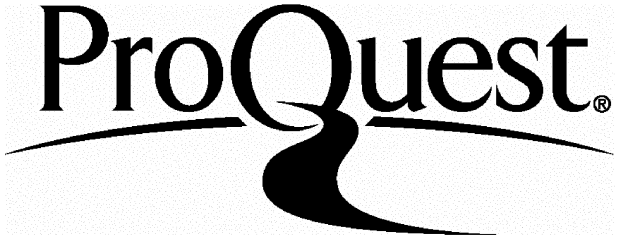
CHARACTERISATION OF HUMAN D-GLYCERATE DEHYDROGENASE/GLYOXYLATE REDUCTASE.

by Chrysanthi Foteini Giafi

for the Doctorate of Philosophy

University College London



ACKNOWLEDGEMENTS

This work was funded by the Sir Jules Thorn Charitable Trust. I would like to thank my two supervisors Dr Gillian Rumsby and Mr Colin Samuel for their helpful guidance and support. I would also like to thank Mrs E. Young and Dr Ros Hastings for supplying me with human fibroblasts, Dr B. Portman for the human liver samples and Dr P. Rumsby for the cultured HepG2 cells.

ABSTRACT

The enzyme D-glycerate dehydrogenase (D-GDH), which is also believed to have gloxylate reductase (GR) activity, plays a role in serine catabolism and glyoxylate metabolism and deficiency of this enzyme is believed to be the cause of primary hyperoxaluria type 2 (PH2).

The pH optima and kinetic parameters of D-GDH and GR in human liver have been determined and assays developed for their measurement. A reference range was established for the activity of both enzymes in normal liver. D-GDH activity in kidney, leucocytes and fibroblasts fell within the range of values seen in the liver but GR activity was approximately 30% in the kidney and barely detectable in leuc ocytes and fibroblasts. Analysis of liver and leucocyte samples from patients with PH2 showed that GR activity was either very low or undetectable, while D-GDH activity was reduced in liver but within the normal range in leucocytes.

D-GDH was partially purified from human liver. The purification methods included negative adsorption to DEAE Sephadex pH 7, precipitation with ammonium sulphate, elution from CM Sephadex pH 6 with 150 mM

sodium chloride, chromatofocusing using a pH gradient 7.2-5 and fractionation on a 2'5' ADP Sepharose column.

D-GDH separated into two peaks on the chromatofocusing column. The first peak, with D-GDH activity only, was not retained by the column, whereas the second peak with D-GDH and GR activity eluted with a pI ~ 6.7. These results suggest that liver contains more than one protein with D-GDH activity. These observations were reinforced by the finding of only a single, non-retained peak of D-GDH activity when a leucocyte preparation was applied to the chromatofocusing column.

Antibodies were raised in guinea pigs against the mixture of six proteins which eluted together from ADP sepharose. In non-denatured samples the antibodies recognised a protein of 71 kDa that was observed only faintly in the liver of a patient with PH2. On denaturation the signal at 71 kDa disappeared and a weaker signal at 37 kDa appeared.

Peroxisomes and mitochondria of HepG2 cells, rat hepatocytes and human leucocytes were isolated. In all cases over 80% of D-GDH and GR activity was found in the cytosol.

TABLE OF CONTENTS:

INDEX OF FIGURES

INDEX OF TABLES

CHAPTER 1: INTRODUCTION.

1.1: Hyperoxaluria and oxalosis.

The role of oxalic acid in living organisms is variable. For example, in plants, oxalate can have both structural and defensive functions as well as an important role in calcium homeostasis (Rose, 1988, Hodginson, 1977), whereas in animals in general, and mammals in particular, oxalate has no useful function. In fact, in mammals oxalate is potentially lethal mainly because of the physical properties of its calcium salt, which is very insoluble at physiological pHs. Crystallisation of calcium oxalate can cause obstruction of the renal tubular lumen, disruption of intercellular and possibly intracellular interactions and communications, in addition to killing of cells within which or adjacent to which crystallisation occurs. Calcium oxalate can also be deposited in soft tissues as seen in primary hyperoxaluria, chronic renal failure and certain small bowel diseases.

Oxalate is a relatively small dicarboxylic acid with a pKa_1 1.23 and pKa_2 4.19. Although the free acid is soluble to 8.7 g/100 g H_2O at 20°C (Williams and Smith, 1983), calcium oxalate is poorly soluble within

the physiological pH range of urine (pH 4.5-7.5) with a solubility of 0.67 mg/100 g H_2O at pH 7 at 13°C. Supersaturation of the urine with calcium oxalate happens frequently, but precipitation is inhibited by a number of compounds present in urine, such as urea, citrate, sulphate, pyrophosphate, lactate, magnesium, copper, iron, zinc, adenine, certain colloids and small molecular weight polypeptides (reviewed by Williams and Wilson, 1990). Calcium oxalate usually crystallizes as a monohydrate or a dihydrate but can also form a tetrahydrate. Renal stones are the ultimate result of crystals that have been retained in the kidney because of their dimensions or by their association with the renal tubular epithelium, allowing them to increase in size further (Oswald and Hautman, 1979, Menon and Koul, 1992, Kok and Khan, 1994). It has been estimated that 66% of all renal calculi contain oxalate (Prien and Prien, 1968).

The major determinant of calcium oxalate crystallisation is the oxalate concentration (Robertson and Peacock, 1980, Larson and Tiselius, 1987), since calcium homeostasis in the body is such that changes in the concentration of Ca^{++} ions *in vivo* are tightly regulated. The amount of oxalate found in urine is

determined by dietary intake, gut absorption, endogenous production and renal handling.

1.2: Primary and secondary hyperoxaluria.

Hyperoxaluria reflects increased plasma oxalate levels which can arise from primary, i.e. inherited causes or secondary, i.e. environmental and acquired. Secondary hyperoxaluria can be caused by i. increased ingestion and/or absorption of oxalate and its precursors, e.g. vitamin C, because of surgery, dietary changes, intestinal disease, or changes in intestinal flora; ii. increased endogenous synthesis of oxalate because of the production of excess oxalate precursors; and iii. decreased oxalate clearance due to renal failure. The causes of hyperoxaluria can be interactive; for example renal oxalosis due to increased oxalate absorption or synthesis can result in renal failure which can itself lead to systemic oxalosis.

The term primary hyperoxaluria describes at least two inherited diseases, primary hyperoxaluria type I (PH1) and primary hyperoxaluria type II (PH2, L-glyceric aciduria). Other hyperoxaluric syndromes that could be included in this category are type III

hyperoxaluria that involves primary enteric hyperabsorption (Watts and Mansell, 1988, Yendt and Cohanim, 1986) and familial oxalate transport defects described in red cells (Baggio *et al.*, 1985, Baggio *et al.*, 1986, Baggio *et al.*, 1984). An unrecognised primary disorder might also be the underlying cause in some patients with idiopathic oxalate stone disease (Robertson and Peacock, 1980, Yendt, 1988). This project concentrates on PH2 and therefore only this condition will be discussed at length.

1.3: Oxalate determination in urine.

To diagnose hyperoxaluria it is recommended to use samples from daily urinary excretion, though spot urines may be used in pediatric settings. The correct management of samples upon collection is crucial for reliability of the results. The most important interference in oxalate determination involves ascorbate, which may affect oxalate assays in different ways before and during analysis. In neutral and alkaline environments, ascorbate degrades spontaneously and produces oxalate and threonate, thereby increasing the measurable oxalate (Harris, 1976). It has therefore

been suggested that urine should be stored frozen in the presence of acid.

Oxalate can be measured by chemical, enzymatic and chromatographic procedures. Chemical techniques include atomic absorption spectrometry and colorimetry. Atomic absorption spectrometry requires precipitation of calcium oxalate and colorimetry requires preliminary purification of oxalate either by solvent extraction or precipitation. These techniques are not currently used, the former because precipitation of oxalate is generally incomplete and poorly reproducible (Mazzachi *et al.*, 1984) and the latter because more accurate and simple enzymatic or ion chromatographic techniques have been developed.

The analytical use of commercially available specific oxalate-degrading enzymes has simplified sample processing and enhanced reliability. Oxalate oxidase from moss, barley or other vegetable sources, at a pH of around 4, catalyses the oxidation of oxalate into carbon dioxide and hydrogen peroxide which is activated by peroxidase to produce hydroxide radicals. These are coupled to chromogen substrates to form highly absorbing quinoneimine dyes (Gochman and Schmitz, 1971). The use of simple pre-treatments, such as charcoal adsorption or selective oxidation of

ascorbate or of other reducing interferences is necessary when assaying unprocessed urine because reducing substances present in the sample react with the free radicals, decreasing the colour yield (Kasidas and Rose, 1985). Oxalate oxidase is also used for blood plasma determinations, in spite of the fact that in normals oxalate concentration is two orders of magnitude lower than in the urine (Wilson and Liedtke, 1991, Petrarulo *et al.*, 1994).

Oxalate decarboxylase from fungal cultures (Collubia velutipes) catalyses the decarboxylation of oxalate with formation of carbon dioxide and formic acid with optimum pH around 3. In the presence of formate dehydrogenase and NAD, the formate produced is oxidised with concurrent formation of NADH, which is detected spectrophotometrically. Although the assay of unprocessed urines gives poor results, the addition of EDTA overcomes this drawback (Cannon *et al.*, 1983).

Gas-liquid chromatography (GLC) has been used since the seventies (Duburque *et al.*, 1970, Gelot *et al.*, 1980) and recent advances in separation and detection technologies have enhanced sensitivity and accuracy. Oxalate has to be extracted and esterified in an anhydrous environment before the chromatographic assay. Capillary GLC is particularly useful in

assessing oxalate concentration in body fluids (Wolthers and Hayer, 1982, Lopez *et al.*, 1985), but tedious sample preparation and the requirement for special equipment and expertise hinder widespread adoption of this technique.

Anion exchange chromatography with suppressed conductivity detection is a new and important addition to the oxalate assays (Stevens and Davis, 1981). Suppression of eluent conductivity greatly enhances the sensitivity of conductimetric detection, which, coupled with chromatographic separation using selective and low capacity resins, is particularly suitable for the quantification of small anions. Without sample pre-treatment, the use of alkaline eluents promotes the conversion of ascorbate to oxalate inside the column. It has been suggested that overestimation can be prevented by either buffering the sample with boric acid (Robertson and Scurr, 1984), eliminating ascorbate (Petrarulo *et al.*, 1990), or lowering the eluent pH (Dionex Corporation, 1992). Ion chromatography is one of the most simple and reliable techniques, but the need for automated instrumentation still limits its widespread use.

1.4: Oxalate transport across intestinal and renal epithelia.

Oxalate has been reported to vary from 70 to 930 mg/day in a typical western diet (reviewed by Williams and Wilson, 1990). It is found in most plant tissues and in India, where the diet contains a large percentage of vegetables, the daily intake of oxalate can reach 2,000 mg/day. The most oxalate-rich foods are spinach and rhubarb, while tea, chocolate and cocoa contain generous amounts (reviewed by Williams and Wilson, 1990).

1.4.1: Intestinal handling of oxalate.

The proportion of oxalate in urine that is derived from dietary sources is highly variable and it is estimated by various studies to be from 2% to 50% of the total dietary load (in Hatch and Freel, 1995). The concentration of intraluminal oxalate available for absorption is partly dependent on the presence of oxalate-degrading bacteria in the gut which can degrade oxalate at rates ranging from 0.1 to 4.8 μmol/g faeces/hour (reviewed by Williams and Wilson, 1990).

Oxalate absorption appears also to depend on the solubility of its salt in the gut lumen. Sodium oxalate is more soluble than calcium oxalate and a larger percentage of it is absorbed than calcium oxalate. Furthermore, in patients with steatorrea, a condition where gut luminal calcium forms soaps with fatty acids leaving less ionised calcium to form calcium oxalate, oxalate absorption rate increases almost threefold, as determined by urinary oxalate output after oral sodium oxalate loading (Rampton *et al.*, 1979). Another cation, magnesium, has been found to decrease oxalate absorption, as measured by a decrease in urinary oxalate, possibly by forming a magnesium oxalate complex (Barilla *et al.*, 1978, Berg *et al.*, 1986).

The site and mechanism of oxalate absorption from the intestinal lumen is not fully determined. Although oxalate can be absorbed by all segments of the intestine, most of the oxalate from food is absorbed from the upper part of the intestinal tract (Barilla *et al.*, 1978, Marangella *et al.*, 1982, Earnest *et al.*, 1974).

1.4.2: Oxalate absorption in the small intestine.

Oxalate uptake in everted sacs of rat and rabbit duodenum, jejunum and ileum was measured (Binder, 1974, Caspary, 1977). Oxalate flux appeared to be only concentration-dependent and not affected by metabolic inhibitors or temperature changes and it was therefore concluded that intestinal absorption occurred by passive diffusion (Binder, 1974). This was confirmed in another study where it was found that decreasing net water transport in the jejunum and ileum also inhibits oxalate absorption (Saunders *et al.*, 1975). In contrast, *in situ* experiments demonstrated that the jejunum of nephrectomised rats secreted oxalate into the lumen against a concentration gradient (Dobson and Finlayson, 1973). More recent studies of oxalate transport across isolated, intact sheets of small intestine from rats and rabbits clearly showed a net secretion of oxalate into the gut (Hatch and Vasiri, 1994a, Hatch *et al.*, 1994, Hatch and Vasiri, 1994b). In the absence of an electrochemical gradient the serosal to mucosal flux of oxalate is significantly greater in the jejunum compared to ileum in rabbits (Hatch and Vasiri, 1994a), whereas in rats, net secretion of

oxalate was much greater in the ileum when compared to the jejunum (Hatch *et al.*, 1994).

Absorption of oxalate also occurs in the small intestine. Because of the presence of "leaky" epithelia the major route for the passive movements across epithelia is paracellular since the resistance of tight-junctions to solute flow is very low (Frizzell and Schultz, 1972). Hence, oxalate will be predominantly absorbed paracellularly when intraluminal oxalate concentrations are high.

The presence of oxalate carriers was investigated using membrane vesicle preparations. In rabbit ileal brush membrane vesicles two anion exchangers were identified: an oxalate(SO_4)-OH and an oxalate-Cl carrier (Knickelbein *et al.*, 1985a, Knickelbein *et al.*, 1985b). The specificity of the first system was determined based upon the fact that outwardly directed OH gradients stimulated oxalate and SO_4 uptake and because both of the divalent anions *cis*-inhibited and *trans*-stimulated one another (Knickelbein *et al.*, 1985a). Specificity of the second system was determined likewise (Knickelbein *et al.*, 1985b). Both systems are DIDS (4-4'-diisothiocyanostilbene-2,2'-disulphonic acid) sensitive and electroneutral (Woolffram *et al.*, 1988).

Oxalate uptake into brush border membrane vesicles from pig jejunum occurs by an oxalate-SO_4 process (Wolffram *et al.*, 1988). Only one mechanism has been suggested for the movement of oxalate across the basolateral membrane of ileal enterocytes: an oxalate(SO_4)-HCO_3 exchange, which is DIDS sensitive and electroneutral (Knickelbein and Dobbins, 1990).

The mechanics of oxalate secretion in the small intestine are not yet known, but may be similar to those governing cAMP-induced chloride secretion (Halm and Frizzell, 1990). It has been shown that an exogenous cAMP analog stimulates oxalate secretion in isolated, short-circuited segments of the rabbit distal ileum. Ileal brush border membrane vesicles were shown to possess conductive pathways for oxalate and chloride that exibited some of the characteristics of a pore or channel (i.e. activation energies of a non-mediated diffusion process and poorly saturable) (Freel *et al.*, 1995).

1.4.3: Oxalate absorption in the large intestine.

Oxalate transport across the large intestine was also considered to be via passive diffusion since studies using everted gut sacs did not indicate

involvement of a carrier-mediated process (Binder, 1974, Caspary, 1977). Later studies, nevertheless, demonstrated that oxalate was actively transported by the large intestine of rats and rabbits (Freel *et al.*, 1980, Hatch *et al.*, 1984). A net absorption of oxalate and chloride in isolated segments of rat colon immersed in a calcium-containing solution has been demonstrated (Freel *et al.*, 1980). Upon removal of the calcium, transmucosal resistance was decreased twofold and net oxalate transport was ceased. It was concluded that calcium was necessary to maintain conductive pathways across colonic epithelia. In *in vivo* studies with rats, the degree of oxalate absorption by rat caecum was shown to correlate with luminal pH (Diamond *et al.*, 1988). When the intraluminal pH was dropped from 7.1 to 4.9, urinary oxalate excretion was significantly elevated consistent with an increase in caecal transport due to diffusion of the free acid.

Differences in oxalate transport were also demonstrated within the colon. In the isolated, short-circuited proximal colon of the rabbit, oxalate transport is coupled to Na-H and Cl-HCO_3. The addition of cAMP enhanced the basal net secretion of oxalate across this segment (Hatch et al., 1993). As demonstrated in both the proximal and distal (see

below) segments of the colon, the direction of net oxalate flux can be regulated by neuro-hormones which are known to affect enteric handling of major plasma electrolytes.

In the rabbit distal colon, where oxalate is absorbed under short-circuit conditions, the movements and sensitivities of oxalate paralleled those of chloride, and were Na-dependent but Cl-independent (Hatch *et al.*, 1994, Hatch and Vasiri, 1994a). Like the proximal part of the rabbit colon, net flux of oxalate across the distal colon was sensitive to secretory stimuli, and cAMP reversed the net absorptive flux to a net secretory flux. It was proposed that oxalate was accumulated intracellularly via a Na-K-2Cl cotransport system on the basolateral membrane and crossed the apical membrane possibly via a Cl channel (Hatch *et al.*, 1994).

While there are data regarding oxalate transport in isolated colonic tissues, the transport systems suggested to account for net secretion or absorption of oxalate across the colon have not been tested using isolated membrane fractions. In rat colonic apical membrane vesicles, oxalate produced a modest cis-inhibition of HCO_3 or OH gradient driven Cl uptake, suggesting the possibility that Cl-OH or Cl-HCO_3

exchangers mediate oxalate absorption in this tissue (Hatch *et al.*, 1994, Mascolo *et al.*, 1991).

1.4.4: Importance of intestinal oxalate handling in disease states.

Enteric hyperoxaluria is the term used for hyperoxaluria resulting from enhanced absorption of oxalate secondary to a wide variety of gastrointestinal diseases, ileal resection, or jejuno-ileal bypass. The common finding among these disorders is malabsorption of bile salts and fatty acids. Using a variety of techniques including everted gut sacs (Binder, 1974), perfused loops of rat intestine (Dobbins and Binder, 1976), and perfused colonic segments from humans (Fairclough *et al.*, 1977), it was concluded that oxalate absorption from the colon is markedly enhanced when excessive concentrations of bile salts and fatty acids are present in the lumen. Two hypotheses have been proposed to explain the mechanism: one deals with changes in the permeability of colonic epithelia and the other with oxalate solubility and its availability in fecal contents. Low concentrations of bile salt applied to sheets of rabbit colon resulted in a large increase in passive permeability of brush membranes to

oxalate (Hatch *et al.*, 1982). *In vitro* experiments examining oxalate solubility in the presence of fatty acids and calcium showed that the affinity of calcium for fatty acids was greater than for oxalate, leaving more soluble oxalate available for absorption (Binder, 1974, Earnest, 1977).

It can be concluded that enteric hyperoxaluria can be attributed to a combination of enhanced mucosal permeability that allows large increases in the paracellular passive movement of oxalate along its concentration gradient and increased oxalate solubility. The contribution of active transporters of oxalate will be negligible under the circumstances.

Absorptive or 'dietary' hyperoxaluria.

Some oxalate stone-formers have enhanced intestinal absorption of oxalate that is not secondary to steatorrhea or bile salt malabsorption. In studies of patients with an inheritable anomaly of red blood cell oxalate transport, it was suggested that the erythrocyte defect might also be present in enterocytes (Baggio *et al.*, 1986, Borsatti, 1991). This is because male stone-forming patients with the underlying red cell defect excreted significantly higher amounts of

oxalate in urine following an oxalate load when compared to their brothers who were characterized as having normal red cell transport of oxalate (Baggio *et al.*, 1986). It has also been reported that vitamin B_6 deficiency in rats is associated with an increase in oxalate uptake in intestinal rings (Farooqui *et al.*, 1981) and isolated apical membrane vesicles (Gupta *et al.*, 1988). *In vitro* studies do not permit the drawing of conclusions on *in vivo* transport of oxalate in stone-forming patients because excretion of oxalate from the kidney cannot be excluded and intraluminal oxalate availability cannot be definitively controlled in these types of studies. Furthermore, a decrease in the intestinal secretion of oxalate, rather than, or in addition to an increase in the absorption of oxalate could explain absorptive hyperoxaluria.

Intestinal secretion in chronic renal failure.

Until recently oxalate excretion was thought to occur exclusively via the kidneys but new information regarding alternative routes for the elimination of oxalate has come to light. Enteric elimination of oxalate was shown to occur in rats with chronic renal failure (CRF) in two different studies (Comici *et al.*,

1982, Costello *et al.*, 1992). This, together with the observation that an increased population of oxalate-degrading microorganisms was found in the large intestine of patients with CRF (Comici *et al.*, 1982), provide evidence that colonic excretion of oxalate is potentially significant in patients with CRF.

1.4.5: Renal handling of oxalate.

Animal studies reveal that oxalate is freely filtered at the glomerulus and that oxalate undergoes bidirectional transport along the renal tubule (reviewed by Hatch and Freel, 1995). However, there is conflicting information as to whether the bidirectional transport of oxalate results in tubular secretion or reabsorption. Oxalate clearance studies employing ^{14}C oxalate in chickens, sheep, dogs, rats, rabbits and man have shown that oxalate clearance exceeds the clearance of an extracellular marker like inulin or creatinine, indicating net secretion of oxalate by the kidney(reviewed by Hatch and Freel, 1995). In contrast, recent studies in humans (Kasidas, 1988, Hatch, 1993) and rats (Costello *et al.*, 1992, Hatch *et al.*, 1994), in which clearance was determined by measurement of plasma and urinary oxalate concentrations, show

oxalate/creatinine clearance ratios less than unity, implying renal reabsorption of oxalate.

Micropuncture studies indicate that oxalate secretion occurs in the proximal convoluted tubule (Greger *et al.*, 1978, Knight *et al.*, 1979, Weinman *et al.*, 1978, Knight *et al.*, 1981). In isolated, perfused proximal tubular segments from the rabbit kidney the rates of oxalate secretion were markedly different between proximal tubules from superficial and juxtamedullary nephrons indicating that in this species there is heterogeneity of oxalate handling within the kidney (Senekjian and Weinman, 1982).

The secretion of oxalate from the proximal tubule exhibits some of the characteristics of an active, carrier-mediated transport process. In rats, simultaneous perfusion of the proximal tubule and the peritubular capillary indicated that oxalate secretion was a saturable process that was reduced by sodium cyanide and, at capillary oxalate concentrations less than 4 mM oxalate, was inhibited by the organic anion transport inhibitor probenecid (Knight *et al.*, 1981).

Reabsorption of oxalate is also considered to occur in the proximal tubule. Using different microperfusion techniques in rats, it was concluded that 7% to 30% of the filtered load of oxalate is

reabsorbed along the early to mid portions of the proximal tubule (Greger *et al.*, 1978).

Using *in situ* tubular microperfusion or stop-flow peritubular capillary methods it was demonstrated that oxalate interacts with a luminal, Na-dependent SO_4 cotransport system in the rat proximal tubule (David and Ullrich, 1992). It was also shown that inclusion of oxalate in peritubular capillary perfusates inhibited SO_4 uptake as well, but in a Na-independent manner, suggesting a SO_4-oxalate exchange process on the contraluminal membrane (Ullrich *et al.*, 1987).

According to membrane vesicle studies, in rabbit renal microvillus (luminal) membranes there are at least two distinct anion exchangers that accept oxalate as one of the counterions, the oxalate-Cl exchanger and the SO_4-HCO_3 exchanger. Three modes of oxalate transport are possible via the oxalate-Cl exchanger: oxalate-Cl, oxalate-formate, and oxalate-OH (Karniski and Aronson, 1987). An oxalate-OH(Cl) has also been identified in rat renal cortical brush border epithelia, with a different stoichiometry from the exchanger already mentioned, since the exchange process was electroneutral (Yamakawa and Kawamura, 1990).

In the rabbit a luminal oxalate exchanger that utilizes either SO_4 or HCO_3 has been proposed. This

exchanger is reported to be electroneutral and has little affinity for Cl (Aronson, 1989). A SO_4-HCO_3 exchanger has also been identified in rat renal luminal membranes, but this exchanger is Na-dependent (Bästlein and Burchhardt, 1986), and therefore not a simple anion exchanger.

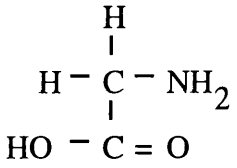
Oxalate transport across rabbit renal basolateral membrane vesicles has also been shown to occur via a sodium-independent, electroneutral SO_4-HCO_3 exchanger (Kuo and Aronson, 1988). Oxalate has been shown to stimulate SO_4 uptake into rat renal basolateral membrane vesicles. This transport process is strongly Na-dependent and can utilize a wider range of anions (including Cl and PO_4) (Bästlein and Burchhardt, 1986).

1.5: Endogenous oxalate production.

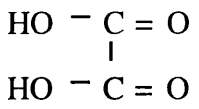
The endogenous production of oxalate is not fully elucidated. It is believed that oxalate is a metabolic end product excreted in the urine and possibly secreted into the gut. Most of the studies investigating metabolic pathways of oxalate precursors have been performed in animals raising the question which precursors are the most important in humans. Dietary precursors of oxalate are mainly sugars and amino acids

(reviewed by Danpure and Purdue, 1995). Most oxalate precursors are metabolised via glyoxylate and/or glycolate (see fig. 1.1 and 1.2). It is thought that the two major immediate precursors of oxalate are ascorbic acid and glyoxylate.

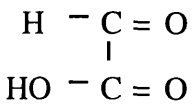
40% of urinary oxalate is derived from ascorbic acid (Crawhall *et al.*, 1959, Atkins *et al.*, 1963). An increase in the daily intake of ascorbic acid results only in a limited increase of oxalate. Intake of 4 g/day of asorbic acid produces an increase in urinary oxalate, but when daily intake of ascorbic acid is increased to 10 g, less than 1% of the extra ascorbate is metabolised to oxalate. *In vivo* studies using ^{14}C ascorbic acid show that urinary oxalate is derived from carbon atoms 1 and 2, suggesting cleavage of the C_2-C_3 carbon bond Ascorbic acid is not metabolised to CO_2, since only 1% to 5% of C^{14}-labelled ascorbic acid is converted to $^{14}CO_2$ (reviewed by Williams and Wilson, 1990). To establish the metabolic pathway by which ascorbic acid is converted to oxalate in vivo studies were done in rats using 2-^{14}C ascorbic acid (Gibbs and Watts, 1973). No ^{14}C urinary intermediates were found, indicating that ascorbic acid is converted directly to oxalate in a nonenzymic manner.



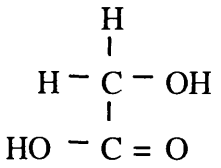
Glycine



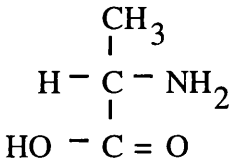
Oxalic acid



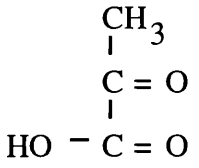
Glyoxylic acid



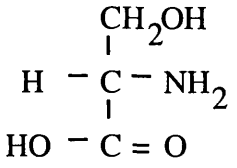
Glycolic acid



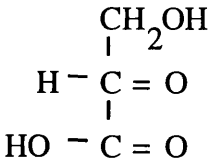
Alanine



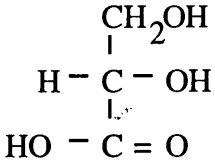
Pyruvic acid



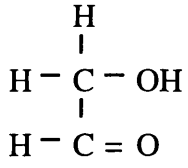
Serine



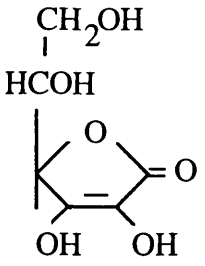
Hydroxypyruvic acid



Glyceric Acid



Glycolaldehyde



Ascorbate

Fig. 1.1: Structures of some of the 2 and 3 carbon compounds important in PH1 and PH2.

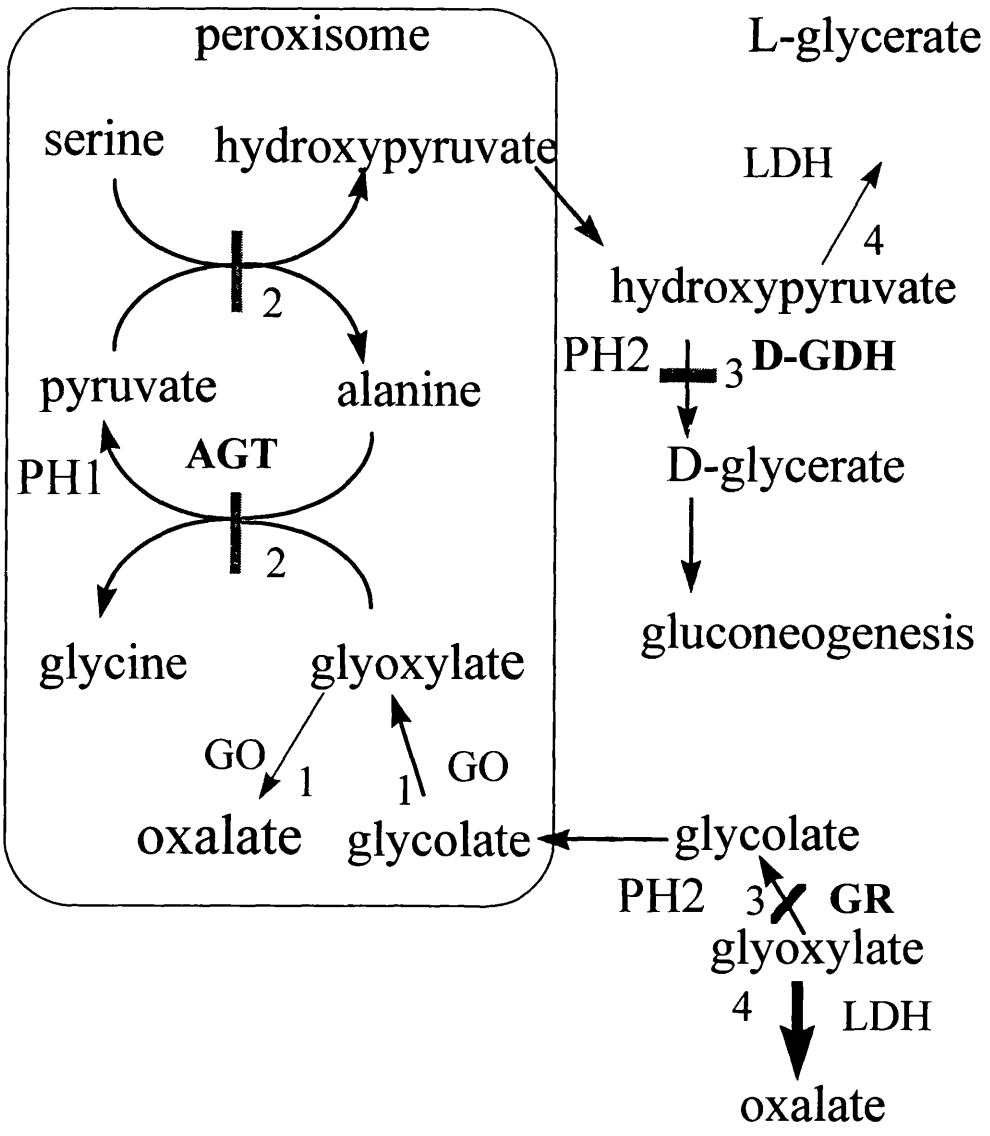


Fig. 1.2: Pathways involved in oxalate, glyoxylate and glycolate metabolism in the human hepatocyte.

Peroxisomal enzymes: 1. L-2-hydroxy acid oxidase A/glycolate oxidase (GO, EC 1.1.3.1); 2. alanine:glyoxylate aminotransferase (AGT)/serine:pyruvate aminotransferase (EC 2.6.1.44/2.6.1.51). *Cytosolic enzymes:* 3. D-glycerate dehydrogenase (D-GDH)/glyoxylate reductase (GR) (EC 1.1.1.29/1.1.1.79); 4. lactate dehydrogenase (LDH, EC 1.1.1.27). PH1: primary hyperoxaluria type 1; PH2: primary hyperoxaluria type 2.

1.5.1: Metabolic pathways involved in the synthesis of glyoxylate.

The most important immediate precursors of glyoxylate are glycine and glycolate (see fig. 1.2). The oxidative deamination of glycine to glyoxylate is catalysed by D-amino acid oxidase (DAO, glycine oxidase) in the presence of flavin adenine nucleotide, producing hydrogen peroxide in the process. DAO is found in many tissues, the activity in the kidney being greater than that in the liver (Neims and Hellerman, 1962, Dixon and Kleppe, 1965). Experiments with isolated perfused rat livers, where competitive inhibition of DAO leads to a marked inhibition of glycine to oxalate metabolism (Liao and Richardson, 1972) may indicate that DAO is important for oxalate synthesis from glycine. However, in guinea pigs, the enzyme has a Michaelis constant (60 mM) (Neims and Hellerman, 1962) for oxidation of glycine approximately 4 times greater than the determined physiological level (~ 15 mM) (Hosaki *et al.*, 1985), suggesting that the glycine oxidising activity is low.

The importance of glycine as an oxalate precursor is disputed. Studies have shown that the proportion of glycine converted to oxalate ranges from less than 1% (Elder and Wyngaarden, 1960), to 40% (Crowhall *et al.*, 1959, Atkins *et al.*, 1964). Only small quantities of glycine were converted to glyoxylate and oxalate by isolated guinea pig peroxisomes (Poore *et al.*, 1997). Moreover, the effect of glycine loading (5.4 g/day) was investigated in normal and stone forming patients and no change was found in plasma or urine oxalate levels in either study group (Butz *et al.*, 1980). This agrees with the observation that in humans, an intravenous infusion of 22 g of glycine failed to alter urinary oxalate excretion (Hahn and Sikk, 1994). These experiments, together with the low activity of DAO, suggest that glycine is not likely to be a significant source of glyoxylate.

Tryptophan and serine are also reported to be metabolised to oxalate via glycine (Gambardella and Richardson, 1978). Hydroxyproline catabolism produces α-keto-β-hydroxy-glutaric acid, which is another precursor of glyoxylate. Both pathways contribute minimally to oxalate production.

The oxidation of glycolate to glyoxylate is catalysed by L-2-hydroxy acid oxidase A (GO, glycolate oxidase), Purified human liver glycolate oxidase requires flavin mononucleotide or flavin adenine nucleotide for activity (Fry and Richardson, 1979b) and yields hydrogen peroxide as a toxic by-product. GO exists as two isozymes, A and B, of which only the liver specific isozyme A can use glycolate as substrate (Nakano *et al.*, 1968). Isozyme B, which is found mainly in nonhepatic tissues, especially the kidney, is inactive toward glycolate (McGroarty *et al.*, 1974). Both GO and DAO are peroxisomal enzymes (De Duve and Bauduin, 1966). Their intracellular location facilitates the rapid removal of the toxic hydrogen peroxide, catalysed by peroxisomal catalase.

1.5.2: Oxidation of glyoxylate to oxalate.

Glyoxylate can be metabolised to oxalate by GO, xanthine oxidase and lactate dehydrogenase (LDH). Xanthine oxidase is however a minor contributor in oxalate production (Gibbs and Watts, 1973) as demonstrated by the observation that allopurinol, a xanthine oxidase

inhibitor, did not cause a change in oxalate excretion in patients with gout (Gibbs and Watts 1966).

GO is likely to oxidise glycolate, rather than glyoxylate *in vivo,* as revealed by kinetic studies (Yanagawa *et al.*, 1990). On the other hand, glyoxylate is as good a substrate as lactate for oxidation catalysed by LDH (Sawaki *et al.*, 1967) although the lactate concentration in the cytosol of liver cells is orders of magnitude greater than that of glyoxylate (Funai and Ichiyama, 1986).

Various *in vitro* studies have suggested that LDH is the major enzyme catalysing glyoxylate oxidation (Smith *et al.*, 1971, Gibbs and Watts, 1973, Williams and Smith, 1983). However, in such systems the NAD concentration is a major determinant of LDH activity (Bais *et al.*, 1987). In fact, the role of LDH *in vivo* must be limited, since patients deficient in various LDH isozymes have normal urinary oxalate excretion (Yanagawa *et al.*, 1990).

In isolated perfused rat livers the GO inhibitors phenyllactate and *n*-heptanoate completely inhibit glyoxylate to oxalate oxidation indicating that glycolate oxidase is the major enzyme catalysing the oxidation of glyoxylate to oxalate (Liao and Richardson, 1973).

Phenyllactate has the same effect in isolated rat hepatocytes (Rofe *et al.*, 1976). Furthermore, urinary excretion of oxalate in rats correlates with the levels of GO, not LDH or xanthine oxidase (Richardson, 1964). However, glyoxylate is a much poorer substrate for GO than glycolate (Yanagawa *et al.*, 1990) and glycolate actually inhibits GO-catalysed glyoxylate oxidation (Bais *et al.*, 1987).

In other studies, phenyllactate and the LDH inhibitor NAD-pyruvate adduct inhibit both glyoxylate and glycolate oxidation in isolated rat hepatocytes, implicating both GO and LDH in the oxidative processes (Bais *et al.*, 1989). It is predicted that the steady-state glyoxylate concentration in the peroxisomes is low, but if it were to increase, for example in PH1, then the glyoxylate would leak out into the cytosol to be oxidised by LDH.

In experiments with guinea pigs, when their response to hyperglucagonemia was examined, urinary oxalate excretion was increased concomitant with a decrease in the liver levels of alanine and lactate, suggesting that alanine and lactate may play a role in regulating oxalate synthesis (Holmes *et al.*, 1995). A decrease in alanine concentration may reduce the transamination of glyoxylate

to glycine catalysed by AGT, leaving more glyoxylate to be oxidised to oxalate. On the other hand, in experiments with isolated guinea pig peroxisomes and purified LDH (Poore *et al.*, 1997), although lactate inhibited both GO- and LDH-catalysed oxidation of glyoxylate to oxalate, its inhibition of the reaction when both GO and LDH were present was minimal.

1.5.3: Oxidation of glycolate to oxalate.

Glycolate is an immediate precursor both of glyoxylate and oxalate. Glycolate can be oxidised directly to oxalate by glycolate dehydrogenase (Fry and Richardson, 1979a). Tyrosine and phenylalanine are metabolised to oxalate via glycolate (Gambardella and Richardson, 1977). Glyoxylate can be reversibly reduced to glycolate by LDH (Banner and Rosalki, 1967) or glyoxylate reductase (GR) (Zelich and Gotto, 1962), which are both cytosolic enzymes (McGroarty *et al.*, 1974).

Hydroxypyruvate, an intermediate in gluconeogenesis from serine is decarboxylated and oxidised to form glycolate or glyoxylate respectively. In isolated perfused rat livers, 12% of administered hydroxypyruvate was

recovered as glycolate (Liao and Richardson, 1978). On the other hand, in the intact rat, the major metabolic intermediate recovered from ^{14}C-hydroxypyruvate was ^{14}C-glyoxylate, not ^{14}C-glycolate (Gambardella and Richardson, 1978).

1.5.4: Transamination of glyoxylate to glycine.

Glyoxylate can be converted to glycine by transamination catalysed by alanine:glyoxylate aminotransferase (AGT) (Thomson and Richardson, 1967) and glutamate:glyoxylate aminotransferase (GGT) (Kamoda *et al.*, 1980). The reaction requires pyridoxal phosphate as cofactor and a number of studies have shown that pyridoxine deficiency leads to an increase in oxalate synthesis (in Danpure and Purdue, 1995). In humans AGT is a liver-specific (Kamoda *et al.*, 1980) peroxisomal enzyme (Noguchi *et al.*, 1979), whereas GGT activity is found in many tissues and is localised mainly in the cytosol (Noguchi *et al.*, 1977). The activity of AGT in human liver is substantially greater than that of GGT (Thomson and

Richardson, 1966) and it appears that AGT is the most important enzyme in the transamination of glyoxylate in mammals (Roswell *et al.*, 1972).

1.6: PH1.

PH1 is the most common and best characterised of the primary hyperoxalurias. Like PH2, PH1 is an autosomal recessive disorder. The disease is characterised by increased oxalate and glycolate production. Increased oxalate production leads to recurrent urolithiasis and nephrocalcinosis. For most patients, chronic renal failure occurs in mid-to-late childhood or early adulthood, although in some cases the disease is neonatal.

Alanine:glyoxylate aminotransferase (AGT) deficiency occurs in individuals with PH1 (Danpure and Jennings, 1988). In humans, AGT is liver-specific (Kamoda *et al.*, 1980) and solely peroxisomal (Noguchi and Takada, 1979) in most normal individuals, although in some people 8-10% is also found in the mitochondria (Purdue *et al.*, 1990). AGT also possesses serine:pyruvate aminotransferase activity (Noguchi and Takada, 1978), which is also deficient in PH1 patients (Danpure, 1991). AGT-deficient subjects are

unable to convert glyoxylate to glycine. This leads to incresed levels of peroxisomal glyoxylate, which is either converted to oxalate by GO in the peroxisomes, or diffuses into the cytosol where it is oxidised to oxalate by LDH and reduced to glycolate by GR (Danpure and Jennings, 1986).

PH1 is manifested at the enzymic level in three ways: i. immunoreactive AGT protein and catalytic activity are absent (CRM-/ENZ-), accounting 32.1% of patients, ii. immunoreactive AGT is present, but AGT catalytic activity is absent (CRM+/ENZ-), accounting for 24.1% of patients, and iii. both immunoreactive AGT and AGT catalytic activity are present (CRM+/ENZ+), the latter up to a level of nearly 50% of the mean control value (Danpure and Jennings, 1988, Danpure, 1991, Danpure *et al.*, 1994). Although in most CRM+/ENZ- PH1 patients AGT is localised within the peroxisomes (Cooper *et al.*, 1990), in most CRM+/ENZ+ patients 90% of AGT is localised in the mitochondria and only about 10% in the peroxisomes (Danpure *et al.*, 1989a).

The gene encoding AGT (AGXT) has been mapped to the end of the long arm of chromosome 2 at 2q37.3 (Purdue *et al.*, 1991). AGXT is composed of eleven exons, ranging in

size from 65 bp to 407 bp, and its total size is approximately 10 kb of genomic DNA. The gene translates into a 392 amino acid protein with an approximate size of 43 kDa. To date five polymorphisms (Purdue *et al.*, 1990, Purdue *et al.*, 1991, Danpure *et al.*, 1994, von Schnakenburg and Rumsby, 1997) and eleven mutations have been described in the gene (Purdue *et al.*, 1990, Purdue *et al.*, 1991, Nishiyama *et al.*, 1991, Minatogawa *et al.*, 1992, Purdue *et al.*, 1992, Danpure *et al.*, 1993, von Schnakenburg and Rumsby, 1997).

In one of the polymorphisms a more common "major AGXT allele" differs from the "minor AGXT allele" in at least three positions (Purdue *et al.*, 1990, Purdue *et al.*, 1991). Normal individuals homozygous for the major AGXT allele have excusively peroxisomal AGT, whereas homozygotes for the minor allele target 5-10% of AGT to the mitochondria. The most frequent mutation (G630A) (Purdue *et al.*, 1990) causes mitochondrial mistargeting of AGT in homozygotes for the minor allele. G630A occurs in about 25% of PH1 alleles. The second most common mutation found to date is a point mutation in exon 7 that has an allelic frequency of 9% (von Schnakenburg and Rumsby, 1997). Only 40% of the patients studied to date have one

of the defined mutations (Tarn *et al.*, 1997), leaving a large number of alleles without any known mutation detected.

Diagnosis of PH1 is suggested by a combination of renal failure in a child or young adult, recurrent urolithiasis, a family history of urolithiasis, and nephrocalcinosis. Measurement of oxalate and glycolate in the urine can establish PH1 (reviewed by Barratt and Danpure, 1994). However, renal failure diminishes the excretion of oxalate and reduces excreted amounts to normal values (Morgan *et al.*, 1987). In such cases diagnosis can be established by liver biopsy, measurement of the enzyme activity (Danpure *et al.*, 1987), immunoblotting (Wise *et al.*, 1987) and, in case of patients that cannot be distinguished from carriers by enzyme activity alone, immunoelectron microscopy.

Prenatal diagnosis of PH1 by oxalate and glycolate measurements in amniotic fluid is not possible because their levels are not affected by the phenotype of the fetus (Leumann *et al.*, 1986). Instead, fetal liver AGT is measured, although analysis of subcellular location is not possible because of the limited amount of material available (reviewed by Danpure *et al.*, 1989b). Molecular

genetic prenatal diagnosis using either mutational analysis or linkage analysis (Rumsby *et al.*, 1994) can also be employed. Mutational analysis is limited to screening of PH1 patients for the G603A mutation. Linkage analysis can be performed for a second pregnancy provided DNA is available from the affected child and the parents. Intron 1 and intron 4 polymorphisms have been used as linkage markers for prenatal diagnosis successfully.

PH1 is treated symptomatically by good hydration, administration of pyridoxine and the administration of inhibitors of CaOx crystallisation, such as magnesium. Kidney failure is followed by haemodialysis and renal transplantation. However, since the metabolic defect is not rectified, CaOx deposition reoccurs. Liver transplantation is the definite treatment for PH1 and can be carried out successfuly in case of residual renal function of the patients' kidneys (Cochat *et al.*, 1989). Otherwise, combined kidney and liver grafting is recommended to avoid the risk in liver transplantation caused by severe renal dysfunction (Ellis *et al.*, 1986). Complete resolution of oxalate crystals in the body as well as complete biochemical correction after liver or combined liver-kidney transplantations have been

documented (Mc Donald *et al.*, 1989, Toussaint *et al.*, 1993). Since AGT is expressed almost entirely in the liver , this makes PH1 an ideal candidate for gene therapy in the future.

1.7: Enzymologic defects of PH2.

PH2 is characterised by increased urinary excretion of oxalate and L-glycerate, while excretion of glycolate and glyoxylate is normal (Williams and Smith, 1968a). L-glycerate is not normally detectable in urine (Tolbert, 1981), and its appearance in PH2 patients suggests a failure to metabolise hydroxypyruvate by its other metabolic routes. Peripheral blood lymphocytes from PH2 patients were found to lack D-glycerate dehydrogenase (D-GDH) activity (Williams and Smith, 1968a, Chalmers et al., 1984). D-GDH is a cytosolic enzyme in mammals that catalyses the reduction of hydroxypyruvate to D-glycerate. D-GDH also possesses glyoxylate reductase (GR) activity (see fig. 1.2) (Willis and Sallach, 1962, Dawkins and Dickens, 1965). Deficiency of both GR and D-GDH has been observed in livers of patients with PH2 (Mistry et al, 1988, Seargeant et al., 1991, Chelbeck et al., 1994).

D-GDH/GR can use both NAD(H) and NADP(H) as cofactors. The reaction catalysed by D-GDH is weighted heavily toward the reduction reaction. Therefore, a deficiency of D-GDH allows hydroxypyruvate to be reduced to L-glycerate by LDH in the presence of NADH (Williams and Smith, 1968a, Williams and Smith, 1968b). Although there is no disagreement about this hypothesis, many theories have been put forward to explain increased oxalate synthesis in PH2.

The first theory is based on the fact that D-GDH is the same enzyme as GR. The lack of GR activity is thought to decrease the conversion of glyoxylate to glycolate. The subsequent conversion of glyoxylate to oxalate by LDH would then account for the hyperoxaluria characteristic of PH2 (Williams and Smith, 1968a). The second hypothesis has been developed using the experimental models of isolated rat liver perfusions (Liao and Richardson, 1978) and isolated rat liver hepatocytes (Fry and Richardson, 1979a). This hypothesis proposes that the build up of hydroxypyruvate due to the deficiency of D-GDH causes more to be decarboxylated to glycolaldehyde (see fig. 1.2), which is then oxidised to glycolate and oxalate by glycolate dehydrogenase without glyoxylate as an intermediate. Against this theory is the fact that high

levels of hydroxypyruvate have never been demonstrated in PH2, and it is likely that the vast majority is reduced by LDH to L-glycerate, as suggested by the L-glyceric aciduria found in PH2 patients. Furthermore, PH2 is not accompanied by hyperglycolic aciduria (Williams and Smith, 1968b), as would be expected if glycolate was an intermediate in the conversion of hydroxypyruvate to oxalate.

The third hypothesis suggests that the buildup of hydroxypyruvate has a direct effect on LDH, increasing its oxidative role and diminishing its reductive role, due to a shift in NADH:NAD ratios (Williams and Smith, 1971). The third theory is cast into doubt because it has been found that hydroxypyruvate inhibits both GO- and LDH-catalysed oxidation of glyoxylate in human liver, whether using purified LDH (Raghavan and Richardson, 1983a) or isolated perfused rat livers (Liao and Richardson, 1978).

The fourth hypothesis suggests hydroxypyruvate could autooxidise to oxalate nonenzymatically (Raghavan and Richardson, 1983b). However, there are no experimental data to support this hypothesis. Apart from the first theory all the others do not take into account that both D-GDH and GR activities are deficient in PH2 patients. These facts make the first theory the most plausible.

1.8: Clinical presentation and prognosis of PH2.

PH2 is a rare disease with only 24 patients reported in the literature so far. However, it should be considered in any patient presenting with urolithiasis or nephrocalcinosis in whom secondary causes of hyperoxaluria have been excluded. It should also be considered in cases of suspected PH1 without hyperglycolluria who account for up to 30% of patients (Latta and Brodehl, 1991, Cochat *et al.*, 1995, Danpure and Purdue, 1995). Diagnosis can be established by increased urinary excretion of oxalate above 0.46 mmol/1.73 m^2 BSA/day or >2SD above established oxalate/creatinine ratios (Leumann *et al.*, 1990, Barratt *et al.*, 1991, Schnakenburg *et al.*, 1994) and L-glycerate above 28 µmol/mmol creatinine (Chlebeck *et al.*, 1994, Seargeant *et al.*, 1995). However, hyperoxaluria in PH2 tends to be less pronounced than in PH1, which is important since reference data for oxalate excretion vary considerably, especially in small infants (Leumann *et al.*, 1990, Barratt *et al.*, 1991, Schnakenburg *et al.*, 1994). Oxalate and L-glycerate measurements in plasma have also

been used for diagnosis in patients presenting with renal insufficiency (Marangella *et al.*, 1995).

The classical presentation of PH2 is urolithiasis (Williams and Smith, 1968a, Hicks *et al.*, 1983, Chalmers *et al.*, 1984, Seargeant *et al.*, 1991, Chlebeck *et al.*, 1994, Marangella *et al.*, 1994, Marangella *et al.*, 1995, Mansell, 1995). In 17 of 24 published patients urinary stone disease developed. Nephrocalcinosis in PH2 is rare: in two patients unilateral and in one bilateral nephrectomy was performed. Patients with PH2 are susceptible to complications from urolithiasis such as infection and obstruction which may cause further kidney damage. Other symptoms include urinary tract infection and leucocyturia (Barratt and Danpure, 1994, Kemper and Müller-Wiefel, 1996). Although nephrocalcinosis is rare, it is recurrent. In two of the patients who progressed to end-stage renal failure and renal transplantation was carried out, there was evidence of nephrocalcinosis in the transplanted organ (Mansell, 1995). Systemic oxalosis has been documented in only one patient who had excessive melanin deposition due to retinal oxalate deposition and radiological evidence revealed oxalate deposition in bone

and vessels (Marangella *et al.*, 1994, Marangella *et al.*, 1995).

It is generally assumed that at least part of the clinical biochemistry associated with PH1 and PH2 causes the disease pathology. Although dysfunction at the organ level in the primary hyperoxalurias is due to the physical consequences of calcium oxalate crystallisation, the cause of damage at the cellular level has not yet been elucidated. Of the metabolites known to accumulate in PH1 and PH2, oxalate, and especially glyoxylate, are toxic in the biochemical sense. However, glycolate (in PH1) and L-glycerate (in PH2) are not known to be damaging.

Oxalate is known to inhibit LDH, pyruvate kinase and pyruvate carboxylase *in vitro* (reviewed by Danpure and Purdue, 1995). Oxalate is taken up by mitochondria, where it appears to bind to the inner membrane. It competitively inhibits mitochondrial uptake and oxidation of malate and succinate and may affect membrane integrity and permeability.

Glyoxylate is a highly reactive molecule known to be toxic to animals. In the presence of oxaloacetate, glyoxylate inhibits aconitate and citrate oxidation (reviewed by Danpure and Purdue, 1995) and may also

inhibit the 2-oxoglutarate:glyoxylate carboligase activity of 2-oxoglutarate dehydrogenase. However, in PH1 and PH2 glyoxylate is expected to be converted instantaneously to oxalate.

Whether any of the aforementioned biochemical toxicities of oxalate and glyoxylate play any role in the pathology associated with PH1 and PH2 is currently unknown. In any case, such putative effects are overshadowed by the obvious physical effect of calcium deposition.

Treatment of PH2 is supportive with high fluid intake and prevention of complications. Prompt treatment is needed on presentation of urinary tract infections and urolithiasis to prevent pyelonephritis and secondary obstruction. For patients at risk chemoprophylaxis with nitrofurantoin or trimethoprim may be used (Kemper and Müller-Wiefel, 1996). In contrast to PH1, pyridoxine (vitamin B6) has not been documented to be beneficial (Yendt and Cohanim, 1985, Barratt and Danpure, 1994, Chlebeck *et al.*, 1994). In 5 patients with PH2, vitamin B6 and orthophosphate decreased urinary calcium oxalate crystallisation (Milliner *et al.*, 1994). Unfortunately the study was not controlled and did not distinguish the

effects between individual drugs. The crystallisation inhibitor potassium citrate has proved successful in sustaining renal function and preventing the development of urolithiasis in PH1 patients (Leumann *et al.*, 1993) and might be beneficial in PH2 as well.

The definitive treatment of the metabolic defect and the clinical manifestations in PH1 is combined liver and kidney transplantation or, if performed early, preemptive liver transplantation alone (Cochat and Schärer, 1993). Two PH2 patients with end-stage renal failure have undergone 3 renal transplantations so far with minor success (Mansell, 1995), since in both there was evidence of recurrence of nephrocalcinosis, hyperoxaluria and L-glyceric aciduria. Therefore, renal transplantation alone does not seem to correct the metabolic defect and combined kidney and liver or preemptive liver transplantation for PH2 in analogy to PH1 has been suggested (Mansell, 1995, Toussaint, 1995).

It is difficult to make a prognosis for PH2 patients especially since there are probably some unreported cases (Chlebeck *et al.*, 1994, Cochat *et al.*, 1995). Until recently long-term prognosis in PH2 was thought to be favourable compared to PH1 (Chlebeck *et al.*, 1994), where

patients progress to end-stage renal failure in early or late childhood (Latta and Brodehl, 1991). Up to 1994 only one published patient progressed to end-stage renal failure requiring dialysis (Chlebeck *et al.*, 1994). However, end-stage renal disease has been documented in 3 of 24 patients, with onset of dialysis treatment at 23, 38 and 50 years of age (Kemper *et al.*, 1997) and reduced renal function has been documented in one additional patient. All these data therefore indicate that PH2 has to be viewed as a disease of considerable morbidity which merits improvement in diagnosis and treatment.

1.9: Feline PH2.

PH2 has also been described in cats (Blakemore et al., 1988, McKerrell et al., 1989). The disease is characterised by acute renal failure due to intratubular deposition of calcium oxalate crystals, severe hyper-L-glyceric aciduria and intermittent hyperoxaluria. Enzymic analysis of the livers of affected cats revealed D-GDH and GR deficiency (Danpure et al., 1989c). The condition is inherited in an autosomal recessive fashion and heterozygotes have levels of D-GDH/GR intermediate between

the levels found in in unrelated normals and affected cats (Danpure et al., 1989c).

The use of feline PH2 as a clinical model for the human disease is limited for the following reasons: firstly, the pathology of the feline disease is slightly different from that of the human. In addition, the disease has neurologic manifestations in cats (McKerrell et al., 1989) that are not found in humans. Secondly, the biochemistry of the disease in cats differs, in that hyperoxaluria is much less than in humans and intermittent. The causes of these differences are not known, but they could arise from differences in the metabolism of oxalate and oxalate precursors in cats, this being predicted as a consequence of the different subcellular distribution of AGT (Noguchi et al., 1978, Okuno et al., 1979, Takada and Noguchi, 1982).

1.10: Physiological function of mammalian D-GDH and GR.

The effect of various metabolites closely related to serine metabolism was investigated on bovine D-GDH (Rosenblum *et al.*, 1971). D-GDH activity was inhibited by

ATP (Rosenblum *et al.*, 1971, Sugimoto *et al.*, 1972) and GTP in a non-competitive fashion with either hydroxypyruvate or NADH. At high concentrations of hydroxypyruvate (0.3 mM) and NADH (50 μM) UTP and CTP also acted as competitive inhibitors. Of other metabolites closely related to serine metabolism, e.g. hydroxypyruvate, pyruvate, serine-P and serine, only hydroxypyruvate gave significant inhibition (Rosenblum *et al.*, 1971, Sugimoto *et al.*, 1972) in a non-competitive fashion. Other glycolytic intermediates such as α-D-fructose, 1,6-diphosphate, 3-phospho-D-glycerate, 2,3-diphospho-D-glycerate and 2-phospho-D-glycerate strongly inhibited the enzyme activity in the concentration range 0.1-1 mM (Sugimoto *et al.*, 1972). In the presence of 1 mM pyruvate the activity of D-GDH was greater with NADP (NADPH) than with NAD (NADH), the reverse of that observed in its absence (Sugimoto *et al.*, 1972).

The addition of hydroxypyruvate or glyoxylate to suspensions of rat hepatocytes stimulated the pentose-phosphate pathway (Van Shaftingen *et al.*, 1989), whose function is to generate NADPH to be used as reducing power for biosynthetic processes. This, together with the findings that at high phosphate concentrations the enzyme

shows greater activity with NADPH than with NADH and the NADPH/NADP system is more sensitive to substrate and feedback inhibition suggest that liver D-GDH functions physiologically as an NADPH-specific reductase.

In mammals D-GDH is involved in one pathway by which the carbon chain of serine can be converted to glycolytic intermediates (Roswell *et al.*, 1969, Cheung *et al.*, 1969). For an overview of the pathway of serine biosynthesis and catabolism see fig. 1.3. Thus, inhibition of D-GDH by the glycolytic intermediates mentioned above could be viewed as feedback control of the enzyme participating in a pathway leading to the formation of glycolytic intermediates from serine.

Mammalian D-GDH also possesses GR activity (Willis and Sallach, 1962, Dawkins and Dickens, 1965). The enzyme catalyses the reduction of glyoxylate to glycolate and can thus contribute to the production of endogenous glycolate. It is distinct from LDH in that it fails to act on pyruvate, D-lactate or L-lactate (Dawkins and Dickens, 1965).

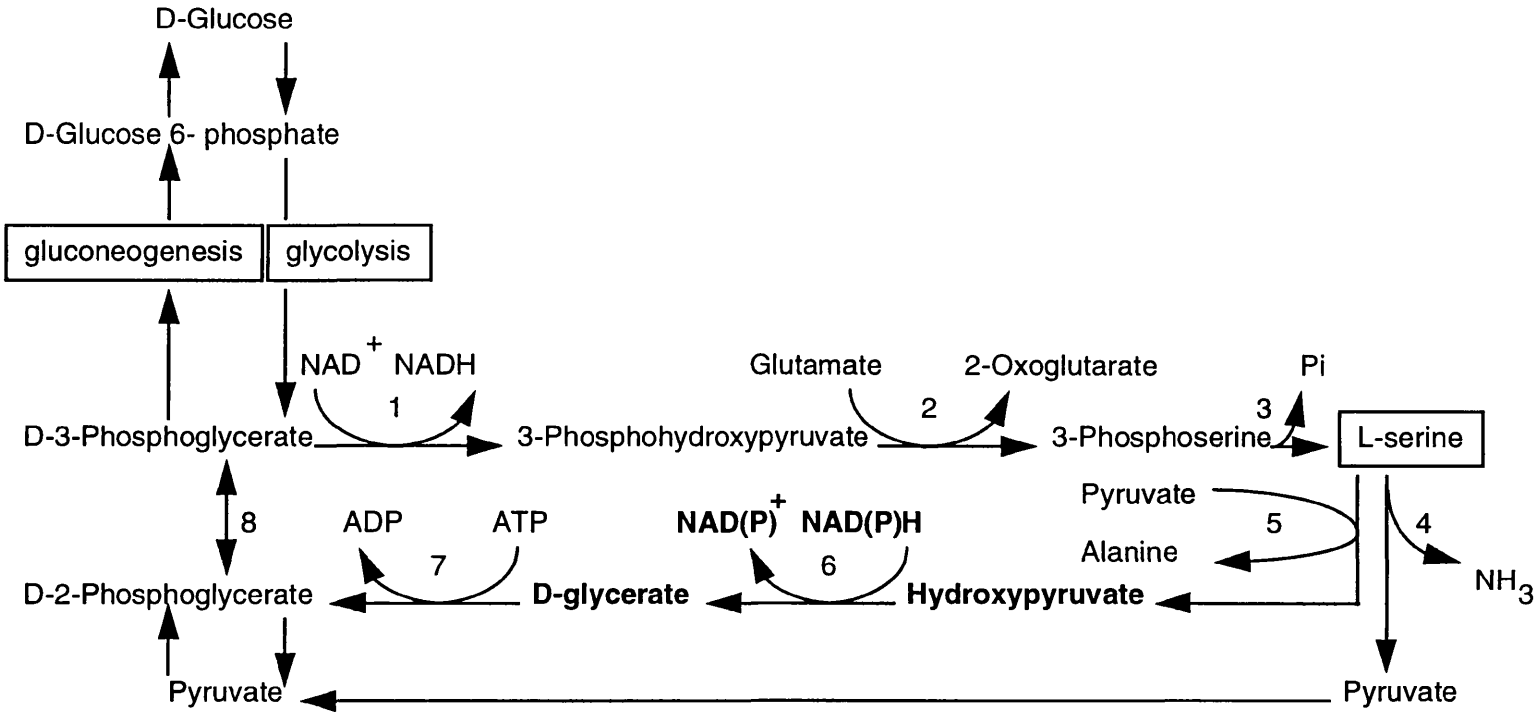


Fig. 2.3: Pathways of serine biosynthesis and catabolism. 1. D-3-phosphoglycerate dehydrogenase; 2. 3-phosphoserine-2-oxoglutarate aminotransferase; 3. 3-phosphoserine phosphatase; 4. L-serine dehydratase; 5. L-serine-pyruvate aminotransferase; 6. D-glycerate dehydrogenase; 7. D-glycerate kinase; 8. phosphoglyceromutase; Pi = orthophosphate

the enzyme participating in a pathway leading to the formation of glycolytic intermediates from serine.

Mammalian D-GDH also possesses GR activity (Willis and Sallach, 1962, Dawkins and Dickens, 1965). The enzyme catalyses the reduction of glyoxylate to glycolate and can thus contribute to the production of endogenous glycolate. It is distinct from LDH in that it fails to act on pyruvate, D-lactate or L-lactate (Dawkins and Dickens, 1965).

1.11: Hydroxypyruvate and glyoxylate reductase activities in leaves of higher plants.

Both glyoxylate and hydroxypyruvate are metabolites involved in the pathway of carbon in photorespiration. Hydroxypyruvate and glyoxylate reductase activities occur in the leaves of a variety of higher plants (Zelich, 1955, Stafford *et al.*, 1954). At least three reductases utilising hydroxypyruvate and/or glyoxylate as substrates have been identified. Both the NADH-preferring HPR (HPR-1) and NADPH-specific HPR (HPR-2) can utilize glyoxylate as an alternative substrate, while the NADPH-preferring glyoxylate reductase (GR) is specific for glyoxylate (Kohn and Warren, 1970, Kleczkowski et al., 1986, Kleczkowski and

Randall, 1988). Peroxisomes are the sole location of HPR-1 as judged by leaf and protoplast fractionation techniques (Tolbert *et al.*, 1970, Givan *et al.*, 1988), and by immunochemical localisation studies (Sautter, 1986). Both hydroxypyruvate and glyoxylate reduction by HPR-1 are reversible (Kohn and Warren, 1970). HPR-1 comprises two equal subunits of molecular weight 41 kD (Kleczkowski *et al.*, 1986). The high Km of HPR-1 for glyoxylate (Km = 20 mM) indicates that reduction of glyoxylate is a side reaction with no physiological importance.

A different glyoxylate-reducing enzyme (GR) was first described by Zelich and Gotto (Zelich and Gotto, 1962). It was distinguished from HPR-1 by its strong preference for glyoxylate (Km = 0.13 mM and 0.32 mM for spinach and tobacco leaves respectively) (Zelich and Gotto, 1962) and immunologically (Kleczkowski *et al.*, 1986, Kleczkowski *et al.*, 1990).

A glyoxylate-reducing enzyme (GR) was later purified to homogeneity from spinach leaves (Kleczkowski *et al.*, 1986). GR was found to be a tetramer of 125 kD with identical subunits of molecular weight 33 kD. It was described as being different from HPR-2 because it uses both NADPH and NADH for the reduction of glyoxylate, although NADPH-dependent

activity is predominant. The activity of this enzyme with hydroxypyruvate as substrate is detectable but is only about 2% of the rate of glyoxylate.

Glyoxylate-reducing activity is now known to be mainly cytosolic, with 10% present in chloroplasts in pea compared to 20% in spinach (Givan *et al.*, 1988, Kleczkowski *et al.*, 1988). In unfractionated soluble extracts of pea and spinach leaves and in lysates of whole pea-leaf chloroplasts, NADPH-dependent GR activity exceeds the NADH-dependent rates by approximately 2-fold. The NADPH-dependent activity of purified chloroplasts is far less than that of total pea-leaf extracts.

HPR-2 prefers NADPH over NADH (Kleczkowski and Randall, 1988, Kleczkowski *et al.*, 1990). HPR-2, which has been purified to near homogeneity, is composed of two subunits each having a molecular weight of 38 kD. The enzyme can react with glyoxylate in addition to hydroxypyruvate, but kinetic experiments have shown that the activity with hydroxypyruvate and NADPH is at least double than with any other pair of substrates. In crude leaf extracts HPR-1 activity predominates (Kleczkowski and Edwards, 1989, Kleczkowski *et al.*, 1990).

The formation of hydroxypyruvate in leaves takes place through the action of a peroxisome-localised serine-glyoxylate aminotransferase (Husic *et al.*, 1987). This enzyme transaminates glyoxylate to glycine and produces hydroxypyruvate as the other product. As mentioned earlier, hydroxypyruvate is readily reduced to D-glycerate in peroxisomes. In the pathway of carbon in photorespiratory metabolism (Somerville and Ogren, 1982, Husic *et al.*, 1987, Givan *et al.*, 1988) there does not seem to exist any necessary role for extraperoxisomal glyoxylate or hydroxypyruvate reduction in photorespiring leaves, at least not if the substrates are localised in the peroxisomes as it is presumed, based on the peroxisomal location of the enzymes that process them.

It seems reasonable to assume that cytosolic glyoxylate-reducing enzymes trap the compound, so that any glyoxylate escaping transamination to glycine in the peroxisomes will neither be lost from the carbon-oxidation cycle nor enter the chloroplasts and have a deleterious effect on photosynthesis. Millimolar concentrations of glyoxylate inhibit photosynthesis (Lawyer *et al.*, 1983, Mulligan *et al.*, 1983), but the physiological significance of this inhibition is

difficult to assess in the absence of reliable measurements of intrachloroplastic glyoxylate levels.

Although hydroxypyruvate is thought to be confined to the peroxisomes (Heupel *et al.*, 1991), the ability of the cytosolic HPR-2 to carry out hydroxypyruvate reduction in the HPR-1-deficient barley mutant suggests that the compartmentation of hydroxypyruvate within the peroxisomes is not absolute. In the case of the HPR-1-lacking barley mutant, hydroxypyruvate escaping from the peroxisomes is converted to D-glycerate by HPR-2 which can then be transferred to chloroplasts so that the photosynthetic carbon oxidation cycle is left largely intact.

The presence of an extraperoxisomal HPR may also enable the cell to make use of reduced pyridine nucleotides outside the peroxisomes. Plant biochemists have had considerable difficulty in accounting for the supply of NADH required in the reduction of hydroxypyruvate within peroxisomes (Yu and Huang, 1986) and perhaps the availability of intraperoxisomal NADH does limit the reduction in situ. Therefore, the additional option made possible by the cytosolic HPR-2 may be important.

1.12: Hydroxypyruvate reductase in methylotrophic bacteria.

Hydroxypyruvate reductase activity has been found in the facultative methylotroph Methylobacterium extorquens AM1. M. extorquens AM1 is a gram-negative bacterium capable of growth on methanol, methylalanine, and a variety of multicarbon compounds which assimilates C_1 units via the serine cycle during growth on C_1 substrates (Peel and Quayle, 1961, Large and Quayle, 1963).

The serine cycle functions by the condensation of methylene tetrahydrofolate and glycine to generate serine, conversion of serine to phosphoenolpyruvate via several steps, and carboxylation of phosphoenolpyruvate to generate oxaloacetate. The oxaloacetate is then converted to malyl coenzyme A (CoA), which is cleaved to acetyl-CoA and glyoxylate. The acetyl-CoA is oxidised to glyoxylate and this is then transaminated to glycine.

As in humans, bacterial HPR catalyses the reduction of hydroxypyruvate to glycerate, one of the key steps in the conversion of serine to phosphoenolpyruvate in the serine cycle. It has also been suggested to carry out the reduction of glyoxylate

to glycolate in cell extracts, but the significance of this reaction in vivo is not known (Dunstan *et al.*, 1972, Anthony, 1982). HPR-deficient mutants were unable to grow on both C_1 and C_2 compounds (Dunstan *et al.*, 1972). This suggests a second role for hydroxypyruvate reductase involving C_2 compounds (Dunstan *et al.*, 1972).

HPR from M. extorquens has been purified to homogeneity (Chistoserdova and Lidstrom, 1991). It has a molecular weight of about 71 kD, and it consists of two identical subunits of about 37 kD in size. The enzyme uses both NADH (Km = 0.04 mM) and NADPH (Km = 0.06 mM) as cofactors, uses hydroxypyruvate (Km = 0.1 mM) and glyoxylate (Km = 1.5 mM) as the only substrates for the forward reaction, and carries out the reverse reaction with D-glycerate (Km = 2.6 mM) only. Kinetics and inhibitory studies of the enzyme from M. extorquens suggest that HPR is not a site for regulation of the serine cycle at the level of enzyme activity.

HPR is also found in other bacteria, such as Hyphomicrobium methylovorum GM2 (Izumi *et al.*, 1990), Pseudomonas acidovorans (inducible and constitutive) (Utting and Kohn, 1975) and Paracoccus denitrificans (Dunstan *et al.*, 1972). The enzymes show some common features and some differences (table 1.1).

M. extorquens is most similar to the HPR from P. denitrificans, although that enzyme uses NADPH as the preferred cofactor. The other enzymes show different substrate specificities, being unable to utilise glyoxylate or unable to carry out the reverse reaction with glycerate.

Table 1.1: Comparison of hydroxypyruvate reductase enzymes from bacteria.

Source	M. extorquens AM1	H. methylovorum GM2	Pseusomonas acidovorans		Paracoccus denitrificans
			inducible	constitutive	
Mass (kD)	71	70	85	75.6	
Subunits	2 x 37	2 x 38	2 x 44		
pH optimum	4.5	6.8	5.5-8	5.3	5
Cofactor(s)	NADH[b], NADPH	NADH	NADH[b], NADPH	NADH, NADPH[b]	NADH, NADPH[b]
Km hydroxypyruvate (mM)[a]	+(0.1)	+(0.175)	+(8)	+(0.13)	+(0.1)
Km glyoxylate (mM)[a]	+(1.5)	+(10.8)	-	-	+
Km glycerate (mM)[a]	+(2.6)	-	+(0.76)	-	+

[a] determined with the preferred cofactor; + or - indicates presence or absence of activity

[b] preferred

Surprisingly, M. extorquens shows some significant differences from the other HPR that has been purified from a serine cycle methylotroph, the enzyme from H. methylovorum GM2. The latter has a pH optimum near neutral, does nor use NADPH as cofactor, and does not carry the reverse reaction with glycerate. In addition, the Km of the H. methylovorum enzyme for glyoxylate is an order of magnitude higher than that for the M. extorquens AM1 enzyme, suggesting that the former enzyme may not be involved in glyoxylate reduction in vivo. H. methylovorans is an obligate methylotroph, and strain GM2 is a glycine-resistant mutant that excretes serine (Izumi *et al.,* 1990). It is possible that the differences in the two HPR enzymes reflect the different physiology of these two serine cycle methylotrophs.

1.13: Aims of the project.

This project aims to characterise human D-GDH/GR in order to improve the methods of diagnosis for PH2 and advance our understanding of the disease at the molecular level. This will be achieved firstly by detailed analysis of the kinetics of the reaction,

taking into account the pH-optima for the reactions and the Km of each substrate, and by development of assays for the D-GDH forward (hydroxypyruvate→D-glycerate), reverse (D-Glycerate→hydroxypyruvate) and GR reactions. This will not only minimise the tissue required for enzymic diagnosis of PH2, but will also facilitate tracing D-GDH/GR during purification by measuring enzyme activity.

Although D-GDH has been purified from a variety of mammalian, plant and bacterial sources, it has never been purified from humans. An attempt will be made to purify D-GDH from human liver in order firstly to raise polyclonal antibodies and diagnose PH2 immunologically and secondly to determine the peptide sequence for future genetic analysis.

The tissue distribution of the enzyme will also be studied both by enzymic and by immunological means in order to identify the tissues for potential transplantation. The subcellular distribution of D-GDH and GR activities will also be determined in various tissues so that a comparison can be made between different species and different human tissues.

CHAPTER 2: KINETIC ANALYSIS OF HUMAN D-GLYCERATE DEHYDROGENASE/GLYOXYLATE REDUCTASE.

2.1: Introduction.

D-GDH from rat (Dawkins and Dickens, 1965) and bovine liver (Willis and Sallach, 1962a) is a cytosolic enzyme that catalyses the reduction of hydroxypyruvate to D-glycerate and of glyoxylate to glycolate. Under comparable conditions hydroxypyruvate was reduced 5 times faster than glyoxylate by D-GDH. The enzyme can also oxidise D-glycerate in the presence of either NAD or NADP, although the equilibrium was strongly in favour of hydroxypyruvate reduction (Dawkins and Dickens, 1965). D-GDH can utilise either NADH or NADPH for both reactions. Lysates used for kinetic studies were purified to a stage where they were free of LDH to avoid interference by LDH which can also reduce glyoxylate to oxalate and hydroxypyruvate to glycerate, but in this case to the L- isomer of glycerate using NADH as cofactor.

In rat liver, using acetate buffer at pH 4, D-GDH displayed greater affinity for NADPH (Km=0.017 mM) than it did for NADH (Km=0.031 mM) (Dawkins and Dickens, 1965). Similarly, D-GDH from bovine liver was found to

have greater activity with NADPH (Km=0.02 mM) than with NADH (Km= 0.045mM) using phosphate buffer at pH 6.8 (Heinz *et al.*, 1962). Similar results were obtained by Van Schaftingen (Van Schaftingen *et al.*, 1989) who showed that D-GDH from rat and bovine liver displayed much greater affinity for NADPH (Km= 0.0018 mM and greater than 0.0004 mM for bovine and rat enzymes respectively) than for NADH (0.128 mM and 48 mM for bovine and rat enzymes respectively). However, other studies contradict these findings. D-GDH from rat liver catalysed the reduction of hydroxypyruvate with NADPH (Km=0.025 mM) and NADH (Km=0.025 mM) with almost equal efficiency at pH 7.4 in phosphate buffer (Dawkins and Dickens, 1965). Furthermore, in bovine liver (Rosenblum *et al.*, 1971, Sugimoto *et al.*, 1972) NADP and NADPH were less effective coenzymes for D-GDH than NAD and NADH. In rat D-glycerate was oxidised at a faster rate by NAD than by a comparable amount of NADP at all conditions tested (Dawkins and Dickens, 1965). These contradictory results could arise from differences in salt content of the reactions.

NADP was over 1000-fold more potent than NAD in inhibiting the D-GDH forward and GR reactions in rat liver (Van Shaftingen *et al.*, 1989), suggesting that it is the cofactor used *in vivo*. In rat, D-glycerate

oxidation by both NAD and NADP was inhibited by 1 mM hydroxypyruvate and 10 mM L-glycerate caused considerable inhibition of D-glycerate oxidation by NAD but not by NADP (Dawkins and Dickens, 1965).

Both the bovine (Willis and Sallach, 1962b, Heinz *et al.*, 1962) and rat (Dawkins and Dickens, 1965) enzymes are activated in the presence of either coenzyme by inorganic anions and particularly Cl^-, with monovalent and divalent cations NO_3^{2-}, and SO_4^{2-} to different degrees depending on pH and substrate concentration (Coderch *et al.*, 1979). The presence of NaCl affected the Km, activity and pH optimum of the D-GDH forward reaction in cattle (reduction of hydroxypyruvate) with NADH as cofactor, and increased the concentration of hydroxypyruvate necessary to cause substrate inhibition (Coderch *et al.*, 1979). The reaction was inhibited by high concentrations of NaCl and activated by low salt concentrations. An increase in the NaCl concentration caused a displacement of the optimum pH towards acidic values. NaCl did not affect the D-GDH reverse reaction (oxidation of D-glycerate) with NAD as cofactor. p-chloromercuribenzoate inhibited the reduction of hydroxypyruvate in rat, suggesting a dependence on free thiol groups for activity. Oxalate (1 μM) and oxamate also inhibited hydroxypyruvate

reduction in rat by either coenzyme. EDTA (10 mM) had little effect on D-GDH activity with either coenzyme, suggesting that enzyme activity does not require divalent cations such as calcium. In all cases inhibitors of the rat enzyme had greater effect in the presence of NADH-NAD than with NADPH-NADP.

Little is known of the kinetics of the human enzyme and the assay currently used for the enzymatic diagnosis of PH2 in liver and lymphocytes is based on that established on bovine liver (Sugimoto *et al.*, 1972). As the kinetics of the enzyme vary between different species, I have sought to characterise human D-GDH and determine the reaction kinetics and assay conditions for measurement of human D-GDH and GR. The reactions investigated were the following:

hydroxypyruvate + NADPH → D-glycerate + NADP

D-glycerate + NADP → hydroxypyruvate + NADPH

glyoxylate + NADPH → glycolate + NADP

NADPH and NADP were used as cofactors because the assay was developed for measuring D-GDH and GR in biopsies where LDH is present and cannot be removed.

2.2: Materials And Methods.

All chemicals were purchased from BDH, Lutterworth, Leicestershire, U.K., or Sigma, Poole, Dorset, U.K., and were of analytical grade unless otherwise stated.

Phosphate buffers were prepared from equimolar solutions of di-potassium hydrogen orthophosphate and potassium dihydrogen orthophosphate. The water used was protease-free distilled deionised water from a Milli-Q RG Ultra-Pure Water System (R4EM55253).

2.2.1: Lowry procedure for the estimation of protein.

MATERIALS:

1. Bovine serum albumin 1 mg/ml
Stock 2 mg/ml solution (Pierce and Warriner, Chester, U.K.) diluted 1:1
2. 0.1 M sodium hydroxide

Reagent A

2% sodium carbonate anhydrous in 0.1 M sodium hydroxide

Reagent B

0.5% copper II sulphate-5-hydrate in 1% trisodium citrate

Reagent C

50 ml of reagent A were mixed with reagent B.

Reagent D

Commercial Folin Ciocalteu regent (Fisons, Loughborough, U.K.) diluted 1:1. It was discarded if the absorbance at 660 nm (A_{660}) was greater than 0.2.

PROCEDURE:

1. The following standards were prepared using the diluted BSA (see table 2.1):

Table 2.1: BSA standards for the estimation of protein using the Lowry method.

STD (µg/tube)	0	5	10	25	50	75	100
water (µl)	200	195	190	175	150	125	100
BSA 1 mg/ml (µl)	0	5	10	25	50	75	100

2. The samples were diluted with distilled water, for example 2% (weight/vol) solutions were diluted 1:20. Both samples and standards were run in duplicate.

3. 1 ml of reagent C was added to each tube containing 200 µl standard or diluted sample. The tubes were vortexed and allowed to stand for at least 10 min at room temperature.

4. 100 µl of reagent D were added to each tube. The tubes were vortexed and were allowed to stand for 30 min at room temperature. The absorbance was read at 660 nm against zero standard.

5. The absorbances of standards were plotted against µg of BSA/tube. The standard curve was used to determine the µg of protein per tube and the figures were converted into mg of protein/ml.

An example of a standard curve is shown in figure 2.1.

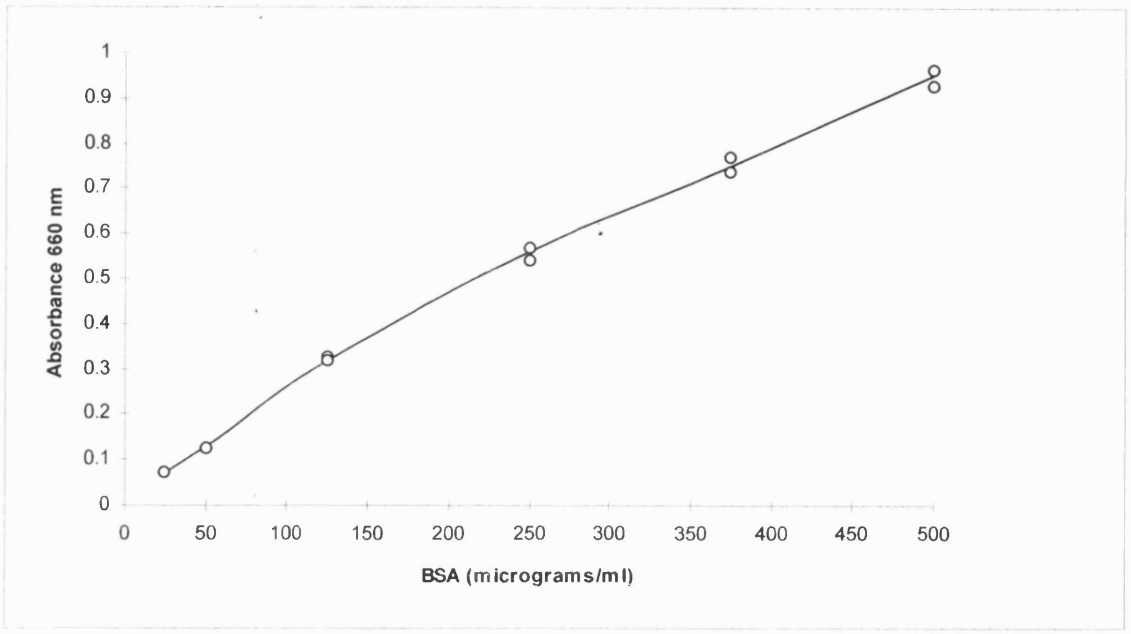


Fig.2.1: Standard curve of BSA concentration versus absorbance at 660 nm for the estimation of protein concentration by the Lowry method.

2.2.2: Human tissues.

Human liver was obtained from cadaveric organ donors free of prior systemic disease. Ethical approval for these studies was obtained from the Joint UCLH committees on the Ethics of Human Research. Written confirmation was obtained that the donor was not positive for hepatitis B antigen or HIV.

2.2.3: Homogenisation procedure for kinetic analysis experiments.

MATERIALS:

1. 50 mM potassium phosphate pH 7

2. Ultra-Turrax homogeniser TP18-10 (Janke and Kunkel, Staufen i. Breisgau, Scandinavia)

3. Sorval angle rotor centrifuge

4. Cellu•Sep Regenerated Cellulose Tubular Membrane T2 (10mm) with M.W. cut-off of 8,000-10,000 (1215-10) or Cellu•Sep Regenerated Cellulose Tubular Membrane T3 (45mm) with M.W. cut-off of 12,000-14,000 (1215-45) (Pierce Warriner)

PROCEDURE:

A 30% (w/v) lysate solution was made for D-GDH/GR kinetic analysis experiments. Human liver was homogenised in 50 mM potassium phosphate buffer pH 7 using an Ultra-Turrax homogeniser. The lysate was homogenised in 10 sec bursts with 30 sec intervals on ice to allow cooling. The lysate was centrifuged at 12,000 g for 30 min. and the supernatant was retained. The lysate was passed over glass wool which retains insoluble lipids. The solution was dialysed overnight against homogenising buffer.

Subsequently, the lysate was centrifuged again at 12,000 g for 30 min. to discard the remaining debris.

2.2.4: D-glycerate dehydrogenase (D-GDH) and glyoxylate reductase (GR) assays for kinetic analysis.

The initial conditions were obtained from Danpure et al., 1989.

MATERIALS:

D-GDH forward reaction (hydroxypyruvate→ D-glycerate)

STOCK SOLUTIONS:
1. 75 mM hydroxypyruvate stock in distilled deionised water
2. 11.25 mM NADPH stock in 50 mM potassium phosphate pH 7.6
(Boehringer Mannheim, Lewes, East Sussex, U.K.)
3. 100 mM potassium phosphate pH 6
4. 30% w/v liver lysate

REACTION MIXTURE:
500 µl 100 mM potassium phosphate pH 6 (50 mM final concentration)
200 µl 2.25 mM NADPH stock (0.45 mM final concentration)
200 µl 35 mM hydroxypyruvate stock (7 mM final concentration)
25 µl 6% w/v liver lysate (88 µg liver protein/reaction mixture)
75 µl water
final reaction volume: 1 ml

D-GDH reverse reaction (D-glycerate→ hydroxypyruvate)

STOCK SOLUTIONS:
1. 330 mM D-glycerate stock in distilled deionised water
2. 12.5 mM NADP stock in 50 mM potassium phosphate pH 7.6
3. 4 mM hydrazine sulphate, pH 8 (with NaOH)
4. 250 mM Tris pH 8
Made up of 60 ml 250 mM Tris-HCl and 40 ml Trizma base
5. 30% w/v liver lysate;

REACTION MIXTURE:

500 µl 250 mM Tris pH 8 (final concentration in assay 125 mM)
200 µl 2.5 mM NADP stock (0.5 mM final concentration)
200 µl 250 mM D-glycerate stock (50 mM final concentration)
50 µl 4 mM hydrazine sulphate pH 8 (final concentration 0.2 mM)
50 µl 6% w/v liver lysate (176 µg liver protein/reaction mixture)
final reaction volume: 1 ml

Glyoxylate reductase reaction

STOCK SOLUTIONS:
1. 75 mM glyoxylate stock in distilled deionised water
2. 11.25 mM NADPH stock in 50 mM potassium phosphate pH 7.6
3. 100 mM potassium phosphate pH 7.6 (final concentration in assay 50 mM)
4. 30% w/v liver lysate

REACTION MIXTURE:
500 µl 100 mM potassium phosphate pH 7.6 (50 mM final concentration)
200 µl 2.25 mM NADPH stock (0.45 mM final concentration)
200 µl 30 mM glyoxylate stock (6 mM final concentration)
25 µl 6% w/v liver lysate (88 µg liver protein/reaction mixture)
75 µl water
final reaction volume: 1 ml

PROCEDURE:

All the reactions were carried out in duplicate and the change in absorbance at 340 nm read against a tissue blank (without second substrate) using a Kontron (Watford, England) 922 double beam spectrophotometer. In each case the reaction was started by the addition of the non-nucleotide substrate (D-glycerate and hydroxypyruvate for the D-GDH reverse and forward

of the non-nucleotide substrate (D-glycerate and hydroxypyruvate for the D-GDH reverse and forward reactions respectively and glyoxylate for the GR reaction. Enzyme activity was calculated as nmoles of NADPH oxidised (or reduced) per minute; the specific activity was calculated as $nmol.min^{-1}.mg\ protein^{-1}$.

2.2.5: Estimation of pH optima for the D-GDH (forward and reverse) and for the GR reactions.

All measurements were performed in duplicate. For the forward D-GDH reaction the pH of the potassium phosphate buffer was varied from 5.8 to 7.8. The pH of the Tris buffer was varied between 7.4 and 9.0 for the estimation of the optimal pH of the D-GDH reverse reaction. The changes of GR activity with pH were investigated by varying the pH of the potassium phosphate buffer from 6.4 to 8.0.

2.2.6: Calculation of Km values of the substrates of D-GDH and GR.

The enzyme activity was calculated from the linear part of the plot of NADPH oxidised or formed against time. During that time the reaction is at a steady

state and the Michaelis-Menten equation applies (in Segel, 1993).

$$v = Vmax[S]/([S]+Km) \quad (1),$$

where v is the velocity of the reaction, Vmax the maximal velocity of the reaction when the enzyme is saturated with substrate, [S] the substrate concentration and Km the Michaelis constant.

The Michaelis constant is defined as the substrate concentration at which enzyme velocity is half maximal. In theory, when the substrate concentration is considerably greater than Km (so that $Km+[S] \approx [S]$), the Michaelis-Menten equation becomes:

$$v = Vmax[S]/[S]$$

$$v = Vmax \quad (2)$$

The Km of both substrates for each reaction was calculated in order to determine the optimal substrate concentrations to achieve Vmax. The substrates are considered to be in saturation at 10xKm.

The Km for each substrate was calculated by plotting $[S]/v$ against [S] (Hane's plot). By inverting (1)

$$1/v = ([S]+Km)/Vmax[S]$$

$$\Leftrightarrow [S]/v = ([S]+Km)/Vmax$$

$$\text{or } [S]/v = (1/Vmax) \cdot [S] + Km/Vmax \quad (3)$$

The x-axis intercept of the plot of (3) is -Km and the y-axis intercept is Km/Vmax.

2.2.7: Linearity of the D-GDH and GR reactions.

Total liver protein was varied from 50 µg to 425 µg per ml reaction volume for the D-GDH (forward) and GR reactions.

2.2.8: Intra-assay variation.

The intra-assay variation was estimated by determining the coefficient of variation (CV) for both D-GDH (forward) and GR assays in 10 replicates.

$$CV = \frac{\text{Standard Deviation} \times 100}{\text{mean}} \%$$

2.2.9: Minimum detectable activity.

The minimum detectable activity, taken as the mean plus 3 standard deviations of a suitable blank, was calculated by taking 10 measurements of a heat-treated sample (70° C for 40 minutes) and reading it against a heat-treated tissue blank.

2.3: Results.

2.3.1: Determination of the optimal pH for the human D-GDH/GR enzyme assays.

i. D-GDH forward reaction.

The results are depicted in fig. 2.2a. Maximal activity was observed at pH 6.0 falling rapidly to a trough at pH 7.4. The non-enzymatic destruction of NADPH was observed at a pH less than 7 (see table 2.2).

Table 2.2: Spontaneous degradation of NADPH at different pH in the blanks of the D-GDH/GR reaction. The blanks did not contain any tissue.

pH	-ΔAbsorbance 340 nm/min
5.8	0.0330
6.0	0.0302
6.4	0.0230
6.8	0.0105
7.0	0.0138
7.4	0.0089
7.8	0.0014

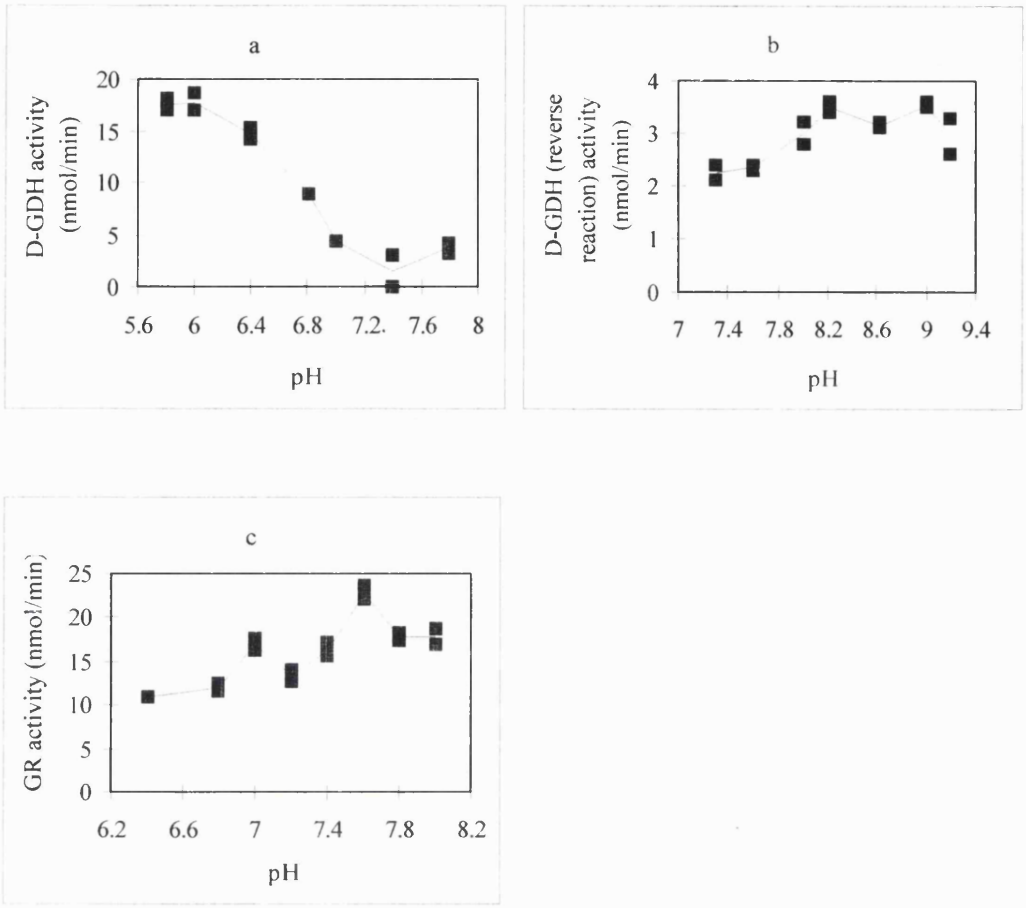


Fig. 2.2: pH-dependence of (a) D-GDH forward, (b) reverse and (c) GR reactions.

ii. D-GDH reverse reaction.

Enzyme activity of this reaction was less affected by variations of pH within the range 7.4 to 9 (fig. 2.2b). Nevertheless, the enzyme activity is greater between pH 8-9 than between pH 7.4-8. A pH of 8 was selected for the assay because it is closer to physiological pH than pH 9 currently used.

iii. GR reaction.

Two peaks of activity were observed for the GR reaction, one at pH 7 and another at pH 7.6, the one at pH 7.6 being the greater. This indicates that there may be more than one enzyme with GR activity in human liver.

2.3.2: Determination of the Km of the substrates for the D-GDH and GR reactions.

Substrate concentrations used for determination of Km values were taken from literature (Danpure *et al.*, 1989).

i. D-GDH forward reaction.

The experiments were carried out in 50 mM potassium phosphate buffer pH 6. The Km of NADPH for the D-GDH reaction was determined by measuring the activity of D-GDH at NADPH concentrations between 0.1-1.6 mM, while keeping hydroxypyruvate at 7 mM (fig. 2.3a). The activity reached a plateau at 0.4 mM NADPH and strong substrate inhibition was observed after 0.8

mM NADPH. From the x-axis intercept of the Hane's plot of [NADPH] versus [NADPH]/D-GDH activity the Km of NADPH was calculated to be 0.08 mM (fig. 2.3b).

To determine the Km of hydroxypyruvate the activity of D-GDH was measured at concentrations of hydroxypyruvate varying between 0.5-12 mM (see fig. 2.3c), while NADPH was kept at 0.45 mM. NADPH was not at saturating concentration because of inhibition of NADPH at higher concentrations and therefore the Km of hydroxypyruvate is Km apparent (Bergmeyer, 1983). A plateau in activity was observed between 4-8 mM hydroxypyruvate with a slight decrease in activity at 12 mM possibly the result of substrate inhibition. The Km apparent of hydroxypyruvate was calculated to be 0.5 mM (see fig. 2.3d).

ii. D-GDH reverse reaction.

The experiments were carried out at pH 8. For the estimation of the Km of D-glycerate, D-GDH activity was measured at concentrations of D-glycerate between 10 and 50 mM, while NADP was kept at 0.5 mM. The activity increased in a linear fashion and substrate inhibition was not observed (fig. 2.4a) although at glycerate concentrations greater than 50 mM a white precipitate formed in the cuvette, which interfered with the absorbance readings. It seems that precipitation is

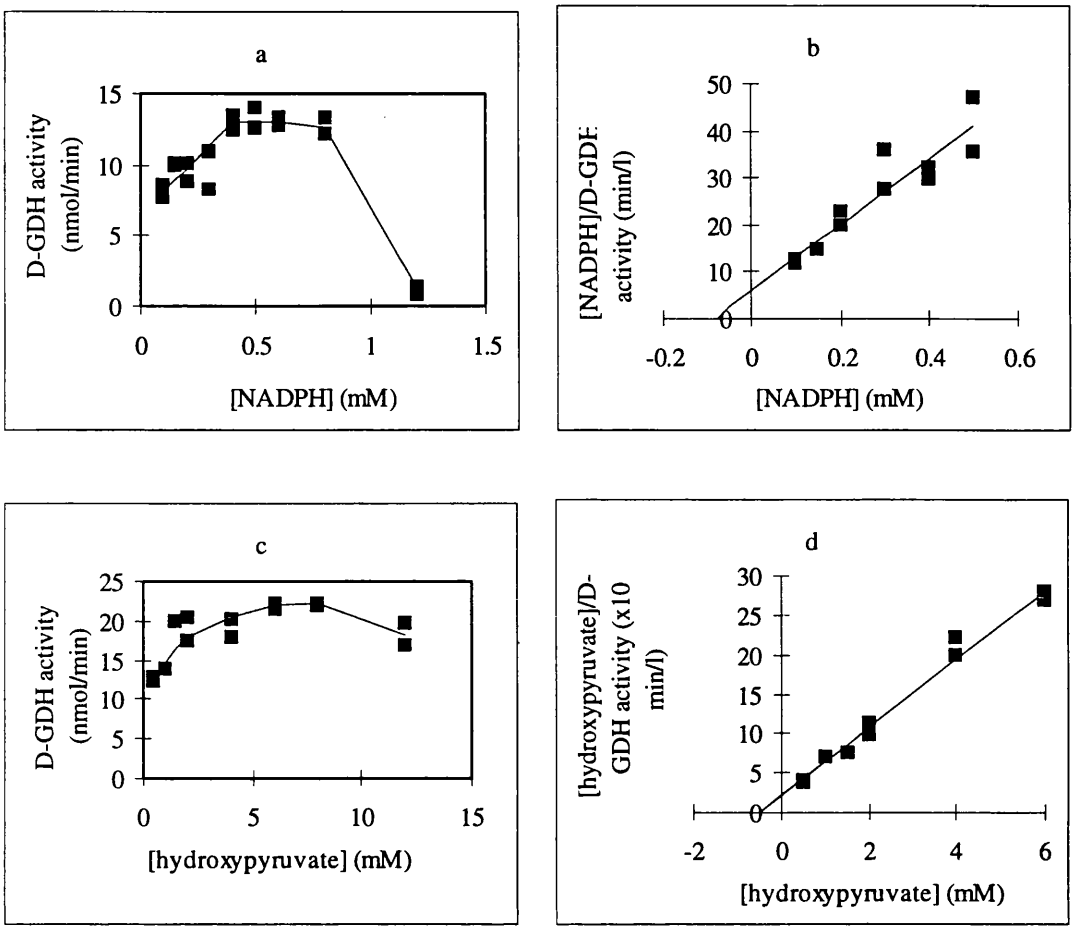


Fig. 2.3: Dependence of D-GDH activity (forward reaction) on the concentration of NADPH (a) and hydroxypyruvate (c); Hanes-Woolf plots for the determination of the Km of NADPH (b) and Km apparent of hydroxypyruvate (d) for the same reaction.

caused partly by the insolubility of D-glycerate in a reaction mixture that contains protein in high concentration and partly by the aggregation of protein at that concentration and temperature.

The Km of D-glycerate was calculated from the Hane's plot to be 20 mM (fig. 2.4b).

The effect of varying NADP concentration from 0.1-0.8 mM is shown in fig. 2.4c. The activity reached a plateau after 0.2 mM NADP and substrate inhibition was not observed for the concentrations of NADP used. From the Hane's plot of [NADP] versus [NADP]/D-GDH activity the Km apparent of NADP was estimated to be 0.03 mM (see fig. 2.4d).

iii. GR reaction.

The experiments were carried out at pH 7.6. To determine the Km of NADPH the activity of GR was measured for NADPH concentrations 0.1-1.0 mM (see fig. 2.5a), while glyoxylate was kept at 6 mM. A peak in activity was reached at 0.6 mM, followed by substrate inhibition. The Km apparent of NADPH was determined from the Hane's plot of [NADPH] versus [NADPH]/GR activity to be 0.33 mM (fig. 2.5b).

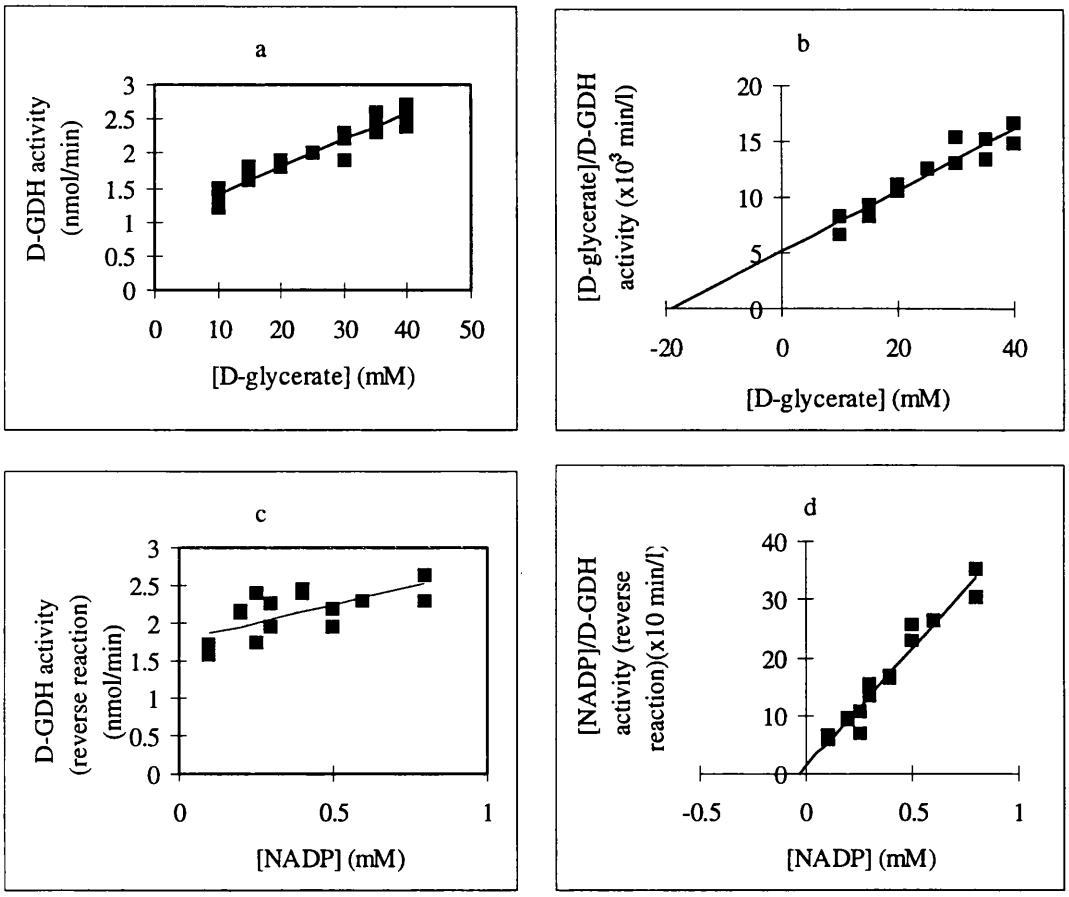


Fig. 2.4: Variation of D-GDH activity (reverse reaction) with D-glycerate concentration (a) and Hanes-Woolf plot for the determination of the Km of D-glycerate (b). Variation of D-GDH activity (reverse reaction) with NADP concentration (c) and Hanes-Woolf plot for the determination of the Km apparent of NADP (d).

GR activity was measured at concentrations of glyoxylate from 1-20 mM (see fig. 2.5c), while NADPH was kept constant at 0.45 mM. GR activity reached a plateau at 6 mM glyoxylate. Substrate inhibition was not observed for glyoxylate concentrations up to 20 mM. The Km apparent of glyoxylate was calculated from fig. 2.5d to be 1.25 mM.

The results are summarised in table 2.3.

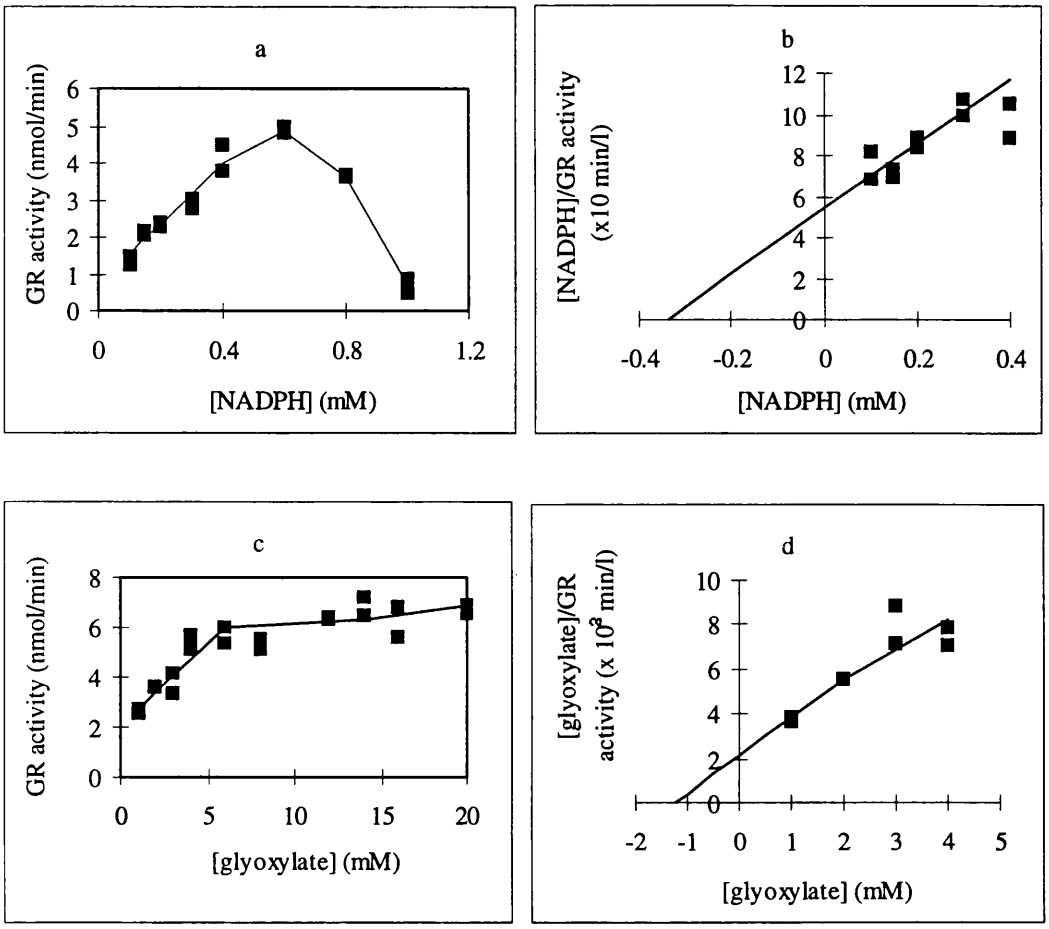


Fig. 2.5: Variation of GR activity on the concentration of NADPH (a) and glyoxylate (c); Hanes-Woolf plots for the determination of the Km apparent of NADPH (b) and glyoxylate (d) for the same reaction.

Table 2.3: Km values and reaction conditions for the D-GDH (forward and reverse) and GR reactions.

Reaction	Buffer	pH optimum	Substrate	Km (mM)	[S] at which inhibition occurs (mM)	[S] in assay (mM)
D-GDH forward	50 mM potassium phosphate	6	hydroxypyruvate NADPH	0.5 0.08	12 0.8	5 (10xKm app) 0.6 (7.5xKm)
D-GDH reverse	125 mM Tris	8	D-glycerate NADP	20 0.03	not observed at 50 mM* not observed at 0.8 mM	not used
GR	50 mM potassium phosphate	7.6	glyoxylate NADPH	1.25 0.33	not observed 0.8	6 (4.8xKm app) 0.6 (1.8xKm app)

* at higher concentrations a precipitate is formed

2.3.3: Linearity of the D-GDH and GR reactions.

Using the reaction conditions outlined in table 2.3, the D-GDH (forward) and GR reactions were semi-automated as described in 3.2.3 using a Cobas Bio centrifugal analyser. The D-GDH forward reaction was linear up to 2.8 mg protein/ml reaction volume (activity 1,469 nmol/min). In experiments conducted before automation, at 6 mM glyoxylate the GR reaction was linear only up to 300 µg/ml reaction volume (34 nmol/min). When, using the Cobas centrifugal analyser, glyoxylate concentration was increased to 25 mM, the GR reaction was linear up to 2.8 mg protein/ml reaction volume (activity 362 nmol/min). The amount of protein used routinely would be the equivalent of 100 µg liver protein per ml of reaction volume.

2.3.4: Intra-assay variation.

The CV of the semi-automated method was 8.6% at an activity of 233 $nmol.min^{-1}.mg\ protein^{-1}$ for the D-GDH (forward) reaction and 10.5% at 85 $nmol.min^{-1}.mg\ protein^{-1}$ for the GR reaction.

2.3.5: Minimum detectable activity.

The minimum measurable activity was 2.1 and 2.0 $nmol.min^{-1}.mg\ protein^{-1}$ for D-GDH (forward) and GR respectively.

2.4: Discussion.

2.4.1: Discussion on the determination of assay conditions for human D-GDH/GR.

A number of problems were encountered in development of assays for measurement of human D-GDH/GR activities. The optimum pH for the D-GDH (forward) reaction was 6. At this pH NADPH is oxidised rapidly at room temperature, the rate being a linear function of hydrogen ion concentration and this non-enzymatic destruction is increased in the presence of phosphate (Lowry *et al.*, 1961). Replacement of the phosphate buffer in the D-GDH forward reaction with citrate at pH 6 was unsuccessful either because citrate inhibits the reaction or more probably because phosphate enhances D-GDH activity (Kohn and Warren, 1970). Thus the effect of NADPH degradation was minimised by measuring D-GDH activity over the first 5 minutes against a tissue blank. For a typical normal human liver biopsy, the change in absorbance per minute for the D-GDH assay was 0.2, while the tissue blank had a $\Delta A.min^{-1}$ of 0.03.

The reverse D-GDH reaction (glycerate to hydroxypyruvate) previously used by other groups (Williams and Smith, 1968a, Mistry *et al.*, 1988) was not practical for two reasons. Firstly, the high protein concentrations necessary to measure detectable activity in combination with insolubility of D-glycerate resulted in formation of a precipitate. Secondly, the activity of this reaction was extremely

low and undetectable in 2% (w/v) sonicates making it unsuitable for routine analyses.

The starting absorbance for the D-GDH (forward) and GR assays is approximately 3, because of the high concentrations of NADPH needed to satisfy the requirement of substrate concentrations at least 10 times greater than the Km values. This does not cause a problem for the routine assays, since the sensitivity of the spectrophotometer is acceptable at absorbance readings of 4. However, the observed inhibition of the D-GDH (forward) and GR reactions at higher NADPH concentrations might be due to the inability of the spectrophotometer to read such high absorbances.

It is notable that previous methods documenting the D-GDH reverse reaction used spectrofluorometric detection of cofactor (Williams and Smith, 1968a, Sugimoto *et al.*, 1972, Chalmers *et al.*, 1984) to increase sensitivity. However, as the reaction is not believed to be relevant physiologically (Snell, 1986) it was not investigated further.

It was not possible to use optimal substrate concentrations for the measurement of GR, because NADPH at concentrations greater than 0.8 mM was found to inhibit the reaction and the absorbance of NADPH at concentrations greater than 0.6 mM exceeds the linearity of most spectrophotometers. These restrictions limited the theoretical range of the GR reaction, but in practice the amount of protein used would be unlikely to exceed the 300 µg limit per ml of reaction buffer.

Since the reactions catalysed both by D-GDH and GR involve two substrates, the velocities of the reactions depend on both substrates. The reactions are therefore

second order. In two-substrate reactions, the reaction mechanism and the resulting velocity equation can vary. In order to use the Michaelis-Menten equation for the estimation of the Km values, two assumptions were made: firstly that the concentration of the substrate that was kept constant was saturating, and secondly that only the initial velocity- before any significant formation of product- was being studied. Inability to use [S]>> Km due to practical limitations has an effect both on V and on Km. For a certain ratio v/V it follows from equation (1) (see 2.2.6) that

$$\frac{v}{V} = \frac{1}{1 + Km/[S]} \; ; \; \frac{V}{v} - 1 = Km/[S] \quad (4)$$

$$[S] = \frac{v}{V-v} \times Km = F \times Km$$

where v is the reaction velocity under substrate concentration [S], V is the maximum reaction velocity and

$$F = \frac{v}{V-v} \quad (5).$$

F is a measure of the approximation of v to V at the substrate concentrations used (Bergmeyer, 1983) and can be expressed in terms of [S] and Km by the equation:

$$F = \frac{[S_1][S_2]}{Km^{S1}[S_2] + Km^{S2}[S_1] + Ks^{S1}\, Km^{S2}} \quad (10)^1$$

where Km^{S1} is the Michaelis-Menten constant of S_1, Km^{S2} is the Michaelis-Menten constant of S_2 and Ks^{S1} the dissociation constant of the enzyme-S_1 complex.

From equation (5) it follows that $v = V \times \frac{F}{1 + F}$. (11)

The reaction velocity of the GR assay could be increased by using a higher glyoxylate concentration (25 mM) and this approach has since been adopted for the assay used in the Primary Hyperoxaluria diagnostic service.

[1] see appendix 1 for derivation of equation 10

2.4.2: Comparison of the reaction kinetics for human D-GDH/GR with D-GDH/GR from other sources.

In the present study on human liver, Hane's plots for the substrates and cofactors of the D-GDH (forward and reverse) and the GR reactions were linear, indicating the reactions follow Michaelis-Menten kinetics. In contrast, double reciprocal plots of specific activity versus substrate concentration were not linear for the D-GDH reverse reaction in partially purified bovine liver (Rosenblum *et al.,* 1971) using either NAD or NADP as substrate and in the forward D-GDH reaction with NADH as substrate. However the

double reciprocal plots of velocity versus substrate concentration of the bovine D-GDH forward reaction with NADPH as cofactor, were linear indicating kinetics of the Michaelis-Menten type. Later studies contradicted the latter results (Sugimoto *et al.*, 1972, Coderch *et al.*, 1979), finding that the D-GDH forward and reverse reactions with NADH/NAD as cofactors both follow Michaelis-Menten kinetics.

The forward D-GDH and the GR reactions in rat liver were both inhibited by high (≥0.5 mM), unphysiological concentrations of hydroxypyruvate or glyoxylate much more strongly in the presence of NADPH than NADH (Van Shaftingen *et al.*, 1989). However, in the presence of NADPH the human enzyme was not inhibited by glyoxylate at 20 mM and was only inhibited by hydroxypyruvate at 10 mM. In human liver, NADP did not inhibit the D-GDH reverse reaction at 0.8 mM whereas it did inhibit the reaction in bovine liver at ~ 0.5 mM. Furthermore, human D-GDH forward and GR reactions were inhibited by NADPH at 0.8 mM. However, it is difficult to determine the significance of these results *in vivo* since the cytosolic concentrations of the aforementioned substrates are not known. Both in bovine (Rosenblum *et al.*, 1971) and human liver D-glycerate did not inhibit the D-GDH reverse reaction in the presence of NADP.

The pH optima and calculated Km values of the D-GDH (forward and reverse) and GR reactions in cattle, rat and human are tabulated in table 2.5. To allow direct comparison with the human enzyme, only the results with NADPH and NADP as cofactors are included, since it appears that when using NADH and NAD substantial differences arise in the reaction kinetics.

Although the pH optima of the D-GDH forward reaction in cattle and rat are similar, in human the pH optimum was more acidic. The pH optima of the GR reaction are dissimilar in all species. There appears to be similarity in the pH optima of the D-GDH reverse reaction in human and rat; however, this reflects the fact that in human there was no difference in enzyme activity across the pH range 8-9.

The Km for hydroxypyruvate is much less in bovine than in rat or human liver, indicating a more active enzyme. In contrast the Km values for hydroxypyruvate in rat and human and glyoxylate in all three species are of the same order of magnitude. The Km for D-glycerate in rat is less than that in human. However, it is still much greater than the Km of hydroxypyruvate, suggesting that in rat, as in cattle and human liver, reduction of hydroxypyruvate is favoured *in vivo*.

The dissimilarities in kinetic behaviour of the D-GDH and GR reactions between species may indicate

differences in enzyme structure. This lack of homology between species is justified by the differences in the diet and thus in the intake of oxalate precursors and gluconeogenesis between species.

Table 2.5: Comparison of existing Km values of substrates for the D-GDH (and HPR) forward and reverse reactions and for the GR reaction in bovine and rat liver with those obtained from human liver.

	Source of D-GDH		
	bovine liver[1]	rat liver[2]	human liver
D-GDH forward reaction			
Optimal pH	6.8	7 (NADPH)	6
Km hydroxypyruvate (with NADPH)	0.02 mM	0.25 mM	0.5 mM
Km NADPH (with hydroxypyruvate)			0.08 mM
D-GDH reverse reaction			
Optimal pH for the D-GDH reverse reaction		9	8-9
Km D-glycerate (with NADP)		1.7-3.3 mM	20 mM
Km NADP (with D-glycerate			0.03 mM
GR reaction			
Optimal pH	6.8	6	7.6
Km glyoxylate (with NADPH)	0.25 mM	0.5 mM	1.25 mM
Km NADPH (with glyoxylate)			0.33 mM

References: [1]Heinz et al., 1962, [2]Dawkins and Dickens, 1965

CHAPTER 3: PURIFICATION OF HUMAN D-GDH/GR.

3.1: Introduction.

While the properties of human D-GDH are largely unknown, the enzyme has been purified to homogeneity from bovine liver, and partially from rat liver and beef spinal cord (Feld and Sallach, 1975).

Partial purification of the enzyme was performed from bovine (Coderch *et al.*, 1979) and rat (Dawkins and Dickens, 1965) liver to remove contaminating lactate dehydrogenase and thus allow study of the effects of various ions on D-GDH activity. Generally these procedures used delipidation of homogenate followed by precipitation with ammonium sulphate and negative adsorption on calcium phosphate gel. In the case of the bovine enzyme this was followed by adsorption on CM sephadex pH 7. D-GDH from rat liver was also partially purified by Van Schaftingen *et al.* (Van Schaftingen *et al.* 1989) by adsorption to DEAE Trisacryl and gel filtration.

Purification to homogeneity has been described for bovine liver (Rosenblum *et al.*, 1971, Sugimoto *et al.*, 1972). Following delipidation and ammonium sulphate precipitation a variety of ion exchange and size fractionation columns were used.

Rosenblum *et al.* purified D-GDH from bovine liver based on a method developed by Sallach (Sallach, 1966). Purification consisted of treatment with cetyltrimethylammonium chloride (CTAB) to remove lipids, precipitation in the 33-55% ammonium sulphate fraction, chromatography on phosphocellulose, negative adsorption on calcium phosphate gel, chromatography on SE-sephadex pH 6.5, and chromatography on DEAE sephadex pH 7. A single band was observed when the purified enzyme was subjected to disc gel and slab polyacrylamide electrophoresis. Sedimentation studies were indicative of a single protein being present. From gel filtration experiments using sephadex G-200 and Bio-Gel P-300, the molecular weight of the enzyme was estimated to be between 65,000 and 70,000. When purified enzyme was subjected to SDS gel electrophoresis under denaturing conditions one band was observed of molecular weight 34,000.

Sugimoto *et al.* (Sugimoto *et al.*, 1972) purified D-GDH from bovine liver by delipidation with Mn^{2+}, precipitation in the 30-50% ammonium sulphate fraction, adsorption to DEAE sephadex pH 7 and CM sephadex pH 7 and gel filtration with sephadex G-150. The final enzyme preparation was proven to be homogeneous by ultracentrifugation and zone

electrophoresis. The molecular weight of the enzyme was calculated to be 72,000.

Antibodies have been raised against purified D-GDH from beef liver (Feld and Sallach, 1975). In a comparison made between purified D-GDH from beef spinal cord and hog spinal cord the enzymes were shown to be immunologically different by immunodiffusion and immunoelectrophoresis when tested with a rabbit antibody raised against D-GDH from beef liver (Feld and Sallach, 1975).

Using published methods as a guide my aim was to purify D-GDH/GR from human liver in order to raise antibodies against it and to obtain partial amino acid sequence of the protein.

3.2: Materials And Methods.

Protein elution from the columns was followed by measuring the absorbance at 280 nm. Quantitation of protein in the collected fractions from the purification experiments was performed using the Lowry method (see 2.2.1), apart from those fractions collected from the chromatofocusing and 2'5' ADP sepharose columns. Unless otherwise stated all the purification procedures were carried out at 4°C.

3.2.1: Human tissues.

Human liver was obtained from cadaveric organ donors free of prior systemic disease.

3.2.2: Homogenisation procedure for purification of D-GDH/GR.

Materials are described in 2.2.3.

PROCEDURE:

A 30% (w/v) lysate solution was made by homogenisation of 15 g of human liver at 12,000 g in 20 mM potassium phosphate buffer pH 7 using an Ultra-Turrax homogeniser. 50 ml of lysate were homogenised in 30 sec bursts with 60 sec intervals on ice between

bursts to allow cooling. The lysate was centrifuged at 12,000 g for 30 minutes and the supernatant was retained. The lysate was passed over glass wool to retain insoluble lipids and dialysed overnight against the homogenisation buffer.

Subsequently, the lysate was centrifuged again at 12,000 g for 30 minutes.

3.2.3 : Semi-automated D-GDH/GR assays.

Reactions were carried out in a Cobas semi-automated spectrophotometer (Roche, Welwyn Garden City, Hertfordshire, U.K.)

MATERIALS:

D-GDH forward reaction (hydroxypyruvate→ D-glycerate)

1. Reagent A: 50 mM hydroxypyruvate stock in distilled deionised water (5 mM final concentration)
2. Reagent B: 1.2 mM NADPH stock (Boehringer Mannheim, Lewes, East Sussex, U.K.) (final concentration 0.6 mM) in
3. 100 mM potassium phosphate pH 6 (final concentration 50 mM)
4. Liver protein from 12.5 µg to 106.2 µg per 250 µl reaction volume.

final reaction volume: 250 µl

Glyoxylate reductase reaction

1. Reagent C: 50 mM glyoxylate in distilled deionised water (5 mM final concentration)
2. Reagent B: 1.2 mM NADPH (Boehringer Mannheim, Lewes, East Sussex, U.K.) (final concentration 0.6 mM) in
3. 100 mM potassium phosphate pH 6 (final concentration 50 mM)
4. Liver protein from 12.5 µg to 106.2 µg per 250 µl reaction volume.

final reaction volume: 250 µl

PROCEDURE:

1. 150 µl of tissue sample were put in a Cobas cup. Reagent B was placed into the large well of reagent boat and reagents A or C into the small well of the reagent boat.
2. Reaction mixture: Reagent B:125 µl, start Reagent A or C: 30 µl, sample: 15 µl, diluent: 80 µl. The reaction was carried out at 37°C. Absorbance readings were taken every 30 seconds at 340 nm.
3. Calculation:

The reading at 4.5 minutes was subtracted from the reading at 0 minutes and wad divided by 4.5 (to give

change in absorbance/min). The blank, calculated the same way, was subtracted. That was multiplied by factor (determined from extinction coefficient of NADPH and volumes used, see note) to give activity in nmol NADP formed per minute. Activity was divided by protein (mg/ml) to give specific activity in nmol NADP formed/min/mg protein.

Note.

Factor derived as follows:

A = 3.1778 at pH 6 and 3.2519 at 37°C

sample volume in Cobas 15 µl

total volume 250 µl

from A = Ecl

molar absorption coefficient = A/cl

at pH 6 (D-GDH) = $\frac{3.1778}{0.6 \times 10^{-3} \times 1}$ cm^2/mol

E = 5296

activity = $\frac{\Delta A/min \times 0.25 \times 1}{0.015 \times 5296}$ mol/L therefore for nmol/ml

divide by 10^{-6}

$= \Delta A/min \times 3147$

At pH 7.6, 37°C (GR assay) absorbance was 3.2519 and E = 5420

activity = ΔA/min x 3075

3.2.4: Ammonium sulphate precipitations.

Ammonium sulphate (164 g/litre of lysate) was added gradually while stirring to lysate homogenised as described in 3.2.2 to achieve 30% saturation. The sample was left for 2 hours and then it was centrifuged at 12,000 g for 10 minutes. Ammonium sulphate (181 g/litre) was added gradually to the supernatant while stirring to achieve 60% saturation. The sample was left for 2 hours and then it was centrifuged at 12,000 g for 10 minutes. The pellet was resuspended in a volume equal to the starting one and it was dialysed for 40 hours against 20 mM potassium phosphate pH 5.8 with 0.1 mM EDTA changing the buffer once. Activity of the fractions was measured after removing ammonium sulphate by dialysis against 50 mM potassium phosphate pH 7.

3.2.5: Gel filtration by sephadex G-200.

The gel, equilibration buffer and elution buffer were degassed before use.

MATERIALS:

1. Sephadex G-200 (Pharmacia 17-0080-01)

2. 29 x 1.5 cm column

Swelling of dry gel

distilled deionised water

Equilibration

3. 50 mM potassium phosphate pH 7 with 0.1 mM EDTA

Storage

0.04% (w/v) sodium azide in 50 mM potassium phosphate pH 7 with 0.1 mM EDTA

Standardisation

0.5 mg cytochrome c (250 µl of 2 mg/ml solution)

0.5 mg bovine serum albumin (BSA) (500 µl of 1 mg/ml solution)

250 µl haemoglobin made by diluting red blood cells 1:2, 45 minutes later centrifuging at 12,000 g for 20 minutes and collecting the supernatant

PROCEDURE:

Sephadex G-200 was suspended in distilled deionised water, was placed in a boiling water bath for 3 hours and was degassed at 4°C before packing. The slurry was poured down the side of the column using a glass rod with the bottom end open. Once the

column was filled, the flow rate was adjusted to 0.3 ml/min and as the slurry settled the column was continuously topped up with sephadex G-200. When the column was filled to 3 cm from the top, it was connected to an equilibration buffer supply and the slurry was equilibrated with 150 ml of buffer at a flow rate 0.3 ml/min. To load the column the buffer on top of the slurry was removed, the sample (0.5-1 ml) was applied without disturbing the bed and the column was run at a rate 0.2 ml/min until the sample entered the column. The column was topped up with equilibration buffer, it was connected to the buffer supply and it was run at 0.2 ml/min.

A 1 ml solution containing cytochrome c (12.4 kD), BSA (68 kDa) and haemoglobin (64.5 kDa) was loaded on the column to provide an elution profile of proteins of known molecular weight. 0.7 ml aliquots were collected. Haemoglobin was detected by its ruby red colour and by measuring the absorbance at 420 nm.

To estimate the molecular weight of D-GDH, crude lysate was prepared as described in 3.2.2 and 1 ml was loaded on the column. 0.7 ml aliquots were collected. D-GDH was detected by measuring enzyme activity.

3.2.6: DEAE sephadex ion exchange chromatography.

MATERIALS:

1. DEAE Sephadex A-50 from (Pharmacia Biotech 17-0180-01)

Swelling of dry gel

2. 1 M NaCl

3. 500 mM potassium phosphate pH 7

3. 20 mM potassium phosphate pH 7

Equilibration

4. 0.1 mM EDTA in 20 mM potassium phosphate pH 7

Storage

5. 0.04% (w/v) sodium azide in 20 mM potassium phosphate pH 7

PROCEDURE:

1. An adequate amount (20-25 ml of bed per g of dry weight) of gel was soaked in 1 M NaCl for 5 min. An equal volume of DEAE sephadex was used to the lysate volume (50 ml).

2. The gel was transferred to excess 500 mM potassium phosphate, pH 7 for 1-2 days at room temperature.

3. The supernatant was replaced with 20 mM potassium phosphate buffer pH 7 four times to remove broken beads.
4. DEAE sephadex was equilibrated in 20 mM potassium phosphate buffer pH 7 with 0.1 mM EDTA using a Buchner funnel. The volume of the buffer used was 8-10 times the bed volume.
5. 950 mg of protein were added to the equilibrated slurry and were left at 4°C stirring occasionally. The sample was collected using a Buchner funnel and the slurry was washed with an equal volume of equilibration buffer that was collected as well.
6. The unused swollen gel was kept in 20 mM potassium phosphate pH 7 at 4°C. For storage over long periods of time 0.04% (w/v) sodium azide was added.

3.2.7: CM sephadex ion exchange chromatography.

MATERIALS:

1. CM Sephadex C50 (Sigma 9047-08-9)

Swelling of dry gel

Swelling was achieved using steps 2 and 3 in 3.2.6. Approximately 3 g were required for the column size given below.

Equilibration

4. 0.1 mM EDTA in 20 mM potassium phosphate pH 5.8

5. 40 x 2.6 cm column from Pharmacia

Elution

5. 150 mM NaCl in 20 mM potassium phosphate pH 5.8 containing 0.1 mM EDTA

Storage

reagent 5 of 3.2.6

PROCEDURE:

Dry gel was swollen as described in 3.2.6 procedure steps steps 1-3.

4. 60 ml of CM sephadex were equilibrated in a column with 20 mM potassium phosphate buffer pH 5.8 with 0.1 mM EDTA at 5 ml/min. The volume of the buffer used was 8-10 times the bed volume.

5. The sample (580 mg protein approximately in a volume of 140 ml) was loaded at a rate 3.3 ml/min and 10 ml aliquots were collected. The column was washed with 2 column volumes of equilibration buffer at 5 ml/min.

6. D-GDH/GR was eluted with 150 mM NaCl in 20 mM potassium phosphate pH 5.8 containing 0.1 mM EDTA at 5 ml/min.

7. Unused CM sephadex was stored as described in 3.2.6 procedure step 6.

3.2.8: Chromatofocusing chromatography.

The gel, equilibration buffer and elution buffer were degassed before use.

MATERIALS:

1. PBE 94 polybuffer exchanger (Pharmacia Biotech, 17-0712-01) received as a slurry.

Equilibration

2. 25 mM imidazole.HCl brought to pH 7.2 with HCl

3. 36 cm x 16 mm Pharmacia column

Elution

4. Polybuffer 74 (Pharmacia Biotech 17-0713-01) pH 5 (with concentrated HCl) diluted 1:8 with water

Regeneration

5. 1 M NaCl

Storage

6. 0.04% sodium azide in 20 mM imidazole.HCl pH 5

PROCEDURE:

1. 70 ml of PBE 94 were equilibrated with 20 mM imidazole.HCl pH 7.2 at 1 ml/min.
2. 25 mg of protein (maximum) were applied on the column at 0.5 ml/min. The sample was washed with 5 ml equilibration buffer. 2 ml aliquots were collected.
3. D-GDH/GR was eluted with Polybuffer 74 pH 5 at a rate 0.5 ml/min. 310 ml were required for elution of D-GDH/GR collected in 2 ml aliquots.
4. PBE 94 was regenerated with three column volumes 1 M NaCl at a rate 0.5 ml/min. For long periods of time it was stored in equilibration buffer with 0.04% sodium azide.

3.2.9: 2'5' ADP sepharose.

MATERIALS:

1. 5 g 2'5' ADP Sepharose 4B (35 ml final volume)(Pharmacia Biotech 17-0700-01)

Swelling

2. 100 mM potassium phosphate pH 7

Equilibration

3. 0.1 mM EDTA in 10 mM potassium phosphate pH 7.2
4. 8.5 cm x 1.5 cm Pharmacia column

Elution

5. 5 mM, 10 mM NADP (Boehringer Mannheim) in 10 mM potassium phosphate containing 0.1 mM EDTA

Regeneration

6. 100 mM NaCl, 0.1 mM EDTA

7. 250 mM Tris.HCl pH 8.5 at 4°C made by mixing 60 ml 250 mM Tris.HCl and 40 ml 250 mM Trizma base or

8. 250 mM sodium acetate pH 5.5

Storage

9. 0.04% sodium azide in 10 mM potassium phosphate pH 7.2 + 0.1 mM EDTA

PROCEDURE:

1. 7 g powder (3.5-5 ml/g freeze-dried material) were suspended in 100 mM potassium phosphate pH 7 for 5 minutes. The material was washed on sintered glass filter with 700 ml of the same buffer (100 ml/g dry powder), added in several aliquots.

2. The gel was resuspended in equilibration buffer so that the settled gel was 70% v/v of the slurry and was degassed at 4°C before pouring into the column.

2. 2'5' ADP Sepharose 4B was equilibrated in degassed 10 mM potassium phosphate pH 7.2 with 0.1 mM EDTA using 3-5 times the bed volume at 1 ml/min.

3. To determine the capacity of the column and the concentration of NADP necessary for elution of D-GDH/GR ~ 15 mg of protein from liver lysate homogenised as described in 3.2.2, precipitated by ammonium sulphate (see 3.2.4) and eluted from a CM sephadex column (see 3.2.7) were loaded. For the purification of D-GDH/GR ~5 mg of protein were applied. The loading, washing and elution rate was 0.6 ml/min and 2 ml aliquots were collected.Before elution the column was washed with two column volumes equilibration buffer.

4. D-GDH/GR was eluted with 5 mM NADP and 10 mM NADP in equilibration buffer. Once it was established that D-GDH/GR is fully eluted from the column with 10 mM NADP, that concentration was used.

5. The gel was regenerated by washing with alternating 2-3 bed volumes 0.1 M Tris.HCl pH 8.5 + 0.5 M NaCl and 0.1 M sodium acetate + 0.1 M NaCl, pH 5.5. This was repeated 3 times followed by re-equilibration with 3-5 bed volumes of equilibration buffer.

3.2.10: Wessel/Flügge extraction of proteins.

This method allows the quantitative recovery of protein in a dilute solution in the presence of detergents and lipids. It was used for concentrating

protein solutions while removing salts prior to SDS-PAGE.

MATERIALS:

1. methanol/chloroform 4:1 fresh daily
2. distilled deionised water
3. methanol

PROCEDURE:

As detailed in table 3.1.

Table 3.1: Wessel/Flügge extraction of proteins.

Sample volume	100 µl
Methanol/chloroform 4:1 fresh daily	500 µl
vortex	
water	300 µl
vortex; spin for 1 min at 13,000 rpm; discard the aqueous phase and leave the interphase which contains the protein, and the organic phase in the tube	
add methanol to the tube	300 µl
vortex; spin for 5 min at 13,000 rpm; remove all the supernatant and air dry pellet. Resuspend in sample buffer (see 3.2.12) at the required concentration (5-30 µg/6 µl)	

3.2.11: Concentration of protein samples using sucrose.

MATERIALS:

1. sucrose
2. Cellu·Sep Regenerated Cellulose Tubular Membrane T2 (10 mm) with M.W. cut-off of 8,000-10,000 (1215-10) or
3. Cellu·Sep Regenerated Cellulose Tubular Membrane T3 (45 mm) with M.W. cut-off of 12,000-14,000 (1215-45) (Pierce Warriner)

PROCEDURE:

Whenever it was required to concentrate the protein sample without losing D-GDH activity the sample was placed in dialysis tubing in a bed of sucrose for a maximum of 24 hours at 4°C.

3.2.12: Separation of proteins using SDS-Polyacrylamide Gel Electrophoresis (PAGE).

MATERIALS:

1. Cleangel 48S from Pharmacia (18-1031-56)
2. SDS buffer kit pH 8 from Pharmacia (18-1031-60)
3. Multiphor II electrophoresis unit from Pharmacia (18-1018-06)

4. MultiTemp III Refrigerated Bath Circulator, 100-120 V (18-1102-77)

5. Electrode strips 255 mm x 50 mm from Pharmacia (382546)

6. GelPool from Pharmacia (18-1031-58)

7. PaperPool from Pharmacia (18-1031-59)

8. sample buffer

0.06 g Tris in 8 ml distilled deionised water (final concentration 0.05 M) adjusted to pH 7.5 with approximately 28 µl acetic acid. 0.08 g SDS (0.8% final concentration) were added and a few grains of bromophenol blue. Before use 0.005 g of DTT were added. The buffer was stored at 4°C.

9. Rainbow markers 14K to 200K from Amersham, Buckinghamshire, U.K. (RPN 756)

stored as 10 µl aliquots at -20°C.

PROCEDURE:

1. The gel was cut to size while still dry so as to obtain enough wells for the experiment.

2. It was rehydrated at room temperature in the GelPool for 1 hour with SDS buffer pH 8.

2. The electrode strips were cut to the size of the gel and were immersed one in anode buffer and the other in cathode buffer in the PaperPool.

3. 15 minutes before running the gel the thermostatic circulator, which was connected to the

electrophoresis unit was set at 10°C and was switched on.

4. The rainbow markers were diluted 1:2 in sample buffer. Protein samples treated by the Wesssel/Flügge method to remove salts were resuspended in sample buffer. The samples and the markers were heated for 2 minutes at 98°C.

4. 1 ml of light paraffin oil was spread onto the cooling plate to aid contact between gel and plate.

5. The gel was wiped with the edge of a filter paper and was placed in the centre of the cooling plate.

6. The cathodic and anodic electrode strips were applied to the appropriate edges of the gel so that the strips overlapped the gel by 5 mm.

7. The samples were loaded. Approximately 10 µg protein /well were required for Coomassie blue detection, less for detection by silver staining. 30 µg protein/well were required for immunodetection of D-GDH/GR in non-purified samples. 500 ng, 1 µg and 2 µg protein/well were used for immunodetection of eluted proteins from 2'5' ADP sepharose. 1 µl of rainbow markers was loaded.

8. A full gel was run at 200 V, 70 mM, 40 W for 10 minutes and at 600 V, 100 mM, 40 W for approximately 80 minutes. For half of a gel, the current and the power settings were divided by two.

3.2.13: Protein detection by Coomassie blue.

MATERIALS:

1. fixing solution

400 ml ethanol, 100 ml acetic acid, distilled deionised water to 1000 ml

2. Coomassie blue staining solution

0.5 g coomassie blue, 800 ml methanol, 140 ml acetic acid, 1060 ml water

3. Destaining solution

250 ml ethanol, 80 ml acetic acid, distilled deionised water to 1000 ml

PROCEDURE:

The procedure was carried out at room temperature as described in table 3.2.

Table 3.2: Coomassie blue staining of SDS Cleangels.

Step number	Solution	Time (min)	Temperature °C
1.	fixing solution	20	23
2.	destaining solution	2	23
3.	staining solution	10	60
4.	destaining solution	20	23
5.	destaining solution	30	23

3.2.14: Protein detection by silver staining.

MATERIALS:

1. Fixation solution

30 ml ethanol, 100 ml acetic acid, distilled deionised water to 1000 ml

2. incubation solution

40 ml of 25% gluteraldehyde stock

3. silvering solution

0.5 g $AgNO_3$ (0.2% w/v final concentration), 50 µl of 37% w/v formaldehyde stock (7.4% w/v final concentration), distilled deionised water to 250 ml

4. developer

9 g Na_2CO_3 (3% w/v final concentration), 150 µl of 37% formaldehyde stock (18.5% w/v final concentration), distilled deionised water to 300 ml

5. stop solution

2.5 g glycine (1% final concentration) to 250 ml distilled deionised water

6. preserving solution

25 ml of 87% glycerol w/v (8.7% w/v final concentration), distilled deionised water to 250 ml

PROCEDURE:

The procedure was carried out at room temperature as described in table 3.3.

Table 3.3: Silver staining of SDS Cleangels.

Step number	Solution	Volume (ml)	Time (min)
1.	fixing solution	200	> 30
2.	incubation solution	100	20
3.	washing with water	3 x 200	3 x 5
4.	silvering solution	100	20
5.	development solution	100	1
6.	development solution	200	3-7
7.	stop solution	200	10
8.	preserving solution	200	30

3.2.15: Immunodetection of D-GDH/GR.

I. Preparation of protein samples.

MATERIALS:

1. 20 mM potassium phosphate pH 7 + 0.1 mM EDTA

PROCEDURE:

0.15 g of human liver from a normal control and from a patient with PH2 were homogenised as described

in 3.2.2, negatively adsorbed on DEAE sephadex as described in 3.2.6 and precipitated by ammonium sulphate as described in 3.2.4. Protein of all the fractions was measured by Lowry and 120 µg of protein from each fraction was precipitated by the Wessel/Flügge method and were resuspended in 24 µl sample buffer without DTT (see 3.2.12).

Protein from the crude lysates mentioned above and those treated with DEAE sephadex was precipitated as before, but was resuspended in denaturing sample buffer. 2% w/v kidney lysate was prepared as described in 2.2.3 but the homogenisation buffer and the dialysis buffer was 20 mM potassium phosphate pH 7 + 0.1 mM EDTA. 40 ml of human lymphocytes were isolated as described in 4.2.2, were resuspended in 1 ml 20 mM potassium phosphate pH 7+ 0.1 mM EDTA, were processed as described in 2.2.3. and were dialysed against 20 mM potassium phosphate pH 7 + 0.1 mM EDTA. 300 µl were kept of the kidney and lymphocyte lysates and 200 µl of DEAE sephadex prepared as described in 3.2.7 were added to the remaining lysates. The samples were rotated on a daisy wheel for 30 minutes, the slurry was allowed to settle and the supernatant was retained. Protein content of the samples was determined by the method of Lowry as described in 2.2.1. 120 µg of protein from each fraction were

precipitated by the method of Wessel/Flügge and were resuspended in 24 µl denaturing sample buffer.

ii. Western blotting.

MATERIALS:

2. electroblotting buffer

72 g glycine (final concentration 192 mM), 15 g Tris (final concentration 25 mM), distilled deionised water to 4 litres, 1 litre methanol

3. nitrocellulose by Schleicher and Schuell (BA83)

4. 3 mm filter paper

5. Trans-blot SD Semi-Dry Transfer Gel from Biorad

PROCEDURE:

1. The gel (see 3.2.13) was rinsed twice with electroblotting buffer and was washed in electroblotting buffer for 15 minutes on a rocker.

2. Two pieces of 3 mm filter paper and one piece of nitrocellulose were cut to the size of the gel and were soaked in electroblotting buffer. Excess buffer was removed by absorbent paper.

3. The gel, pieces of filter paper and nitrocellulose were placed in the Trans-blot SD Semi-Dry Transfer Gel as instructed in the manual. The proteins were

blotted onto the nitrocellulose by applying 15 V for 30 minutes.

iii. Immunodetection of D-GDH/GR.

MATERIALS:

6. guinea pig anti-human antiserum raised to a partially purified preparation of D-GDH/GR. Stored at 4°C.

7. anti-guinea pig IgG Peroxidase Conjugate from Sigma (A 9167)

8. PBSA

8 g NaCl, 0.2 g KCl, 2.894 g $Na_2HPO_4.12H_2O$ (or 1.174 g anhydrous), 0.2 g KH_2PO_4, distilled deionised water to 1 litre

9. PBSA-T

400 µl Tween-20 (0.05% v/v final concentration) in 800 ml PBSA

10. low fat milk powder

11. blocking solution

5% milk powder in PBSA-T

12. ECL western blotting detection reagents from Amersham (RPN 2109)

13. Hyperfilm ECL from Amersham (RPN 2103)

14. Ilford Hypam Rapid Fixer diluted 1:5 (758249)

15. Universal Developer diluted 1:20 from the Paterson Photax Group, West Midlands, U.K. (062001)

PROCEDURE:

The procedure was carried out at room temperature.

1. The nitrocellulose from the western blotting procedure was blocked by incubation in blocking solution for 1 hour.

2. The membrane was rinsed twice in PBSA-T, incubated once for 15 minutes and twice for 5 minutes.

3. The membrane was incubated in guinea pig anti-human D-GDH/GR antibody diluted 1:50 in PBSA-T for at least 1 hour.

4. The membrane was rinsed twice with PBSA-T and incubated once for 15 minutes and twice for 5 minutes.

5. The membrane was incubated with anti-guinea pig IgG Peroxidase Conjugate diluted 1:2,500 in PBSA-T for 1 hour.

6. The membrane was rinsed three times with PBSA-T and was incubated for 40 minutes once and for 5 minutes four times.

7. Equal volumes of detection reagent 1 and detection reagent 2 were mixed and a few drops (1 ml) were added to the nitrocellulose which was placed on filter paper. The detection reagent was spread evenly over the nitrocellulose using SaranWrap. After ensuring that the SaranWrap was not creased and air pockets were excluded, the nitrocellulose was

completely wrapped in SaranWrap and placed in an X-ray cassette. The whole procedure took 1 minute.

8. Under safety lights Hyperfilm-ECL was placed on top of the blot for 25 seconds. It was immediately replaced by another film that was exposed for 3 minutes.

9. The film was developed according to the manufacturer's instructions.

3.3: Results.

3.3.1: Preliminary studies for determination of the protocol for purification of D-GDH/GR.

The experiment was carried out to determine the percentage of ammonium sulphate that was required to precipitate D-GDH. Liver lysate was prepared and ammonium sulphate at 20%, 40%, 60% and 80% saturation was added to different aliquots. The activities of both the pellet and the supernatant were measured (see table 3.4).

Table 3.4: Fractionation of D-GDH by ammonium sulphate precipitations.

Percentage (%) of ammonium sulphate	D-GDH specific activity in supernatant (nmol/min/mg)	D-GDH specific activity in pellet (nmol/min/mg)
0	139	
20	139	0
40	140	109
60	41	176
80	0	122

D-GDH activity was observed neither in the pellet of 20% ammonium sulphate precipitate nor in the supernatant of 80% precipitate. The pellet of the

40% precipitate had 2/3 of the specific activity of the supernatant. The supernatant of the 60% precipitation had 1/4 of the specific activity of the pellet. A clear cut-off was however not found.

In another experiment two aliquots of liver lysate were precipitated one with 25-65% ammonium sulphate and the other with 30-60% ammonium sulphate as described in 3.2.4 (see table 3.5). D-GDH specific activity was greatest in the 30-60% ammonium sulphate precipitate and this was used for purification of the enzyme.

Table 3.5: Comparison of D-GDH specific activity in fractions precipitated with different percentages of ammonium sulphate.

Protein sample % ammonium sulphate	D-GDH specific activity (nmol/min/mg)
0	124
25-65	172
30-60	325

To determine the pH at which D-GDH adheres to CM and DEAE sephadex, CM sephadex was equilibrated in 20 mM potassium phosphate pH 5.5, 6, 6.5, 7, 7.5, and 8 and DEAE sephadex in the same buffer between pH 6-8. Human liver protein precipitated by ammonium sulphate

(30-60%) was equilibrated in the same buffers and 7 mg of protein (0.5 ml) was added to 1 ml of slurry. The samples were rotated on a daisy wheel for 30 minutes, the beads were allowed to settle and the activity of the supernatants was determined. The percentage of D-GDH activity adsorbed corrected for volume is shown in table 3.6.

Although 100% of D-GDH adhered to CM sephadex at pH 5.5 it did not elute with 0.5 M NaCl, suggesting that the protein was irreversibly bound or damaged. At pH 6 D-GDH was eluted with 0.1 M NaCl but adhered to CM sephadex partially. As a compromise it was decided to use pH 5.8.

Table 3.6: Percentage of D-GDH activity adsorbed onto CM and DEAE sephadex.

pH	% D-GDH adsorbed on DEAE sephadex	% D-GDH adsorbed on CM sephadex
5.5	-	100
6.0	27	65
6.5	54	68
7.0	61	51
7.5	62	54
8.0	58	42

D-GDH/GR was adsorbed to 2'5' ADP sepharose in 0.1 mM potassium phosphate pH 7.2 and could be eluted with 5 and 10 mM NADP. Most of the protein was eluted at 5 mM NADP, but since some was eluted with 10 mM NADP it was decided to use the latter concentration in order to maximise the yield (see figure 3.1).

3.3.2: Determination of the molecular weight of D-GDH by gel filtration on sephadex G-200.

An elution profile of proteins of known molecular weight which served as reference was determined and is shown in fig. 3.2. The void volume of the column was 12.5 ml. Haemoglobin and BSA have similar molecular weights and eluted together between 24 and 36 ml. End of elution occurred after 42-45 ml. D-GDH eluted after 23-32 ml, indicating that its molecular weight is similar to that of BSA and haemoglobin, probably towards the highest end of that range.

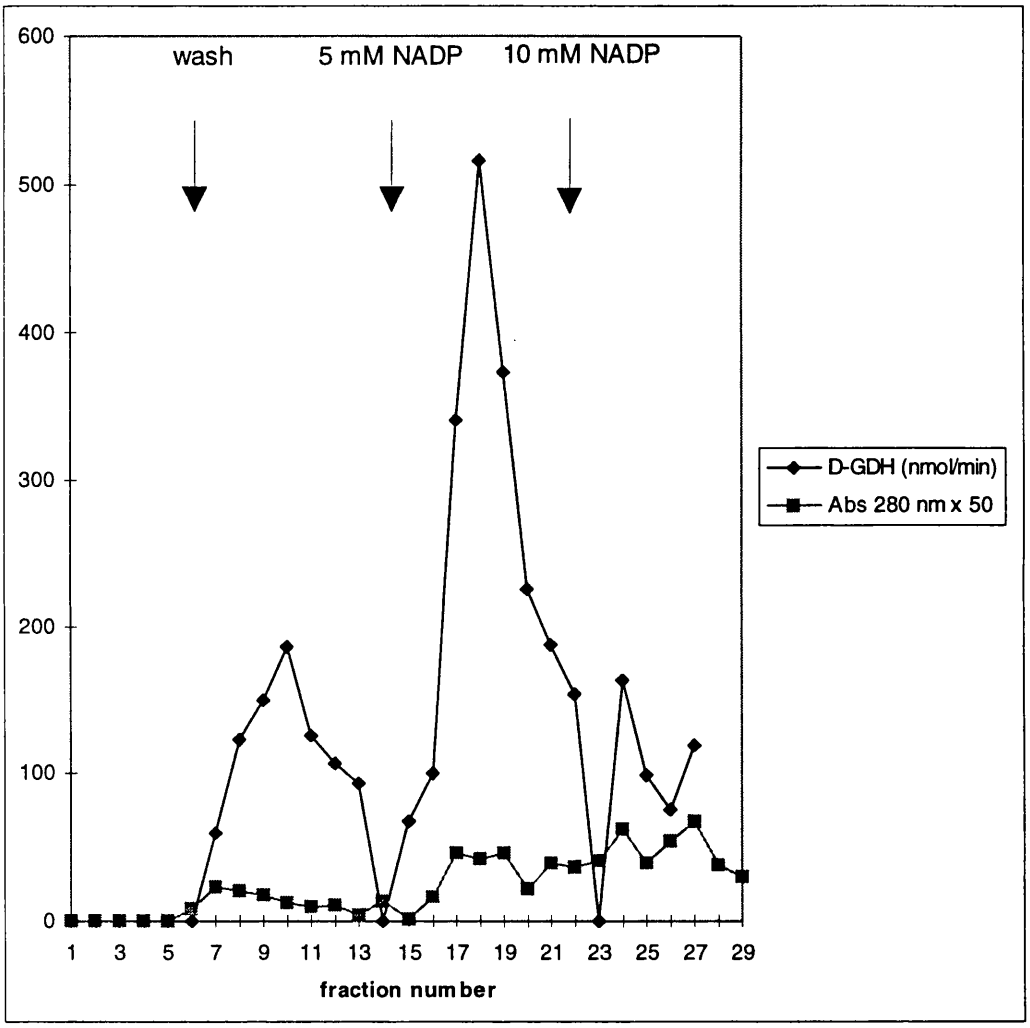


Fig. 3.1: Determination of conditions for elution of D-GDH from 2'5' ADP sepharose.

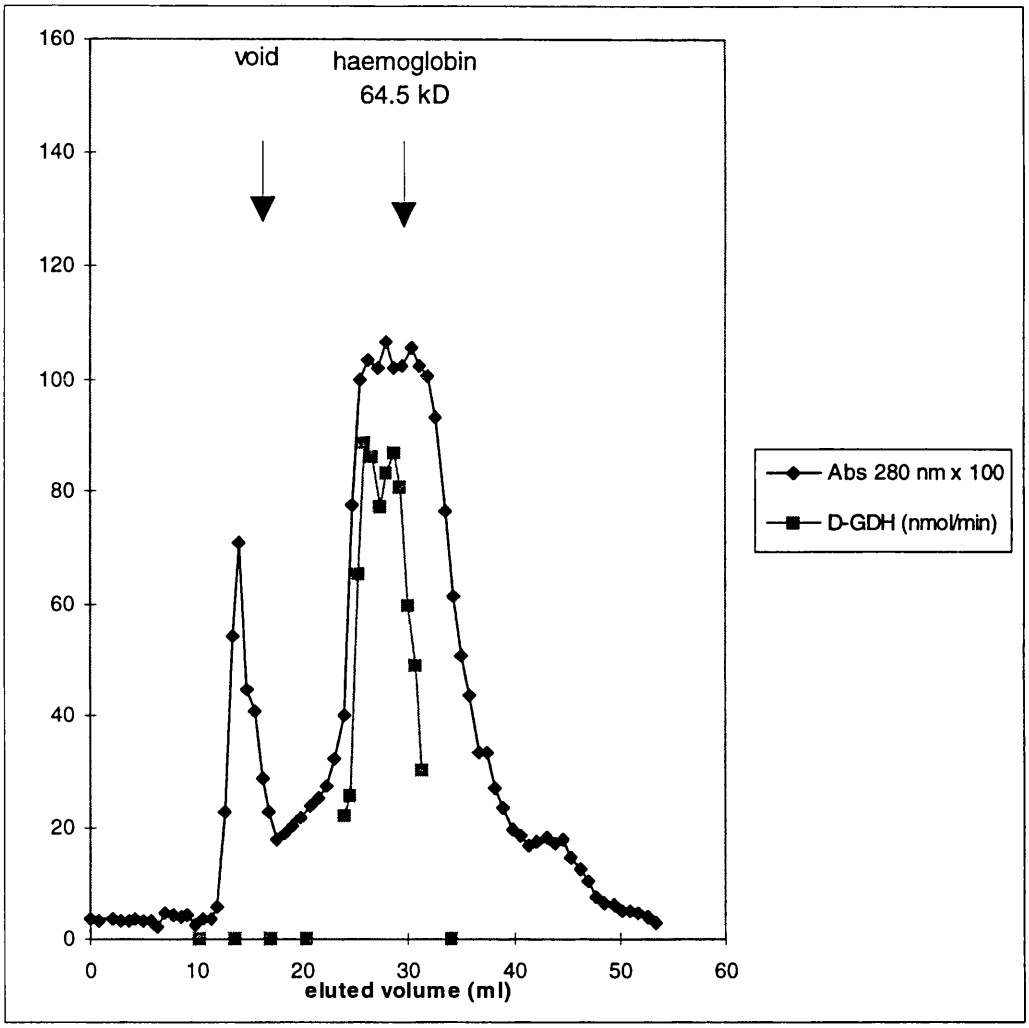


Fig. 3.2: Estimation of the molecular weight of D-GDH/GR by gel filtration using sephadex G-200.

3.3.3: Purification of D-GDH/GR.

Using the conditions established in the previously described experiments, the procedure used to purify human D-GDH/GR is shown below.

homogenisation

⇓

negative adsorption on DEAE sephadex pH 7

⇓

precipitation by ammonium sulphate (30-60%)

⇓

adsorption on CM sephadex pH 5.8

elution with 150 mM NaCl

⇓

binding to chromatofocusing column at pH 7.2

elution with Polybuffer 74 pH 5

⇓

adsorption on 2'5'ADP sepharose pH 7.2

elution with 10 mM NADP

After a negative adsorption step on DEAE sephadex and precipitation by ammonium sulphate (30-60%), the resulting dialysate was adsorbed on CM sephadex, with the elution profile shown in fig. 3.3. GR data was not available on the eluates from this column but data from an earlier non-optimised experiment

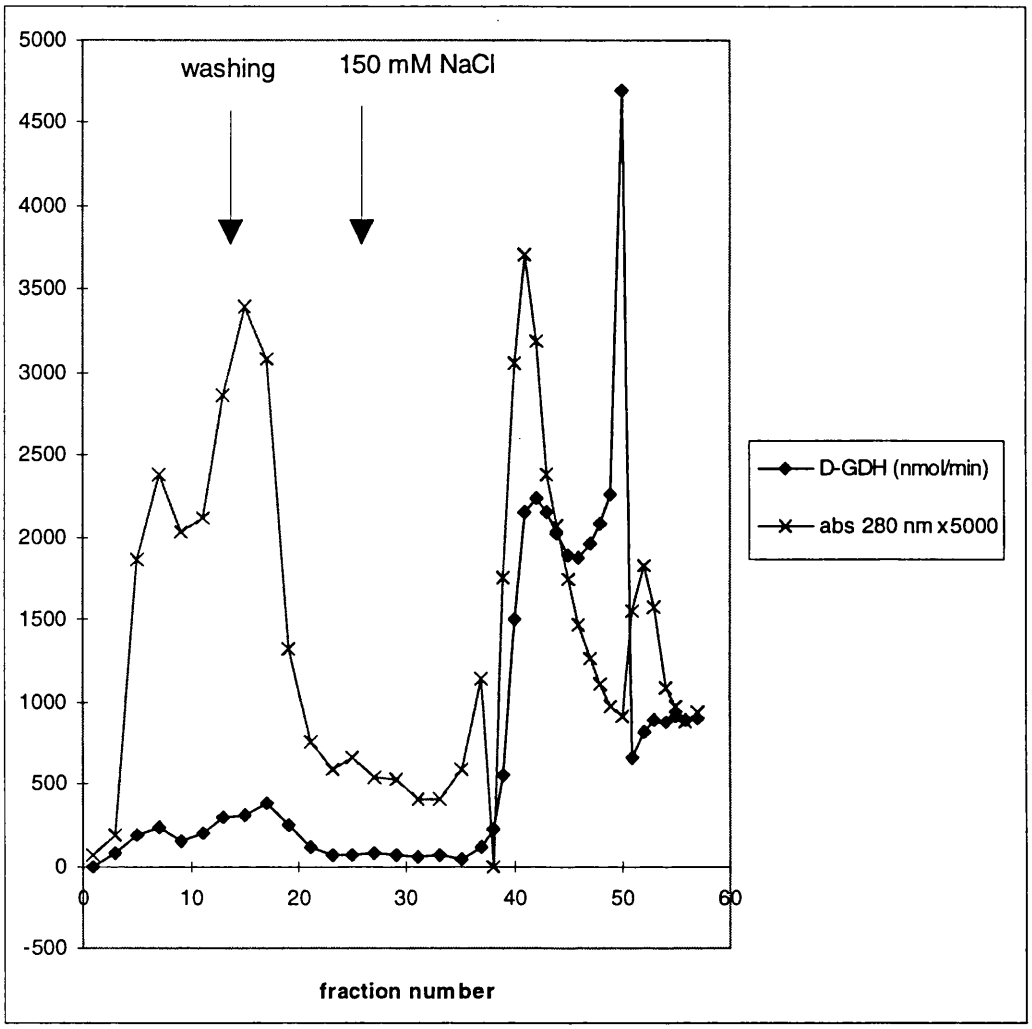


Fig. 3.3: Elution profile of D-GDH from CM sephadex.

(fig. 3.4) showed that D-GDH and GR were eluted together from this column, the early peak of D-GDH/GR activity reflecting overloading of the column.

Fractions 40-56 in fig. 3.3 were pooled, dialysed against 25 mM imidazole pH 7.2 and applied to a chromatofocusing column. The elution profile is shown in fig. 3.5. Two peaks of activity were present. The first peak at fractions 30-50 (pH 7.2) was not retained by the column and had D-GDH activity only, whereas the second peak with D-GDH and GR activity eluted at a pH of ~ 6.7 as measured with a pH meter. Thus the pI of these two proteins are approximately 7.2 or greater and 6.7 respectively. Fractions 145-181 were pooled, dialysed against 10 mM potassium phosphate pH 7.2 and loaded on a 2'5' ADP sepharose column. The elution profile is shown in fig. 3.6. Fractions 89-98 had D-GDH and GR activity. Protein samples from all the purification steps were extracted by the Wessel-Flügge method and 10 µg of each sample applied to SDS-PAGE and electrophoresed before staining with Coomassie blue. The pooled fractions with D-GDH and GR activity which eluted from the final 2'5' ADP sepharose column consisted of 6 proteins as can be seen in fig. 3.7.

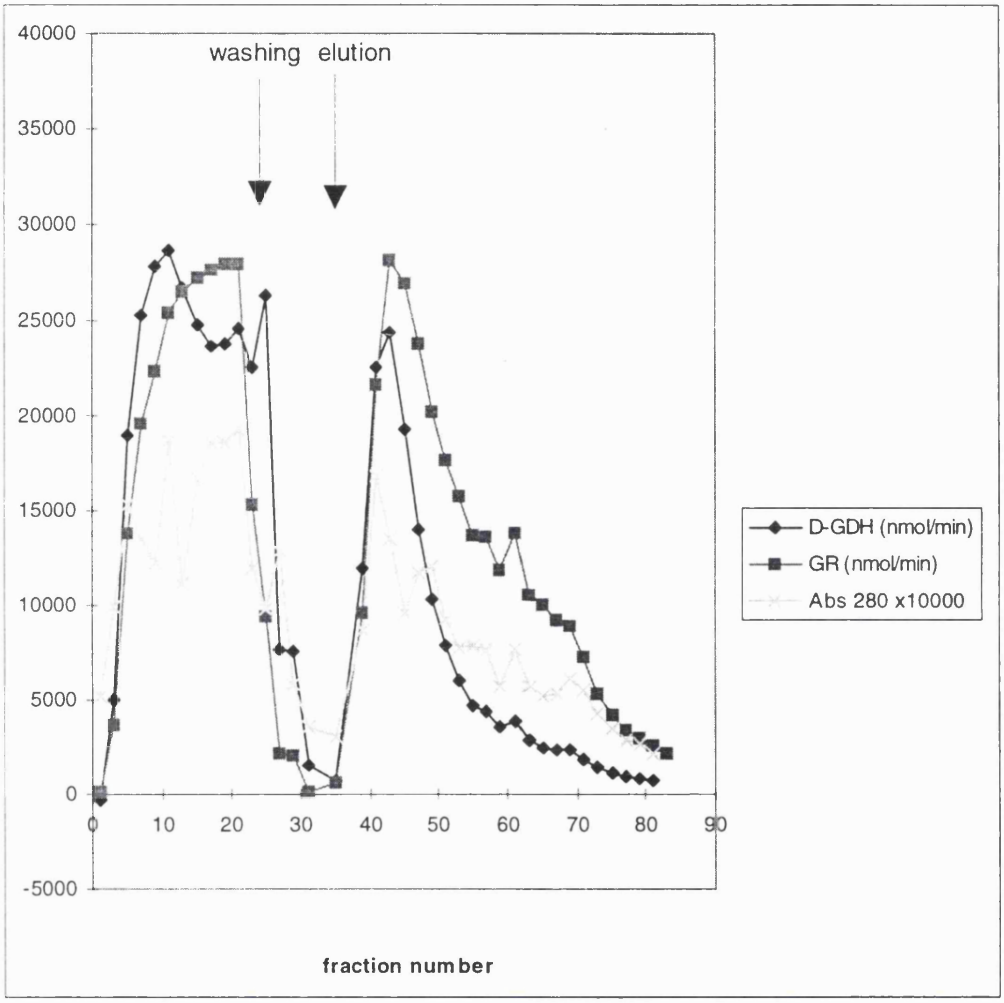


Fig. 3.4: Elution profile of D-GDH and GR activities from CM sephadex.

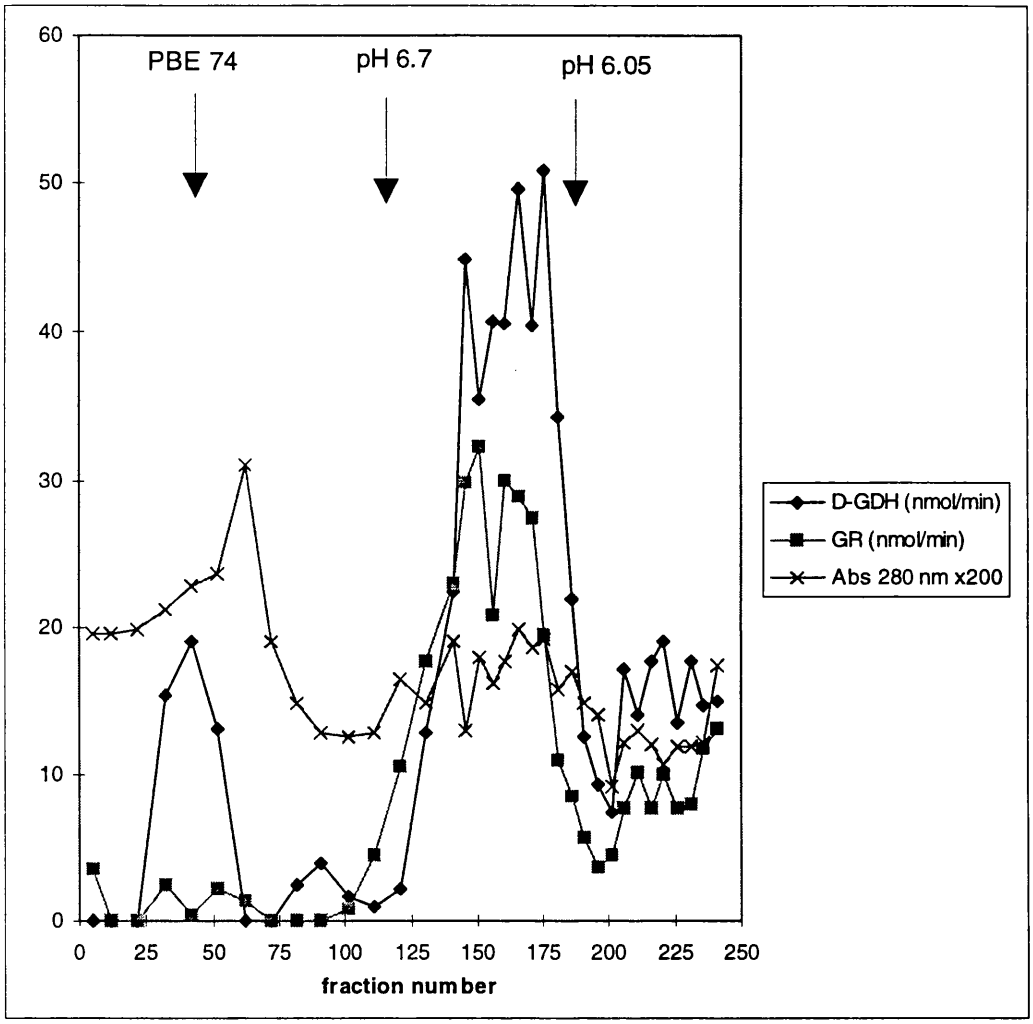


Fig. 3.5: Elution profile of D-GDH/GR from a chromatofocusing column.

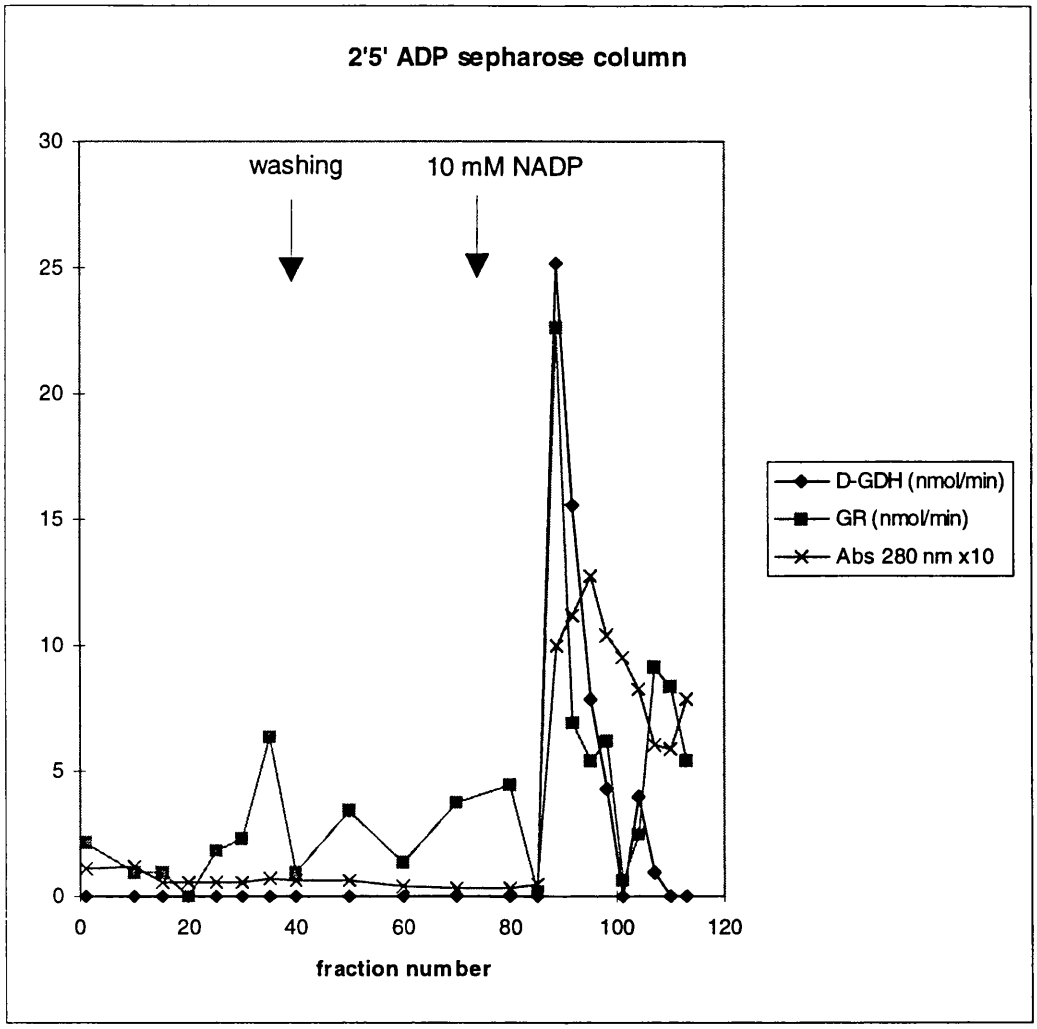


Fig. 3.6: Elution profile of D-GDH/GR from 2'5' ADP sepharose.

In an attempt to ascertain which of the six proteins was D-GDH/GR, a separate experiment was performed as above but taking aliquots from the peak four fractions (89-92) of the 2'5'ADP sepharose column showing D-GDH/GR activity. Protein was extracted by the Wessel-Flügge method and 5 µg applied to SDS-PAGE under denaturing conditions 3 proteins were detected by silver staining estimated to be of size p35, p40 and p60. The results are shown in figure 3.8. A summary of the purification procedure is shown in table 3.7. Data was not available for GR activity of the crude, DEAE sephadex, ammonium sulphate precipitations and CM sephadex fractions from that experiment.

The ratio of D-GDH:GR in liver biopsies is approximately 4:1 (see chapter 4), whereas after purification the ratio is 1:1. While this can be accounted for in part by the resolution of 2 different enzymes with hydroxypyruvate reductase activity, one of which is not retained by the chromatofocusing column, it does not fully explain the reduction in D-GDH activity. The remaining loss may be explained by denaturation of the protein that affects D-GDH activity preferentially over GR.

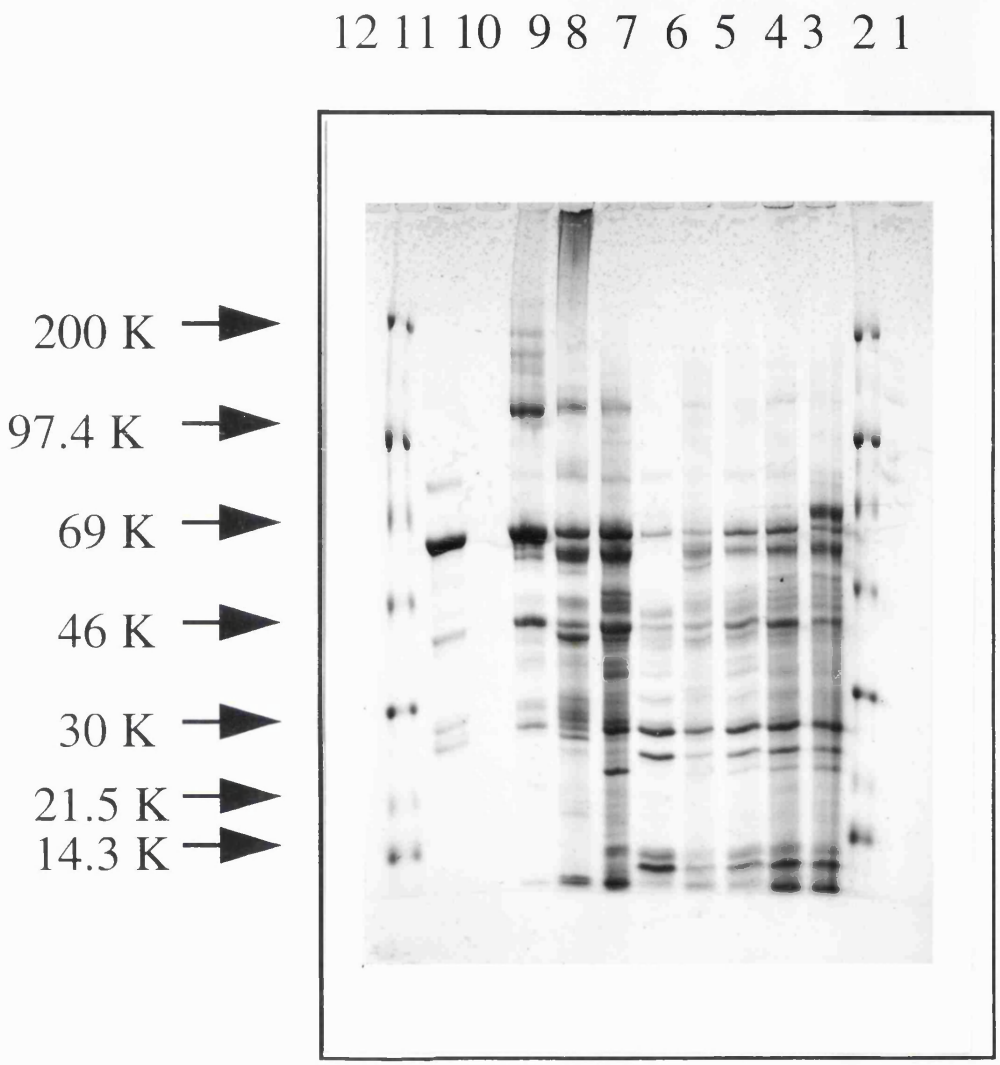


Fig. 3.7: SDS-PAGE of the fractions from the purification procedure of D-GDH/GR stained by coomassie blue. Lanes 1: M.W. markers, 2: crude lysate, 3: after DEAE sephadex, 4: 30% $(NH_4)_2SO_4$ supernatant, 5: 30% $(NH_4)_2SO_4$ pellet, 6: 30-60% % $(NH_4)_2SO_4$ supernatant, 7: 30-60% % $(NH_4)_2SO_4$ pellet, 8: after CM sephadex, 9: after chromatofocusing, 10: blank, 11: after 2'5'ADP sepharose, 12: M.W. markers.

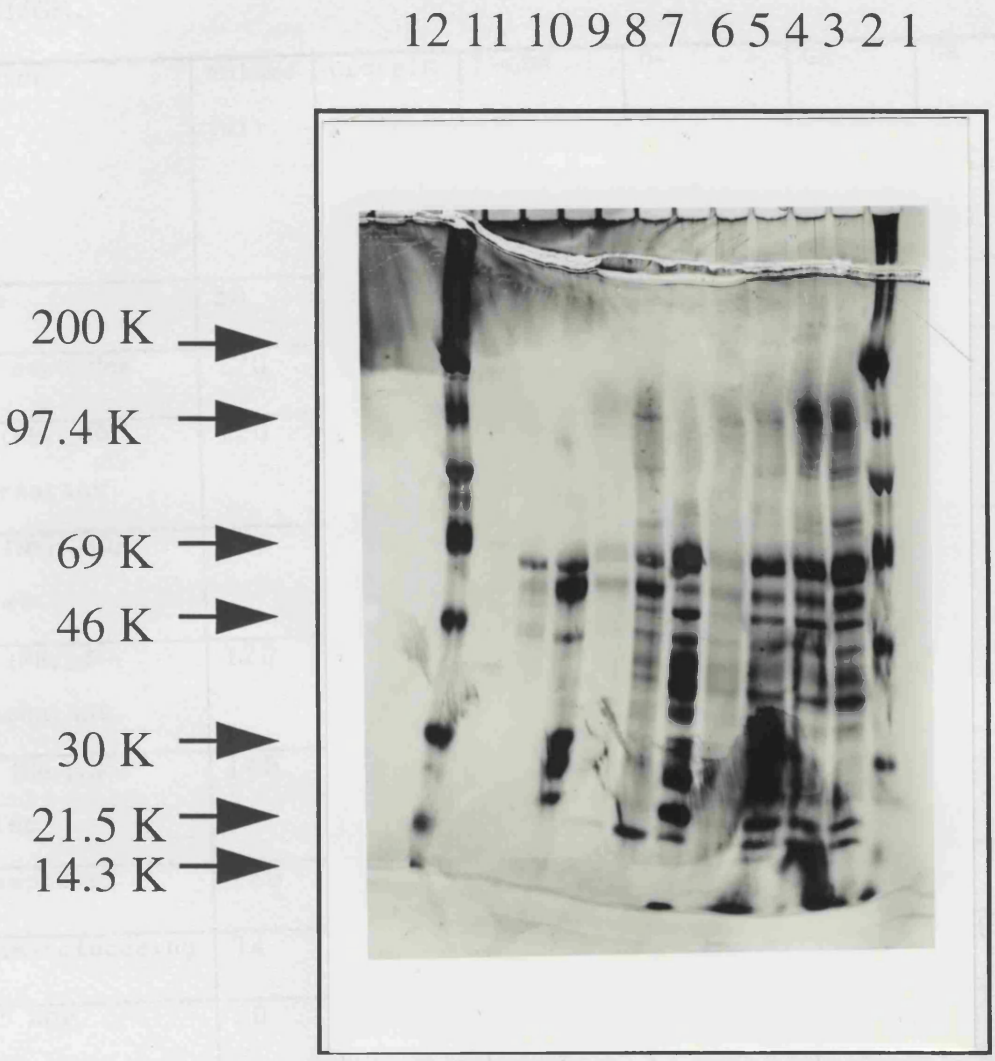


Fig. 3.8: SDS-PAGE of the fractions from the purification procedure of D-GDH/GR stained by silver. Lanes 1: M.W. markers, 2: crude lysate, 3: after DEAE sephadex, 4: 30% $(NH_4)_2SO_4$ supernatant, 5: 30% $(NH_4)_2SO_4$ pellet, 6: 30-60% % $(NH_4)_2SO_4$ supernatant, 7: 30-60% % $(NH_4)_2SO_4$ pellet, 8: after CM sephadex, 9, 10: after chromatofocusing, 11: after 2'5'ADP sepharose, 12: M.W. markers.

Table 3.7: Outline of the purification procedure of D-GDH/GR.

Fraction	volume (ml)	Protein (mg)	D-GDH (nmol/ min)	D-GDH(nmol /min/mg)	GR (nmol/ min)	GR (nmol /min/ mg)
crude	50	950	850752	895		
DEAE sephadex	120	564	1106580	1926		
30% $(NH_4)_2SO_4$ supernatant	120	303	337468	1113		
30% $(NH_4)_2SO_4$ pellet	120	37.2	10507	282		
60% $(NH_4)_2SO_4$ supernatant	120	108	100518	930		
60% $(NH_4)_2SO_4$ pellet	140	217	231938	1068		
CM sephadex	160	52	29865	574		
chromatofocusing	74	5	1384	276	819	163
2'5' ADP sepharose	20	0.2	132	660	102	510

The experiment was repeated six times and consistency was observed in the elution profiles. Consistency was also observed in the detected proteins when they were separated by SDS-PAGE electrophoresis, provided the experiment was performed in the minimum amount of time and protein degradation did not occur.

The yield of this purification procedure is 0.02% and it should be kept in mind that this figure includes contaminating proteins present. The purification factor (i.e. the degree of purification of the protein with respect to other proteins present) is 73%. However, this was calculated by means of specific activity of the starting and final sample, and does not take into account D-GDH/GR with reduced or absent catalytic activity in the final sample.

3.3.4: Raising of antibodies against D-GDH/GR.

The partially purified protein was used to raise antibodies. Three guinea pigs were used. Four injections of approximately 30 µg were administered to each guinea pig at monthly intervals. At the end of that interval two of the guinea pigs had developed antibodies against all six proteins as determined by immunodetection with horseradish peroxidase-labelled anti-guinea pig antiserum.

3.3.5: Immunodetection of D-GDH/GR in human liver.

30 µg of each sample of human liver in non-denaturing sample buffer were electrophoresed on an SDS-polyacrylamide gel. The proteins were blotted on nitrocellulose incubated with antibody from guinea pig 1 and after washing detected with horseradish peroxidase-labelled second antibody. The antibodies recognised a protein of 71 kDa that was observed only faintly in the liver of a patient with PH2 (fig. 3.9).

Samples from liver, kidney and leucocytes were analysed on denaturing SDS-PAGE followed by immunodetection with the same antiserum. In liver samples the signal at 71 kDa disappeared and a weaker signal at 38 kDa was detected. However, under these conditions there was no longer a difference in intensity between normal liver and liver from a patient with PH2. The 38 kDa band was also present in kidney but not in leucocytes. Results of the immunoblot are shown in fig. 3.10.

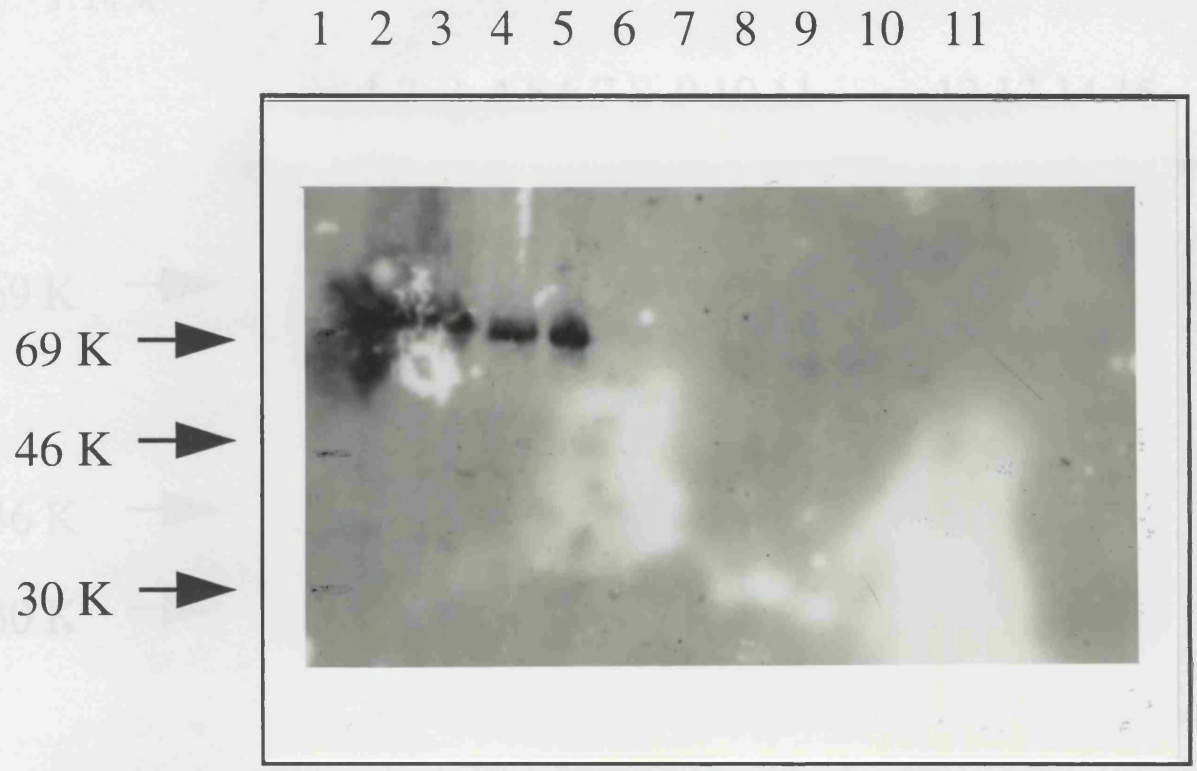


Fig. 3.9: 25 second exposure of immunoblot of normal liver (lanes 2, 3, 4, 5) and liver from a PH2 patient (lanes 8, 9, 10, 11) hybridised to antibodies raised against partially purified D-GDH/GR. Lanes 2, 8: crude lysate, 3, 9: following DEAE negative adsorption, 4, 10: 30% $(NH_4)_2SO_4$ supernatant, 5, 11: 30-60% % $(NH_4)_2SO_4$ pellet, 6, 7: blank

fig. 3.10.a

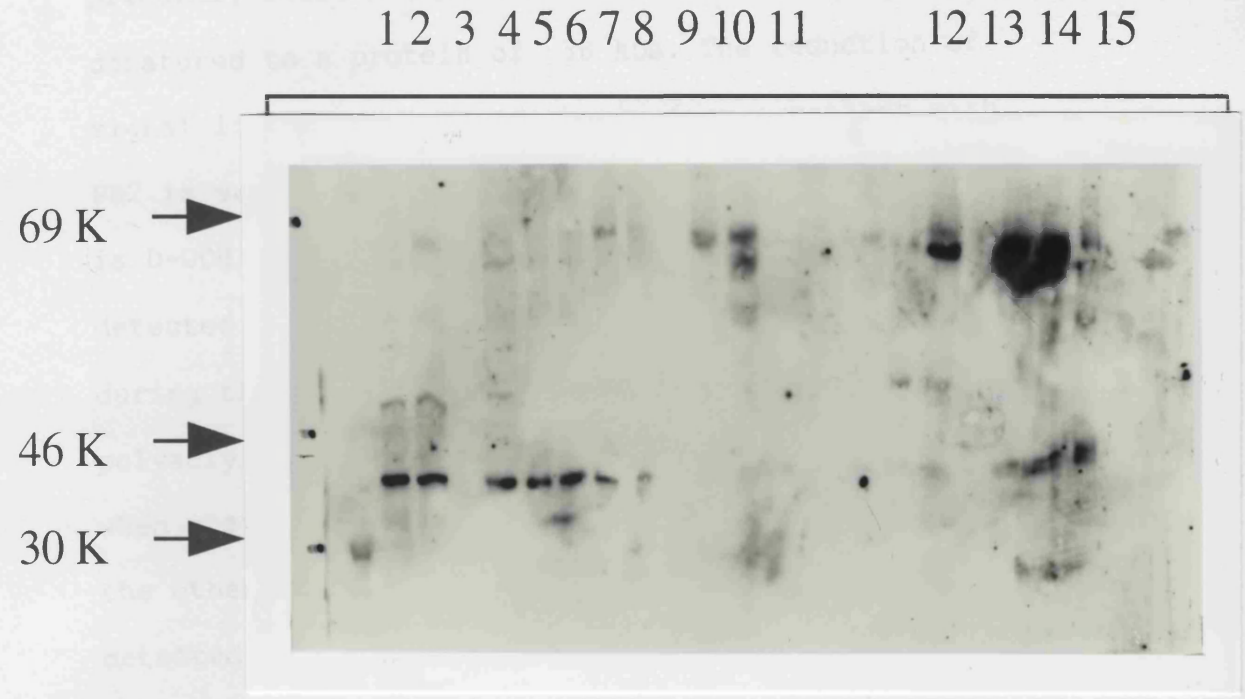


fig. 3.10.b

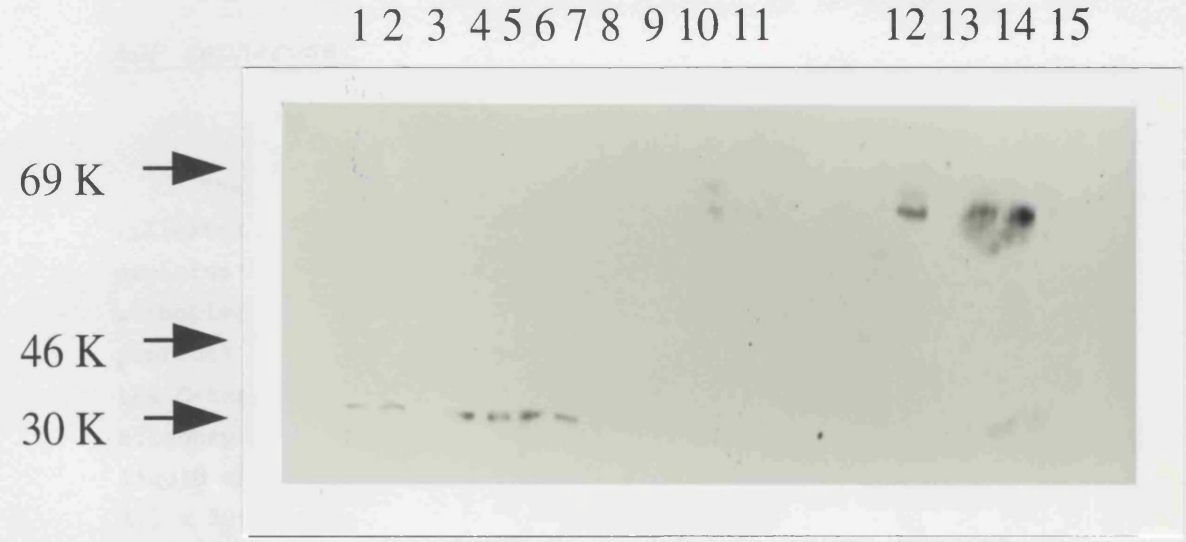


Fig. 3.10: a. 25 second exposure of immunoblot of protein from liver of PH1 patients (lanes 1, 2), blank (lane 3), liver of a PH2 patient (lanes 4, 5, 6), kidney (lanes 7,8), blank (lane 9), lymphocytes (lanes 10, 11), udenatured protein from liver of a PH2 patient (lane 12), blank (lane 13), undenatured protein from liver of PH1 patients (lanes 14, 15). b. 20 second exposure of the same immunoblot.

These results suggest that the antibody primarily detects a protein of 71 kDa which can be denatured to a protein of ~38 kDa. The reduction of signal in the undenatured sample from a patient with PH2 is suggestive but not conclusive that this enzyme is D-GDH/GR. Furthermore, no band at 71 kDa was detected on the lane of the 2'5' ADP sepharose eluate during the purification procedure on the SDS-polyacrylamide gels run under denaturing conditions, when stained either with Coomassie blue or silver. On the other hand, a protein at approximately 38 kDa was detected on the gel stained by Coomassie blue and a protein at approximately 39 kDa was observed on the gel stained by silver.

3.3.6: Sequencing of proteins eluted from 2'5' ADP sepharose.

The sample collected following ADP-sepharose purification (illustrated in fig. 3.6) was fractionated into individual proteins by SDS-PAGE and partial peptide sequencing was attempted on proteins migrating at 28, 29, and 35 kDa. The peptides were digested with trypsin, which cleaves proteins on the C-terminal side of lysine and arginine and the resultant oligopeptides were separated by reverse phase high-performance liquid chromatography (HPLC) using an Applied Biosystems OD.300 3.1 x 100 mm column, with a linear gradient of acetonitrile and monitored by a diode array detector (HP.1090/1040A). Peptides were sequenced using an Applied Biosystems 477A with modified chemistry and analysis cycles (Drs N. Tottie and A. Sterling, Ludwig Institute, U.C.L.). Sequence data were obtained on three peptides, p28, p29 and p35 (table 3.8). p28 and p29 have parts

of peptide sequences 20 and 10 and 45 and 13 in common and appear to be the same protein. The peptide sequences were used to search for homology to known sequences within the human genome Swiss Prot database.

Peptides from p28/p29 have homology to the mitochondrial precursor of human enoyl-CoA hydratase (EC 4.2.1.17) from liver (fig. 3.11). They also have 100% homology to the Expressed Sequence Tag (EST) 78012 (fig. 3.11), which currently has an unknown function. The clone was obtained from the human genome project and sequenced, and the complete nucleotide sequence was used to search the Swiss Prot database. Expressed EST 78012 has homology to human enoyl-CoA hydratase.

Enoyl-CoA hydratase is an enzyme involved in fatty acid oxidation that adds a molecule of water across the double bond of trans-enoyl-CoA to give an L-3-hydroxyacyl-CoA.

$$R.CH{=}CH.CO.SCoA + H_2O \rightarrow R.CH(OH).CH_2CO.SCoA$$

trans-enoyl-CoA L-3-hydroxyacyl-CoA

Enoyl-CoA hydratase is a homohexamer with subunits of ~31 kDa size, whereas the apparent subunit molecular weight of D-GDH/GR is ~38 kDa. The fact that enoyl-CoA hydratase is found in mitochondria, whereas human D-GDH/GR in liver is cytosolic suggests that D-GDH/GR and enoyl-CoA are not the same enzyme. Moreover,

and enoyl-CoA are not the same enzyme. Moreover, enoyl-CoA hydratase is present in liver, fibroblast and muscle but not in spleen and kidney, whereas D-GDH/GR is not present in fibroblast and present in kidney (see chapter 4).

The peptide sequence of p35 has 95% homology to fructose-1,6-bisphosphate aldolase (fig. 3.11). However, the amino acid differences between the two peptides are not conservative and are not described polymorphisms. Fructose-1,6-bisphosphate aldolase is a homotetramer with ~39 kDa subunits, involved in the pathway of fructose catabolism. It splits fructose 1-phosphate to glyceraldehyde and dihydroacetone phosphate. It is highly unlikely that fructose bisphosphate aldolase possesses D-GDH/GR activity, since deficiency of fructose bisphosphate aldolase results in a disease named hereditary fructose intolerance, which manifests itself as potentially severe hypoglycaemia after ingestion of fructose, a symptom not present in PH2 patients. Furthermore, fructose bisphosphate aldolase is a cytoskeletal protein, and its three forms are found in muscle, liver and brain. The tissue and subcellular distribution differences (chapter 4), as well as the lack of 100% homology, suggest that fructose 1,6-bisphosphate aldolase and D-GDH/GR are not the same protein. Moreover, the high homology of p35 to a

protein involved in a different metabolic pathway that does not utilise NAD(P)H as cofactor, indicates that p35 is not D-GDH/GR. It is concluded that p28/p29 and p35 are contaminating proteins, and sequencing of the other purified peptides at ~ 39 kDa is necessary to obtain a partial sequence of D-GDH/GR.

Table 3.8: Peptide sequences obtained by digesting proteins eluted by the 2'5'ADP sepharose column during purification of D-GDH/GR.

protein	Fraction	peptide sequence
P28	20	**GLLPGAGGTQR***
	35	ALASEAVM
	45	G**LMNDIAAFPA**P**T**IAM-GFAL*
P29	2	VAKDAR
	4	FVGH-
	14	*NALGNVFVSELLETLAQL***
	6	AASEIQVRVR
	13	X**LMNDIAAFPA**X**T**-*
	10	**GLLPGAGGTQR***
	15	*NALGNVFVSELLETLAQL*R**
	12	VLLFRAHVL
P35	23	FILSTDESVK
		LDVGEAPLAGXNK
	19	EATEEAFMK

* common sequences between p28 and p29

** identical peptides

ENOYL-COA HYDRATASE, MITOCHONDRIAL PRECURSOR (EC 4.2.1.17)

MAALR**VLL**SC **A**RGPLRPPVR CPAWRPFASG ANFEYIIAEK RGKNNTVGLI QLNRPKALNA

LCDGLIDELN QALKIFEEDP AVGGIVLTGG DKAFAAGADI KEMQNLSFQD CYSSKFLKHW
GHLTQVKKPV IAAVNGYPFG GGCELAMMCD IIYAGEKAQF AQPEILIGTI **PGAGGTQR**LT

RAVGKSLELE MVLTGDAISA QDAKQAGLVS KICPVETLVE EAIQCAEKIA SNSKIVVAMA
KESVNAAFEM TLTEGSKLEK KLFYSTFATD DRKEGMTAFV EKRKANFKDQ

PARTIAL AMINO ACID SEQUENCE OF CLONE 78012 (EST)

MNRPSAR**NALGNVFVSELLETLAQLR**EDRQVR**VLLFR**SGVKGVFCAGADLKEREQMSEAEVGV FVQRLRG**LMNDIAAFPAPTIA**AMGWGLPWAGGLGALPWGLLTFRVGQLSFGQFMGIDLRPRRR GFLPGGH

FRUCTOSE-BISPHOSPHATE ALDOLASE B (EC 4.1.2.13) (LIVER)

AHRFPALTQE QKKELSEIAQ SIVANGKG**IL** AA**DESV**GTMG NRLQRIKVEN TEENRRQFRE

ILFSVDSSIN QSIGGVILFH ETLYQKDSQG KLFRNILKEK GIVVGIK**LD**Q **GGAPLAGTNK**

ETTIQGLDGL SERCAQYKKD GVDFGKWRAV LRIADQCPSS LAIQENANAL ARYASICQQN
GLVPIVEPEV IPDGDHDLEH CQYVTEKVLA AVYKALNDHH VYLEGTLLKP NMVTAGHACT
KKYTPEQVAM ATVTALHRTV PAAVPGICFL SGGMSEEDAT LNLNAINLCP LPKPWKLSFS
YGRALQASAL AAWGGKAANK **EATQEAFMK**R AMANCQAAKG QYVHTGSSGA ASTQSLFTAC

YTY

Fig. 3.11: Sequences of human enoyl-CoA hydratase, mitochondrial precursor, and EST 78012 which have homology to p28/p29, and human fructose bisphosphate aldolase B, which has homology to p35. Bold letters indicate the homologous sequences.

3.4: Discussion on the purification of D-GDH/GR.

From the purification procedure deductions can be made as to some properies of D-GDH/GR in human liver. From a preliminary experiment on sephadex G-200, the protein was in the region of 70 kDa. The pI of the protein was determined on the chromatofocusing column to be ~6.7. This finding agrees with adsorption to CM sephadex at pH 6 when the protein would be positively charged. One would perhaps expect adsorption to DEAE sephadex at either pH 7 or 8. However, it should be kept in mind that adhesion to ion exchangers is a competitive process and will also depend on the presence of competing proteins. It can be deduced that many of the amino acids on the surface of the protein are not negatively charged at pH 7-8, for example cysteine (pK=8.5), tyrosine (pK=10), lysine (pK=10) and arginine (pK=12).

The properties of human D-GDH/GR are different to that from rat and beef liver in that bovine and rat proteins both bind to CM and DEAE sephadex at pH 7, which suggests that at that pH on their surface there are both negatively charged amino acids, i.e. aspartic and glutamic acids (pK=4.4) and histidine (pK=6.5) as well as positively charged amino acids. D-GDH/GR from beef liver was estimated to be ~70 kDa consisting of two identical subunits of an

approximate molecular weight 34 kDa by Rosenblum (Rosenblum *et al.*, 1971) and 72 kDa by Sugimoto (Sugimoto *et al.*, 1972). Thus the molecular weight of human D-GDH/GR is similar to the bovine one.

It was not possible to add another purification step after 2'5' ADP sepharose, for example gel filtration with sephadex G-200 because D-GDH and GR activities were so low at this point that they could not be reliably measured. Scaling up the amount of material used is an option, but a balance must be kept between the time it takes to perform a step and consequently the activity lost and the increase in protein collected. It is not a problem to scale up the DEAE and CM sephadex steps since the materials are cheap, have a high capacity and high elution rates. If necessary bulk procedures using a Buchner funnel rather than a column can be used for CM sephadex as well. 2'5' ADP sepharose has a high elution rate but a low capacity and the cost could be prohibitive. Chromatofocusing has a relatively low elution rate, but its capacity is higher than that of ADP sepharose. An additional step using a specific antibody coupled to an affinity column could now be introduced, followed by chromatofocusing. Gel filtration on sephadex G-200 is useful for size fractionation because the amount of protein loaded is not important. Nevertheless, the volume of the sample

is important (1-2% total column volume) and concentration by sucrose can be achieved only until saturation of the sample occurs.

Another option would be to use cultured HepG2 cells instead of liver as a source of D-GDH/GR and thus avoid the problem of contamination by blood proteins. However, D-GDH/GR specific activity is not as high in HepG2 cells as in native liver (see 4.3.1).

In retrospect, the retention of contaminating proteins by the use of bulk procedures in the CM-Sephadex step is preferable to the loss of D-GDH/GR activity that occurs during the two days it takes to run the column, because the time gained allows the introduction of another step. Furthermore, if the DEAE-Sephadex step was performed at the same pH as the CM-Sephadex, adhesion of D-GDH/GR to the DEAE-Sephadex would be minimised. Adhesion of D-GDH/GR was performed at pH 7, because at pH 5.8 activity is lost rapidly. However, there is evidence to suggest that inactive D-GDH/GR still binds to the materials that follow, and it may be preferable to collect more D-GDH/GR even if it is partially denatured. If these changes are made to the protocol, the size of the chromatofocusing and the 2'5' ADP-Sepharose columns would have to increase to cope with the greater amounts of protein. The resulting greater amounts of protein after the 2'5' ADP-Sepharose column could be used either to introduce another purification step, or for direct sequencing of peptides with subunit molecular weight of 35-40 kDa.

The specific activity of D-GDH/GR did not increase as the protein was purified probably because of loss of activity with time and because D-GDH was fractionated into two separate enzymes in the chromatofocusing column. The increase in activity observed after negative adsorption on DEAE sephadex is probably because the linearity of the reaction is exceeded in the measurement of the crude fraction.

Antisera raised against the purified protein detected a protein of 71 KDa in undenatured liver sonicates from normal liver. The intensity of the band was substantially reduced compared to the same amount of liver protein from a patient with PH2 suggesting that this might be the D-GDH/GR protein. On denaturation a band of 38 kDa was observed, while the 71 kDa band was not detectable, indicating the protein was composed of more than one subunit. D-GDH from mammalian (Rosenblum *et al.*, 1971), plant (Titus *et al.*, 1983, Kleczkowski *et al.*, 1986) and bacterial

et al., 1983, Kleczkowski *et al.*, 1986) and bacterial (Izumi *et al.*, 1990, Chistoderova and Lidstrom, 1991) sources is known to be a homodimer of similar size. For example, hydroxypyruvate reductase (HPR) from *Methylobacterium Extorquens* AM1 has a molecular weight of about 71 kDa and consists of two identical subunits with a molecular weight of 37 kDa (Chistoserdova and Lidstrom, 1991), HPR from *Hyphomicrobium methylovorum* GM2 has a molecular weight of about 70 kDa and is a homodimer with subunits of molecular weight 38 kDa (Yoshida *et al.*, 1994), HPR from cucumber cotyledons is a homodimer with native molecular weight 91-95 kDa and subunit molecular weight ~41 kDa (Titus *et al.*, 1983). The molecular weight of D-GDH/GR as estimated by immunoblotting agrees with the size of approximately 68 kDa estimated by sephadex G-200.

From the peptide sequencing data, homology of p28/p29 with enoyl-CoA hydratase is too low to draw any conclusions on the function of p28/p29. However, from the western blot results it was concluded that p28/p29 is unlikely to be D-GDH/GR because of its size, since anti-D-GDH/GR antibodies recognise a protein ~39 kDa in size. p35 is more likely to be D-GDH/GR in terms of size, but because of its high homology to fructose-bisphosphate aldolase, which has a different subcellular and intracellular

distribution, does not use NAD(P)H as cofactor, and is involved in a different metabolic pathway, it is unlikely to be D-GDH/GR. In future, anti-D-GDH/GR antibody could be attached to an affinity column in order to purify more D-GDH/GR and obtain more sequencing data which would allow the design of oligonucleotide probes to screen a cDNA library for the D-GDH/GR gene. The peptide sequence might even identify already sequenced cDNA, which exist as ESTs with no assigned function. Alternatively, immunoscreening of libraries with anti-D-GDH/GR antibody could be attempted to identify the D-GDH/GR gene and subsequently raise antibodies to D-GDH/GR by molecular biology methods. Raising antibodies by expression of the cloned gene would also sort out the problem of the low yield of purified D-GDH/GR which is likely to exist even after introduction of the antibody affinity column in the purification procedure.

An additional, unexpected finding was the presence of a second protein with D-GDH activity which was discovered only on chromatofocusing. This protein did not adhere to the chromatofocusing column and has therefore a pI of, or greater than, 7.2 and no associated GR activity. One way to accurately determine the pI of this protein would be to increase the pH of the equilibrating buffer of the

chromatofocusing column so that the protein adheres to the column and determine its pI by eluting it from the column. The significance of this second protein will be discussed in chapter 4.

CHAPTER 4: TISSUE DISTRIBUTION AND SUBCELLULAR DISTRIBUTION OF HUMAN D-GLYCERATE DEHYDROGENASE/ GLYOXYLATE REDUCTASE.

4.1: Introduction.

Although some data is available on the presence of D-GDH in tissues of various species, nothing is known of the tissue distribution of GR. In mammals, D-GDH activity has been found in the liver of cattle, rat, rabbit and humans (Willis and Sallach, 1961, Heinz and Lambrecht, 1961, Dawkins and Dickens, 1965, Cheung *et al.*, 1962, Williams and Smith, 1968a), human lymphocytes (Williams and Smith, 1968a), rat kidney, rat brain and bovine and porcine spinal cord (Snell, 1983, Feld and Sallach, 1974a, Feld and Sallach, 1975, Feld and Sallach, 1974b). All that is known about tissue distribution of human GR is that activity is present in the liver. Present controversy on whether PH2 patients can be treated by kidney, liver or bone marrow transplants can only be resolved by the investigation of tissue distribution of D-GDH/GR in humans.

In human liver, D-GDH (for the forward and reverse reaction) and GR activity is found mainly in the

enzymes are able to catalyse the conversion of pyruvate to hydroxypyruvate, AGT (Thompson and Richardson, 1967) and glutamate:glyoxylate aminotransferase (GGT) (Thompson and Richardson, 1966). AGT, a liver specific enzyme (Kamoda *et al.*, 1980), is localised in the peroxisomes (Noguchi and Tagada, 1979) while GGT is found in various tissues and is localised mainly in the cytosol (Noguchi *et al.*, 1977). The activity of AGT in human liver is substantially greater than that of GGT (Thomson and Richardson, 1966) and this raises the question whether hydroxypyruvate is produced in the peroxisomes and is transported into the cytosol or whether gluconeogenesis from serine occurs purely in the cytosol.

Using the methods established in chapter 2, D-GDH (forward) and GR activities were determined in human liver, kidney, lymphocytes and fibroblasts. In addition, the subcellular distribution of these enzyme activities was determined in a HepG2 liver cell line and lymphocytes using rat liver as a control. In addition, the discovery of a protein with D-GDH activity only detected during chromatofocusing of proteins from human liver (chapter 3, fig. 3.5) led to the investigation of lymphocytes and kidney by the same method.

4.2: Materials And Methods.

Protein was estimated by the Lowry procedure as described in 2.2.1.

4.2.1: Human Tissues.

i. for tissue distribution studies

Human liver samples were from donor samples as previously described and were kindly supplied by Dr B. Portman, King's College Hospital. Kidney samples were obtained from the UCLH frozen tissue bank. Samples from patients with PH1, PH2, and other forms of hyperoxaluria were obtained from referrals to the University College Hospitals Primary Hyperoxaluria Service. ~~Lympho~~ leuc cytes were obtained from healthy adult volunteers. Normal fibroblasts were obtained from primary cultures and were kindly supplied by Mrs E. Young (Institute of Child Health) and Dr Ros Hastings (University College Hospitals). I gratefully acknowledge Dr P. Rumsby for the cultured HepG2 cells. Tissue was stored at -70°C until analysis.

ii. for subcellular distribution studies

Leucocytes were obtained from 1 week old blood samples from the Chemical Pathology Department, UCLH. Rat liver was obtained from adult rat killed by cervical dislocation. Liver was removed onto ice. Lymphocytes and HepG2 cells used in the cell fractionation experiments were stored at 4°C.

4.2.2: Isolation of leucocytes from blood.

MATERIALS:

1. Red Blood Cell (RBC) lysis solution

8.4 g ammonium chloride, 1 g $KHCO_3$, 2 ml EDTA 0.5 M pH 8, distilled deionised water to 1 litre

PROCEDURE:

1. 25 ml or 150 ml of whole blood were collected into EDTA tubes for the tissue distribution and the subcellular fractionation experiments respectively. It was transferred as quickly as possible to 50 ml falcon tubes in 10-20 ml aliquots.
2. The tubes were filled with cold lysis buffer and were left on ice for 20-30 minutes.
3. The tubes were spun at 1,500 rpm in a swing out head on MSE for 10 minutes at 4°C to pellet leukocytes.

4. The supernatant was discarded and further 20 ml of cold lysis buffer were added. The pellet was resuspended gently.

5. Step 3 was repeated.

6. The supernatant was discarded and the tubes were left inverted for a few minutes on absorbent paper.

7. Leucocytes (approximately 4 mg) for tissue distribution experiments were resuspended in 0.5 ml 50 mM potassium phosphate pH 7; leucocytes (25 mg approximately) for subcellular fractionation experiments were resuspended in 5 ml homogenising buffer.

4.2.3 : Homogenisation procedure for tissue distribution experiments.

MATERIALS:

1. Sonication medium

240 mM sucrose in 100 mM potassium phosphate pH 8

2. Microson XL Heat System sonicator

3. 0.1 mM EDTA in 50 mM potassium phosphate pH 7

4. Cellu•Sep Regenerated Cellulose Tubular Membrane T2 or T3 (as in 2.2.3)

PROCEDURE:

Biopsy material was suspended in sonication medium aiming for a final suspension of 2% w/v. The suspended tissue was disrupted with four 10 second bursts using a Microson XL sonicator with 30 second intervals on ice between bursts. The non-suspended fibrous material was removed by centrifuging at 2,000 g for 10 minutes. The resulting supernatant was dialysed overnight against 50 mM potassium phosphate pH 7 with 0.1 mM EDTA to remove endogenous NADH that would otherwise interfere with the D-GDH assay. The dialysate was centrifuged again at 12,000 g for 15 minutes and the supernatant analysed for D-GDH and GR activity.

4.2.4 : Kidney and leucocyte chromatofocusing.

Experiments were carried out as described in 3.2.8. 40 µg of kidney protein or 25 µg of leucocyte protein were loaded on the chromatofocusing column.

4.2.5: Subcellular distribution experiments.

MATERIALS:

1. rat liver, isolated human leucocytes or HepG2 cells
2. Teflon/glass homogeniser

3. MOPS buffer

523.25 mg MOPS (5 mM final concentration), 186.12 mg EDTA (1 mM final concentration), 0.5 ml ethanol (0.1% v/v final concentration), distilled deionised water to 500 ml. Brought to pH 7.4 with NaOH

4. homogenising buffer

42.75 g sucrose in 500 ml MOPS buffer (250 mM final concentration)

5. 25%, 30%, 40%, 50% Nycodenz (Robins Scientific, Solihull, U.K., 227610) in MOPS buffer

6. swing out head on Sorvall RC3C centrifuge

7. Quick-Seal centrifuge tubes 16 x 76 mm from Beckman, Palo Alto, California, U.S.A. (342413)

8. Beckman angle centrifuge (8 x 50)

PROCEDURE:

1. Adult rat (approximately 200-250 gm weight) was killed by cevical dislocation and its liver was removed onto ice. It was minced and homogenised with two movements in a Teflon/glass homogeniser in 5 ml homogenising buffer aiming for a 2% w/v solution (8 mg protein after homogenisation). 9 x 10^7 HepG2 cells (9.1 mg protein after homogenisation) and ~ 25 mg protein from a leukocyte preparation were homogenised in the same volume.

2. The broken cells were spun at 1,200 rpm in swing out head on Sorvall RC3C for 10 minutes at 4°C.
3. The supernatant was spun in angle rotor (8 x 50) at 19.5 K rpm for 25 minutes at 4°C. The cytosolic supernatant was retained for measurement of enzyme activity.
4. The pellet was resuspended in homogenising buffer (4 ml for rat liver, 1 ml for lymphocytes and 0.5 ml for HepG2 cells) and 0.5 ml was loaded on to the gradient.
5. In the case of lymphocytes the gradient was formed by layering 1 ml 50% Nycodenz, 1.5 ml 40% Nycodenz, 7 ml 30% Nycodenz and 2.5 ml 25% Nycodenz in a Quick-Seal tube. In the case of HepG2 cells the gradient consisted of 2 ml 50% Nycodenz, 5 ml 40% Nycodenz, 4 ml 30% Nycodenz and 1.5 ml 25% Nycodenz.
6. The tubes were spun on an ultracentrifuge with the brake off at 50 K for 65 minutes at 4°C.
7. 0.5 ml fractions were collected from the bottom of the tube.

4.2.6 : D-GDH/GR assays for tissue distribution studies, patient diagnosis, and subcellular distribution studies.

Reactions were carried out as described in 3.2.3.

4.2.7: Catalase and cytochrome oxidase assays.

i. catalase assay (Peters *et al.*, 1972)

MATERIALS:

1. substrate

500 mg BSA fraction V (0.1% w/v final concentration) from Boehringer Mannheim (775860), 5 ml 0.2 M imidazole/HCl pH 7 (0.02 M final concentration), 75 µl 30% H_2O_2 (0.045% final concentration), distilled deionised water to 50 ml.

2. «$TiSO_4$»

1.7 g titanium sulphate (Aldrich, Gillingham, Dorset, U.K., 26.849-6) in 500 ml 2N H_2SO_4 (concentrated $H_2SO_4 \approx$ 36 N $\therefore$ 55.6 ml concentrated H_2SO_4 to 1 litre distilled deionised water $\approx$ 2 N)

Reflux for 2 hours, cool and filter through Whatman paper number 1 and Whatman paper number 42.

PROCEDURE:

The sample was diluted 1:40 for rat liver or used neat for HepG2. 50 µl was added to 250 µl substrate in a micro cuvette and left at room temperature for 10

minutes. The reaction was stopped with 2 ml «$TiSO_4$»; the absorbance was read at 405 nm against air. The blank contained water instead of sample. The absorbance of the samples was subtracted from the blank.

ii. cytochrome oxidase assay (Cooperstein and Lazarow, 1951)

MATERIALS:

1. substrate

1.7×10^{-5} M cytochrome c from horse heart (Sigma, C-2506) made as 0.0303 g/50 ml in 30 mM potassium phosphate pH 7.4

Reduced by addition of 166.6 µl of 1.2 M sodium hydrosulphite

2. 1.2 M sodium hydrosulphite

0.2089 g/ml distilled deionised water

3. potassium ferricyanide

PROCEDURE:

100 µl of sample was added to 1000 µl of reduced cytochrome c. The blank contained phosphate buffer in place of tissue extract. The change in absorbance at 550 nm was recorded for 2 minutes at room temperature.

place of tissue extract. The change in absorbance at 550 nm was recorded for 2 minutes at room temperature. A grain of potassium ferricyanide was added to oxidise cytochrome c fully.

Calculation:

A: absorbance at 0 min

B: absorbance at 2 min

C: absorbance after addition of ferricyanide

absorbance drop/min/ml =

$$= \frac{\log_{10}(A-C) - \log_{10}(B-C)}{2} - \text{control } \frac{\log_{10}(A-C) - \log_{10}(B-C)}{2}$$

4.2.8: D-GDH and GR assays in rat.

Reaction conditions were obtained from Van Schaftingen *et al.* (Van Schaftingen *et al.*, 1989) and calculations of enzyme activity were carried out as described in 3.2.3. The conditions of the assay for human D-GDH and GR were not used because the concentrations of hydroxypyruvate and glyoxylate would be inhibitory to the rat enzyme. The starting absorbance of the blank was

A = 0.4048 at pH 7.2 at 37°C

4.3: Results.

4.3.1: Reference range and tissue distribution of D-GDH and GR.

The tissue distribution of D-GDH (forward) and GR are shown in fig. 4.1a and 4.1b respectively. D-GDH and GR activities in 13 control livers ranged from 350-940 (median 547) nmol.min^{-1}.mg protein^{-1} and 129-209 (median 145) nmol.min^{-1}.mg protein^{-1} respectively. Six control kidney samples had D-GDH (forward) and GR activity of 859-1229 (median 985) nmol.min^{-1}.mg protein^{-1} and 23-61 (median 40) nmol.min^{-1}.mg protein^{-1} respectively. D-GDH (forward) activity in 9 leuc ocyte and 5 fibroblast preparations ranged from 327-585 (median 469) and 539-779 (median 672) nmol.min^{-1}.mg protein^{-1} respectively (fig. 4.1a), while GR in the same samples was 7-22 (median 11) and 21-32 (median 29) nmol.min^{-1}. mg protein^{-1} respectively (fig. 4.1b). A summary of the results including a comparison of the ratios of D-GDH (forward):GR activity between different tissues is shown in table 4.1.

Table 4.1: D-GDH (forward) and GR activity in human liver, kidney, leucocytes and fibroblasts. Activity of both enzymes is nmol.min^{-1}.mg protein^{-1}.

Tissue	n	D-GDH (forward) range	D-GDH (forward) median	GR range	GR median	D-GDH (forward) : GR
liver	13	350-940	547	129-209	145	4:1
kidney	6	859-1229	985	23-61	40	24:1
lymphocytes	9	327-585	469	7-22	11	42:1
fibroblasts	5	539-779	672	21-32	29	23:1

D-GDH (forward) and GR activity in livers from 12 patients with PH1 ranged from 387-950 nmol.min^{-1}.mg protein^{-1} and 92-296 nmol.min^{-1}.mg protein^{-1} respectively, that is, within the reference range for normal livers. Three liver samples from PH2 patients previously diagnosed by the finding of raised urinary L-glycerate were analysed. Significant D-GDH (forward) activity was present although in all three cases it was below the normal range (342, 230, and 142 nmol.min^{-1}.mg protein^{-1} respectively) but GR activity in the same samples was either very low (18, n=2) or undetectable (n=1). Interestingly, lymphocyte D-GDH (forward) from two PH2 patients showed activity within the normal range (fig. 4.2). GR in these samples was undetectable

Figure 4.1a

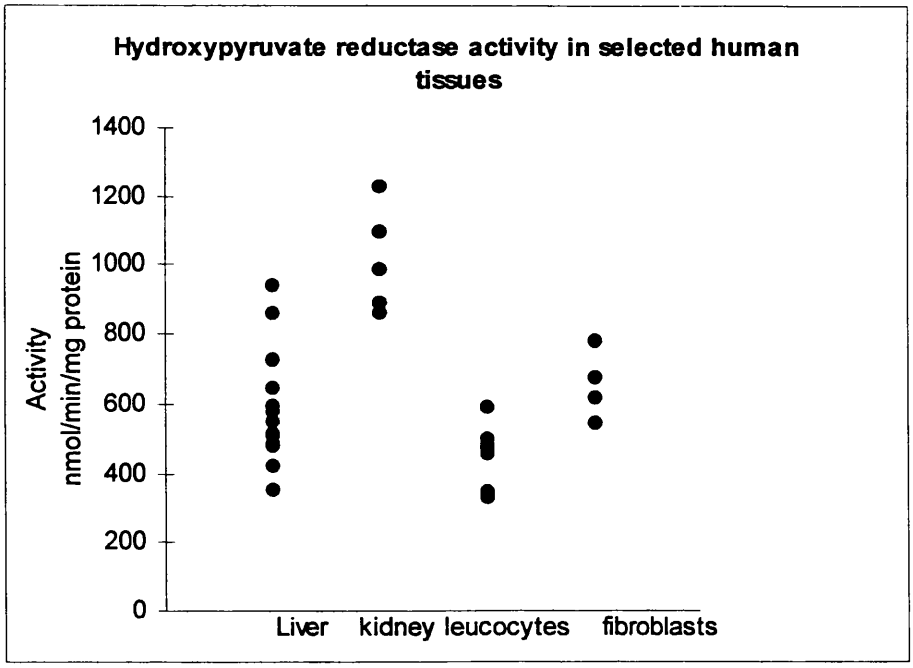


Figure 4.1b

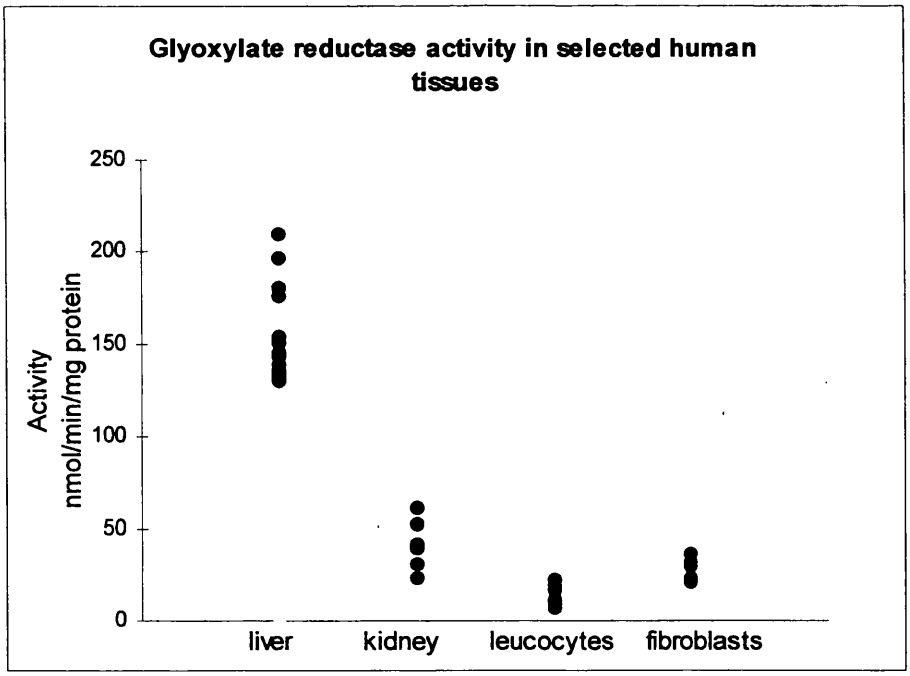


Fig. 4.1: Tissue distribution of D-GDH (a) and GR (b).

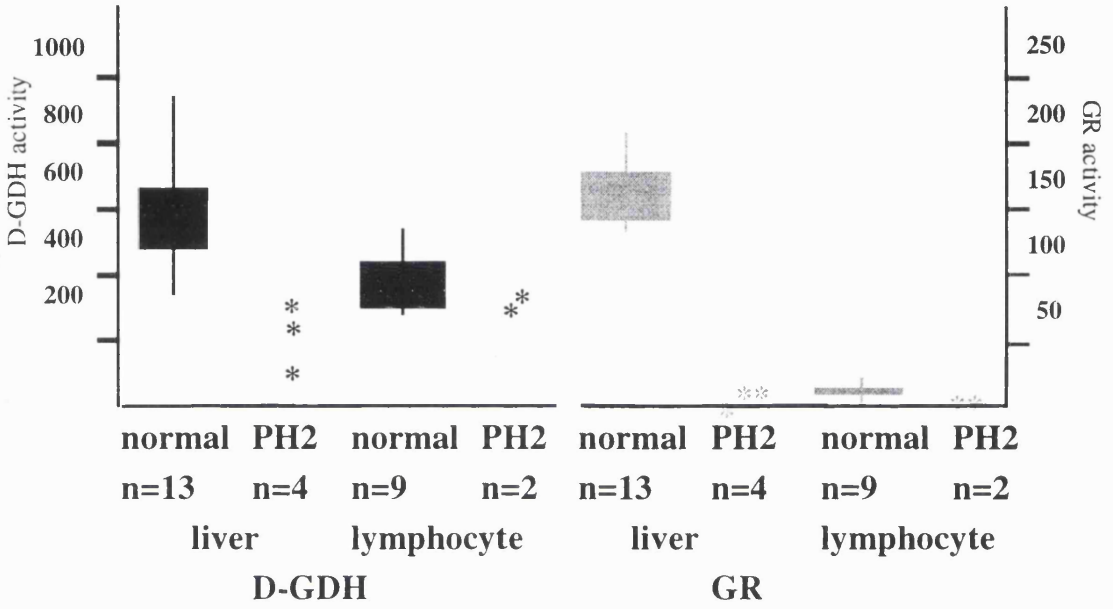


Fig 4.2: D-GDH (forward) (solid boxes) and GR (shaded boxes) activity in liver and leucocytes from controls and patients with PH2. Boxes represent 25^{th}-75^{th} percentiles and lines the range. Activity of both enzymes is nmol.min^{-1}.mg $protein^{-1}$.

although it must be remembered that GR activity is very low in normal leucocytes.

4.3.2: Chromatofocusing of human GDH/GR.

The leucocyte preparation applied on the chromatofocusing column had total activity 564 $nmol.min^{-1}$ and 70 $nmol.min^{-1}$ for D-GDH and GR respectively. D-GDH activity separated in two peaks, a major one at pH 7.2 which was not retained by the column and a minor one at pH 6.7 (fig. 4.3). In liver, two peaks were again found but in this case the second peak was more pronounced (fig. 3.5). In both leucocyte and liver, no GR activity was associated with the first peak but GR activity was detected in the second peak from liver. The kidney preparation applied on the chromatofocusing column had total activity 29,754 $nmol.min^{-1}$ and 964 $nmol.min^{-1}$ for D-GDH and GR respectively. Kidney chromatofocusing (fig. 4.4) resembled liver chromatofocusing regarding the proportion of D-GDH activity eluted in the two peaks, but D-GDH and GR activities were both reduced in the second peak.

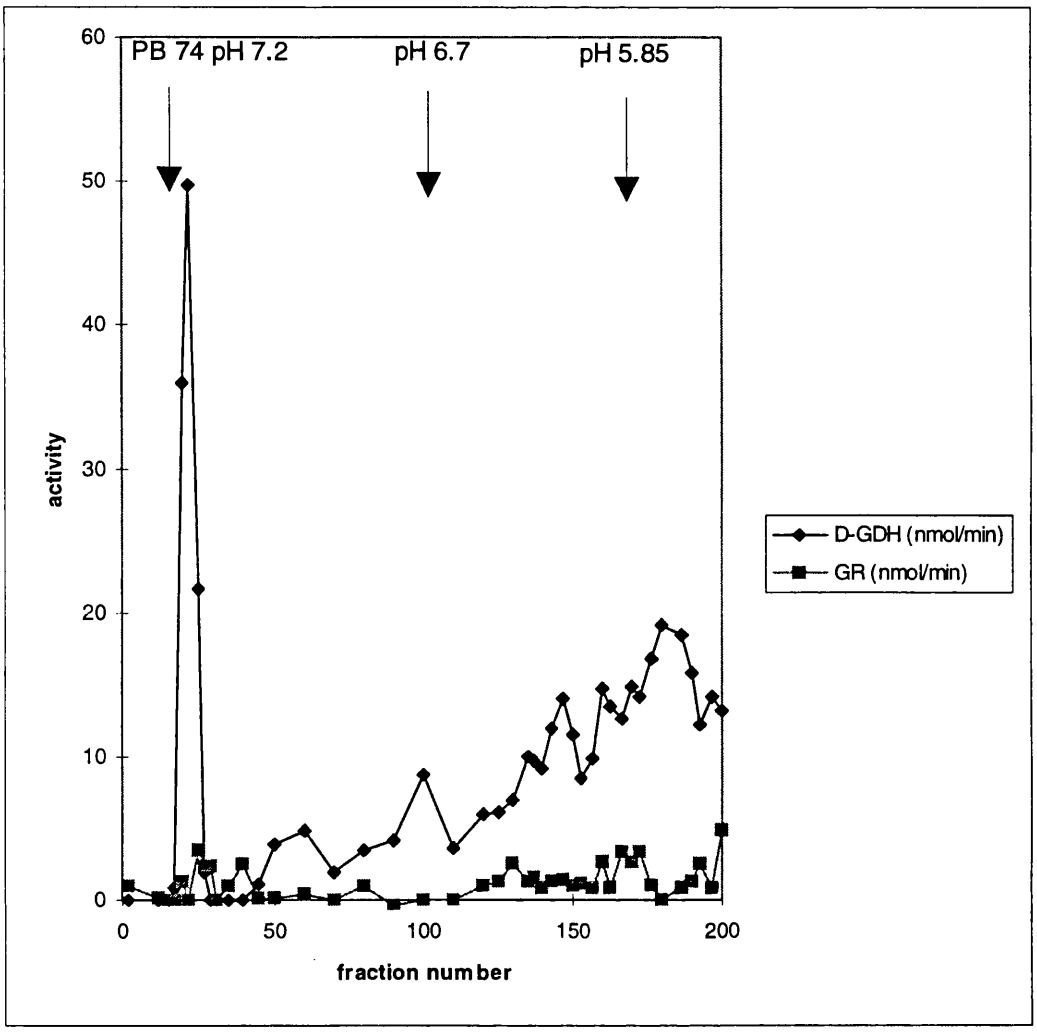


Fig. 4.3: Elution profile from a chromatofocusing column of human leucocyte D-GDH and GR activity.

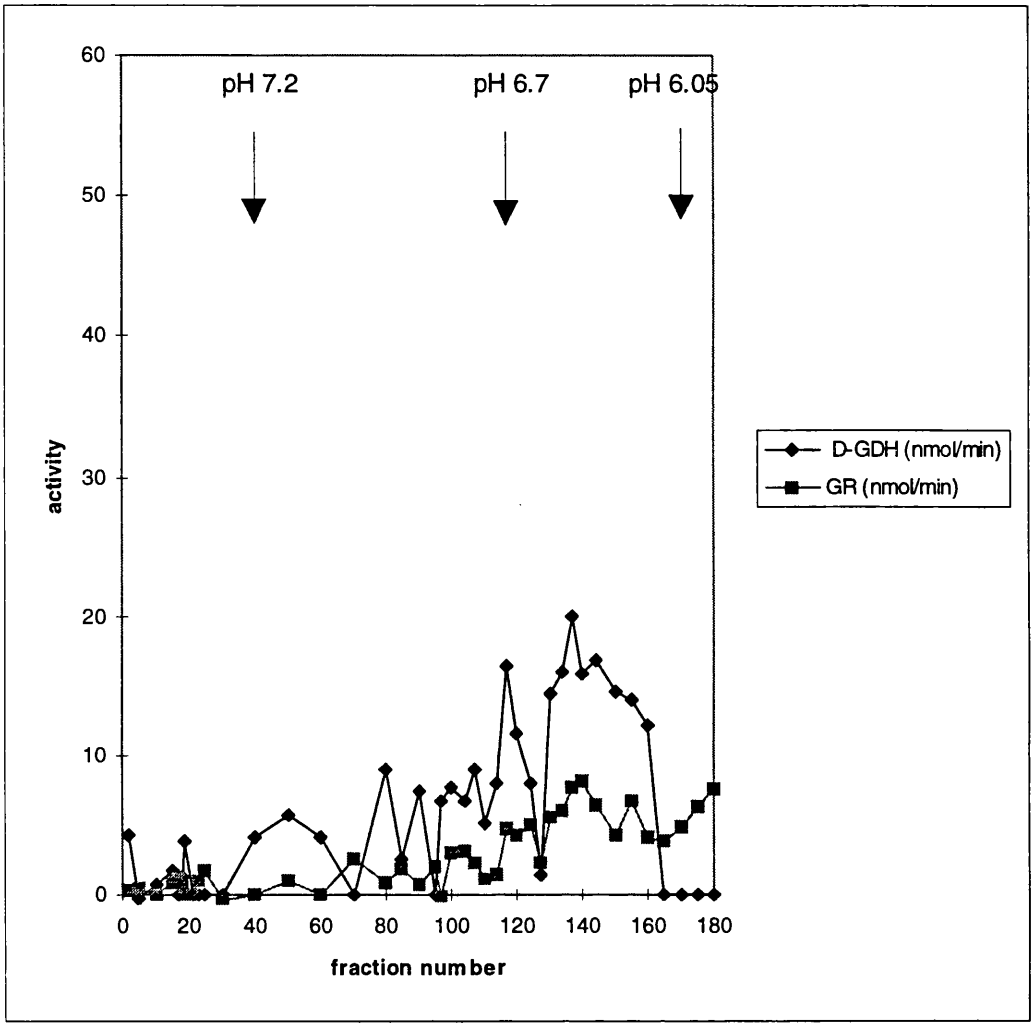


Fig. 4.4: Elution profile from a chromatofocusing column of human kidney D-GDH and GR activity.

4.3.3: Intracellular distribution of human D-GDH/GR.

Three preparations of HepG2 cells were separated into cytosolic and particulate fractions in separate occasions. 85% (range 78-96%) of D-GDH and 89% (range 81-96%) of GR was found in the cytosol. The HepG2 preparation that was applied on a Nycodenz gradient had total D-GDH and GR activity of 1,895 $nmol.min^{-1}$ and 106 $nmol.min^{-1}$ respectively.

Rat liver had a completely different ratio of D-GDH:GR total activity of 1:1 with total D-GDH and GR activity 999 $nmol.min^{-1}$ and 1109 $nmol.min^{-1}$ respectively. 89% of D-GDH and 85% of GR activity was found in the cytosol.

Human leucocytes were separated in cytosolic and particulate fractions in four separate occasions. 88% (range 85-94%) of D-GDH and 83% (range 68-93%) of GR activity was found in the cytosol. The human leucocyte preparation applied on a Nycodenz column had total D-GDH and GR activities of 2388 $nmol.min^{-1}$ and 149 $nmol.min^{-1}$ respectively. The mean percentages and ranges of D-GDH and GR cytosolic activities as well as the D-GDH and GR activities of a typical subcellular distribution for rat liver, human leucocytes and HepG2 cells are summarised in table 4.2.

Table 4.2: Subcellular distribution of D-GDH (forward) and GR activity in human leucocytes, HepG2 cells and rat liver. Activity is expressed in $nmol.min^{-1}$. and refers to whole samples. Values in brackets represent the range.

	human leucocytes n=4		HepG2 cells n=3		rat liver n=1	
	D-GDH (forward)	GR	D-GDH (forward)	GR	D-GDH (forward)	GR
activity in cytosol	2253 94% (85-94%)	102 68% (68-93%)	3288 81%(78-96%)	379 100% (81-100%)	890 (89%)	949 (85%)
activity in particulate	117	below sensitivity of method	374	49	32	25
activity in whole cells	2388	149	4056	379	999	1109

Separation of HepG2 and leucocyte peroxisomes and mitochondria by Nycodenz gradient is depicted in fig. 4.5 and 4.6 respectively. Fraction 1 is at the bottom of the tube. In the HepG2 separation, catalase activity, denoting peroxisomes, peaked at fractions 3, 8 and 13 and cytochrome oxidase activity, a marker for mitochondria, peaked at fraction 4. Although it appears from fraction 3 in fig. 4.5 that GR is peroxisomal, the measured activity is below the sensitivity of the method. D-GDH peaked at fraction 21, where cytochrome oxidase and catalase activity were undetectable. In the leucocyte fractionation, catalase peaked in fractions

7, 21 and 23 and cytochrome oxidase in fractions 2, 7 and 22. Again, both D-GDH and GR activities were too low to measure.

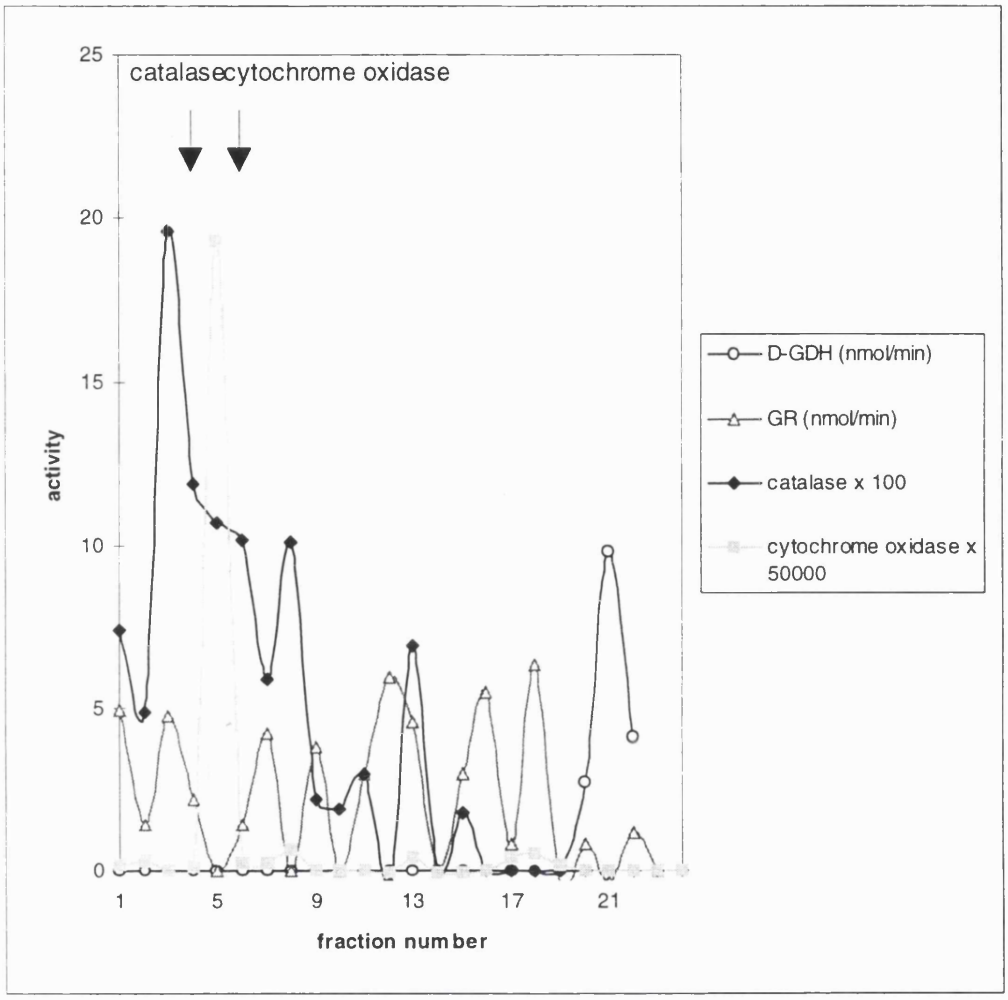


Fig. 4.5: Subcellular fractionation of a HepG2 cell line using Nycodenz.

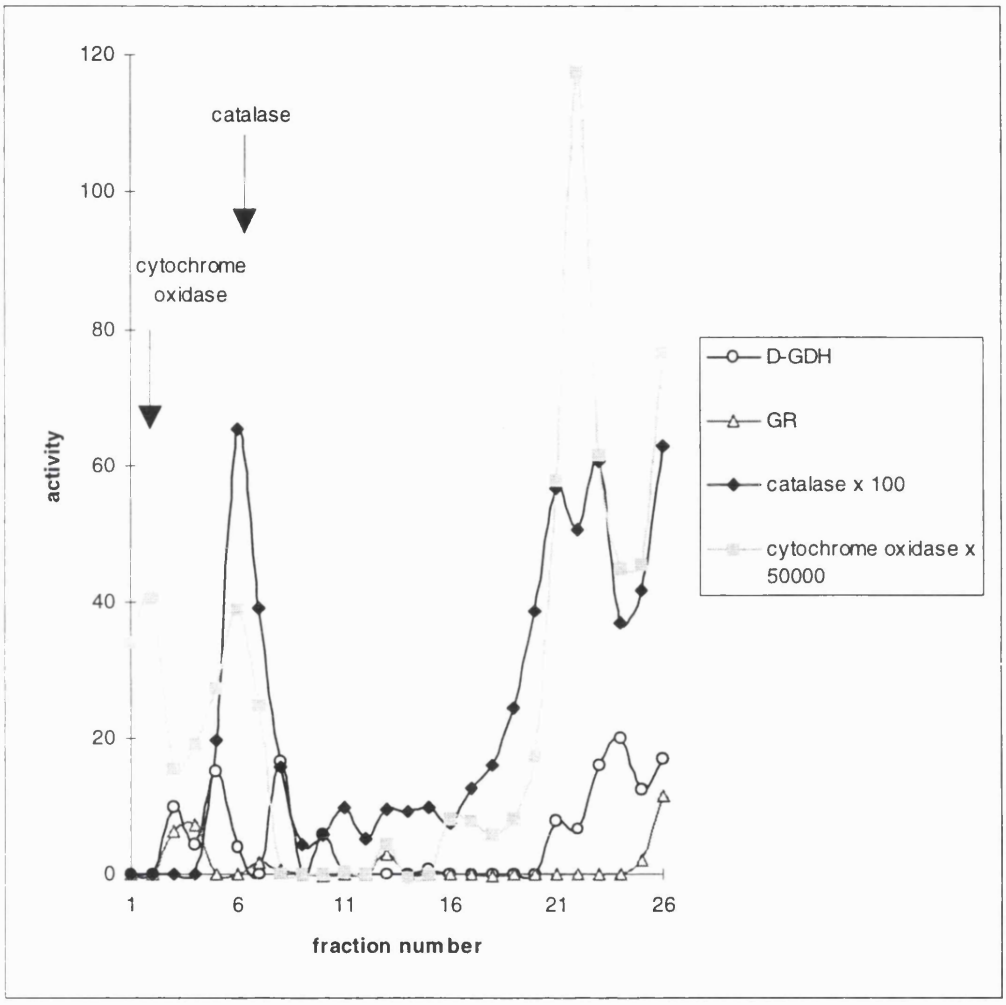


Fig. 4.6: Subcellular fractionation of human leucocytes using Nycodenz.

4.4: Discussion.

D-GDH (forward) and GR activity was determined in a number of livers from normal subjects in addition to samples from patients with PH1 and PH2. The samples from patients with PH1 had activities within the reference range whereas those from 3 subjects with PH2 had measurable D-GDH (forward) activity although in all cases below the reference range. GR in the PH2 samples was less than 15% of the median.

Tissue distribution studies yielded some interesting if rather unexpected findings. D-GDH (forward) activity was found in all tissues tested, that is liver, kidney, leucocyte and fibroblast (fig. 4.1a) as well as in HepG2 cells (4.3.3). GR activity on the other hand was present in liver but substantially less was present in kidney, leucocytes, fibroblasts (fig 4.1b) and HepG2 cells (4.3.3). Leucocytes from 2 PH2 patients had D-GDH (forward) activity within the reference range but undetectable GR.

There are a number of possible explanations for these findings. Firstly, the same protein may not have both D-GDH and GR activities, but since D-GDH and GR from human (chapter 3) and rat liver (Dawkins and Dickens, 1965) co-purify and reduced GR and D-GDH

activity has been documented in liver from patients with PH2 both in this study and those of others (Mistry *et al.*, 1988, Seargeant *et al.*, 1991, Chlebeck *et al.*, 1994) this is unlikely. Secondly, an inhibitor of GR activity may be present in the different tissues, although by mixing kidney and liver tissue and measuring D-GDH and GR before and after mixing we have found no evidence to support this theory. Thirdly, based on the results of chromatofocusing presented in this thesis (fig. 3.5), two peaks of D-GDH activity at pH 7.2 and 6.7 have been observed. We propose that there are at least two enzymes with hydroxypyruvate-reducing activity but only the one with a pI of approximately 6.7 also has the ability to reduce glyoxylate. The latter is predominantly hepatic in origin, with lesser amounts in kidney, fibroblasts and HepG2 cells and, according to the lymphocyte chromatofocusing elution profile (fig. 4.3), negligible amounts in lymphocytes.

To avoid the requirement for obtaining fresh human liver for subcellular distribution experiments, HepG2 cells were used. However, the difference in the ratio D-GDH:GR between hepatocytes (4:1) and HepG2 cells (10:1) suggests that more of the protein without GR

activity is present in HepG2 cells relative to liver and this appears to be predominantly cytosolic.

In rat the cytosolic fraction contains most D-GDH activity (94%) measured by oxidation of D-glycerate (Suzuki *et al.*, 1972). Likewise, in humans Mistry *et al.* (Mistry *et al.*, 1988) found 90% of liver D-GDH (both forward and reverse) and GR activities to be cytosolic. The same D-GDH and GR activity distribution was observed in human leucocytes and HepG2 cells. As shown from the chromatofocusing elution profile, the predominant protein with D-GDH activity in leucocytes does not possess GR activity and is not the same as in liver. From the subcellular distribution experiments it can be concluded that this newly found D-GDH is also primarily cytosolic. From the similarity in D-GDH:GR activity ratio and in the subcellular distribution, it may be assumed that the predominant protein with D-GDH activity in HepG2 cells is the same as in leucocytes.

the cytosol as is the case with human liver. It should however be remembered that the predominant D-GDH enzyme in HepG2 cells and leucocytes is a protein without GR activity.

Another enzyme with glyoxylate-reducing activity has been reported in rat (EC 1.1.1.26) (Vandor and Tolbert, 1970), which utilises NADH and glyoxylate, also reduces hydroxypyruvate (with NADH as cofactor only) and pyruvate but has no D-glycerate dehydrogenase activity. The enzyme was first reported to be located in the peroxisomes(Vandor and Tolbert, 1970). However, when Suzuki *et al.* (Suzuki *et al.*, 1973) subjected rat liver to differential centrifugation in 0.15 M KCl instead of 0.25 M sucrose, as was the case with the previous experiments, the cytosolic fraction contained most (94%) activity. This led to the conclusion that ionogenic interaction of the enzyme with the peroxisomal membranes took place during homogenisation in 0.25 M sucrose and raises the question whether a different homogenising buffer would alter the subcellular distribution of GR in leucocytes and HepG2 cells.

The observed tissue distribution has significance for the diagnosis and treatment of PH2. The accumulation of L-glycerate as a result of the action

of lactate dehydrogenase on excess hydroxypyruvate probably has little pathological consequence as it is relatively soluble. Deficient GR activity however, with the subsequent increase of oxalate production *in vivo*, will lead to oxalate accumulation and precipitation. From our analyses, GR expression is highest in the liver and thus, to prevent the endogenous overproduction of oxalate, the definitive treatment for PH2 would appear to be liver transplantation as recommended for PH1. Furthermore, the subcellular distribution results suggest that for the diagnosis of PH2, the measurement of both GR and D-GDH (forward) is preferable to D-GDH alone.

CHAPTER 5: DISCUSSION.

The kinetic characteristics of human liver D-GDH/GR were determined and a normal reference range was established. It was verified that liver from PH2 patients had undetectable GR activity and D-GDH activity below the normal reference range, while PH1 patients have D-GDH and GR activity within the reference range.

The experimental strategy used for the estimation of Km values of the substrates for the D-GDH and GR reactions has the limitation that the concentration of the substrate kept constant cannot be increased to 10-20 times Km. As a consequence, inaccuracy is introduced in the estimation of Km values. Instead, the experimental design should involve sequential variation of the concentrations of two substrates at non-saturating levels and analysis of the results with primary and secondary graphs (Cornish-Bowden, 1995).

Human liver D-GDH/GR was partially purified. It was established that the same enzyme has D-GDH and GR activity as it is the case for rat (Dawkins and Dickens, 1965) and bovine (Heinz *et al.*, 1962) liver. By western blotting it was determined that the purified protein is not recognised in PH2 patients, while it is present in PH1 patients, and that it has an approximate molecular weight of 71 kD.

From the chromatofocusing step during the purification procedure it was discovered that two proteins with D-GDH (forward) activity were present in human liver and only one had GR activity. Tissue distribution studies showed that the ratio of D-GDH:GR GR activity ranged from 4:1 in liver to 42:1 in leucocytes. The presence of another D-GDH enzyme lacking GR activity would explain the presence of D-GDH (forward) enzyme activity in leucocytes from patients

(forward) enzyme activity in leucocytes from patients with PH2. The absence of D-GDH/GR in leucocytes and its reduced presence in kidney was also supported by western blotting experiments. Antibodies raised against partially purified D-GDH/GR detected reduced amounts of the protein in kidney compared to liver, and did not detect it at all in leucocytes. By subcellular fractionation of HepG2 cells it was established that the new D-GDH is mainly cytosolic.

It was established that GR activity in humans is found mainly in the liver. Since it is thought that the buildup of glyoxylate causes increased oxalate synthesis and deposition in PH2 patients, the definitive treatment for PH2 would apppear to be liver transplantation.

In the future, antibodies against D-GDH/GR can be used to construct a sepharose affinity column that would enable further purification of D-GDH/GR. After sequencing D-GDH/GR, databases can be searched for homology to already sequenced peptides. In case of 100% homology, cDNA probes can be used to perform northern blots and examine the expression of the gene in various tissues and in PH2 patients, and genomic probes can be used to perform Southern blots in order to obtain information on the size of genomic DNA. In case no

constructed from the peptide sequence in order to screen a cDNA library from liver, sequence the positive clones and convert the nucleotide sequence to amino acid sequence so that a comparison can be made with the known peptide sequence. Finally, in both cases DNA from patients with PH2 could be sequenced to search for mutations.

The fact that PH1 patients are deficient of AGT, but have D-GDH/GR activity (Giafi and Rumsby, 1998a, Giafi and Rumsby, 1988b), while PH2 patients are deficient of D-GDH/GR but have AGT activity (Mistry *et al.,* 1988) indicates that PH1 and PH2 are a peroxisomal and cytosolic disease of glyoxylate metabolism independent of each other. It is believed that pathology in PH2 is caused by the deficiency of GR resulting in a buildup of glyoxylate which is converted to oxalate by LDH. To verify this hypothesis and to elucidate whether hydroxypyruvate is also converted to oxalate in PH2 patients, when the gene is identified and sequenced, experiments can be performed in cell culture where the D-GDH/GR gene is knocked out and labelled glyoxylate or hydroxypyruvate is added.

Further experiments could include the enzymic characterisation of the new D-GDH (forward) enzyme as well as the determination of coenzyme preference for D-GDH/GR and the new enzyme, after the removal of LDH. Finally it could be determined whether D-GDH/GR and HPR are part of the pathway of gluconeogenesis by examining whether feedback inhibition with "downstream" metabolites, such as ATP, 2-phosphoglycerate, 3-phosphoglycerate, glucose-6-phosphate, and glucose, and enzyme activation by serine occurs.

Appendix 1.

The NADH or NADPH-dependent dehydrogenases follow a two-substrate mechanism called ordered bi-bi (Cornish-Bowden, 1995), whereby the enzyme binds each substrate in an obligatory order and releases each product sequentially. The rate equation for the ordered bi-bi mechanism is:

$$v = \frac{V}{1 + \frac{Km^{S1}}{[S_1]} + \frac{Km^{S2}}{[S_2]} + \frac{Ks^{S1}\,Km^{S2}}{[S_1][S_2]}} \quad . \quad (6)$$

where is Km^{S1} the Michaelis-Menten constant of S_1, Km^{S2} is the Michaelis-Menten constant of S_2 and Ks^{S1} is the dissociation constant of the enzyme-S_1 complex. S_1 could be ATP, NADH, acetyl-CoA, etc.

$$\frac{v}{V} = \frac{1}{1 + \frac{Km^{S1}}{[S_1]} + \frac{Km^{S2}}{[S_2]} + \frac{Ks^{S1}\,Km^{S2}}{[S_1][S_2]}} \quad . \quad (7)$$

$$\frac{V}{v} - 1 = \frac{V-v}{v} = \frac{Km^{S1}}{[S_1]} + \frac{Km^{S2}}{[S_2]} + \frac{Ks^{S1}\,Km^{S2}}{[S_1][S_2]} \quad (8)$$

$$\text{or} \quad \frac{1}{F} = \frac{Km^{S1}[S_2] + Km^{S2}[S_1] + Ks^{S1}\,Km^{S2}}{[S_1][S_2]} \quad (9)$$

$$\text{Thus } F = \frac{[S_1][S_2]}{Km^{S1}[S_2] + Km^{S2}[S_1] + Ks^{S1}\,Km^{S2}} \quad (10)$$

REFERENCES:

1. Anthony C., The biochemistry of methylotrophs, Academic Press, Inc., New York, 1982.

2. Aronson P. S., The renal proximal tubule: a model for diversity of anion exchangers and stilbene-sensitive anion transporters, Annu. Rev. Physiol. 51:419, 1989.

3. Atkins G. L., Dean B. M., Griffin W. J., Scowen E. F., Watts R. W., Primary hyperoxaluria. The relation between ascorbic acid and the increased urinary excretion of oxalate, Lancet 2:1096, 1963.

4. Atkins G. L., Dean B. M., Griffin W. J., Scowen E. F., Watts R. W., Quantitative aspects of ascorbic acid metabolism in man, J. Biol. Chem. 239:2975, 1964.

5. Baggio B., Gambaro G., Marchini F., Cicerello E., Borsatti A., Raised transmembrane oxalate flux in red blood cells in idiopathic calcium oxalate nephrolithiasis, Lancet 2:12, 1984.

6. Baggio B., Gambaro G., Marchini F., Cicerello E., Borsatti A., Red blood cell transmembrane oxalate flux in calcium-oxalate nephrolithiasis, Contrib. Nephrol. 49:118, 1985.

7. Baggio B., Gambaro G., Marchini F., Cicerello E., Tenconi R., Clementi M., Borsatti A., An inheritable

anomaly of red-cell oxalate transport in "primary" calcium nephrolithiasis correctable with diuretics, N. Engl. J. Med. 314:599, 1986.

8. Bais R., Nairn J. M., Rofe A. M., Conyers R. A., Enzymology of endogenous oxalate formation, Adv. Clin. Enzymol. 5:43, 1987.

9. Bais R., Rofe A. M., Conyers R. A., Inhibition of endogenous oxalate production: Biochemical considerations of the roles of glycollate oxidase and lactate dehydrogenase, Clin. Sci. 76:303, 1989.

10. Banner M. R. and Rosalki S. B., Glycolate as a substrate for lactate dehydrogenase, Nature 213:726, 1967.

11. Barratt T. M. and Danpure C. J., Hyperoxaluria, in Pediatric Nephrology, Williams and Wilkins, Baltimore, chap. 29B, 1994.

12. Barratt T. M., Kasidas G. P., Murdoch I., Rose G. A., Urinary oxalate and glycolate excretion and plasma oxalate concentration, Arch. Dis. Child 66:501, 1991.

13. Barilla D. E., Notz C., Kennedy D., Pak C. Y., Renal oxalate excretion following oral oxalate loads in patients with ileal disease and with renal and absorptive hypercalciurias. Effect of calcium and magnesium, Am. J. Med. 64:579, 1978.

14. Bästlein C. and Burchardt G., Sensitivity of rat renal luminal and contraluminal sulphate transport systems to DIDS, Am. J. Physiol. 250:F226, 1986.
15. Berg W., Bothor C., Pirlich W., Janitzky V., Influence of magnesium on the absorption and excretion of calcium and oxalate ions, Eur. Urol. 12:274, 1986.
16. Bergmeyer H. U., Establishing optimum measurement conditions, in Methods of Enzymatic Analysis. Fundamentals 1:114, 1983.
17. Binder H. J., Intestinal oxalate absorption, Gastroenterology, 67:441, 1974.
18. Blakemore W. F., Heath M. F., Bennett M. J., Cromby C. H., Pollitt R. J., Primary hyperoxaluria and L-glyceric aciduria in the cat, J. Inherited Metab. Dis. (suppl. 2) 11:215, 1988.
19. Borsatti A., Calcium oxalate nephrolithiasis: defective oxalate transport, Kidney Int. 39:1283, 1991.
20. Butz M., Hoffmann N., Kohlbecker G., Dietary influence on serum and urinary oxalate in healthy subjects and oxalate stone formers, Urol. Int. 35:309, 1980.
21. Cannon G. D., Eaton R. H., Glen A. C. A., Enzymic determination of urinary oxalate, with EDTA added to improve recovery, Clin. Chem. (letter) 29:1855, 1983.

22. Caspary W. F., Intestinal oxalate absorption. I. Absorption *in vitro*, Res. Exp. Med. (Berl.) 171:13, 1977.

23. Chalmers R. A., Tracey B. M., Mistry J., Griffith K. D., Green A., Winterborn M. H., L-glyceric aciduria (primary hyperoxaluria type 2) in siblings in two unrelated families, J. Inherited Metab. Dis. 7 [Suppl. 2]: 133, 1984.

24. Cheung G. P., Cotropia J. P., Sallach H. J., The effects of dietary protein on the hepatic enzymes of serine metabolism in the rabbit, Arch. Biochem. Biophys. 129:672, 1969.

25. Chistoserdova L. V. and Lidstrom M. E., Purification and characterisation of hydroxypyruvate reductase from the facultative methylotroph *Methylobacterium extorquens* AM1, J. Bacteriol. 173:7228, 1991.

26. Chlebeck P. T., Milliner D. S., Smith L. H., Long-term prognosis in primary hyperoxaluria type II (L-glyceric aciduria), Am. J. Kid. Dis. 23:255, 1994.

27. Cochat P., Deloraine. Rotily M., Olive F., Liponski I., Deries N., Epidemiology of primary hyperoxaluria type 1, Nephrol. Dial. Transplant 10 [Suppl. 8]:3, 1995.

28. Cochat P. and Schärer K., Should liver transplantation be performed before advanced liver insufficiency in primary hyperoxaluria type 1?, Pediatr. Nephrol. 7:212, 1993.

29. Cochat P., Faure J. L., Divry C. J., Danpure C. J., Descos B., Wright P., Tavkorian P., Floret D., Single liver transplantation in primary hyperoxaluria type I, Pediatr. Nephrol. 3:C153, 1989.

30. Coderch R., Lluis C., Bozal J., Effect of salts on D-glycerate dehydrogenase kinetic behavior, *Biochim. Biophys. Acta* 566:21, 1979.

31. Comici M., Balestri P. L., Lupetti S., Colizzi V., Falcone G., Urinary excretion of oxalate in renal failure, Nephron 30:269, 1982.

32. Cooper P. J., Danpure C. J., Wise P. J., Guttridge K. M., Immunocytochemical localisation of human hepatic alanine:glyoxylate aminotransferase in control subjects and patients with primary hyperoxaluria type 1, J. Histochem. Cytochem. 36:1285, 1988.

33. Cooperstein S. J. and Lazarow A., A microspectrophotometric method for the determination of cytochrome oxidase, J. Biol. Chem. 189:665, 1951.

34. Costello J. F., Smith M., Stolarski C., Sadovnic M. J., Extrarenal clearance of oxalate increases with

Cornish-Bowden A., (1995) Fundamentals of Enzyme Kinetics, 2nd edn., Portland Press, London.

progression of renal failure in the rat, J. Am. Soc. Nephrol. 3:1098, 1992.

35. Crawhall J. C., De Mowbray R. R., Scowen E. F., Watts R. W. E., Conversion of glycine to oxalate in a normal subject, Lancet 2:810, 1959.

36. Danpure C. J., Molecular and clinical heterogeneity in primary hyperoxaluria type 1, Am. J. Kidney Dis. 17:366, 1991.

37. Danpure C. J., Birdsey G. M., Rumsby G., Lumb M. J., Purdue P. E., Allsop J., Molecular characterisation and clinical use of a polymorphic tandem repeat in an intron of the human alanine:glyoxylate aminotransferase gene, Hum. Genet. 87:394, 1991.

38. Danpure C. J., Cooper P. J., Jennings P. R., Wise P. J., Penketh R. J., Rodeck C. H., Enzymatic prenatal diagnosis of primary hyperoxaluria type I: potential and limitations, J. Inherited Metab. Dis. 12 [Suppl. 2]:286, 1989b.

39. Danpure C. J., Cooper P. J., Wise P. J., Jennings P. R., An enzyme trafficking defect in two patients with primary hyperoxaluria type 1: peroxisomal alanine/glyoxylate aminotransferase rerouted to mitochondria, J. Cell Biol. 108:1345, 1989a.

40. Danpure C. J. and Jennings P. R., Peroxisomal alanine:glyoxylate aminotransferase deficiency in primary hyperoxaluria type 1, FEBS Lett. 201:20, 1986.
41. Danpure C. J. and Jennings P. R., Further studies on the activity and subcellular distribution of alanine:glyoxylate aminotransferase in the livers of patients with primary hyperoxaluria type 1, Clin. Sci. 75:315, 1988.
42. Danpure C. J., Jennings P. R., Fryer P., Purdue P. E., Allsop J., Primary hyperoxaluria type 1: genotypic and phenotypic heterogeneity, J. Inherit. Metab. Dis. 17:487, 1994.
43. Danpure C. J., Jennings P. R., Mistry J., Chalmers R. A., McKerrell R. E., Blakemore W. F., Heath M. F., Enzymological characterisation of a feline analogue of primary hyperoxaluria type 2: a model for the human disease, J. Inher. Metab. Dis. 12:403, 1989c.
44. Danpure C. J., Jennings P. R., Watts R. W. E., Enzymologic diagnosis of primary hyperoxaluria type I by measurement of the alanine:glyoxylate aminotransferase activity, Lancet I:289, 1987.
45. Danpure C. J. and Purdue P. E., Primary Hyperoxaluria, in The Metabolic and Molecular Basis of Inherited Disease, 7th edn. McGraw Hill, New York, 2385, 1995.

46. Danpure C. J., Purdue P. E., Fryer P., *et al.*, Enzymological and mutational analysis of a complex primary hyperoxaluria type 1 phenotype involving alanine:glyoxylate aminotransferase peroxisome-to-mitochondrion mistargeting and intraperoxisomal aggregation, Am. J. Hum. Genet. 53:417, 1993.
47. David C. and Ullrich K. J., Substrate specificity of the luminal Na(+)-dependent sulphate transport system in the proximal renal tubule as compared to the contraluminal sulphate exchange system, Pflügers Arch. 421:455, 1992.
48. Dawkins P. D. and Dickens F., The oxidation of D- and L-glycerate by rat liver, Biochem. J. 94:353, 1965.
49. De Duve C. and Baudhuin P., Peroxisomes (microbodies and related particles) Physiol. Rev. 46:323, 1966.
50. Diamond K. L., Fox C. C., Barch D. H., Role of cecal pH in intestinal oxalate absorption in the rat, J. Lab. Clin. Med. 112:352, 1988.
51. Dionex Corporation, Determination of oxalate in urine by ion chromatography, in Application note no. 36, Dionex Corporation U.S.A., Sunnivale CA, 1992, 1.
Dixon M. and Kleppe K., D-amino acid oxidase. II Specificity, competitive inhibition and reaction sequence, *Biochim. Biophys. Acta,* 96:368, 1965.

52. Dobbins J. W. and Binder H. J., Effect of bile salts and fatty acids on the colonic absorption of oxalate, Gastroenterology, 70:1096, 1976.

53. Dobson D. M., and Finlayson B., Oxalate transport from plasma to intestinal lumen in the rat, Urol. Surg. 540, 1973.

54. Duburque M. T., Melon J. M., Thomas J., Thomas E., Pierre R., Charransol J., Desgrez P., Dosage et identification de l' acide oxalique dans les milieux biologiques, Ann. Biol. Clin. 28:95, 1970.

55. Dunstan P. M., Anthony C., Drabble W. T., The role of glyoxylate, glycolate and acetate in the growth of *Pseudomonas* AM1 on ethanol and on C-1 compounds, Biochem. J. 128:107, 1972.

56. Earnest D. L., Perspectives on incidence, etiology, and treatment of enteric hyperoxaluria, Am. J. Clin. Nutr. 30:72, 1977.

57. Earnest D. L., Johnson G., Williams H. E., Admirand W. H., Hyperoxaluria in patients with ileal reaction: an abnormality in dietary oxalate absorption, Gastroenterology 66:1114, 1974.

58. Elder D. and Wyngaarden J. B., The biosynthesis and turnover of oxalate in normal and hyperoxaluric subjects, J. Clin. Invest. 39:1337, 1960.

59. Ellis D., Avner E. D., Starzl T. E., Renal failure in children with hepatic failure undergoing liver transplantation, J. Pediatr. 108:393, 1986.
60. Fairclough P. D., Feest T. G., Chadwick V. S., Clark M. L., Effect of sodium chenodeoxycholate on oxalate absorption from the excluded human colon - a mechanism for "enteric" hyperoxaluria, Gut 16:543, 1975.
61. Farooqui S., Mahmood A., Nath R., Thind S. K., Nutrition and urolithiasis: Part I - intestinal absorption of oxalate in vitamin B6 deficient rats, Indian J. Exp. Biol. 19:551, 1981.
62. Feld R. D. and Sallach H. J., D-glycerate dehydrogenase from hog spinal cord, Methods Enzymol. 41:289, 1974.
63. Feld R. D. and Sallach H. J., The regulation of D-glycerate dehydrogenase from porcine spinal cord, Brain Res. 73:558, 1974.
64. Feld R. D. and Sallach H. J., Comparison of D-glycerate dehydrogenases from beef liver, beef spinal cord and hog spinal cord, Arch. Biochem. Biophys. 166:417, 1975.
65. Freel R. W., Hatch M., Earnest D. L., Goldner A. M., Oxalate column across the isolated rat colon. A re-examination, *Biochim. Biophys. Acta* 600:838, 1980.

66. Freel R. W., Hatch M., Vaziri N. D., Conductive pathways for oxalate (Ox) and chloride in rabbit ileal brush border membrane vesicles (BBMV), FASEB J. A87, 1995.

67. Fry D. W. and Richardson, Isolation and characterisation of glycolic acid dehydrogenase from human liver, *Biochim. Biophys. Acta* 567:482, 1979a.

68. Fry D. W. and Richardson, Isolation and characterisation of glycolic acid oxidase from human liver, *Biochim. Biophys. Acta* 568:135, 1979b.

69. Frizzel R. A. and Schultz S. G., Ionic conductances of extracellular shunt pathway in rabbit ileum, J. Gen. Physiol. 59:318, 1972.

70. Fukushima M., Aihara Y., Ichiyama A., Immunochemical studies on induction of rat liver mitochondrial serine:pyruvate aminotransferase by glucagon, J. Biol. Chem. 253:1187, 1978.

71. Funai T. and Ichiyama A., High-performance liquid chromatographic determination of glyoxylate in rat liver, J. Biochem. (Tokyo) 99:579, 1986.

72. Gambardella R. L. and Richardson K. E., The pathways of oxalate formation from phenylalanine, tyrosine, tryptophan and ascorbic acid in the rat, *Biochim. Biophys. Acta* 499:156, 1977.

73. Gambardella R. L. and Richardson K. E., The formation of oxalate from hydroxypyruvate, serine, glycolate and glyoxylate in the rat, *Biochim. Biophys. Acta* 544:315, 1978.

74. Gelot M. A., Lavoue G., Belleville F., Nabet P., Determination of oxalates in plasma and urine using gas chromatography, *Clin. Chim. Acta* 106:279, 1980.

75. Gibbs D. A. and Watts R. W., An investigation of the possible role of xanthine oxidase in the oxidation of glyoxylate to oxalate, Clin. Sci. 31:285, 1966.

76. Gibbs D. A. and Watts R. W., The identification of the enzymes that catalyse the oxidation of glyoxylate to oxalate in the 100,000 g supernatant fraction of human hyperoxaluric and control liver and heart tissue, Clin. Sci. 44:227, 1973.

77. Givan C. V., Joy K. W., Kleczkowski L. A., A decade of photorespiratory nitrogen cycling, Trends Biochem. Sci. 13:433, 1988.

78. Gochman N. and Schmitz J. M., Automated determination of uric acid, with use of a urease-peroxidase system, Clin. Chem. 17:1154, 1971.

79. Greger R., Lang F., Oberleithner H., Deetjen P., Handling of oxalate by the rat kidney, Pflügers Arch. 374:243, 1978.

Giafi C.F. and Rumsby G., Kinetic Analysis and Tissue Distribution of Human D-Glycerate Dehydrogenase/Glyoxylate Reductase, Ann. Clin. Biochem. 35:104, 1998.

Giafi C.F. and Rumsby G., Primary Hyperoxaluria Type 2; Enzymology, J. Nephol. 11 Suppl 1:29, 211 1998.

80. Gupta R., Sidhu H., Rattan V., Thind S. K., Nath R., Oxalate uptake in intestinal and renal brush-border membrane vesicles (BBMV) in vitamin B6-deficient rats, Biochem. Med. Metab. Biol. 39:190, 1988.
81. Hahn R. G. and Sikk M., Glycine loading and urinary oxalate excretion, Urol. Int. 52:14, 1994.
82. Halm D. R. and Frizzel R. A., Intestinal chloride secretion, in Textbook of Secretory Diarrhea, Lebenthal E. and Duffey M., Raven Press, Ltd., New York, 47, 1990.
83. Hatch M., Oxalate status in stone-formers. Two distinct hyperoxaluric entities, Urol. Res. 21:55, 1993.
84. Hatch M. and Freel R. W., Oxalate transport across intestinal and renal epithelia, in Calcium Oxalate in Biological Systems, Eds. CRC Press, Boca Raton, Florida, U.S.A., 217, 1995.
85. Hatch M., Freel R. W., Goldner A. M., Earnest D. L., Effect of bile salt on active oxalate transport in the colon, in Colon and Nutrition, Kasper H. and Goebell H., MTP Press Ltd., Lancaster, 299, 1982.
86. Hatch M., Freel R. W., Vaziri N. D., Mechanisms of oxalate absorption and secretion across the rabbit distal colon, Pflügers Arch., 426:101, 1994.

87. Hatch M., Freel R. W., Vaziri N. D., Intestinal excretion of oxalate in chronic renal failure, J. Am. Soc. Nephrol. 5:1339, 1994.

88. Hatch M. and Vaziri N. D., Do thiazides reduce intestinal oxalate absorption?: a study *in vitro* using rabbit colon, Clin. Sci. 86:353, 1994a.

89. Hatch M. and Vaziri N. D., Enhanced enteric excretion of urate in rats with chronic renal failure, Clin. Sci. 86:511, 1994b.

90. Heinz F., Bartelsen K., Lamprecht W., D-glycerat-dehydrogenase aus leber, Z. Physiol. Chem. 329:222, 1962.

91. Heupel R., Markgraf T., Robinson D. G., Heldt H. W., Compartmentation studies on spinach leaf peroxisomes. Evidence for channeling of photorespiratory metabolites in peroxisomes devoid of intact boundary membrane, Plant Physiol. 96:971, 1991.

92. Hicks N. R., Cranston D. W., Charlton C. A. C., Fifteen-year follow up on primary hyperoxaluria type II, N. Engl. J. Med. (letter) 309:796, 1983.

93. Hodgkinson A., Oxalic acid in biology and medicine, Academic, New York, 1977.

94. Holmes R. P., Hurst C. H., Assimos D. G., Goodman H. O., Glucagon increases urinary oxalate excretion in

the guinea pig, Am. J. Physiol. 269 (Endocrinol. Metab. 32):E568, 1995.

95. Hosaki Y., Nishina H., Ubuka T., Free amino acid contents in guinea pig tissues, *Acta Med.* Okayama 39: 425, 1985.

96. Husic D. W., Husic H. D., Tolbert N. E., The oxidative photosynthetic carbon cycle or C_2 cycle, CRC Crit. Rev. Plant. Sci. 5:45, 1987.

97. Izumi Y., Yoshida T., Kanzaki H., Toki S., Miyazaki S. S., Yamada H., Purification and characterisation of hydroxypyruvate reductase from a serine-producing methylotroph, *Hyphomicrobium methylovorum* GM2, Eur. J. Biochem. 190:279, 1990.

98. Kamoda N., Minatogawa Y., Nakamura M., Nakanishi J., Okuno E, Kido R., The organ distribution of human alanine-2-oxoglutarate aminotransferase and alanine-glyoxylate aminotransferase Biochem. Med. 23:25, 1980.

99. Karniski L. P., and Aronson P. S., Anion exchange pathways for Cl-transport in rabbit renal microvillus membranes, Am. J. Physiol. 253:F513, 1987.

100. Kasidas G. P., Assaying of oxalate in plasma, in Oxalate metabolism in relation to urinary stone, Rose G. A., Springer, Berlin, 26, 1988.

101. Kasidas G. P. and Rose G. A., Spontaneous *in vitro* generation of oxalate from L-ascorbate in some assays

for urinary oxalate and its prevention, in Urolithiasis and Related Clinical Research, Swille P. O., Smith L. 102. H., Robertson W. G., Vahlensieck W., Eds. Plenum Press, New York, 653, 1985.
103. Kemper M. J., Conrad D. E., Müller Wiefel D. E., Primary hyperoxaluria type 2, Eur. J. Pediatr. 156:509, 1997.
104. Kemper M. J. and Müller Wiefel D. E., Nephrocalcinosis in a patient with primary hyperoxaluria type 2. Pediatr. Nephrol. 10:442, 1996.
105. Kitagawa Y., Katayama H., Sugimoto E., Identity of mitochondrial and cytosolic glycerate kinases in rat liver and regulation of their intracellular localisation by dietary protein, *Biochim. Biophys. Acta* 582:260, 1979.
106. Kitagawa Y. and Sugimoto E., Possibility of mitochondrial-cytosolic cooperation in gluconeogenesis from serine via hydroxypyruvate, *Biochim. Biophys. Acta* 582:276, 1979.
107. Kleczkowski L. A. and Edwards G. E., Identification of hydroxypyruvate and glyoxylate reductases in maize leaves, Plant Physiol. 91:278, 1989.
108. Kleczkowski L. A., Edwards G. E., Blackwell R. D., Lea P. J., Givan C. V., Enzymology of the reduction of

hydroxypyruvate and glyoxylate in a mutant of barley lacking peroxisomal hydroxypyruvate reductase, Plant Physiol. 94:819, 1990.

109. Kleczkowski L. A., Givan C. V., Hodgson J. M., Randall D. D., Subcellular location of NADPH-dependent hydroxypyruvate reductase activity in leaf protoplasts of *Pisum sativum* L. and its role in photorespiratory metabolism, Plant Physiol. 88:1182, 1988.

110. Kleczkowski L. A. and Randall D. D., Purification and characterisation of a novel NADPH(NADH)-dependent hydroxypyruvate reductase from spinach leaves, Biochem. J. 250:145, 1988.

111. Kleczkowski L. A., Randall D. D., Blevins D. G., Purification and characterisation of a novel NADPH(NADH)-dependent glyoxylate reductase from spinach leaves, Biochem. J. 239:653, 1986.

112. Knickelbein R. G., Aronson P. S., Dobbins J. W., Substrate and inhibitor specificity of anion exchangers on the brush border membrane of rabit ileum, J. Membr. Biol. 88:199, 1985a.

113. Knickelbein R. G., Aronson P. S., Schron C. M., Seifter J., Dobbins J. W., Sodium and chloride transport across ileal brush border. II. Evidence for $Cl\text{-}HCO_3$ exchange and mechanism of coupling, Am. J. Physiol. 249:G236, 1985b.

114. Knickelbein R. G. and Dobbins J. W., Sulphate and oxalate exchange for bicarbonate across the basolateral membrane of rabbit ileum, Am. J. Physiol. G807, 1990.
Knight T. F., Sanson S. C., Senekjian H. O., Weinman E. J., Oxalate secretion in the rat proximal tubule, Am. J. Physiol. 240:F295, 1981.
115. Knight T. F., Senekjian H. O., Weinman E. J., Effect of para-aminohippurate on renal transport of oxalate, Kidney Int. 15:38, 1979.
116. Kohn L. D. and Warren W. A., The kinetic properties of spinach leaf glyoxylic acid reductase, J. Biol. Chem. 245:3831, 1970.
117. Kok D. J. and Khan S. R., Calcium oxalate nephrolithiasis, a free or fixed particle disease, Kidney Int. 46:847, 1994.
118. Latta K. and Brodehl J., Primary Hyperoxaluria 1, Eur. J. Pediatr. 149:518, 1991.
119. Larsson L. and Tiselius H. G., Hyperoxaluria, Min. Electr. Metab. 13:242, 1987.
120. Lawyer A. L., Cornwell K. L., Gee S. L., Bassham J. A., Glyoxylate and glutamate effects on photosynthetic carbon metabolism in isolated chloroplasts and mesophyll cells of spinach, Plant Physiol. 72:420, 1983.

121. Large P. J. and Quayle J. R., Microbial growth on C1 compounds. 5. Enzyme activities in extracts of *Pseudomonas* AM1, Biochem J. 87:386, 1963.

122. Leumann E. P. Dietl A., Matasovic A., Urinary oxalate and glycolate excretion in healthy infants and children, Pediatr. Nephrol. 4:493, 1990.

123. Leumann E. P., Hoppe B., Nehaus T, Management of primary hyperoxaluria: efficacy of oral citrate administration, Pediatr. Nephrol. 7:207.

124. Leumann E. P., Matasovic A., Niederwieser A., Primary hyperoxaluria: oxalate and glycollate unsuitable for prenatal diagnosis, Lancet II:340, 1986.

125. Liao L. L. and Richardson K. E., The metabolism of oxalate precursors in isolated perfused rat livers, Arch. Biochem. Biophys. 153:438, 1972.

126. Liao L. L. and Richardson K. E., The inhibition of oxalate biosynthesis in isolated perfused rat liver by DL-phenyllactate and n-heptanoate, Arch. Biochem. Biophys. 154:68, 1973.

127. Liao L. L. and Richardson K. E., The synthesis of oxalate from hydroxypyruvate by isolated perfused rat liver, *Biochim. Biophys. Acta* 538:76, 1978.

128. Lopez M., Tuchman M., Scheinman J. I., Capillary gas chromatography measurement of oxalate in plasma and urine, Kidney Int. 28:82, 1985.

129. Lowry O. H., Passonneau J. V., Rock M. K., The stability of pyridine nucleotides, J. Biol. Chem. 236:2756, 1961.
130. Mansell A., Primary Hyperoxaluria type 2, Nephrol. Dial. Transplant. 10 [Suppl. 8]:58.
131. Marangella M., Fruttero B., Bruno M., Linari F., Hyperoxaluria in idiopathic calcium stone disease: further evidence of intestinal hyperabsorption of oxalate, Clin. Sci. 63:381, 1982.
132. Marangella M., Petrarulo M., Cossedu D., End-stage renal failure in primary hyperoxaluria type 2, N. Engl. J. Med. (letter) 330:1690, 1994.
133. Marangella M., Petrarulo M., Cossedu D., Vitale C., Cadario A., Portigliatti Barbos M., Gurioli M., Linari F., Detection of hyperoxaluria type 2 (L-glyceric aciduria) in patients with maintained renal function or end-stage renal failure, Nephrol. Dial. Transplant. 10 [Suppl. 8]:1381, 1995.
134. Mascolo N., Rajendran V. M., Binder H. J., Mechanism of short-chain fatty acid uptake by apical membrane vesicles of rat distal colon, Gastroenterology, 101:331, 1991.
135. Mazzachi B. C., Teubner J. K., Ryall R. L., Factors affecting measurement of urinary oxalate, Clin. Chem. 30:1339, 1984.

136. McDonald J. C., Landreneau M. D., Rohr M. S., De Vault G. A. Jr., Reversal by liver transplantation of the complications of primary hyperoxaluria as well as the metabolic defect, N. Engl. J. Med. 321:1100, 1989.
137. McGroarty E., Hsieh B., Wied D. M., Gee R., Tolbert N. E., Alpha hydroxy acid oxidation by peroxisomes, Arch. Biochem. Biophys. 161:194, 1974.
138. Mc Kerrel R. E., Blakemore W. F., Heath M. F., Plumb J., Bennett M. J., Pollitt R. J., Danpure C. J., Primary hyperoxaluria (L-glyceric aciduria) in cat: A newly recognised inherited disease, Vet. Rec. 125:31, 1989.
139. Menon M. and Koul H., Calcium oxalate nephrolithiasis, J. Clin. Endocrin. Metab. 74:703, 1992.
140. Minatogawa Y., Tone S., Allsop J., Purdue P. E., Takada Y., Danpure C. J., Kido R., A serine-to-phenylalanine substitution leads to loss of alanine:glyoxylate aminotransferase caralytic activity and immunoreactivity in a patient with primary hyperoxaluria type 1, Hum. Mol. Genet. 1:643, 1992.
141. Mistry, J., Danpure, C.J., Chalmers, R.A., Hepatic D-glycerate dehydrogenase and glyoxylate reductase deficiency in primary hyperoxaluria type 2, Biochemical Society Transactions 16:626, 1988.

142. Morgan S. H., Purkiss P., Watts R. W. E., Oxalate dynamics in chronic renal failure, Nephron 46:253, 1987.

143. Mulligan R. M., Wilson B., Tolbert N. E., Effects of glyoxylate on photosynthesis by intact chloroplasts, Plant Physiol. 72:415, 1983.

144. Nakano M., Ushijima Y., Saga M., Tsutsumi Y., Asami H, Aliphatic L-alpha-hydroxyacid oxidase from rat livers. Purification and properties, *Biochim. Biophys. Acta* 167:9, 1968.

145. Neims A. H. and Hellerman L., Specificity of the D-amino acid oxidase in relation to glycine oxidase activity, J. Biol. Chem. 237:976.

146. Nishiyama K., Funai T., Katafuchi R., Hattori F., Onoyama K., Ichiyama A., Primary hyperoxaluria type 1 due to a point mutation of T to C in the coding region of the serine:pyruvate aminotransferase gene, Biochem. Biophys. Res. Commun. 176:1093, 1991.

147. Noguchi T. and Takada Y., Peroxisomal localisation of serine:pyruvate aminotransferase in human liver, J. Biol. Chem. 253:7598, 1978.

148. Noguchi T. and Takada Y., Peroxisomal localisation of alanine:glyoxylate aminotransferase in human liver, Arch. Biochem. Biophys. 196:645, 1979.

149. Noguchi T., Takada Y., Kido R., Glutamate-glyoxylate aminotransferase in rat liver cytosol. Purification, properties and identity with alanine-2-oxoglutarate aminotransferase, Hoppe Seyler Z. Physiol. Chem. 358:1533, 1977.

150. Noguchi T., Takada Y., Oota Y., Intraperoxisomal and intramitochondrial localisation and assay of pyruvate (glyoxylate) aminotransferase from rat liver, Hoppe Seyler Z. Physiol. Chem. 360:919, 1979.

151. Okuno E., Minatogawa Y., Nakanishi J., Nakamura M., Kamoda N., Makino M., Kido R., The subcellular distribution of alanine:glyoxylate aminotransferase and serine:pyruvate aminotransferase in dog liver, Biochem. J. 182:877, 1979.

152. Osswald H. and Hautmann R., Renal elimination kinetics and plasma half-life of oxalate in man, Urol. Int. 34:440, 1979.

153. Peel D. and Quayle J. R., Microbial growth on C-1 compounds, Biochem. J. 81:465, 1961.

154. Peters T. J., Muller M., De Duve C., Lysosomes of the arterial wall 1. Isolation and subcellular fractionation of cells from normal rabbit aorta, J. Exp. Med. 136:1117, 1972.

155. Petrarulo M., Cerelli E., Marangella M., Cossedu D., Vitale C., Linari F., Assay of plasma oxalate with soluble oxalate oxidase, Clin. Chem. 40:2030, 1994.
156. Petrarulo M., Marangella M., Bianco O., Marchesini A., Linari F., Preventing ascorbate interference in ion-chromatographic determinations of urinary oxalate: four methods compared, Clin. Chem. 36:1642, 1990.
157. Poore R. E., Hurst C. H., Assimos D. G., Holmes R. P., Pathways of hepatic oxalate synthesis and their regulation, Am. Physiol. Soc. C289, 1997.
158. Prien E. L. and Prien E. L. Jr, Composition and structure of urinary stones, Am. J. Med. 45:654, 1968.
159. Purdue P. E., Lumb M. J., Allsop J., Minatogawa Y., Danpure C. J., A glycine to glutamate substitution abolishes alanine:glyoxylate aminotransferase catalytic activity in a subset of patients with primary hyperoxaluria type 1, Genomics 13:215, 1992.
160. Purdue P. E., Lumb M. J., Fox M., Griffo G., Hamon Benais C., Povey S., Danpure C. J., Characterisation and chromosomal mapping of a genomic clone encoding human alanine:glyoxylate aminotransferase, Genomics 10:34, 1991.
161. Purdue P. E., Takada Y., Danpure C. J., Identification of mutations associated with peroxisome-to-mitochondrion mistargeting of alanine/glyoxylate

aminotransferase in primary hyperoxaluria type 1, J. Cell Biol. 111:2341, 1990.

162. Raghavan K. G. and Richardson K. E., Hydroxypyruvate-mediated regulation of oxalate synthesis by lactate dehydrogenase and its relevance to primary hyperoxaluria type II, Biochem. Med. 29:101, 1983a.

163. Raghavan K. G. and Richardson K. E., Hyperoxaluria in L-glyceric aciduria: Possible nonenzymic mechanism, Biochem. Med. 29:114, 1983b.

164. Rampton D. S., Kasidas G. P., Rose G. A., Sarner M., Oxalate loading test: a screening test for steatorrhoea, Gut 20:1089, 1979.

165. Richardson K. E., Effect of testosterone on the glycolic acid oxidase levels in male and female rat liver, Endocrinology 74:128, 1964.

166. Robertson W. G. and Peacock M., The cause of idiopathic calcium stone disease: hypercalciuria or hyperoxaluria?, Nephron 26:105.

167. Robertson W. G. and Scurr D. S., Prevention of ascorbic acid interference in the measurement of oxalic acid in urine by ion chromatography, *Cin. Chim. Acta* 140:97, 1984.

168. Rofe A. M., Chalmers A. H., Edwards J. B., (^{14}C)oxalate synthesis from (U-^{14}C)glyoxylate and (I-

^{14}C)glycollate in isolated rat hepatocytes, Biochem. Med. 16:277, 1976.

169. Rose G. A., Introduction, in Oxalate Metabolism in Relation to Urinary Stone, Springer-Verlag, London, 1, 1988.

170. Rosenblum I. Y., Antkowiak D. H., Sallach H. J., Flanders L. E., Fahien L. A., Purification and regulatory properties of beef liver D-glycerate dehydrogenase, Arch. Biochem. Biophys. 144:375, 1971.

171. Rowsell E. V., Snell K., Carnie J. A., Al Tai A. H., Liver-L-alanine-glyoxylate and L-serine-pyruvate aminotransferase activities: An apparent association with gluconeogenesis, Biochem. J. 115:1071, 1969.

172. Rowsell E. V., Snell K., Carnie J. A., Rowsell K. V., The subcellular distribution of rat liver L-alanine -glyoxylate aminotransferase in relation to a pathway for glucose formation involving glyoxylate, Biochem. J. 127:155, 1972.

173. Rumsby G., Uttley W. S., Kirk J. M., First trimester diagnosis of primary hyperoxaluria type 1, Lancet 344:1018, 1994.

174. Sallach H. J., D-glycerate dehydrogenases of liver and spinach, Methods in Enzymol. 9:221, 1966.

175. Saunders D. R., Sillery J., McDonald G. B., Regional differences in oxalate absorption by rat

intestine: evidence for excessive absorption by the colon in steatorrhoea, Gut, 16:543, 1975.
176. Sautter C., Microbody transition in greening watermelon cotyledons. Double immunocytochemical labelling of isocitrate lyase and hydroxypyruvate reductase, Planta, 167:491, 1986.
177. Sawaki S., Hattori N., Morikawa N., Yamada K., Oxidation and reduction of glyoxylate by lactate dehydrogenase, J. Vitaminol. (Kyoto) 13:93, 1967.
178. Schnakenburg C. von, Byrd D. J., Latta K., Reusz G. S., Graf D., Brodehl J., Determination of oxalate excretion in spot urines of healthy children by ion chromatography, Eur. J. Clin. Chem. Clin. Biochem. 32:27, 1994.
179. Schnakenburg C. von and Rumsby G., Primary hyperuxaluria type 1: a cluster of new mutations in exon 7 of the AGXT gene, J. Med. Genet. 34:489, 1997.
180. Seargeant L. E., Groot G. W. de, Dilling L. A., Mallory C. J., Haworth J. C., Primary hyperoxaluria 2 (L-glyceric aciduria): a rare cause of nephrolithiasis in children, J. Pediatr. 118:912, 1991.
181. Segel I. H., Behaviour and analysis of rapid equilibrium and steady-state enzyme systems, in Enzyme Kinetics, John Wiley, New York, 1993.

182. Senekjian H. O.and Weinman E. J., Oxalate transport by proximal tubule of the rabbit kidney, Am. J. Physiol. 243:F271, 1982.

183. Smith L. H., Bauer R. L., Williams H. E., Oxalate and glycolate synthesis by hemic cells, J. Lab. Clin. Med. 78:245, 1971.

184. Snell K., Mitochondrial-cytosolic interrelationships involved in gluconeogenesis from serine in rat liver, FEBS Lett. 55:202, 1975.

185. Snell K., Enzymes of serine metabolism in normal, developing and neoplastic rat tissues, Adv. Enzyme Regul. 22:325, 1983.

186. Snell K., The duality of pathways for serine biosynthesis is a fallacy, Trends Biochem. Sci. 11:241, 1986.

187. Sommerville C. R. and Ogren W. L., Genetic modification of photorespiration, Trends Biochem. Sci. 7:171, 1982.

188. Stafford H. A., Magaldi A., Vennesland B., The enzymic reduction of hydroxypyruvic acid to D-glyceric acid in higher plants, J. Biol. Chem. 207:621, 1954.

189. Stevens T. S. and Davis J. C., Hollow fiber ion-exchange suppressor for ion chromatography, Anal. Chem. 53:1488, 1981.

190. Sugimoto E., Kitagawa Y., Nakanishi K., Chiba H., Purification and properties of beef liver D-glycerate dehydrogenase, J. Biochem. 72:1307, 1972.
191. Suzuki S., Suga T., Niinobe S., Intracellular localisation of $NADH_2$-glyoxylate reductase in rat liver, J. Biochem. 73:1033, 1973.
192. Takada Y., Kaneko N., Esumi H., Purdue P. E., Danpure C. J., Human peroxisomal L-alanine: glyoxylate aminotransferase. Evolutionary loss of a mitochondrial targeting signal by point mutation of the initiation codon, Biochem. J. 268:517, 1990.
193. Takada Y. and Noguchi T., Subcellular distribution, and physical and immunological properties of hepatic alanine: glyoxylate aminotransferase isoenzymes in different mammalian species, Comp. Biochem. Physiol. 72B:597, 1982.
194. Tarn A. C., von Schnakenburg C., Rumsby G., Primary hyperoxaluria type 1: Diagnostic relevance of mutations and polymorphisms in the alanine:glyoxylate aminotransferase gene (AGXT), J. Inher. Metab. Dis. 20:689, 1997.
195. Thomas J. H., Abstr. 7th FEBS Meet. (Varna) Bulgarian Biochem. Biophys. Soc., Sofia, 160, 1971.

196. Thomson J. S. and Richardson K. E., Isolation and characterisation of glutamate:glycine transaminase from human liver, Arch. Biochem. Biophys. 117:599, 1966.
197. Thomson J. S. and Richardson K. E., Isolation and characterisation of an L-alanine:glyoxylate aminotransferase from human liver, J. Biol. Chem. 242:3614, 1967.
198. Titus D. E., Hondred D., Becker W. M., Purification and characterisation of hydroxypyruvate reductase from cucumber cotyledons, Plant Physiol. 72:402, 1983.
199. Tolbert N. E., Metabolic pathways in peroxisomes and glyoxysomes, Annu. Rev. Biochem. 50:133, 1981.
200. Tolbert N. E., Yamazaki R. K., Oeser A., Localisation and properties of hydroxypyruvate and glyoxylate reductases in spinach leaf particles, J. Biol. Chem. 245:5129, 1970.
201. Toussaint C., Conclusions, Nephrol. Dial. Transplant. 10 [Suppl. 8]:61, 1995.
202. Toussaint C., de Pauw L., Vienne A., Gevenois P. A., Quintin J., Gelin M., Pasteels J. L., Radiological and histological improvement of oxalate osteopathy after combined liver-kidney transplantation in primary hyperoxaluria type 1, Am. J. Kidney Dis. 21:54, 1993.

203. Ullrich K. J., Rumrich G., Fritzsch G., Klöss S., Contraluminal para-aminohippurate (PAH) transport in the proximal tubule of the rat kidney. II. Specificity: aliphatic dicarboxylic acids, Pflügers Arch. 408:38, 1987.

204. Utting J. M. and Kohn L. D., Structural, kinetic and renaturation properties of an induced hydroxypyruvate reductase from *Pseudomonas acidovorans* J. Biol. Chem. 250:5233, 1975.

205. Vandor S. L., Tolbert N. E., Glyoxylate metabolism by isolated rat liver peroxisomes, *Biochim. Biophys. Acta* 215:449, 1970.

206. Van Schaftingen E., Draye J. P., Van Hoof F., Coenzyme specificity of mammalian liver D-glycerate dehydrogenase Eur. J. Biochem. 186:355, 1989.

207. Watts R. W. E. and Mansell M. A., Primary Hyperoxaluria in Oxalate Metabolism in Relation to Urinary Stone, Springer-Verlag, London, 65, 1988.

208. Weinman E. J., Frankfurt S. J., Ince A., Sansom S., Renal tubular transport of organic acids. Studies with oxalate and para-aminohippurate in the rat, J. Clin. Invest. 61:801, 1978.

209. Williams H. E. and Smith L. H., L-glyceric aciduria: a new genetic variant of primary hyperoxaluria, N. Engl. J. Med. 278:233, 1968a.

210. Williams H. E. and Smith L. H., The identification and determination of glyceric acid in human urine, J. Lab. Clin. Med. 71:495, 1968b.
211. Williams H. E. and Smith L. H., Hyperoxaluria in L-glyceric aciduria: Possible pathogenic mechanism, Science 171:390, 1971.
212. Williams H. E. and Smith L. H., Primary hyperoxaluria, in The Metabolic Basis of Inherited Disease, 5th edn. New York, McGraw-Hill, 204, 1983.
213. Williams A. W. and Wilson D. M., Dietary intake, absorption, metabolism and excretion of oxalate, Sem. Nephrol. 10:2, 1990.
214. Willis J. E. and Sallach H. J., Evidence for mammalian D-glyceric dehydrogenase, J. Biol. Chem. 237:910, 1962a.
215. Willis J. E. and Sallach H. J., Activation of mammalian glycerate dehydrogenase by inorganic salts, Biochim. Biophys. Acta 62:443, 1962.
216. Wilson D. M. and Liedke R. R., Modified enzyme-based colorimetric assay of urinary and plasma oxalate with improved sensitivity and no ascorbate interference: reference values and sample handling procedures, Clin. Chem. 37:1229, 1991.
217. Wolffram S., Grenacher B., Scharrer E., Transport of selenate and sulphate across the intestinal brush-

border membrane of pig jejunum by two common mechanisms, Q. J. Exp. Physiol. 73:103, 1988.
218. Wolthers B. G. and Hayer M., The determination of oxalic acid in plasma and urine by means of capillary gas chromatography, *Clin. Chim. Acta* 120:87 1982.
219. Wise P. J., Danpure C. J., Jennings P. R., Immunological heterogeneity of hepatic alanine:glyoxylate aminotransferase in primary hyperoxaluria type 1, FEBS Lett. 222:17, 1987.
220. Yamakawa K. and Kawamura J., Oxalate:OH exchange across rat renal cortical brush border membrane, Kidney Int. 37:1105, 1990.
221. Yanagawa M., Maeda Nakai E., Yamakawa K., Yamamoto I., Kawamura J., Tada S., Ichiyama A., The formation of oxalate to glycolate in rat and human liver, *Biochim. Biophys. Acta* 1036:24, 1990.
222. Yendt E. R., Hyperoxaluria in idiopathic calcium oxalate nephrolithiasis, Mol. Med. J. 37:857, 1988.
223. Yendt E. R. and Cohanim M., Response to physiologic dose of pyridoxine in type 1 hyperoxaluria, N. Engl. J. Med. 312:953, 1985.
224. Yendt E. R. and Cohanim M., Absorptive hyperoxaluria: A new clinical entity-successful treatment with hydrochlorothiazide, Clin. Invest. Med. 9:44, 1986.

225. Yoshida T., Yamaguchi K., Hagishita T., Mitsunaga T., Miyata A., Tanabe T., Toh H., Ohshiro T., Shimao M., Izumi Y., Cloning and expression of the gene for hydroxypyruvate reductase (D-glycerate dehydrogenase) from an obligate methylotroph *Hyphomicrobium methylovorum* GM2, Eur. J. Biochem. 223:727, 1994.
226. Yu C. and Huang A. H. C., Conversion of serine to glycerate in intact spinach leaf peroxisomes: role of malate dehydrogenase, Arch. Biochem. Biophys. 245:125, 1986.
227. Zelitch I., The isolation and action of crystalline glyceric acid reductase from tobacco leaves, J. Biol. Chem. 216:553, 1955.
228. Zelitch I. and Gotto A. M., Properties of a new glyoxylate reductase from leaves, Biochem. J. 84:541, 1962.