in albumin excretion rate in diabetic Lewis rats since Payton and Boulton-Jones (1989) had shown previously that proteinuria was raised in Lewis rats after four weeks of diabetes. In the non-diabetic period of this study, both total protein and albumin excretion were lowered relative to controls after three weeks of EPO diet; after STZ, there was little difference in albumin excretion between the two diet groups at weeks one and three until week six when AER rose in the EPO group. The results at six weeks may be misleading, however, since so few rats remain in the control group: AER might have been raised in the control diet group had the rats survived. That albuminuria levels was not raised during the first few weeks of diabetes compared with pre-STZ levels may be so, or might be related to differences in the methods used to measure albumin before and after STZ. Urine albumin levels were measured by immunoturbidimetry during the non-diabetic period of the study (Figure 2.5.3.11) and were comparable with those in the previous studies (see Figures 2.2.3.7 and 2.3.3.2), if a little lower; urine albumin levels measured by ELISA during the diabetic phase were within a similar range to those in the pre-STZ period.

In relation to possible discrepancies in urine albumin levels before and after STZ, additional points to consider are that (i) turbidimetry has a greater degree of imprecision and tends to overestimate albumin concentration compared to radioimmunoassay (McElderry *et al*, 1982, Bakker, 1988, Ermann *et al*, 1988, Rowe *et al*, 1990), (ii) normal rat urine is highly coloured and concentrated whereas diabetic rat urine is very dilute and almost colourless and this leads to differences in the urine matrix which can influence immune complex formation, (iii) urine volume had increased some ten to twenty fold in diabetic, relative to non-diabetic rats which makes it more difficult to detect small changes in albumin levels, (iv) the ELISA had been newly developed for this study and may not have been entirely accurate or precise and (v) although albumin is stable in urine, the effects of pH, centrifugation and freezing on urine albumin levels are unclear. When it became clear that the ELISA would take some time to develop, diabetic urine samples were frozen (non-diabetic urine samples were not) and some work suggests that defrosting causes protein precipitation and underestimation of real values. Albumin levels were underestimated by some 20% in frozen urine and were significantly higher in fresh urine than the same urine frozen for seven days: the precipitation of exogenous radiolabelled albumin was greater in frozen urine than that kept at 4°C (Erman *et al*, 1988, Elving *et al*, 1989).

In this study, freshly defrosted aliquots of urine were assayed several times by ELISA as shown in Tables 2.5.3.16, 2.5.3.16(a) and 2.5.3.16(b).

The principal objective of the study was to establish whether there was any difference in the rate of increase in albumin excretion between the two diets groups during diabetes. However, albumin excretion remained stable until week six of diabetes, when it rose in the EPO group and there is some suggestion that AER was higher in rats fed EPO than those fed control diet at weeks one and six. The enigma is that there was no rise in AER but an increased number of deaths in diabetic rats fed control diet whereas albumin excretion started to rise but survival was much improved in diabetic rats fed evening primrose oil.

The issue of whether differences in body weight and food consumption relate to the anti-proteinuric effects of PUFA diets arose again in this study, when both parameters were found to be significantly lower at three weeks in non-diabetic rats fed EPO, in line with the fall in AER. At the end of six weeks of diabetes, although there was no difference in food consumption, rats in the EPO group still tended to be lighter than control rats (despite a raised albumin excretion rate) and this might also explain their improved survival.

This was the only study in which the means to measure the effects of a PUFA diet on arterial blood pressure became available and the results showed that evening primrose oil diet did not affect blood pressure in either normal or diabetic rats.

Irrespective of diet, kidneys removed from diabetic rats were heavier than those from normal control rats and the data also suggests that renal hypertrophy may be more pronounced in diabetic rats fed evening primrose oil than those fed a normal diet but the small number of animals in the control group at six weeks makes it difficult to discern whether this is a genuine difference. Dienoic prostanoids, in particular, have been attributed with direct and indirect growth-stimulating effects (Logan *et al*, 1990) and indomethacin prevented hypertrophy in association with reduced PGE_2 and PGI_2 levels in uninephrectomised rats (Logan *et al*, 1986). Hypertrophy and hyperfiltration is an alternative explanation for the failure to detect a fall in albumin excretion in EPO-fed diabetic rats although glomerular levels of dienoic PGE_2 and PGI_2 were not changed markedly by evening primrose oil diet in non-diabetic Lewis or DA rats when measured in the first study (see section 2.1.3). Fish oil has been shown to induce renal hypertrophy in normal rats (though independently of increased filtration) and obese Zucker rats (Logan *et al*, 1990, Kasiske *et al*, 1991). The effects of evening primrose oil diet on glomerular blood flow and pressure and filtration rate in this study, are unknown.

The difference in survival between the two diet groups is intriguing: many studies have shown marked improvements in the survival of animals fed PUFA diets (Prickett *et al*, 1981; 1983, Robinson *et al*, 1986, Papanikalou *et al*, 1987) while others have reported increased mortality (Scharschmidt *et al*, 1987). Although one rat died early on in the EPO group, survival was similar between the two groups until week five when there was an sudden increase in the number of deaths in the control diet group, the causes of which were never identified; survival rate in the evening primrose oil diet group was double that in the control diet group. It can be argued that had the study continued, the number of rats in the evening primrose oil group might have started to decline; nevertheless, the increased mortality in the control group was untimely since the study had been designed to last for only six weeks and it would have been pointless to continue with a depleted control group. Hypoglycaemia is the most plausible explanation for the deaths since most occurred within hours of insulin administration. The rats had been given the same dose of insulin every day for some five weeks, in response to assessment of glycosuria, which is not always an accurate reflection of blood glucose levels, and so insulin could have given been given to normo- or even hypoglycaemic rats although this is unlikely. Hyperglycaemia has been cited as a side-effect of PUFA diets in diabetic patients (Glauber *et al*, 1988) and plasma glucose levels were significantly higher in non-diabetic rats after three weeks of evening primrose oil diet (Figure 2.5.3.8) but blood glucose levels were comparable between the two diet groups during diabetes.

Differences in their response to insulin may explain the improved survival in EPO-fed rats. ω-6 fatty acids derived from EPO may have altered lipid domains within the membrane and thus the status of the insulin receptor in relation to changes in number and affinity (Ginsberg *al*, 1981). It has been reported that arachidonic acid stimulates insulin release from pancreatic cells (Metz, 1988) and that diabetic rats fed fish oil have a reduced requirement for insulin (Logan *et al*, 1988). There may also be a generalised deficiency of unsaturated fatty acids in diabetes (Dutta-Roy, 1990); for instance, PGE_1, derived from evening primrose oil, lowers the concentration of insulin required to produce a given effect (Ray *et al*, 1985). Insulin resistance, which occurs in non-insulin dependent diabetes, is associated with a failure of tissue to respond to insulin, rather than an inability to produce insulin. The number and affinity of insulin receptors may be reduced in some individuals, while in others, insulin binding is normal but post-receptor responses such as activation of glucose transport is abnormal. Rats in the EPO group may have become more tolerant of a larger dose of insulin than rats fed control diet. Alternatively, the feeding of evening primrose oil diet prior to receiving STZ, may have spared some pancreatic islet cell function.

The marked differences in urine pH between the diet groups during both phases of the study was interesting. Urine pH fell after one week and remained lowered throughout the diabetic phase of the study in the evening primrose oil diet group whereas in the control diet group, urine pH rose during the non-diabetic period and varied during the diabetic phase. That control Lewis rats are unable to excrete acid offers an alternative explanation for the increased number of deaths in this group whereas the fall in urine pH in the EPO group could be related to higher levels of free fatty acids or changes in tubular permeability to H^+ ions. Urine pH may not accurately represent the pH of tubular fluid which is modified extensively as it flows along the nephron. Filtered proteins are reabsorbed in the proximal tubules as a function of their charge which, as well as anionic binding sites within the tubular brush border, are susceptible to changes in the pH of the proximal tubular fluid. Cationic albumin is not only filtered more readily but is also reabsorbed more avidly than native albumin and Coimbra *et al* (1984) found that raising urine (or tubular fluid) pH with sodium bicarbonate, increased the excretion of exogenous cationic albumin and reduced its nephrotoxicity. Were the fall in urine pH to reflect a fall in proximal tubular fluid pH, albumin would become more cationic, a form in which its tubular reabsorption is enhanced and so urine levels reduced (Christensen and Bjerke, 1986). The possibility of a link between changes in urine pH and the fall in albumin excretion may be misleading since it is difficult to reconcile the fall in albumin excretion rate in non-diabetic rats fed EPO diet with the rise in albumin excretion rate in the later stages of diabetes when urine pH remained lowered. Urine pH was not measured in any of the previous studies although it is significant that the ranging study data shows that the pH of Lewis rat urine pH was around 8 - 8.5 (see Table 2.5.3.1).

It had been hoped that this study would uncover (i) a difference in AER between the control and evening primrose oil diet groups in non-diabetic rats which was sustained during diabetes and (ii) a slower rate of increase in albumin excretion rate in diabetic rats fed EPO compared with those fed control diet. However, although both proteinuria and albuminuria were lowered in non-diabetic rats fed EPO, when the animals were made diabetic, there was no difference in AER between the diet groups until six weeks, when AER rose in the EPO group, at which point, the study was terminated. That EPO did not prevent a rise in AER in diabetic Lewis rats might otherwise be explained by the preferential binding of glucose to serum albumin in diabetic animals, thus blocking the binding of PUFAs which intensifies the anionic charge and so impedes the filtration of albumin.

Summary

Albumin excretion rate in diabetic Lewis rats was not lower in the evening primrose oil than control diet group, despite a difference in the pre-diabetic phase of the study. There was little difference in albumin excretion rate between control and EPO diet rats until week six of diabetes, when levels started to rise in the EPO diet group. It is unclear whether this is a genuine difference because of differences in survival between the diet groups. Competitive binding between glucose and fatty acids on the surface of the albumin molecule might explain the inability to demonstrate a fall in AER in diabetic rats.

Blood pressure was not affected by evening primrose oil diet in normal or diabetic rats.

The contribution of a fall in urine pH to a fall in albumin excretion rate in normal rats fed evening primrose oil diet may be a red herring.

Whether differences in food consumption and body weight account for both the fall in AER during the non-diabetic period and increased survival in the diabetic period is also unclear.

Section 3

CONCLUSIONS and FINAL DISCUSSION

3.1 Conclusions

• Albumin excretion rate tended to be lower in groups of healthy Lewis and DA rats fed PUFA diets than those fed a standard laboratory rat diet. Although both glomerular thromboxane B_2 and renin levels *in vitro* were higher in Lewis rats than DA rats, changes in the levels of these two vasoconstrictors did not correlate with the fall in albumin excretion rate in the PUFA diet groups.

• Compared to DA rats, Lewis rats had a diminished negative charge on the glomerular capillary wall when assessed by glomerular uptake of a cationic (+) marker protein. However, the fall in albumin excretion rate in the PUFA diet groups did not correlate with any increase in the magnitude of the glomerular polyanion. Splenic uptake of the same cationic protein was also lower in Lewis rats compared to DA rats.

• The isoelectric point (pI) of serum albumin from Lewis rats in the PUFA diet groups was lower than that from those in the control diet group.

• Despite having lowered proteinuria and albuminuria during the non-diabetic phase of the study, evening primrose oil diet did *not* prevent a rise in albumin excretion rate in Lewis rats after six weeks of diabetes although survival was improved in this group.

• The explanation for the anti-proteinuric effects of PUFA diets is more likely to be some physical modification of the serum albumin molecule such as a change in its isoelectric point - or even its size or shape - as a result of its binding PUFAs.

3.2 General discussion

In association with many other factors, a rising albumin exretion rate is a strong predictor of glomerular disease progression (see section 1.1) and so it is important to consider the pathogical range and prognostic significance of different levels of albuminuria, the degree to which it reflects the presence of established renal disease and its use both to monitor the progression and regression of renal disease and perhaps define a stage at which early intervention might reverse, retard or prevent nephropathy. The finding therefore, that albumin excretion tended to be lower in two strains of healthy rats with different disease susceptibilities, fed either evening primrose oil, fish oil or olive oil diets was exciting in view of the fact that any fall in proteinuria may indicate an improvement in, or reversal of glomerular injury. In diabetics patients, for example, a fall in albuminuria resulting from antihypertensive treatments, glycaemic control or low protein diets is taken as being indicative of just such an improvement (see section 2.5.1). Persistent proteinuria is also associated with structural damage within the glomerulus, tubulointerstitium and other vascular beds (Deckert *et al*, 1988, Remuzzi and Bertani, 1990).

However, the critical aspect of any fall in urine albumin excretion is whether it is genuine, in relation to the high degree of intra-individual variability of urine protein levels associated with exercise, posture and other diurnal variations. The extent of this variability and necessity for multiple measurements within any individual was highlighted in a study in which albumin levels were measured in twenty 24 hour, timed overnight and short, timed daytime collections from two individuals; although 24 hour collections showed the least intraindividual variation there were even wider variations in overnight and daytime collections (Hutchison and O'Reilly, 1985). It is staggering when one considers that to be 95% confidence of detecting urine albumin levels to within 10% of the true value would require some 144 samples from the same individual and to be within 5% would require 576 samples (Johnston *et al*, 1993). One abnormal urine albumin result is not adequate to classify a patient as microalbuminuric and likewise, a single low urine protein result should not be regarded as conclusive or reliable proof of a genuine fall in albumin excretion and improved glomerular permselectivity. One has to acknowledge, however, that much published data on proteinuria relates to only a small number of animals or humans.

The purpose behind the experimental work carried out in this thesis was to establish the mechanism behind the apparent trend towards a significant lowering of AER in, ultimately, some two hundred male Lewis and DA rats fed PUFA diets. That the fall in albumin excretion occurred in normal healthy rats suggests that the diets may have affected some fundamental aspect of glomerular permselective control and while it is usually assumed that albumin leaks across damaged glomeruli mainly, Yoshioka *et al*

(1988) demonstrated by serial micropuncture, albeit in rats with reduced renal mass, that the filtration of proteins across individual healthy non-sclerotic glomeruli often exceeded that across sclerosed glomeruli.

Dietary manipulation is a common method of modifying disease progression (Mitch, 1984, Barcelli and Pollak, 1985, El Nahas and Coles, 1986). Protein restriction, low phosphate and reduced calorie diets have been shown to be beneficial in the treatment of experimental and human nephropathies (Ibels *et al*, 1978, Zeller, 1987, Harris *et al*, 1988, Ichikawa *et al*, 1985, Tapp *et al*, 1989: see section 1.1.3). Recognition too, of the role of lipids and cholesterol in the initiation and progression of glomerular injury prompted the use of lipid-lowering agents and PUFA diets, particularly those rich in ω-3 EFAs with the capacity to reduce plasma lipid levels (see section 1.4). PUFA diets have been shown both to prevent the onset and lower established proteinuria in experimental and clinical studies - often without further impairing glomerular function, which is important if renal function is already compromised (van der Heide *et al*, 1990, Heifets *et al*, 1987, Weise *et al*, 1993: see section 1.4). PUFA diets can be used to regulate prostanoid metabolism less aggressively than non-steroidal anti-inflammatory drugs because they generate alternative series prostanoids with attenuated activity.

Ever mindful that the fall in albumin excretion may not be genuine, one can still speculate upon how PUFA diets might reduce albumin excretion rate. Potentially, the relationship between PUFA diets and a fall in albumin excretion rate is complex because PUFAs perform many functions (section 1.3) and albumin excretion is controlled by a variety of factors (section 1.2) and this makes most investigators unwilling or reluctant to endorse one single mode of action (section 1.4). It is most likely that the anti-proteinuric actions of PUFA diets are related to altered prostanoid levels, cell membrane physiology or plasma lipid levels.

In the first two studies (2.1 and 2.2), it was speculated, perhaps too hastily, that the anti-proteinuric effects of PUFA diets might be prostanoid-mediated in association with a fall in glomerular or systemic blood flow and pressure. However, the nature of the relationship between not only PUFA diets and blood pressure control but also between blood pressure and protein excretion remains unclear (Dusing *et al*, 1983, Axelrod, 1991). Not all antihypertensive therapies reduce protein excretion; in the main, it is ACE inhibitors which lower proteinuria through a selective reduction in glomerular hypertension. Although there have been several reports of a fall in blood pressure in animals and humans rats fed PUFA diets (Heifets *et al*, 1987, Izumi *et al*, 1986, Clark *et al*, 1990; 1993; 1993a), often no antihypertensive effect is observed

(Barcelli *et al*, 1982; 1990, Wheeler *et al*, 1991, Weise *et al*, 1993) and indeed, PUFA diets effected only a modest fall in blood pressure in patients with lupus nephritis (Clarke *et al*, 1993), chronic glomerulonephritis (de Caterina *et al*, 1993) or diabetic nephropathy (Jensen *et al*, 1989). Indomethacin blocked both the antihypertensive effects of a high linoleic acid diet in rats with salt-induced hypertension and the more pronounced cardiac hypertrophy in rats fed a low linoleic acid diet (Hoffman *et al*, 1982) which may be attributed to inhibitory effects of linoleic acid on renin production (Reddy *et al*, 1987). Vasodilatory prostaglandins can also increase blood pressure by either stimulating renin secretion or increasing cardiac output (Dunn and Grone, 1985) and fish oil raised renal blood flow and GFR in normal humans, independently of any change in systemic blood pressure (Dusing *et al*, 1990).

Whether PUFA diets had any effect on systemic or glomerular glomerular haemodynamics in Lewis and DA rats is not known although arterial blood pressure was shown to be unaffected by EPO diet in Lewis rats in the diabetic study (see section 2.5.3.6). Indices of intraglomerular blood flow and pressure, such as glomerular plasma flow rate, transcapillary hydraulic pressure (ΔP) and ultrafiltration coefficient (Kf) were not measured because they require micropuncture techniques, the facilities for which were not available. Glomerular filtration rate and renal plasma flow could have been assessed by measuring the clearance of inulin and para-amino hippuric acid (PAH), respectively and a femoral line introduced to measure systolic blood pressure, but the additional cannulation steps required for such procedures would have extended the surgical procedure substantially and so were not performed in this, or any subsequent study. Clearly, the non-invasive tail vein plethysmography method used to measure systolic blood pressure in the diabetic study, is more convenient but there may be problems associated with over-heating of the animals.

The finding that glomerular thromboxane and renin levels were raised in Lewis compared to DA rats was interesting. Although there is much evidence of the involvement of prostanoids in abnormal protein excretion, many studies have shown that prostaglandin and thromboxane production does not always correlate with the course of proteinuria (see section 1.3.5): changes in prostanoid levels may well be a consequence, rather than a cause of increased urine protein loss. Reciprocal changes in prostaglandin and thromboxane production modulate systemic and glomerular blood pressure. The proteinuric effects of thromboxane may be related to heightened arterial or intraglomerular blood pressure or a rise in filtration fraction as a result of a fall in glomerular plasma flow but the patterns of lowered proteinuria in sections 2.1 and 2.2 were not reflected by a generalised fall in glomerular thromboxane levels.

Baylis (1987) suggests that thromboxane impairs renal function predominantly through changes in renal vascular resistance and not through contractile actions on the mesangium. Attempts were made in section 2.2.4 to define the role of heightened glomerular renin and its potential relationship with raised glomerular thromboxane levels in Lewis rats but it is difficult to do so with the limited amount of data.

PUFA diets may have affected glomerular permeability in relation to regulation of intraglomerular thrombosis and coagulation by altering the balance between the generation of thromboxane by platelets and prostacyclin by vascular endothelium (Haines *et al*, 1986, Knapp *et al*, 1986, Rondeau *et al*, 1986, Sraer *et al*, 1987, Hansen *et al*, 1989, Vane *et al*, 1989, Clark *et al*, 1993). Suppression of platelet activity dampens not only the procoagulant effects of thromboxane but also the release of a variety of platelet-derived factors with both growth-promoting and hydrolytic properties which impair the permselectivity of the glomerular capillary wall (Habenicht *et al*, 1988, Mene *et al*, 1990, Lianos and Zanglis, 1990). The direct and indirect effects of PUFAs on cell proliferation and hypertrophy (Fox *et al*, 1988, Logan *et al*, 1990, Wight *et al*, 1990, Mene *et al*, 1990), inflammation (Meydani *et al*, 1990), sclerosis (Ross, 1986, Weiner *et al*, 1986) and the immune response (Endres *et al*, 1989) are alternative contributory factors to their anti-proteinuric actions.

Membrane function: Attempts were made in section 2.3, by the best means available, to examine the effects of PUFA diets on glomerular cell membrane phospholipids in relation to changes in the glomerular polyanion. One cannot dismiss the fact that Lewis and DA rats can be distinguished on the basis of glomerular (and splenic) uptake of a cationic protein but must retain some degree of scepticism as to whether the difference in uptake between the strains is entirely charge-dependent - although the uptake of native IgG suggests that it is. One also has to ask whether a single non-specific molecule could accurately quantify the effective net charge on the GCW without more substantial validation of the method. An alternative explanation of the findings is that the GCW of Lewis rats is invested with a greater number of larger pores which allow IgG to escape into the glomerular ultrafiltrate whereas DA rats have no such enlarged pores and so IgG remains trapped within the glomerulus: the differences in glomerular uptake of cationic IgG could, in effect, reflect the magnitude of the shunt pathway. However, such a hypothesis does not help to explain the similarly increased splenic uptake of IgG(+) in DA rats which is more likely to relate to differences in tissue charge, blood flow or reticuloendothelial uptake.

The lipid- and cholesterol- lowering effects of PUFA diets may improve glomerular permselectivity in relation to favourable effects on atherogenic lipid deposition and hypertension caused by changes in plasma viscosity (Mensink *et al*, 1989; 1990, Steinberg *et al*, 1989, Nestel, 1990; see section 1.4). Kasiske *et al* (1989) showed that the PUFA content of renal cortical phospholipids were reduced in Zucker rats relative to their lean littermates and that changes in the degree of saturation of fatty acids may correlate with the extent of renal histological damage. The relationship between altered lipid metabolism and glomerular permselectivity is unclear (Scanu, 1991). Changes in plasma lipid levels may lower protein excretion either directly, by inhibiting hepatic lipoprotein synthesis through substrate diversion (Smit *et al*, 1991) or indirectly, through changes in plasma oncotic pressure (Kaysen, 1991).

That a lowering of the isoelectric point (pI) of serum albumin was the most likely explanation for the fall in albumin excretion rate in all the PUFA diet groups is intriguing but unlikely, therefore, to have any significant effect on glomerular disease progression. The hypothesis forwarded in section 2.4.4 is that albumin excretion was lowered in rats fed PUFA diets due to a heightened negative charge on the albumin molecule as a result of binding PUFAs. However, the important questions of whether (i) the isoelectric point (pI) of DA rat serum albumin is lower than that of Lewis rat serum albumin under normal dietary conditions and (ii) if PUFA diets further reduce the pI of DA rat serum albumin remain unanswered. It would be of interest to compare the pI of both serum and urine albumin in both Lewis and DA rats in control and experimental PUFA diet groups and the relationship between changes in the pI of serum albumin and its clearance rate and the effects of fatty acid binding on the size and shape of the albumin molecule could be developed further - perhaps in humans. The problem with this hypothesis is that hyperlipidaemic subjects might be expected to have low levels of albumin excretion unless, of course, the effect is dependent upon specific fatty acids. It is less likely that the effect is related to the essential nature of the fatty acid, since it was seen in the olive oil group. The fat content of the PUFA diets, in all the studies, was derived exclusively from the PUFA oils whereas the control diet contained 2.4% fat, of which the ratio of saturated to unsaturated fatty acids is unknown.

That albumin excretion rate was reduced as effectively in the olive oil diet group as in the evening primrose oil and fish oil diet groups, may have been overlooked as an early clue that the anti-proteinuric effects of the PUFA diets were not prostanoid-dependent but related instead to some other aspect of fatty acid function. Clark *et al* (1993) drew attention to the markedly similar benefits of fish and olive oil diets in the treatment of patients with lupus nephritis. Although olive oil was, and is used widely

as a 'control' PUFA, its effects on plasma viscosity, red blood cell flexibility and arachidonic acid, VLDL-cholesterol and complement levels were similar to those of fish oil. Pagnan *et al* (1989) demonstrated a substantial degree of incorporation of oleic acid into the red blood cell membrane in normotensive, normolipidaemic volunteers fed olive oil diet for three weeks and observed a significant increase in the flux rate of Na^+/K^+countertransport and a slight, if non-significant reduction in sodium lithium countertransport activity. In an earlier study, the same authors reported a significant reduction in blood pressure in eighty three normotensive subjects fed olive oil diet (Pagnan *et al*, 1986). In all of the studies reported in this thesis, the effects of the olive oil diet were often as potent as the other EFA diets.

Diabetes: In the diabetic study (section 2.5), throughout the diabetic period, there was no difference in AER between the diet groups until six weeks when AER rose in the EPO group. Whether this is a genuine rise compared to the control diet group is difficult to discern since so few rats remain in the control diet group. It is interesting that although at no time during diabetes was AER significantly lower in the EPO than control group, survival was improved. Competition between fatty acids and glucose for binding sites on the albumin molecule may explain the failure of evening primrose oil diet to lower albumin excretion in diabetic rats despite having done so in the non-diabetic phase of the study. That evening primrose oil diet had offered no protection from an increase in albumin excretion rate in diabetic Lewis rats, despite having lowered both proteinuria and albuminuria during the pre-STZ period, was taken as confirmation that the PUFA diet did not target the glomerulus directly. The observed differences in albumin excretion, survival, renal hypertrophy and tolerance of insulin between the diet groups is intriguing and might produce interesting results were the study to be repeated in a larger number of animals for a longer period of diabetes. The transition from IT to ELISA to measure urine albumin levels was unavoidable and might explain possible disrepancies in albumin levels before and after diabetes. To a large extent, the emphasis of the diabetic study shifted towards developing and validating a suitable ELISA method to measure albumin in dilute rat urine and although annoying, the problems encountered provided an excellent grounding in the theory and practice of ELISAs.

Species and strain differences in prostanoid metabolism and protein excretion (Alt *et al*, 1985) and differences in lipoprotein metabolism between nephrotic rats and humans (Gherardi *et al*, 1977, Marshall *et al*, 1990, Joven *et al*, 1990, Fine *et al*, 1991) prompt questions about the relevance of animal studies to humans. Even within the rat, $PGF_{2\alpha}$ metabolism can vary with age, sex and strain (Pace-Asciak, 1975). However, as prostanoid measurement techniques become more sophisticated,

it may become apparent that many of the reported discrepancies are related to experimental artefacts and not to altered tissue differentiation or prostanoid enzyme activity. Yet another consideration is that although some glomerular structural changes can be induced in experimental animal models of diabetes, it is not possible to demonstrate in rats by diabetes alone, progressive renal dysfunction culminating in ESRF which represents the real problem of clinical diabetic nephropathy in humans (Carney *et al*, 1979, Hirose *et al*, 1982, Jensen *et al*, 1987). Often however, extrapolation of animal studies to humans is the only option: for obvious reasons, it would be impossible to measure glomerular prostanoid and renin production *in vitro* and assess GCW charge in humans, using the experimental methods applied in this thesis. On the other hand, apart from possible problems with dietary compliance, it would be relatively easy to study the effects of PUFA supplements on urine protein levels, urine pH and to measure the pI of urine and serum albumin in normal and diabetic humans. One can surely presume however, that the fundamental mechanism controlling protein excretion is unlikely to differ substantially between rats and humans.

Several points arising from the work carried out in this thesis may require further modification and clarification.

Studying two strains of rat in four or five different study groups serves only to cloud and complicate issues. It would have been more prudent to explore the anti-proteinuric effects of one, or even two PUFA diets in a larger group of either Lewis or DA rats; this would have facilitated more frequent monitoring and implementation of a variety of additional analytical parameters. The measurement of many parameters was omitted and others were not explored fully due, principally, to the constraints of time and working alone; these include (i) plasma lipid levels and incorporation of PUFAs into glomerular tissue lipids, (ii) glomerular histological changes, (iii) pair-feeding and establishing the calorie content of the experimental PUFA diets, (iv) serum and urine levels of albumin, sodium, creatinine and pH as appropriate.

Perhaps the most significant omission was the use of fat-deficient diet supplemented with beef tallow (as a source of saturated fatty acids) rather than standard laboratory rat chow, as the control diet. There are various permutations of procedures and methods for PUFA diet studies; for example, many groups merely supplement a standard laboratory diet with PUFA oils and so it can be difficult to compare PUFA diet studies because fatty acid content is not standardised.

It must also be conceded that radioimmunoassay was not the best technique to use for identifying changes in different series prostanoid metabolites in relation to PUFA diet feeding and although HPLC or GC-MS would have been more appropriate, RIA does have the specificity for comparing dienoic prostanoid levels between the two rat strains within a particular diet group. Were the prostanoid studies to be repeated, it would be advisable to re-introduce measurement of PGE_2 and PGI_2 levels and measure serum thromboxane levels at a higher dilution.

Certain aspects of the studies could be developed further.

(i) The effects of dietary protein intake on protein excretion are unclear and there are questions as to whether a reduced dietary protein intake (or even a lower body weight) might account for the observed fall in albumin excretion in PUFA-fed rats
(ii) The duration of the PUFA diet and it's use as either a preventative or therapeutic intervention.
(iii) There is scope for further investigation of the interstrain differences in glomerular thromboxane and renin by cell culture or molecular biological techniques such as Northern blotting.

The problem of sample size underlies all the studies reported in this thesis. Not only is it tiresome, but it also disheartening, to invest time, effort and enthusiasm in studies which, ultimately, cannot be justified on the basis that statistical analyses are inappropriate. However, although the number of experimental rats used seems small in statistical terms, in practice, the problems associated with this number are extensive (see section 2.2.4). Comparisons amongst ten different groups, as in the first study, is ludicrous because (i) sample size must be sacrificed and so there is greater scope for possible type I (false positive; as in the case of urine albumin measurements) and type II (false negative; as in the case of PGE_2results) statistical errors and (ii) it is well known that the greater the number of statistical comparisons performed, the more likely that a significant difference will occur by chance alone (although this problem can be corrected for by applying the Bonferroni formula). For example, a statistically significant correlation between glomerular TxB_2 levels and AER, albeit only in DA rats fed control diet ($r^2 = 0.82$: $p = 0.003$) was uncovered in section 2.2, which may be related simply to statistical chance. There are several instances, in many of the studies, in which results might have attained statistical significance had numbers been larger such as kidney weight and survival data (section 2.5), glomerular PGE_2 levels (section 2.1) and splenic uptake of ^{125}I-IgG^N (section 2.3). It is prudent to apply a power calculation to provide some indication of the optimal number of subjects required to observe a desired effect.

Experimental reproducibility is yet another issue arising from the work in this thesis. It is disconcerting that the results of the first two studies are so different despite the intended similarities between them and while it has been conceded *ad nauseum* throughout, that sample size is small, in many studies published currently, sample size is often not much greater.

A fall in albumin excretion does not necessarily indicate an improvement in glomerular permselectivity. The final amount of protein in the urine is governed by GFR, plasma protein concentration and tubular reabsorption and so although PUFA diets may have reduced urine albumin levels in healthy Lewis and DA rats, the amount of albumin filtered at the glomerulus may not have changed. PUFA diets could have increased tubular reabsorption of albumin, reduced GFR or effectively lowered serum albumin levels. There is some suggestion that the Lewis rat population might be bimodal in terms of albumin excretion (see Figures 2.2.3.7 and 2.3.3.2). However, despite the intraindividual variation in urine protein measurements, comparisons were made between control and PUFA diet groups, assuming the same degree of variability.

It is intriguing that few trials have shown that lowering or elimination of microalbuminuria does, in fact, influence patient mortality or the incidence of ESRF (Rowe *et al*, 1990). Indeed, demonstrating a fall in albumin excretion rate cannot, of itself, be regarded as a measure of improved glomerular function until the contentious issue of whether increased amounts of protein crossing the glomerular barrier actively exacerbates the progression of renal disease is resolved once and for all.

The concept that PUFA diets reduce albumin excretion rate in rats by changing the pI of serum albumin suggests that such diets are unlikely to have a beneficial effect on the course of a progressive glomerular disease such as diabetic nephropathy, although neither does it exclude such a possibility. In the final analysis, the success of PUFA diets may be related to the absence of saturated fats rather than the presence of unsaturated fats.

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