A STUDY OF BIOCHEMICAL GENETIC ABNORMALITIES ASSOCIATED WITH PURINE AND PTERIDINE METABOLISM

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PUBLICATIONS

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Published papers

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Graham GW, Aitken DA, Connor JM. Methylenetetrahydrofolate reductase variant 677C→T and neural tube defects in a Scottish population. (in preparation).

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ABBREVIATIONS

+/+	Homozygous thermolabile MTHFR genotype
+/-	Heterozygous MTHFR genotype
-/-	Homozygous normal MTHFR genotype
5' MTAR	5'-methylthioadenosine
AAWD	Abdominal wall defects
ACHE	Acetylcholinesterase
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AK	Adenylate kinase
AMP	Adenosine monophosphate
AMPDA	Adenylate deaminase
AMPRT	amido phosphoribosyltransferase
AOPCP	α,β-methyleneadenosine diphosphate
APRT	Adenine phosphoribosyl transferase
ATP	Adenosine triphosphate
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
cAMP	cyclic AMP
CBS	Cystathione ß-synthase
CNS	Central nervous system
Co	Cobalt
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
$CuSO_4$	Copper sulphate
cv	coefficient of variation
CV	Chorionic villi
dADO	Deoxy-adenosine
dGMP	Deoxy-guanosine monophosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavine adenine dinucleotide
g	gram

GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HcySH	Homocysteine
HGPRT	Hypoxanthine-guaninephosphoribosyl transferase
Hinf	*Haemophilus Influenzae*
HLA	Human leucocyte antigen
HPLC	High performance liquid chromatography
I	Iodine
IMP	Inosine monophosphate
K_2HPO_4	Dipotassium hydrogen phosphate
$K_3Fe(CN)_6$	Potassium ferricyanide
kb	kilobases
KCN	Potassium Cyanide
KH_2PO_4	Potassium dihydrogen phosphate
$KHCO_3$	Potassium carbonate
K_m	Michaelis constant
KS	Kolmogorov-Smirnov
l	litre
LNS	Lesch-Nyhan Syndrome
M	mole
Mbq	Megabecquerels
mg	milligram
$MgCl_2$	Magnesium Chloride
ml	millilitre
mM	millimole
µl	microlitre
µM	micromole
µg	microgram
mRNA	messenger RNA
MS	Methionine synthase
MSAFP	Maternal serum alphafetoprotein
MTHFR	5,10 methylenetetrahydrofolate reductase
Na_2CO_3	Sodium carbonate
Na_2HPO_4	di-Sodium hydrogen phosphate
NAD	nicotinamide dinucleotide

NADP	nicotinamide dinucleotide phosphate
NaOH	Sodium hydroxide
NTD	Neural tube defect
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylene imine cellulose
pg	picogram
PNP	Purine nucleoside phosphorylase
PRPP-S	Phosphoribosyl pyrophosphate synthetase
PRPP	5'-phosphoribosyl- -1-pyrophosphate
PteGlu	Pteroyl glutamic acid
QC	Quality control
R5P	Ribose 5-phosphate
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RP	Reversed phase
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethione
SAMI	S-adenosyl-methionine
SCID	Severe combined immunodeficiency
SSCP	single-strand conformational polymorphism
Taq	*Thermophilus aquaticus*
TBAHS	Tetrabutylammonium hydrogen sulphate
TCA	Trichloroactic acid
THF	Tetrahydrofolate
TLC	Thin layer chromatography
TRIS	Trisma base
XMP	Xanthine monophosphate
XO	Xanthine oxidase

SUMMARY

Purines and pteridines (more commonly referred to as 'folates') are fundamental components in a number of reactions involved in DNA and RNA synthesis and are inextricably linked to the basic metabolic pathways of the normal functioning cell. The aim of this project was to examine the ways in which existing biochemical and molecular techniques could be used to better effect in the diagnosis of a number of genetic disorders associated with purine and pteridine metabolism. The disorders under study covered a spectrum of inheritance patterns, notably Lesch-Nyhan Syndrome (LNS) and gout (X-linked); adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) deficiency (autosomal) and neural tube defects (multifactorial).

Phase 1: Purines

Lesch-Nyhan Syndrome

The first phase of the project was an investigation of purine metabolism and as a first step, reliable reference ranges in various tissues were established which could be utilised in the diagnosis of patients with specific enzyme deficiencies. Control samples taken from subjects with no known history of metabolic disorders were analysed for the purine enzymes; hypoxanthine-guanine phosphoribosyl transferase (HGPRT); adenine phosphoribosyl transferase (APRT); ADA; PNP and phosphoribosyl pyrophosphate synthetase (PRPP-S) in various tissues, notably adult, fetal and cord red cells; cultured and fresh chorionic villi (CV); fibroblasts and cultured amniotic fluid cells. A critical analysis of the distribution parameters (Kolmogorov-Smirnov (KS) test) confirmed that all enzymes exhibited Gaussian distributions after $\log_{10}$ transformation of the data and this was carried out for all reference ranges.

The method used in this study for measurement of the purine salvage enzymes, was a radiolabelled enzyme assay with separation of the products by thin layer chromatography. This is a simple and cost effective technique and is much less labour intensive than other more sensitive detection systems such as high performance liquid chromatography (HPLC).

Over the period of the study, prenatal diagnosis by analysis of HGPRT and APRT activity was performed on 23 pregnancies in 15 women known or suspected through family history to be carriers for Lesch-Nyhan Syndrome. Three successive pregnancies were investigated in two patients, and two in each of four other obligate carriers, while nine other women had prenatal investigation in only one pregnancy. In 8 of these cases, CV was obtained in the first trimester of pregnancy and in 3 cases in the second trimester while amniotic fluid samples were obtained in the second trimester from 10 cases. No gestation was specified in 2 cases of cultured CV. Normal HGPRT/ APRT ratios were obtained in 19 pregnancies of which 13 were subsequently verified as having a normal outcome; 2 miscarried and 4 failed to have the outcome clinically verified. The remaining 4 pregnancies gave abnormal enzyme ratios with the parents opting for termination of pregnancy. HGPRT activity in the affected pregnancies was observed to be between 0.06 and 13.9% of the mean of the appropriate reference range.

A wide range of reference values was observed for HGPRT, APRT, ADA and PNP in cultured and uncultured tissues. Control ranges for HGPRT and APRT in CV tissue compared well with other reported studies on prenatal diagnosis, although a reduction in enzyme activity in CV material stored frozen which had been reported by other workers, could not be confirmed in this study. Where CV was not available, amniotic fluid cells are a reliable alternative for prenatal diagnosis, with the disadvantages of sampling in the second trimester of pregnancy and the necessity for cell culture prior to diagnosis.

Enzyme analysis were carried out postnatally on 3 male affected cases, 5 obligate carrier mothers of affected children and 33 other members of LNS families. Index cases displayed significantly reduced enzyme levels of HGPRT and the HGPRT/APRT ratio, but significantly elevated levels of ADA and PNP. HGPRT activities were observed to be 0.95 – 4.9% of the reference mean emphasising that residual levels of HGPRT are often found in affected individuals. In the obligate carriers, HGPRT was observed to be significantly different from the mean of the controls. While this reduction may be significant, the degree of overlap between the reference and control ranges, prevent this from being diagnostically useful. However this data was based on a small number of only 5 samples, further data may clarify this. The HGPRT and APRT was found to be significantly reduced in

other family members compared to the controls, but with similar problems of differentiation between the control and patient ranges.

Gout

Blood samples from 54 patients, consisting of 45 males and 9 females, presenting with gout were assayed for HGPRT, APRT and PRPP-S. A significant difference was observed in HGPRT activity in male patients only compared to the control group. The overlap in the distributions suggests that these enzymes cannot be used reliably to distinguish the patient and control populations. Uric acid levels were not evaluated in relation to the purine enzymes, since all patients were undergoing therapy for hyperuricaemia at the time of sampling, and it is unclear whether the various medication regimes employed had any effect on the levels of purine enzymes observed.

Two male patients, FFR and TGR presented with polyarticular gout, (in contrast to the remainder of the population who presented with idiopathic gout of a monoarticular nature), and gave reduced HGPRT levels of around 50% of the control mean. No significant difference was observed in PRPP-S activity between controls and patient groups indicating that PRPP-S superactivity and subsequently increased *de novo* synthesis was not a common cause of the gout. A 2 year old male patient, MHD, presenting with elevated uric acid and dyskinetic cerebral palsy gave PRPP-S activity of 3.7 times the control mean, however due to non-compliance no further investigations could be performed.

ADA and PNP Immunodeficiencies

The activities of ADA and PNP were investigated in 27 patients with suspected ADA deficiency, and in 23 patients with suspected PNP deficiency. Where possible the ADA and PNP analyses were performed in tandem on all samples from these patients presenting with suspected immunodeficiency disorder. Three children were identified with a complete deficiency of ADA activity confirming a diagnosis of ADA-deficient severe combine immunodeficiency (SCID). There was one prenatal diagnostic case based on ADA analysis of cultured amniotic fluid cells which showed that the fetus was unaffected. No evidence was found of ADA carrier levels in any of the patients tested, although 7 were identified where red cell ADA activities were above the upper limit of the reference range.

Around 50% of the cases referred for PNP investigation originated from two families. The index case from family OD was found to have a complete deficiency of red cell PNP activity with ADA activity very high at 4.8 multiples of the mean of the normal reference range. The parents had approximately half normal red cell PNP activities confirming their carrier status, and two sibs of the index case were also identified as PNP carriers on the basis of half-normal enzyme activity. Three prenatal diagnoses were carried out in this family, all based on the enzyme analysis of cultured CV. One affected fetus with zero PNP activity, one possible carrier and one unaffected fetus were diagnosed. ADA activities were found to be within the reference range in each case, and all fetuses were shown by karyotype analysis to be male.

In family FK, PNP deficiency was diagnosed retrospectively in the index case using a re-constituted fibroblast cell line after enzyme analysis in red cells from the parents had shown approximately 50% normal PNP activities, suggesting carrier status. All other patients investigated had PNP activities within the normal reference range, except for one case where carrier levels were found.

Phase 2: Pteridines

The second phase of the project involved a case-control study to examine the role played by the thermolabile mutation (677C→T) of the enzyme 5,10 methylene tetrahydrofolate reductase (MTHFR), in the development of neural tube defects (NTD) in a Scottish population. Assessment of the allelic frequency of the thermolabile variant, and the lymphocyte MTHFR enzyme activity was related to the folate and vitamin B_{12} status in both controls and in populations with a family history of NTD.

A total of 262 controls obtained from the general population, were compared to 4 groups consisting of 40 NTD affected individuals; 85 mothers who had one or more NTD affected pregnancies; 35 fathers of affected pregnancies and 36 other family members. Using PCR, the genotype frequency for the thermolabile allele (+/+) of MTHFR was 11.8% for the controls; 10.0% for the NTD individuals; 15.3% for the mothers; 8.3% for the fathers and 11.1% for the others. This translated to odds ratios (OR) of 0.9, 1.3, 0.7, 0.9 respectively. There was a significant difference ($p<0.01$) in the mothers group compared to the controls.

Lymphocyte MTHFR activities showed a marked reduction associated with the + allele. The combined data for all groups under study suggests that the specific activity in heterozygote (+/-) and +/+ individuals was around 75% and 55% of homozygous normal (-/-) levels respectively. This indicates that the + allele has approximately half of the activity of the normal allele. Consideration of the data within each study group indicated the lowest MTHFR activities were associated with the NTD group where the +/+ NTD affected group showed 21% of -/- control activity. A significant difference in enzyme activity was found for +/+ and +/- genotypes of both mothers and NTD affected individuals compared to the -/- controls. Total heat stable residual lymphocyte MTHFR activity for the -/-, +/- and +/+ genotypes was 38.4%, 37.8%, and 34.0% respectively and under the conditions of the study, the variant allele does not appear to be significantly more heat labile than the normal - allele.

Optimisation of the lymphocyte MTHFR enzyme assay was performed prior to its use in the study. This indicated that a protein and menadione concentration of 2mg/ml and 0.1mM respectively were required to allow for enough material for subsequent testing of the sample and to reduce the blank value of the assay. In addition, optimisation indicated that a 1.5 hour incubation was suitable for maximal separation of test and blank activities. Heating of the enzyme for the thermal stability work was found to be optimal at 46°C for 5 minutes, which was as quoted in the original method. Results suggest that an MTHFR assay for serum was not a viable proposition due to low levels of specific activity and the subsequent difficulties in differentiating these from background hydrolysis of the assay components.

Red cell folate results for each of the respective groups indicates that while +/+ affected , mothers and fathers show reduced folate levels of 75%, 68% and 70% respectively (with an overall reduction of 81% when the combined data for all groups were examined), these are not significantly different from the concentrations in -/- controls. Significantly raised levels of plasma vitamin B_{12} were evident in mothers and fathers of affected individuals. This could have implications on the recommended daily levels of folate and vitamin B_{12} intake for both mothers and fathers to reduce the risk for both first occurrence and recurrent neural tube defects.

In conclusion, the purine study shows the value of careful statistical assessment of the enzyme distribution prior to the establishment of references ranges. Confidence in interpretation of enzyme results in individual patients is improved, particularly in cases such as LNS where residual enzyme activities may be present, and in identification of carriers for ADA and PNP deficiencies. However, for LNS, the determination of carrier status can only be reliably achieved by molecular methods. Also, specific activity appears to be unhelpful in the assessment of the actual causes of gout, although low levels of HGPRT may be of value in differentiating cases of monoacticular and polyarticular gout.

The pteridine study provides some support for the view reported elsewhere that a common mutation of the MTHFR gene is repsonsible for the development of some cases of NTD. While the frequency of the 677C→T mutation was not found to be higher in NTD affected individuals in the Scottish population, the higher frequency found in mothers, along with low MTHFR enzyme activities and low red cell folate, suggest the maternal component may be a potential risk factor in the development of some NTDs.

Future work on purine disorders may concentrate on the analysis of individual intermediates in the salvage pathway, while the role of folate in the aetiology of NTDs, could be extended to the investigation of folate binding proteins, receptor binding and transport during pregnancy.

CHAPTER 1. INTRODUCTION

1.1 Purines and Pteridines

Purines and pteridines (referred to as 'folates') are fundamental compounds necessary for the synthesis of DNA and RNA and as such, have a pivotal role in human development, where these systems are inextricably linked in the basic synthetic and catabolic pathways of the cell. Folate co-enzymes participate in a number of critical 1-carbon transfer reactions including those involved in the synthesis and degredation of purines, where the mediation of a trifunctional peptide links the major source of these carbon units (serine) to the *de novo* synthetic reactions of the purine pathway (Figure 1 - 1).

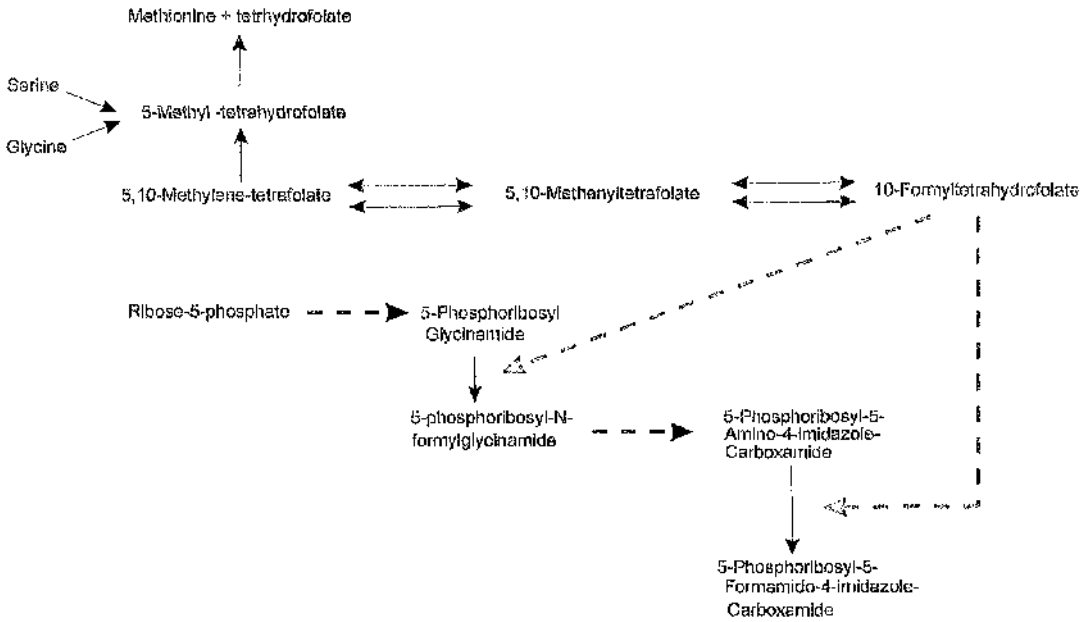


Figure 1 - 1

Relationship between the pteridine (red) and *de novo* purine (purple) metabolic pathways.

A number of other important metabolic pathways link purine and pteridines, notably those of S-adenosylmethionine (SAM) and S-adenosylhomocysteine hydrolase (SAHH). The methyl donor SAM is second only to ATP in reactions in which it serves as a cofactor, where in addition to its role in methylation of nucleic acids, proteins and lipids, is essential for the salvage of the purine adenine from the polyamine pathway, a vital precursor in the synthesis of ATP, while the enzyme S-

adenosylhomocysteine hydrolase (SAHH) is a vital component in the formation of ATP by regulation of the compound deoxyadenosine (Figure 1 - 2).

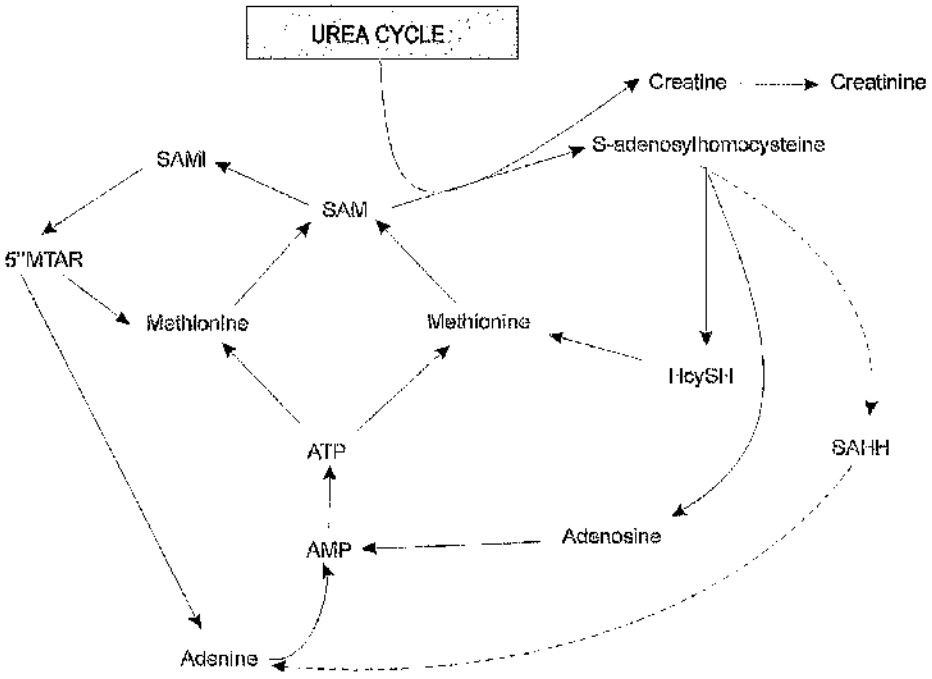


Figure 1 – 2

Relationship between folate (red) and purine (purple) metabolic pathways via the adenine salvage reactions. The dotted line indicates a potential pathway for the formation of adenine from 5-adenosylhomocysteine. Key to abbreviations not in text:- 5'MTAR = 5'methythioadenosine; SAMI = carboxylated S-adenosyl-methionine; HcySH = homocysteine.

Although few disorders of pyrimidine metabolism are known, a surprising number of genetically determined defects in the purine salvage pathway and folate-dependent homocysteine pathway have been identified. A variety of chemical consequences from major congenital malformations to mild or asymptomatic disease may associated with metabolic errors in these pathways.

This study focuses on analysis of the enzymic defects and examines their use in diagnosis, prenatal diagnosis and carrier detection.

1.2 Purines

1.2.1 Metabolism

It has long been recognised that within many biological processes, the role of purines is a crucial one. By far the most important occurrence of purines is in the nucleotides and nucleic acids, compounds which perform some of the most essential functions in metabolism.

Purines play a vital part in intracellular metabolic processes as diverse as energy stores; the driving of chemical processes (AMP, ADP, ATP); membrane signal transduction (GTP, cAMP); cofactors for enzymic reactions (NAD, NADP, FAD) and when combined with pyrimidine molecules, providing the basic building blocks for the deoxyribo and ribonucleic acids, DNA and RNA.

Purine molecules are low molecular weight compounds consisting of a base (adenine or guanine) combining with a pentose sugar of either D-ribose or 2-deoxy-D-ribose, through a glycosidic C-N linkage at position 9 of the base, forming the nucleoside structure (e.g. hypoxanthine, xanthine) . This nucleoside can be further bonded by the C 5 oxygen to one, two or three phosphate units to form the high energy purine nucleotides such as ATP (Figure 1 - 3).

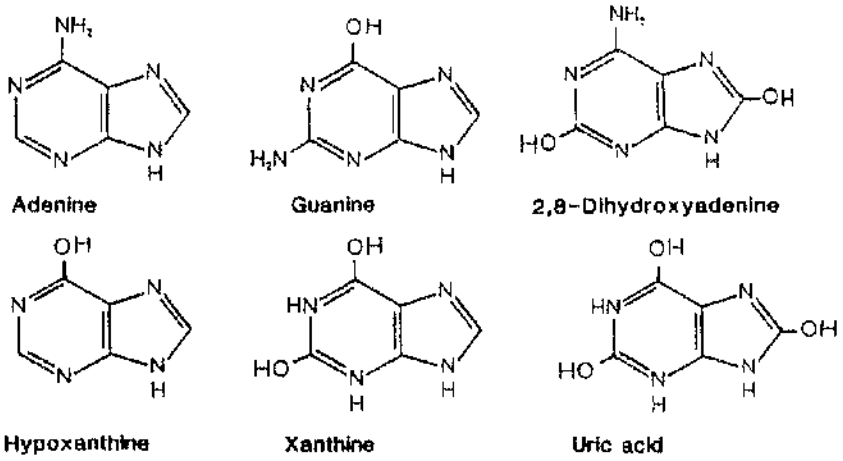
Since purines are required by all cells for survival and growth, there exists within these metabolic processes, a number of pathways for the production of new purines from simple precursor molecules; the interconversion and catabolism of nucleotides and the salvage and re-use of existing purine molecules.

1.2.2 *De novo* production

The 'de novo' biosynthesis of purines involves 11 enzymic reactions , from which the purine ring is constructed from small component molecules beginning with α-D-Ribose 5-phosphate (R5P) and ending with the formation of Inosine mono-phosphate (IMP) (Buchanan and Hartman 1959; Gutman and Yu 1965).

The compound 5-phosphoribosyl-α-1-pyrophosphate (PRPP) is generated from the pyrophosphorylation of R-5-P by the enzyme PRPP- synthetase at the expense of ATP. All in all, 9 high-energy phosphate groups are utilised in the formation of a single molecule of IMP.

a)



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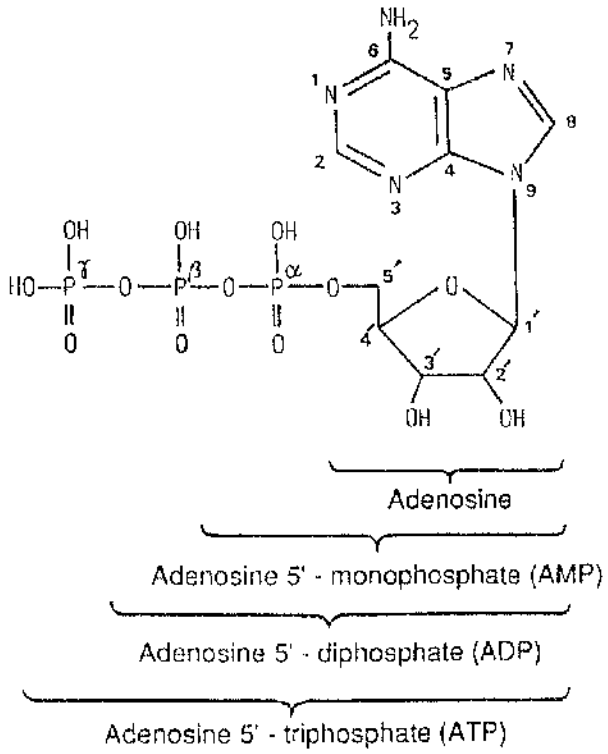


Figure 1 - 3

a) Structural formulae of purines. b) Structural formulae of adenosine, indicating the numbering of the purine and sugar rings and the phosphate groupings in AMP, ADP and ATP. (Diagram taken from Stone and Simmonds 1991).

PRPP is considered to be a key regulatory substrate in the *de novo* purine pathway since its irreversible condensation with L-glutamine to generate 5-β-phosphoribosyl-1-amine catalysed by the enzyme amidophosphoribosyltransferase (AMPRT), is the first committed step in the formation of IMP (Holmes 1980) (Figure 1 - 4).

All newly synthesised purines are ultimately derived from IMP which serves as a branch point for alternative biosynthetic pathways. There appears to be two distinct levels of regulatory mechanisms involved in the *de novo* pathway:-

a) Regulation of the pathway controlling the synthesis of IMP
b) Regulation of the formation of the branch pathways to the adenylates and guanylates.

The enzyme is inhibited at two distinct allosteric sites by the adenylates (AMP,ADP ATP); and the guanylates (GMP,GDP and GTP), as well as IMP. The inhibition appears to be maximal when both groups of nucleotides are present, promoting the formation of a catalytically inactive aggregate (Holmes *et al* 1973).

Cellular levels of PRPP are governed by the relative activity of PRPP-synthetase and the rate of PRPP utilisation. PRPP also activates AMPRT , with high levels of PRPP driving the reaction forward. The other level of regulation of the *de novo* pathway involves the interconversion and catabolism of IMP to AMP and GMP the respective precursors of ADP, ATP GDP and GTP. Each of the end products of these branch pathways, stimulates the other by serving as the reactive phosphate donor. In addition, AMP and GMP inhibit their respective salvage enzymes, thus directly promoting nucleotide catabolism.

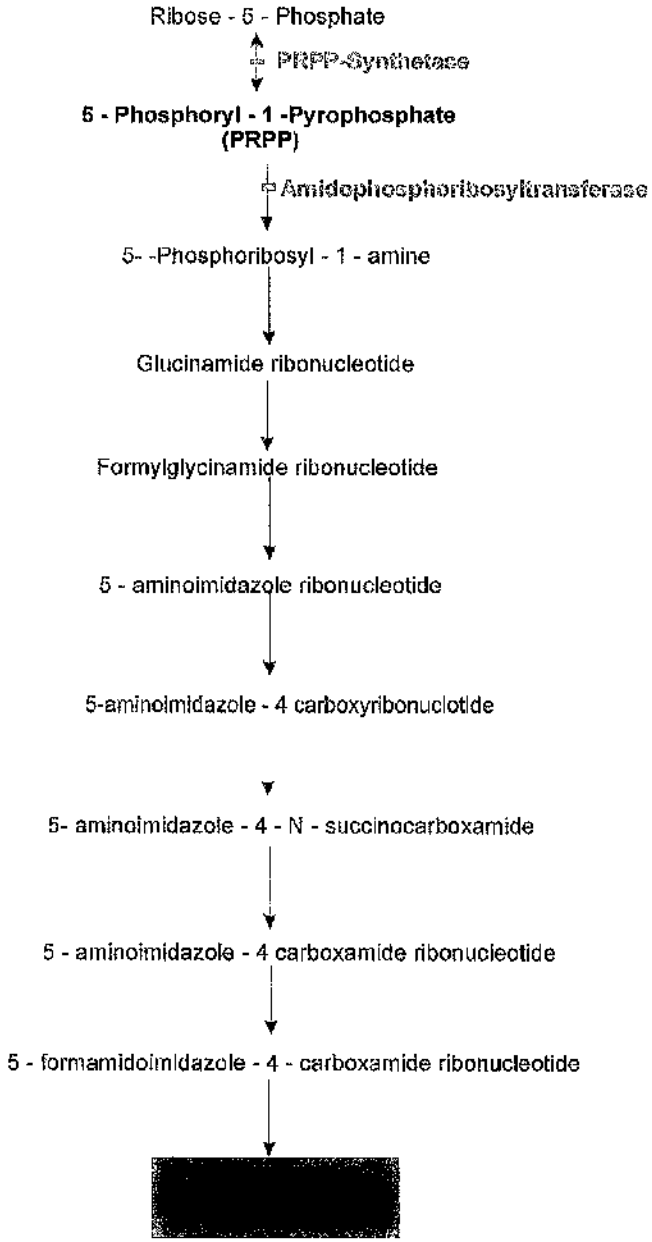


Figure 1 - 4

De novo pathway of purine synthesis indicating the position of PRPP-synthetase (PRPP-S) and amidophosphotransferase (AMPRT).

1.2.3 Catabolism

Catabolism of purine nucleotides involves both the polynucleotides, DNA and RNA as well as the adenine and guanine based nucleotides, ATP and GTP. Mononucleotides released from cells following ischaemia, cell death or extrusion of the erythrocyte nucleus during maturation are further degraded to the purine bases, hypoxanthine or guanine, by a number of different systems.

Adenine based ribonucleotides are deaminated principally via the enzyme adenylate deaminase (AMPDA) to IMP, while the adenine deoxyribonucleotides which cannot be metabolised by this route and must be dephosphorylated by 5'-nucleotidase and deaminated by adenosine deaminase (ADA). Both of these products are ultimately converted to Inosine or deoxyinosine and finally to hypoxanthine by purine nucleoside phosphorylase (PNP). This is in contrast to adenosine which is preferentially converted to AMP by adenosine kinase (AK) because of the Michaelis constant (K_m) for adenosine which favours deamination.

The nucleotides GMP and dGMP are also degraded to the corresponding nucleosides guanosine and deoxyguanosine by 5'-nucleotidase, and subsequently xanthine prior to excretion.

1.2.4 Purine salvage

During normal metabolic processes, the degredation of nucleic acids by nucleases releases nucleotides which undergo enzymic hydrolysis to yield , utimately , the free purine (and pyrimidine) bases.

The salvage pathway (in concert with the processes of nucleotide interconversion) ensures the efficient utilisation of preformed purines, thus conserving much of the cellular energy which might otherwise be consumed in the synthesis of new purines. A small amount of purines catabolised daily are normally excreted, predominantly as uric acid, while the vast majority are actively recycled via this pathway by the action of the phosphoribosyl transferase enzymes, principally hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and adenine-phosphoribosyl transferase (APRT). Another salvage pathway, involves the sequential actions of PNP to convert purine bases to nucleosides, and then of nucleoside kinases, for further phosphorylation to nucleotides. (Murray *et al* 1970).

Other enzymes involved in the reactions include 5' nucleosidase and ADA. The enzymes 5' nucleotidase and PNP are involved in the production of purines that can be either recycled or catabolised to uric acid and excreted. (Figure 1 - 5).

In the early part of this century, Archibald Garrod coined the term' inborn error of metabolism' to describe disruptions of the normal metabolic pathways by the inherited deficiency of an enzyme. While this definition has been modified in the intervening years the general premise of the specific, monogenic nature of these disorders still holds true.

Since all metabolic processes are carried out by these enzymes and are under genetic control, it is possible that some individual will lack any given enzyme because of a genetic defect. Although these specific disorders are individually rare, there are many enzyme pathways and therefore an enormous potential for this enzymic derangement. In specific enzyme deficiencies, substrates will accumulate and at high levels may be converted to alternative products not usually present. Consequently the normal end products of the reaction may be deficient. Since many of these biochemical abnormalities are expressed in a variety of tissues, this allows the diagnosis of purine metabolic disorders to be performed in material as varied as red cells, amniocytes , fibroblasts and cultured or uncultured chorionic villi. Ultra-micro methods of analysis allow for the use of small incubation volumes, favourable signal to noise ratios and long incubation times in the measurement of the low enzyme activites present in affected individuals. These assays may be utilised for small numbers of uncultured cells thus reducing the time taken to perform a prenatal diagnostic assay, or in cases where little cell material is available, in cultured cells in order that confirmatory analysis may be performed.

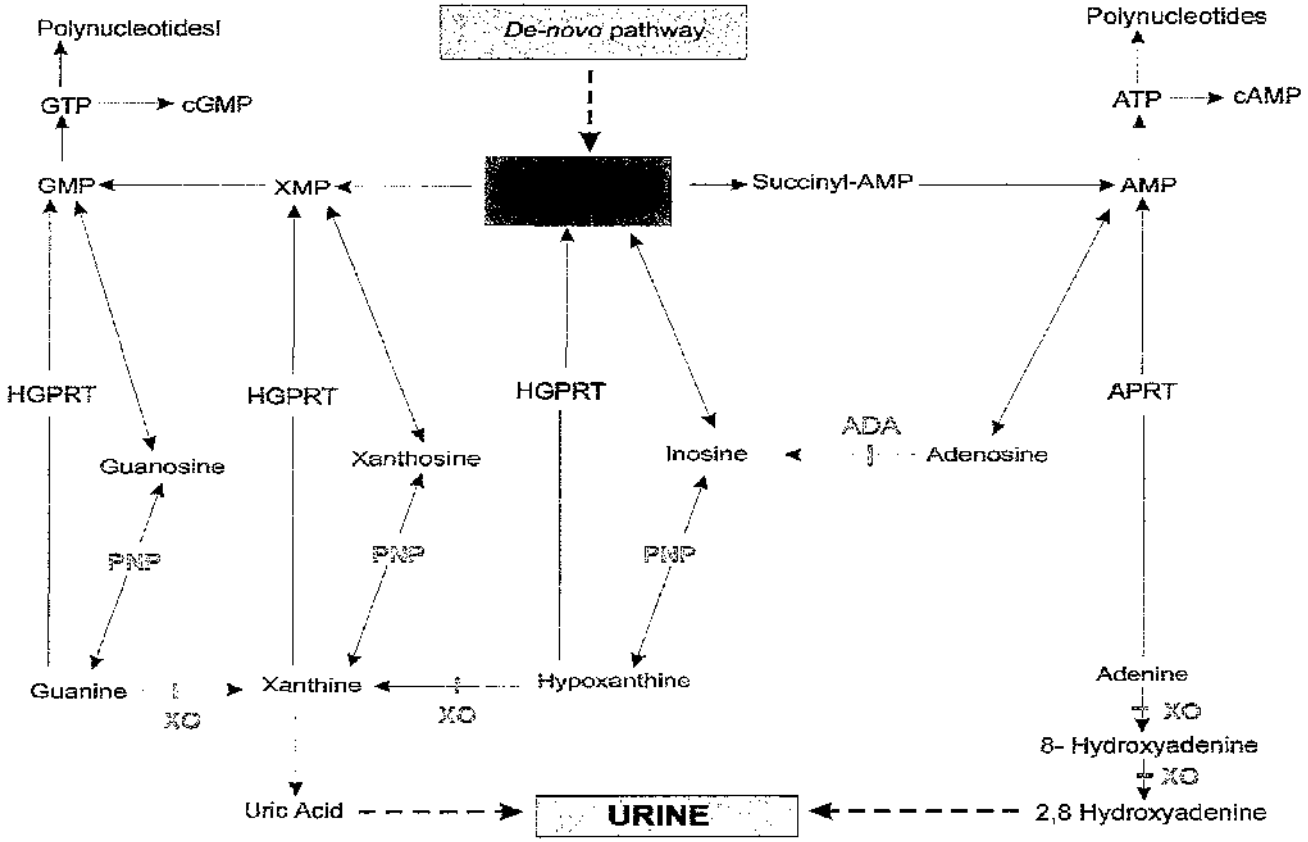


Figure 1 - 5.

Purine salvage pathway

Key to enzymes:- PNP- Purine nucleoside phosphorylase; HGPRT - Hypoxanthine-guanine phosphoribosyl transferase ; XO - Xanthine Oxidase ;APRT - Adenine phosphoribosyl transferase ; ADA - Adenosine deaminase. ; XMP = Xanthine monophosphate.

1.2.5 Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT; HPRT; IMP: pyrophosphate phosphoribosyltransferase; inosinate-guanylate:pyrophosphate; E.C. 2.4.2.8) is a soluble cytoplasmic enzyme widely distributed in all tissues, with the highest activity in found in rapidly dividing tissues such as brain and testes (Kelley and Wyngaarden 1983; Watts *et al* 1987). It catalyses the reaction of purine bases and PRPP to form purine ribonucleotides. The human enzyme has been highly purified and studied extensively with regard to its biochemical characteristics (Bakay and Nyhan 1975). Due to the ease in obtaining material, most of the definitive work has been done on red cells (Krenitsky *et al* 1969; Olsen *et al* 1977)) and has shown the enzyme to be a tetramer composed of identical subunits each consisting of a molecular weight of 24,500 (Holden and Kelley 1978) . The 217 residue amino acid sequence was subsequently elucidated by Wilson *et al* (1982b).

HGPRT binds both 6-oxo or 6-thio compounds such as xanthine, allopurinol, 6-mercaptoethanol, 6-thioguanine as substrates in addition to hypoxanthine and guanine which have a Michaelis constant in erythrocytes of 1.7×10^{-5} and 5×10^{-6} respectively. Kelley *et al* (1967) and Seegmiller *et al* (1967) postulated that the reactions of hypoxanthine and guanine were catalysed by a single enzyme due to identical rates of heat inactivation, electrophoretic mobilities under several conditions and the observation that the loss of one reaction was always accompanied by the loss of the other. Henderson *et al* (1968) substantiated this work by showing on kinetic studies of velocity, product inhibition and isotope exchange of HGPRT that PRPP , the only compound known to serve as a donor for the phosphoribosyl moiety in the reaction exhibited the same Michaelis constants, 2.5×10^{-4} irrespective of whether hypoxanthine or guanine was used as a substrate.

1.2.6 HGPRT Deficiency

A deficiency of the enzyme HGPRT is associated with two clinical syndromes in humans. An almost complete deficiency of the enzyme results in the Lesch-Nyhan Syndrome (Lesch and Nyhan 1964), while a partial deficiency is manifested by a

severe form of gout, with hyperuricaemia and excessive production of uric acid (Seegmiller *et al* 1967; Kelley *et al* 1967). While most patients with the classical syndrome exhibit almost no HGPRT activity, McDonald and Kelley (1971) reported red cell and fibroblast HGPRT activities of between 8% and 34% of normal when assayed with a 10mM concentration of magnesium 5-phosphoribosyl-1-pyrophosphate, as opposed to the normal concentration of 1mM. This ten-fold increase in the PRPP concentration and the increased Michaelis constant for hypoxanthine and guanine, provided the first evidence of the genetic heterogeneity in patients with Lesch-Nyhan Syndrome.

1.2.6.1 Lesch-Nyhan Syndrome (LNS)

LNS is an X-Linked disorder clinically manifested by hyperuricaemia excessive production of uric acid and characteristic neurological features including choreoathetosis, spasticity, mental retardation and self mutilation (Lesch and Nyhan 1964).

The clinical abnormalities associated with HGPRT deficiency are absent at birth (Marks *et al* 1968). Patients may present with hypotonia, recurrent vomiting, difficulties with secretions and delay in motor development which appears at the first 3 to 4 months of life. By the first year, extrapyrimidal signs such as dystonia, chorea, hyper-reflexia and scissoring of the legs begin to appear. A striking feature of LNS is the compulsive self-destructive mutilating behaviour observed between 2 and 16 years of age. Patients exhibit a compulsive tendency to self injurious behaviour observed in 85% of cases, and frequently it is this symptom which results in the correction of initial diagnoses of cerebral palsy (Mizuno *et al* 1970). This behaviour may be so extreme as to warrant restraint of the individual to prevent self injury of the arms or fingers by biting. Patients may also exhibit unusual aggressive behaviour towards others, although the degree to which this self mutilation and aggressiveness occurs may be quite variable, with patients showing mutilative behaviour for weeks or even months at a time before returning to normal behavioural patterns. The degree of self mutilation can also vary on a day to day basis, and may be exacerbated by aggravating environmental events. Patients with partial deficiency of HGPRT do not exhibit mutilatory behaviour. Geerdink *et al* (1973) described an atypical case of an adult patient with gout, but without any demonstrable HGPRT activity in red cells, lymphocytes of fibroblasts.

Few of the neurological abnormalities generally associated in this disease were evident, until further psychological tests disclosed some of the behavioural anomalies associated with LNS , albeit in a concealed form.

The mechanism by which a decrease in HGPRT activity produces the neurologic dysfunction observed in the LNS remains unclear. Rosenbloom *et al* (1967b) demonstrated normal concentrations of uric acid in the CSF of patients examined and the failure of anti-hyperuricaemic therapy to alter CNS function. While elevated concentrations of oxypurines occur in patients with LNS, the finding of similar levels of hypoxanthine and xanthine in the CSF of patients with a partial deficiency of HGPRT, and with no evidence of CNS dysfunction suggests that these compounds are in themselves not responsible for the CNS disease.

While routine studies of cerebral spinal fluid have been reported to be normal in these patients, the levels of specific neurotransmitters and their metabolites have been reported to be altered in cases of LNS (Lloyd *et al* 1981). Other studies have demonstrated decreased levels of homovanillic acid and 3-methoxy-4-hydroxy phenylethelene glycol, suggesting that reduced dopamine and epinephrine turnover may be important in the pathogeneses of the disease (Jankovic *et al* 1988; Silverstein *et al* 1985).

Several animal models which have been developed to examine the neurobiological substrates of this disorder. Jinnah *et al* (1994) described the use of HGPRT deficient mice which had similar, albeit more subtle changes in brain dopamine function and the relationships to the neurological deficits observed in patients with LNS.

Hyperuricaemia is common in patients with a partial or complete HGPRT deficiency and is associated with an markedly increased rates of *de novo* purine synthesis. Hypoxanthine is rapidly converted by HGPRT deficient cells to uric acid, which is excreted dramatically in the range of 24-184 mg/kg body weight per 24 hours (the normal excretion rate being about 1-14 mg/kg of body weight per 24 hours). Patients with a partial deficiency of HGPRT excrete uric acid at levels approaching those observed in LNS patients.

Haematological abnormalities have been associated with the Lesch-Nyhan Syndrome, in particular a number of studies have shown the presence of macrocytic red cells in the peripheral blood and megaloblastic changes in the bone marrow of LNS individuals.

1.2.6.2 Genetics

The initial suggestion that the Lesch-Nyhan syndrome was an X-linked disorder with affected male offspring and carrier females, was provided by Lesch and Nyhan on pedigree analysis in their original report in 1964, and subsequently confirmed by Hoefnagal *et al* (1965), while the finding of patients with a partial deficiency of HGPRT, and who were able to reproduce, enabled Kelley *et al* (1970), to subsequently confirm the X-linked nature of the disease. The studies of (Rosenbloom *et al* 1967$_{a}$) and (Migeon *et al* 1968) demonstrated that obligate heterozygotes for the enzyme defect are mosaics in terms of HGPRT activity and in addition to supporting the X-linked hypothesis, were consistent with the Lyon hypothesis. The incidence of Lesch-Nyhan syndrome is as yet unclear, but has been estimated at between 1:100,000 and 1:380,000 (Seegmiller 1976; Stout and Caskey 1985a).

The use of mouse-human somatic cell hybridisation, demonstrated the location of HGPRT gene to the long arm of the X chromosome, while subsequent mapping using X-autosome translocations in somatic cell hybrids localised it to position Xq26-q27 (Pai *et al* 1980).

In 1968, Hooft *et al* reported a three year old girl with self mutilating behaviour , athetoid movements and mental retardation. The patient had a high urinary uric acid excretion rate , but a normal serum uric acid level. However since HGPRT activity was not measured, there was no confirmatory data on whether this case was LNS.

Hara *et al* (1982) reported a female patient with the characteristic symptoms of LNS, including self mutilation, athetoid movements and mental retardation, in addition to hyperuricaemia, hyperuricosuria and decreased HGPRT activity. Further work on this patient by Ogasawara *et al* (1989) proposed that this disease was caused by a microdeletion of the active maternal X chromosome and inactivation of the HGPRT gene on the paternal X-chromosome. A second patient was described by Yukawa *et al* (1992) with the clinical symptoms of LNS, and with reduced HGPRT and raised APRT levels. The patients family were non-consanguineous, and there were no other family members with LNS, gout or any other psychoneurological disorder. Since the family situation precluded a family study, it was assumed that this case represented a spontaneous mutation.

1.2.6.3 Molecular studies

The cloning of cDNA for the human HGPRT was simplified by the isolation of a mouse neuroblastoma cell-line, NBR4 which expressed high levels of a mutant HGPRT gene (Melton 1981), and subsequently paved the way for differential hybridisation strategies to isolate the first HGPRT cDNA recombinants (Brennand *et al* 1982. Jolly *et al* (1982) using transfection recombinants in mice cells deficient in HGPRT, succeeded in isolating a genomic clone partially encoding human HGPRT, and a year later identified a nearly full length human HGPRT coding sequence by using HGPRT-specific clones in a cDNA library (Jolly *et al* 1983). The isolation of these cDNA recombinants permitted the characterisation of the human HGPRT gene structure which is 44kb long and is composed of 9 exons and 8 introns (Patel *et al* 1986). There are 4 non expressed pseudogenes for HGPRT in human DNA , located on chromosomes 3, 5 and 11 (Dobrovic *et al* 1987; Patel *et al* 1984).

Initially, HGPRT mutations were described on the basis of altered protein or enzymic characteristics , however this approach was limited to those mutants with residual HGPRT activity and excluded those patients with the Lesch-Nyhan Syndrome who exhibited no noticeable activity. The elucidation of the normal HGPRT amino acid sequence allowed structural alterations to be detected by comparative mapping of tryptic peptides and the sequences of these aberrant peptides were determined by manual Edman degredation reactions on protein purified from human red cells (Wilson *et al* 1982a; Wilson *et al* 1982b) .

It was not evident at this time whether the measured lack of HGPRT activity in LNS originated at transcription, with unstable mRNA being transcribed or at translation resulting in the production of a defective enzyme. Prior to the complete elucidation of the nucleotide sequence, Yang *et al* (1984) utilised 4 different restriction endonucleases to examine the genomic DNA from 28 unrelated patients with LNS Results showed that the DNA from 23 patients exhibited a restriction pattern identical to that of the control while the remaining 5 exhibited distinct differences which were reflected in the relative amounts of HGPRT mRNA. This demonstrated for the first time that molecular methods could be used to trace the origins of new mutations. Wilson *et al* (1986) subsequently demonstrated that the deficiency of enzyme in lymphocyte cell line of 24 unrelated patients, was due to an abnormality in enzyme function rather than a deficiency in enzyme structure itself.

The discovery that the mutations in the HGPRT gene were heterogeneous in nature, provoked a surge of activity to define the mutations and their effect on HGPRT function and structure, using a number of novel approaches.

Using the technique first applied by Jolly *et al* (1983) , a number of single base substitutions were defined as HGPRT $_{Flint}$ (Davidson *et al* 1988); HGPRT $_{Ann\ Arbor}$ (Fujimori *et al* 1988); HGPRT $_{Yale}$ (Fujimori *et al* 1989) and HGPRT $_{Ashville}$ (Davidson *et al* 1989a).

Gibbs and Caskey (1987) used a method involving ribonuclease A cleavage with a poly uridinylic acid-paper affinity chromatography step to identify mutational lesions in the HGPRT mRNA of patients with Lesch-Nyhan Syndrome. This method was based on the fact that some single base pair mismatch sites in RNA hybrids with RNA or DNA will be cleaved by RNAase A. Despite its initial promise, the technique only managed to detect 5 mutations out of 14 patients.

A further refinement on these methods has been the ability to automate the processes used in the work on the HGPRT gene. Gibbs *et al* (1989) used automated direct DNA sequence analysis of amplified HGPRT cDNA to detect and characterise 15 independent mutations, including base substitutions, deletions, base insertions and RNA splice errors. Further work by Gibbs *et al* (1990) demonstrated that multiple oligonucleotide primer sets could be constructed for simultaneous in-vitro analysis of all nine HGPRT exons and the products of this multiplex PCR could be analysed by direct automated fluorescent DNA sequencing the identify subtle alterations in the gene.

Tarle *et al* (1991) determined mutations of 17 previously uncharacterised cell lines in 16 patients with LNS using the PCR amplification and direct sequencing method of Davidson *et al* (1989b). In this method, PCR amplified DNA was directly sequenced by denaturing the double stranded and amplified cDNA, annealing the end-labelled sequencing primer to one strand of the DNA and allowing chain termination to proceed through multiple stages of denaturing, annealing and extension. Using this method, (Davidson *et al* 1991) reported that in a study involving 22 subjects in 17 unrelated kindred, a clustering of mutations was evident, particularly in the evolutionarily conserved regions predicted to be involved in hypoxanthine guanine and PRPP binding.

Fujimori *et al* (1990) described an interesting case involving the first nucleotide substitution observed in the Japanese population. A nucleotide substitution at codon 51 (CGA TO TGA) was found to be the same codon but a different

nucleotide substitution to that of $HPRT_{Toronto}$ as described by Wilson *et al* (1983). Whereas the mutation at $HPRT_{Toronto}$ produced a CGA to GGA mutation and a partial deficiency of HGPRT activity, $HPRT_{Fujimi}$ produced symptoms of the Lesch-Nyhan syndrome. This report provided for the first time evidence that two different nucleotide substitutions at the same base position of the human HGPRT gene lead to the generation of two different mutant alleles.

A novel mutation in LNS was reported by Yang *et al* (1988) which showed a duplication of exons 2 and 3 and a second reversion event where most of the duplicated portion of the mutant gene was deleted. This may have accounted for the mild symptoms of the LNS exhibited by this patient.

The examination of a spectrum of phenotypes from classic LNS to clinical gout, and including a number of the more unusual variants of the disease, was carried out by Sege-Peterson *et al* (1992) using automated DNA sequencing of PCR-amplified HGPRT cDNA. This study examined 22 unrelated individuals and a diverse pattern of mutations accounting for the majority of the abnormal HGPRT genes.

1.2.6.4 Carrier Detection

Female carriers of the LNS cannot be reliably detected by clinical evaluation. Attempts at carrier detection by biochemical methods exploited the fact that the Lyon hypothesis postulates inactivation of one of the two X-chromosomes. Cells should therefore be encountered that are either HGPRT positive or HGPRT negative in women heterozygous for HGPRT deficiency, depending on whether the X chromosome carrying the mutant HGPRT gene is the inactive or active X. Dancis *et al* (1969) and Migeon (1970) evaluated this method of carrier detection using skin fibroblasts in which heterozygous females were detected by autoradiographic demonstration of mosaicism of mixed populations of $HGPRT^+$ and $HGPRT^-$ cells. As an alternative to fibroblasts, hair follicles may be used as the majority of hairs arise from a single cell and are therefore clonal in origin. Gartler *et al* (1971) demonstrated that this method for determining carrier status is rapid and relatively inexpensive, but is by no means infallable and that the methodology is subject to a number of pitfalls, a conclusion supported by a number of other studies. (de Bruyn *et al* 1973; de Bruyn *et al* 1974; Silvers *et al* 1972).

An improved biochemical assay for HGPRT heterozygotes was described by Page *et al* (1982) whereby thin-layer chromatography and autoradiography was used on hair roots to provide a fast and sensitive method.

However, despite all previous biochemical methodologies for heterozygote analysis of HGPRT, by far the most reliable method for carrier detection is in instances where DNA analysis is diagnostic. This occurs when the mutation can be detected directly or when an RFLP is informative for the offspring of known heterozyzgotes. When informative, DNA analysis circumvents the problems associated with biochemical tests based on X - inactivation.

1.2.6.5 Prenatal diagnosis

Prenatal diagnosis was first reported by Fujimoto *et al* (1968) who presented evidence that the disease could be diagnosed in the fetus, before 20 weeks gestation, using a method of ^{3}H-hypoxanthine incorporation into amniotic fluid cells. Further workers subsequently reported successful prenatal diagnoses of affected fetuses in the second trimester using amniotic fluid fibroblasts (Singh *et al* 1976; Van Heeswijk *et al* 1972).

Hosli *et al* (1977) successfully used a biochemical ultramicroassay proce dure for prenatal diagnosis of a 15 week male fetus and confirmed the diagnosis on fibroblasts and erythrocytes after termination of pregnancy.

Since diagnosis of LNS based on amniocentesis cannot be carried out before about 15 weeks gestation and cell culture requires a further 2-3 weeks, this leads to the late termination of the affected fetus. This is a very distressing situation for the families concerned, and so alternative procedures were sought.

The first instance of prenatal diagnosis for LNS in the first trimester of pregnancy was reported by Gibbs *et al* (1984), who used chorionic villi (CV) taken at 8-9 weeks gestation in four pregnancies at risk for the disease. Radiochemical assay of HGPRT showed that 3 fetuses were affected, the diagnosis confirmed by analysis of culture fibroblasts after therapeutic abortion in 2 cases and spontaneous abortion in one.

Stout *et al* (1985b) confirmed the advantages early sampling LNS and demonstrated that other enzymes in the metabolic pathway, such as ADA and PNP also appeared amenable to early prenatal diagnosis by this method.

A number of other workers have reported their experience with prenatal diagnosis of LNS in both the first and second trimesters of pregnancy. Zoref-Shani *et al* (1989) reported results of between 5.5-17% of residual HGPRT activities in tissues at-risk of LNS.

A note of caution was introduced by the work of Page and Broock (1990), who drew attention to the possible deleterious effects of long term storage on CV samples, which appear to show reduced HGPRT activities when reassayed. This fact coupled with the presence of substantial residual activities and the relatively low specific activities associated with CV material may lead to difficulties in interpretation of results for pre-natal diagnosis.

1.2.6.6 Hyperuricemia and Gout.

Gout may be regarded as a heterogeneous group of genetic and acquired metabolic aberrations, with many factors promoting and influencing the saturation and deposition of monosodium urate and uric acid crystals in the tissues of individuals.

Kelley *et al* (1967) demonstrated this partial HGPRT deficiency in 5 male patients from 2 different families, with gout, and demonstrated that the characteristics of relative activity and heat stability of the defective enzyme showed similarities within each family, but differed when other families were tested.

Hyperuricaemia in these patients frequently leads to uric acid crystalluria, nephrolithiasis, and obstructive nephropathy. While patients with complete or partial HGPRT deficiency both have hyperuricaemia, it is unusual for patients with LNS to develop gouty arthritis. In contrast, greater that 80% of patients with a partial deficiency, will eventually go on to develop gouty arthritis during the course of the disease. Treatment of the hyperuricaemia can be adequately controlled by the drug allopurinol which inhibits xanthine oxidase activity. The deposition of monosodium urate and uric acid crystals in the tissues of individuals as a result of the saturation of the extracellular fluids with urate, constitutes the main pathophysiological symptom defining gout.

Serum urate concentration varies with age in patterns that are distinctive in men and women. During childhood, men exhibit levels of 3 to 4 mg/100mls which rise through puberty (Munan *et al* 1977) and remain relatively constant thereafter,

while women show little change in this level until the menopause when values approach those of adult men. (Becker *et al* 1995a).

Hyperuricemia is defined as a serum urate concentration exceeding 7.0mg/100mls in men and 6.0mg/100mls in women. Serum values in excess of 7.0mg/100mls confer an increased risk for gouty arthritis (Hall *et al* 1967) and are influenced by factors such as age, sex, body weight, blood pressure and ethnicity. The majority of hyperuricaemic individuals never manifest the clinical features of gout and moderate hyperuricaemia appears to confer no substantial independent risk for the development of chronic renal insufficiency (Langford *et al* 1987; Yu *et al* 1982). The range of clinical manifestations in gout is narrow and includes recurrent attacks of characteristic acute inflammatory arthritis (acute gouty arthritis); accumulation of potentially destructive and deforming crystalline aggregates in connective tissues (tophi) and uric acid urolithiasis. The incidence of gout is estimated at 0.2 to 0.35 per 1000 births with and overall population prevalence of 2 to 2.6 per 1000 (Zalokar *et al* 1981). Only in a minority of hyperuricaemic individuals do the symptoms of gout appear and usually after sustained hyperuricaemia over a number of years. As an untreated disorder, the disease process of gout follows 3 main stages, acute gouty arthritis, intercritical gout and chronic tophaceous gout.

Episodes of painful inflammatory arthritis are the most common manifestations of gout. They are monoarticular in 80-90% of cases with around 50% involving the first metatarsophalangeal joint (Grahame and Scott 1970), although other target joints include midtarsal joints, ankles, wrists and interphalangeal joints. Occurrence of gouty arthritis in men tends to be around 40 to 60 years of age and around 60 to 80 years in women (Lally *et al* 1986; Grahame *et al* 1970). Attacks of gouty arthritis appear to be incited by a range of circumstances, including trauma, excessive ingestion of alcohol of purine rich foods, or certain drugs. In addition to extracellular fluid urate supersaturation, a number of additional factors may contribute to regulate the urate crystal deposition in tissues, including lower temperature, and local increases in urate concentrations in joint structures (Becker *et al* 1995a).

Intercritical gout is described as the intervening periods between the remission and onset of gout attacks. During an intercritical period, the establishment of gout in an individual may be difficult due to the heterogeneous nature of the condition, where with repeated recurrences, the intercritical periods generally diminish in length.

Becker *et al* (1995a) showed that of a series of individuals with gout, only 62% had second attacks within the first year and 78% within 2 years, while 7% were free of recurrences for 10 years or more

An untreated patient may experience a series of severe, longer lasting and polyarticular attacks with sometimes resulting in the disappearance of the intercritical remission stage. Accompanying this, deposition of solid urate crystals or tophi, often occur in articular and other areas of connective tissue, with the accompanying end result of destructive arthropathy. Tophaceous gout is usually associated with an early age of onset, frequent attacks, a high serum urate and a history of upper extremity and polyarticular episodes (O'Duffy *et al* 1975; Nakayama *et al* 1984). Elderly patients in particular women, who may be receiving urico-retentive diuretics and anti-inflammatory drugs, and who may have a mild renal insufficiency, may also be at higher risk for tophus formation and polyarticular gout (Lally *et al* 1986).

1.2.6.7 Diagnosis and Treatment

While a complete deficiency of HGPRT is responsible for LNS, a partial deficiency of this enzyme is responsible for the onset of juvenile or gouty arthritis with only a minority of patients suffering major neurological symptoms. (Kelley *et al* 1969). On the basis of erythrocyte HGPRT activity, a number of studies could not differentiate between patients with LNS and gout (Emerson and Thomson 1973; de Bruyn *et al* 1976). The severity of the neurological symptoms is thought to be related to the enzyme activity but it is uncertain whether these differences in the phenotypic expression of HGPRT deficiency are accompanied in differences in purine metabolism. In order to clarify this, Mateos and Puig (1994) studied the enzyme activities, plasma and oxypurine concentrations and adenine nucleotide degradation in patients with LNS and gout.

These workers found increased purine overproduction and adenine nucleotide degradation compared with normal subjects, suggesting that if the HGPRT activity determines the phenotypic expression, the enzyme defect appeared to be accompanied with an increased purine over production. These workers found similarly decreased HGPRT and increased APRT levels, as well as no substantial difference in plasma and oxypurine levels between LNS and gout patients and as

such could not reliably be used to differentiate between these HGPRT deficient groups.

The aim of treatment in gouty arthritis is specifically to terminate the attack as quickly and effectively a possible, in addition to preventing recurrences and complications resulting from the urate deposition in joints and other soft tissues.

Treatment must be targeted at the effective management of both the acute inflammatory response and the accompanying hyperuricaemia, since the drugs used in reducing the acute inflammation have no value in controlling the hyperuricaemia.

Acute gouty arthritis is usually responsive to a broad array of non-steroidal drugs such as ibuprofen and indomethacin, with the most complete resolution of the symptoms occurring the earlier the commencement of the treatment (Wallace and Singer 1988).

Prophylactic measures may be effective in reducing the frequency of recurrent bouts of gouty arthritis in patients with a prior history of gout, with one of the most widely used methods being the twice daily administration of 0.6mg oral colchicine, or 25mg indomethacin in cases of contraindication. These measures may be used in conjunction with hyperuricaemic drugs early in treatment when patients are at a higher risk for gouty attacks.

Anti-hyperuricaemic therapy generally aims at reducing serum urate levels by enhancing renal excretion of uric acid (uricosuria) or by decreasing uric acid synthesis. The most widely used of these uricosuric drugs are probenicid and sulphinpyrazone, and when used in conjunction with allopurinol, to reduce uric acid synthesis, are effective means of control. While uricosuric drugs and allopurinol are effective treatments for most gouty patients, their use is associated with a number of side effects, notably skin rashes, gastrointestinal intolerance and precipitation of attacks of gouty arthritis.

1.2.7 Phosphoribosyl pyrophosphate synthetase superactivity

Phosphoribosyl pyrophosphate synthetase (PRPP-S; PP-ribose-P synthetase; PRPS; EC 2.7.6.1) catalyses the synthesis of PRPP from MgATP and R-5-P in a reaction requiring inorganic phosphate and free magnesium ions. Human red cell PRPP-S is an allosteric enzyme with at least 3 regulatory sites (Becker *et al* 1975). These sites are defined by 1) competitive inhibition by ADP with respect to MgATP;

2) competitive inhibition by 2,3-diphosphoglycerate with respect to R-5-P and 3) non-competitive inhibiton with respect to its substrates by purines nucleotides, particularly nucleoside diphosphates and triphosphates (Fox and Kelley 1971).
The subunit of the erythrocyte enzyme has a molecular weight 34 kDa and can undergo reversible subunit association to exist as a complex aggregate of up to 32 of these subunits, with the enzyme activity residing in only the 16 and 32 multimers (Meyer and Becker 1977).

1.2.7.1 Clinical

PRPP-S superactivity as a basis of some cases of gout, was first recognised as the cause for purine overproduction in brothers presenting in adolescence with urolithiasis and gout associated with hyperuricaemia and hyperuricosuria (Sperling *et al* 1972). Sperling *et al* (1973) subsequently demonstrated that this was due to a variant form of PRPP-S with normal catalytic activity, but a defect in the feedback inhibition by guanosinediphosphate and adenosinediphosphate. To date, this disorder has been demonstrated in over 20 families with considerable heterogeneity in the kinetic aberrations underlying the excessive enzymic activity, including regulatory defects, catalytic defects, a combination of both of these, and increased affinity for the substrate R-5-P (Becker 1976; Akaoka *et al* 1981; Becker *et al* 1982). Of these, catalytic superactivity is the most commonly reported class of kinetic defect.
PRPP-S superactivity is expressed in two clinical phenotypes, one affecting early childhood and an the other a late-juvenile or early adult onset variety. In families with the more severe early childhood variety, affected hemizygous males show signs of uric acid overproduction in association with neurodevelopmental impairment including sensorial deafness. In four of the five families studied with this disorder, cultured cells of the affected patients showed regulatory or combined defects of the enzyme along with severe functional derangements of PRPP and purine metabolism. (Becker *et al* 1987; Becker *et al* 1988)
The late-juvenile to early onset variety of PRPP-S is restricted to males who show gout and or urolithiasis, but with absent neurological symptoms, and can be associated with catalytic defects of the enzyme (Becker *et al* 1987). This defect should be suspected in any child or young adolescent, of either sex, with marked hyperuricaemia and or hyperuricosuria at a level of between 2 and 4 times normal

HGPRT and APRT in lysed cells. The prognosis for patients presenting in adolescence is good. Allopurinol, as in LNS, is used to treat the condition, with care to avoid xanthine neuropathy. A high fluid intake and alkalisation of the urine may also help and in renal failure the dose of allopurinol must be reduced.

1.2.7.2 Genetics

PRPP-S superactivity is transmitted as an X-linked disorder, with molecular studies studies localising two genes PRPS1 and PRPS2, coding for two isoforms of the enzyme at regions Xq22-q24 and Xp22.2-22.3 respectively (Becker *et al* 1990). Both PRPS1 and PRPS2 genes encode transcripts of 317 amino acids long, which vary in their tissue distribution. In general, growing tissues contain higher levels of PRPS2 than do tissues with little active growth, suggesting that the expression of PRPS2 may be responsive to cell events associated with mitosis and transformation (Becker *et al* 1995a).

The presence of 2 PRPS1 related genes on chromosomes 7 and 9 has also been reported, fuelling speculation that these autosomal genes may play an essential part in the cell's survival in the event of X-inactivation of the PRPS1 and 2 genes during spermatogenesis (Becker *et al* 1990; Taira *et al* 1990).

With the elucidation of the nucleotide sequence of the PRPS1 and 2 genes, an effort was made to differentiate the molecular mechanisms responsible for regulatory and catalytic defects in PRPP-S superactivity.

Becker *et al* (1995b) studied PRPP-S superactivity in 6 families associated with purine nucleotide inhibitor resistance and showed that after reverse transcription and PCR amplification, each PRPS1 cDNA contained a single base substitution, resulting in an amino acid substitution of the transcribed protein. Subsequent kinetic analysis revealed that these point mutations resulted in the alteration of the allosteric mechanisms regulating both the inhibitory and activation feedback system by purine nucleotides and inorganic phosphate.

In a second and more common class of PRPP-S superactivity , this regulation by nucleotide inhibitors and the affinity for its substrates is normal (Becker *et al* 1996), showed that in patients with this deficiency, the catalytic superactivity is not associated with PRPS1 or PRPS2 sequence alterations, but rather the accumulating increases in a PRPS1 isoform with normal sequence, suggested a

post-transitional derangement which regulated the expression of PRPS1 in the catalytic superactivity of PRPP-S.

1.2.8 Adenosine deamininase (ADA) deficiency

The enzyme adenosine deaminase (adenosine aminohydrolase; ADA: EC 3.5.4.4) normally catalyses the deamination of adenosine or 2'-deoxyadenosine to inosine or 2'-deoxyinosine respectively. Deficiency in the activity of red cell ADA was reported by Giblett *et al* (1972) in two females with severe combined immuno-deficiency (SCID) and since then more than a 100 cases have been identified.

1.2.8.1 Clinical

The incidence of ADA deficiency is about 1:1,000,000 and is responsible for 20-30% of cases with recessively inherited SCID (Hirschhorn 1983), a life threatening syndrome that presents during infancy as recurrent opportunistic viral, fungal and protozoal infections, failure to thrive and lymphocytopenia, and frequently present with chronic diahorrea pulmonary infections and perioral candidasis. There is deficiency of both B and T cell-mediated immunity. The age of onset has varied, but is usually within the first 2 years of life (Morgan *et al* 1987), although in about a fifth of ADA deficient patients the immune deficiency is initially less severe and they present later in childhood. Indeed, a few older patients have been diagnosed at 15-40 years of age with chronic pulmonary insufficiency due to recurrent respiratory infections and other manifestations of immune deficiency and dysregulation (Santisteban *et al* 1993; Shovlin *et al* 1993). In the late presenters however the defect may not be easily recognised due to the fact that lymphocyte numbers are not reduced and cellular immunodeficiency may not be so profound.
In addition to red cells, the enzyme deficiency has been demonstrated in serum and lymphocytes (Meuwissen *et al* 1975) and cultured fibroblasts (Hirschhorn *et al* 1976).
The relationship between ADA deficiency and the immunological dysfunction is unknown. It has been suggested that the immunodeficiency results from an accumulation and toxicity of adenosine or adenine nucleotides including ATP and cyclic AMP which inhibit purine biosynthesis (Kredich and Hershfield 1983).

However, Simmonds *et al* (1978) showed that urinary purines from a child with ADA deficiency were deoxyadenosine rather than adenosine. Since deoxy-adenosine is inhibitory at much lower concentrations *in vivo* (1μmol/l compared with 100μmol/l of adenosine), a more convincing possibility was that deoxy-adenosine and not adenosine is toxic to proliferating lymphocytes. This also gave the first evidence of the importance of ADA for the catabolism of these toxic deoxynucleotides.

1.2.8.2 Genetics

Genetic polymorphism of the erythrocyte ADA was discovered by Spencer *et al* (1968) who demonstrated the presence of 3 phenotypes representing homozygous or heterozygous expression, designated ADA1, ADA2 and ADA2-1 of the alleles ADA^1 and ADA^2 . Tissues other than red cells show more complex electrophoretic patterns due to a high molecular weight binding protein, one molecule of which binds two molecules of ADA, with severely affected children having no detectable ADA isoenzymes in any tissues, but exhibiting normal binding protein (Hirschhorn 1983).

Originally assigned to chromosome 20 by the use of mouse/somatic cell hybridisation studies (Tischfield *et al* 1974), the gene was subsequently localised to 20q 13.2-qter and sequenced by Wiginton *et al* (1986). The gene for ADA is encoded as a single 32kb locus containing 12 exons. This information has permitted the analysis of ADA gene regulation and the relationship of phenotype to genotype in ADA deficient patients To date, over 40 distinct mutations have been identified (Hershfield *et al* 1995), with about 75% of these being single base changes causing amino acid substitutions, followed by splicing mutations, then small and a few large deletions (Hirschhorn 1995). Santisteban *et al* (1993) observed that the spectrum of mutations appears to change with clinical severity, but since most of the mutations have occurred in only a single patient or a few patients, establishing a precise correlation has been difficult.

Most obligate heterozygotes have approximately half the normal levels of ADA activities in red cells. Carrier detection is however, unreliable, due to the fact that there is an overlap in ADA activities between obligate heterozygotes and approximately 10 % of the normal population (Scott *et al* 1974). Chen *et al* (1975) studied the expression of ADA activity in cultured skin fibroblasts and amniotic

fluid cells and found that these were unsuitable for heterozygote detection, due to the variability in the ADA levels. Higher levels of residual ADA activity have been found in cells lines from healthy individuals due to a 'partial' ADA deficiency, with this residual activity shown to differ from the normal levels with respect to electrophoretic mobility, heat lability or both. (Hirschhorn and Ellenbogen 1986). Seven ADA mutations have been found in seven individuals with this 'partial' phenotype, and of these five have been shown to be compound heterozygotes. Of these seven 'partial' individuals, one was shown to have a mutation also identified in SCID, a finding which was suggested to be due to a 'null' mutation resulting in the absence of enzyme. (Hirschhorn *et al* 1990)

1.2.8.3 Prenatal Diagnosis

Prenatal diagnosis of ADA deficiency has been reported in cultured amniotic fluid cells (Hirschhorn *et al* 1975; Ziegler *et al* 1981) and chorionic villi (Aitken *et al* 1986; Dooley *et al* 1987),while analysis of fetal blood taken in the second trimester has demonstrated severe lymphopenia with complete absence of T cells, and raised dATP levels indicating the early onset of lymphocyte toxicity (Linch *et al* 1984).

1.2.8.4 Treatment and Therapy

Despite the fact that there appears to be no generally effective therapy in ADA deficiency, a number of different treatment strategies have proven beneficial. Bone marrow transplantation (BMT) when an HLA compatible sibling is available, has been shown to be the only successful long-term treatment (Markert *et al* 1987). Parkman *et al* (1975) and Pahwa *et al* (1978) showed that BMT restored complete immunological function and in addition, evidence of the restoration of ADA activity following BMT was the reduction in erythrocyte deoxy ATP levels (Chen *et al* 1978).

In most cases however, an HLA compatable sibling is not available. In these patients, repeated exchange transfusions of red cell enzyme have been reported to restore some immunological function, but not as completely as in BMT (Hirschhorn *et al* 1980). This therapy regime has been reported by some workers as successful in correcting the immunodeficiency while others have reported failure (Polmar *et al*

1976). Indeed, Ziegler *et al* (1981) suggested that the response to enzyme replacement whether by plasma or erythrocyte infusion, may be more likely when there is already present an appreciable residual ADA activity.

Hershfield *et al* (1987) showed that the intramuscular injection of PEG-ADA (bovine ADA covalently modified with polyethylene glycol) could maintain much higher circulating levels of plasma ADA activity. At weekly or twice weekly doses , immune function has been reported to improve after a lag of several weeks, during which mature lymphocytes differentiate from early progenitors (Weinberg *et al* 1993). Recovery of partial immune function , has been found to be sufficient to sustain resistance to opportunistic infections. To date, 60 patients in 9 countries have been treated with PEG-ADA, with 40 patients receiving it for 2-10.5 years. The candidates for this treatment lack an HLA-matched bone marrow donor and are usually too ill to undergo a mismatched transplantation.

Since 1990, 11 ADA deficient patients receiving PEG-ADA, have entered the first human trials of somatic cell gene therapy, conducted at centres in the United States, Europe and Japan. The therapy involves T-cells (arising as a response to PEG-ADA) or stem-cell enriched preparations isolated from marrow, induced to divide and treated *in vitro* with retroviral vectors carrying ADA cDNA, before being reinfused. Preliminary reports on 10 patients suggest that the efficiency of the ADA gene transduction into target cells was variable and quite low in most cases, as were the levels of expressed ADA in circulating T-cells (Blaese *et al* 1995; Hoogerbrugge *et al* 1996; Kohn *et al* 1995), and 1.5-3 years after the last infusion of ADA gene-treated cells, all patients were still receiving PEG-ADA injections on a weekly basis.

1.2.9 Purine Nucleosidase Phosphorylase (PNP) deficiency

Purine nucleoside phosphorylase deficiency is a rare inherited disease characterised by profound T-cell immunodeficiency. An enzyme of purine metabolism, Purine nucleoside phosphorylase (PNP; EC 2.4.2.1) catalyses the degredation of the nucleosides, inosine and guanosine and their deoxyanalogues to the corresponding base (Kredich and Hershfield 1983 ; Stoop *et al* 1977) and was first described by Giblett *et al* (1975), after screening other SCID patients for errors in the purine metabolic pathway. PNP deficiency is a heterogeneous

disorder which can be considered a subset of SCID, and to date more than 30 patients have been identified.

1.2.9.1 Clinical

All patients so far studied have suffered from recurrent infection, usually beginning in the first year of life, although some patients may be asymptotic until several years of age. The range of infections is similar to that observed in ADA deficiency, including sinusitis, urinary tract infections thrush and diarrhoea in addition to the usual respiratory pathogens *Haemophilus influenzae* and *Streptococcus pneumoniae*. Neurological problems are common in PNP deficient patients with symptoms such as spastic tetraperesis, retardation of motor function, tremor, hypotonia, developmental delay and mild to severe mental retardation. In these patients there is gross overproduction and excretion of purines in the form of nucleosides and deoxynucleotides with uric acid always very low or even absent.

1.2.9.2 Biochemistry

PNP in human erythrocytes is a protein of 289 amino acids composed of 3 identical 32,000 Dalton subunits each with a substrate binding site (Zannis *et al* 1978; Williams *et al* 1984) and an isoelectric point of the native enzyme of 5.0-6.1. The human enzyme has a K_m for inosine in the range of 2.2×10^{-5} to 10.0×10^{-5} M and is found in a range of tissues such as kidney, heart, brain small intestine, lymphoid tissues and in peripheral granulocytes (Carson *et al* 1977).

1.2.9.3 Genetics

Using earlier work based on somatic cell hybridisation and gene dosage studies, which had determined the structural locus of PNP to chromosome 14q13 (Aitken *et al* 1978; Ricciuti *et al* 1973), Remes *et al* (1984) presented additional deletion mapping data to place the location at 14q13.1.

An autosomal recessive mode of inheritance has been demonstrated in kindreds with a deficiency of this enzyme and consanguinity is frequent (Kredich and Hershfield 1983). The gene coding for PNP includes six exons dispersed across 10kb of DNA, the mutant gene cloned from an offspring of a consanguineous

mating showed the deficiency was caused by a single base change in the third exon at position 89, resulting in a G to A transition (Williams *et al* 1987). A number of other mutations in the PNP gene have subsequently been reported (Aust *et al* 1992; Markert *et al* 1997; Williams *et al* 1984).

1.2.9.4 Prenatal Diagnosis

Carapella de Luca *et al* (1986) and Kleijer *et al* (1989) have reported prenatal testing for PNP deficiency in cultured 16 week amniotic fluid cells of women who had previously had affected offspring. One fetus was identified as normal and the other as affected. The purine profile of the normal amniotic fluid showed a normal PNP activity, uric acid in the normal range and undetectable levels of hypoxanthine, xanthine, guanine, inosine, deoxyinosine, guanosine and deoxyguanosine, while the affected fetus showed undetectable levels of PNP activity and raised levels of inosine, deoxyinosine, guanosine and deoxyguanosine. Prenatal diagnosis using chorionic villi has was first reported by Perignon *et al* (1987) and subsequently by Kleijer *et al* (1989) who showed that in the second trimester of pregnancy, there is a significantly higher level of specific activity of PNP in amniocytes than in cultured fibroblasts, and even higher levels in chorionic villi. It would therefore, appear that the latter is an ideal tissue for prenatal diagnosis.

1.2.9.5 Treatment and Therapy

Markert (1991) reviewed the treatment of 33 patients with PNP deficiency, including bone marrow transplantation, red cell transfusion, fetal thymus transplantation, and deoxycytidine or guanine administration.
She concluded that the best available treatment was that of bone marrow transplantation, however even with this treatment there appeared limited success, with only 2 of the first 5 patients treated, still living at the time of the review. Of the 33 patients reviewed, 29 had died with infections, Graft versus Host disease and tumour being the most significant causes of mortality.

1.3 Pteridines

The terms "folic acid" and "folate" are the preferred synonyms for the pteridine compounds pteroylglutamic acid (PteGlu) and pterolglutamate respectively, which are heterocyclic compounds of pteroic acid conjugated with one or more glutamate residues.

1.3.1 Folate metabolism

The term folate is used in the generic sense to designate members of the family of naturally occurring polyglutamate forms having various reduction of the pteridine ring, while folic acid is used to describe the synthetic monoglutamate form . The compound tetrahdrofolate (THF) is the parent compound of all biologically active folates which have substituents at positions 5 or 10 of the pteridine ring, with the predominant folate in serum and in tissues being 5-methyl-THF.

Folate is a key factor in two fundamental biological processes. First, folate acts as a cofactor for enzymes involved in DNA and RNA biosynthesis, where THF accepts single carbon units from a variety of donors, mainly serine, to form a pool of interchangeable cofactors such as 5,10-methylene-THF, 5,10-methenyl-THF, and 10-formyl-THF. While 10-formyl-THF donates its formyl group to two of the enzymes in the *de novo* pathway of purine synthesis forming adenine and guanine, 5,10-methylene-THF donates its C1 group to an enzyme in the *de novo* synthesis of pyrimidines, thymidylate kinase. Since the requirements for folate dramatically increase during periods of rapid growth of the embryo, any deficiency in production or supply will inhibit DNA synthesis and subsequent mitosis, leading to imbalanced cell growth and cell death.

Second, the methyl group of 5-methyl-THF is used by methionine synthase, a vitamin B_{12} dependent enzyme, to methylate homocysteine to methionine, an essential amino acid. A disruption of this remethylation pathway leads to raised homocysteine levels and it is this hyperhomocysteinaeimia or reduced methionine which may cause developmental defects. The disturbance in the remethylation also compromises the cells ability to methylate other important compounds such as proteins, lipids and DNA. It is not clear as yet whether this is due to folate deficiency or a metabolic block in the pathways processing folate.

Methionine is continuously converted to homocysteine via SAM which is then de-methylated to form the S-adenosylhomocysteine (SAH), which is subsequently hydrolysed to adenosine and homocysteine. Significant amounts of methionine are subsequently regenerated via the remethylation of homocysteine, a reaction catalysed by the enzyme methionine synthase (methyltetrahydrofolate: L-homocysteine S-methyltransferase ; EC 2.1.1.13). This reaction requires the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) and is also dependent on the co-factor methylcobalamine, a form of vitamin B_{12}. The majority of homocysteine is not remethylated but catabolised via a pyridoxal-5'-phosphate (Vitamin B_6) - dependent condensation with serine to form cystathionine, catalysed by the enzyme cystathionine β-synthase (CBS; CβS; EC 4.2.1.22), and ultimately metabolised to cysteine and alphaketobutyrate. This is referred to as the transsulphuration pathway (Figure 1 - 6).

Two distinct transport systems have been described for the transport of folate across mammalian cell membranes. (Fan *et al* 1992). One operates in relatively high folate concentrations, ("micromolecular system"), while the other has been characterised using lower folate concentrations ("nanomolecular system"), and both systems may not be operational in all cell types. The folate receptor has been shown to be a membrane protein anchored by glycosylphosphatidylinositol. The gene for the folate binding protein has been mapped to chromosome 11q13.3-q14.1 (Campbell *et al* 1991). These folate receptors are found on the cell surface in clusters in association with the plasmalemmal vesicles in stuctures known as uncoated pits, and have been shown not to be internalised into a vesicular compartment.

Human cells need a critical concentration of intracellular folate to allow activity of folate dependent enzymes. The amount required to maintain an optimal rate of growth varies from about 50nM in fibroblasts to about 1μM in lymphocytes and certain tumour cells (Watkins and Cooper 1983). The K_m for monoglutamate folate of many of the folate-dependent coenzymes is greater than 1μM so that the folate-dependent enzyme reactions cannot progress in cells in the absence of the conversion of folate to the polyglutamate forms. The polyglutamate forms have much lower K_m values for some folate dependent reactions allowing folate metabolism to proceed at the optimal cellular concentration The folate polyglutamates from dietary sources are hydrolysed in the intestine prior to absorption, and the monoglutamates are released into the circulation where they are fully reduced to THF. Two distinct forms of the human pterolypolyglutamate

hydrolase have been described, one in the intestinal brush border and another within the lysosomes.

1.3.2 Folate and NTDs

The possibility that folic acid might be one of the important factors in the aetiology of NTDs, was first suggested by Hibbard (1964).

The first major clinical trial involving folic acid was performed by Smithells *et al* (1981) in which women were supplemented with a multivitamin preparation containing iron and folic acid (Pregnvite Forte F (Bencard)), at a physiological dose of 360µg/day at least 28 days prior to conception and for the first 8 weeks of the pregnancy. The intervention study involved women who were at risk of an affected pregnancy, by virtue of the fact that they had previously had affected children (recurrent NTD). Results showed that the NTD rate in supplemented women was 0.6% compared to 5.0% in unsupplemented women, a reduction of 84%.The results of this study have been criticised on a number of counts. Firstly, that possible bias had been introduced into the study design due to the non-randomised nature of the supplemented group which contained more women from a higher social class than the control group (Wald and Polani 1984). Secondly, that the women from the supplemented group were self-selected while the controls were not, so that the former were self-motivated and thus more likely *per se* to have better compliance with the vitamin supplementation regime. (MRC Vitamin Study 1991)

Despite this, the results were confirmed in a second cohort of women where the recurrence rate was reduced by 82% in supplemented women (Smithells *et al* 1983), while Holmes-Siedle *et al* (1992) independently produced similar results. A second smaller randomised trial by Laurence *et al* (1981) examined the effects of folic acid alone in a pharmacological dose (4mg/day). This study yielded inconclusive results when analysed according to the randomly allocated treatment group, possibly because of the small numbers of individuals studied.

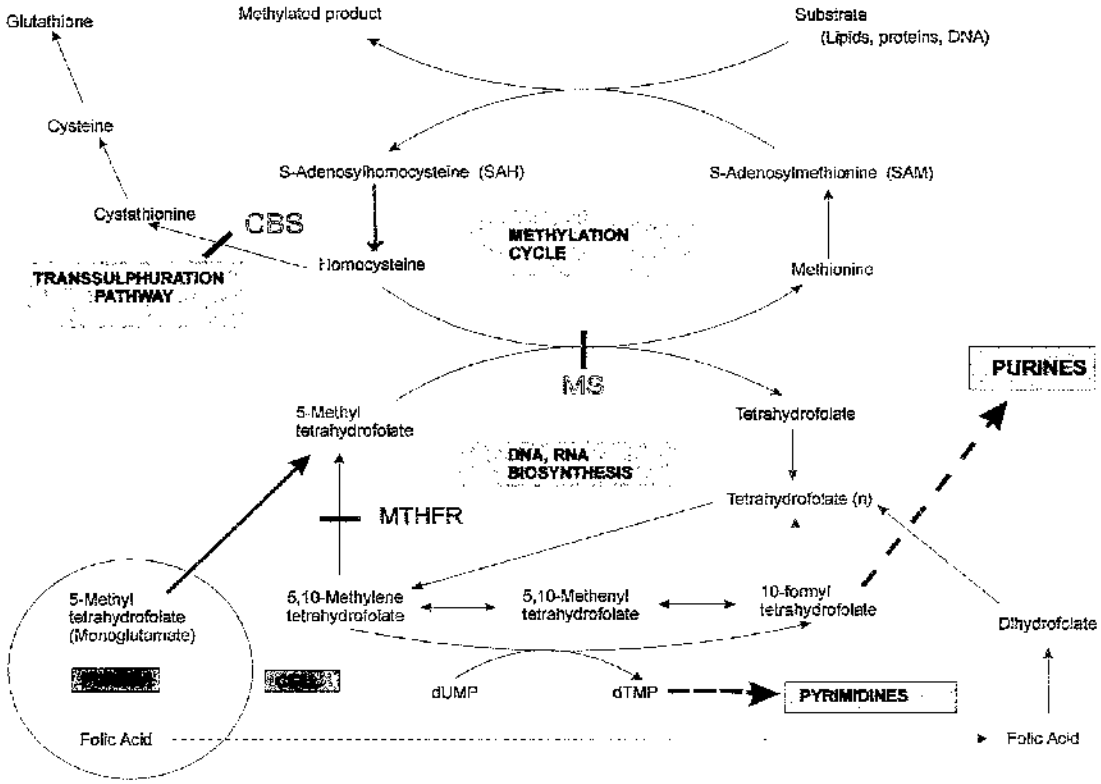


Figure 1 - 6

Methylation and transsulphuration pathways of homocysteine metabolism.

KEY:- MTHFR - 5,10 methylene tetrahydrofolate reductase ; CBS - cystathionine β-synthase ; MS - methionine synthase

In 1983, the Medical Research Council, launched a randomised double-blind prevention trial comprising 33 centres in seven countries to determine whether supplementation with folic acid or a mixture of seven other vitamins (A,B,B_1,B_2,B_6,C and nicotinamide) around the time of conception could prevent NTDs. A total of 1817 women with a recurrence risk were allocated at random to one of four groups, namely folic acid, other vitamins, both or neither. Of 1195 completed pregnancies in which the fetus was known to have or not have an NTD, 27 had an NTD, with 6 in the folic acid group and 21 in the other two groups, a 72% protective effect (MRC Vitamin Study 1991).

Czeizel and Dudas (1992) studied the extent to which vitamin supplementation could reduce the first occurrence of NTD. Women planning the first pregnancy were randomly assigned to receive either a single tablet of vitamin supplement (containing 12 vitamins including 800μg of folic acid, four minerals and three trace elements); or a trace-element supplement (containing copper, manganese, zinc and a very low dose of vitamin C) daily for at least 1 month prior to conception and until the date of the second missed menstrual period. Results showed that in a total of 4753 women who complied, congenital malformations were significantly more prevalent in the trace-element group (22.9/1000), than in the vitamin supplemented group (13.3/1000), and that there were 6 cases of NTD in trace -element group, and none in the group receiving vitamin supplements.

While the results of these randomised trials put beyond doubt the effectiveness of periconceptual folic acid supplementation as a primary means of preventing NTDs, it remains unclear as to the mechanism of this prophylactic effect. Efforts to determine a significant difference in the serum folate and Vitamin B_{12} concentration of mothers carrying an NTD fetus and controls have found that on average, during the first trimester of pregnancy, that levels were lower in cases and controls, but not significantly so. (Yates *et al* 1987; Wald *et al* 1996). Similarly, the work on red cell folate, a more stable indicator of folate intake, have either been inconclusive, or found no significant differences between cases and controls (Economides *et al* 1992;Smithells *et al* 1976; Yates *et al* 1987)

Yates *et al* (1987) investigated women with who had two or more NTD affected infants and demonstrated an association between these offspring and depressed levels of red cell folate in the mothers. Since this could not be entirely attributed to a lower dietary intake of folate, it was postulated that one of the factors predisposing to the occurrence of NTDs was an inherited disorder of folate metabolism.

1.3.3 Neural Tube Defects

The term Neural Tube Defect (NTD) can be applied to any malformation of the developing neural tube. NTDs are a group of morphologically heterogeneous disorders where there may be involvement of the whole of the neural tube or more commonly a localised lesion at some point producing varied abnormalities. Lemire (1988) qualified the terminology of NTDs in terms of whether the lesions are open, (neurulation defects where neural tissue exposed to the surface); or closed, (postneurulation defects which are skin covered). Three general categories of neurulation defects exist from the clinical perspective, notably craniorachischisis, anencephaly and meningomyelocoele. Craniorachischisis is a condition where both the brain and spinal cord are exposed to the surface with many of these cases lost in early spontaneous abortion.

1.3.4 Embryology

The human embryonic period is divided into 23 stages, each comprising 2-3 days and encompasses the first 54-60 days following ovulation (O'Rahilly 1986). In man, neural tube formation occurs around the 17-18th day after conception, when the central part of the ectoderm of the embryonic disc is induced to thicken and differentiate into the neural plate by the underlying mesoderm. Neurulation occurs as the embryonic disc elongates forming a longitudinal groove on its dorsal surface midline, and the lateral edges of the neural plate rise up forming the neural folds, which in turn arch over and gradually meet in the dorsal midline. As the embryo elongates and increases in size, fusion occurs concurrently in both the cephalic and caudal directions. Neurulation is complete when the extreme ends, the neuropores, seal around days 24-25 anteriorly and days 26-28 posteriorly. Failure of the neuropores to fuse, give rise to the NTDs anencephaly and meningomyelocoele.

Anenecephaly exists when brain tissue is exposed to the surface, but with no involvement of the spinal cord. The term meningomyelocoele incorporates spina bifida cystica, and can occur anywhere along the spinal axis, but most commonly in the lumbrosacral region, where 75-80% of cases are open lesions with remainder being closed. Overall, anencephaly accounts for 40% of neutral tube defects, spina bifida for 55% and encephalocoeles for 5%.

1.3.5 Prevalence

NTDs vary not only in morphology but also in aetiology. For a small number of NTDs the cause may be secondary to chromosomal abnormalities such as trisomy 18, trisomy 13 or triploidy, or as part of a recognised genetic disorder as in the case of the autosomal recessive Meckel syndrome. However the vast majority, 90-95%, of NTDs are of unknown origin, and are referred to as the multifactorial type.

Khoury *et al* (1982) found that 80% of all NTDs occurred in the absence of defects in any other body systems, and exhibited different epidemiological characteristics, proposing that these were different aetiologically from those which occurred in conjunction with subsequent abnormalities. Further work (Carter 1974) analysed the association with NTDs and other defects, and found a clustering according to the site of the NTD lesion which conformed to a definite pattern. Hall *et al* (1988) examined the clinical, genetic and epidemiological factors of 512 probands from the population of British Columbia. They found that when data were analysed after grouping the probands by four different classifications with respect to pathological and putative mechanisms, apparently unrelated congenital abnormalities occurred more frequently among probands with craniorachischisis (62%), encephalocoele (30%) or multiple NTDs (25%) than among probands with anencephaly (14.7%) or spina bifida (10%).

There is a marked geographical variation in the pregnancy prevalence of open NTDs, with the British Isles, and the west of Scotland having a prevalence in 1990 of ~2 cases per 1000 pregnancies, compared with a lower rate in England and other countries in Europe. (Dr D. Aitken, personal communication). There is also an observable East-West gradient, highest in the British Isles and lowest in Japan where prevalence is estimated at 0.1-0.6 / 1000 births (Lemire 1988), with the United States being in between. Even within the United States itself, there appears to be a gradient effect with a higher prevalence of open NTDS on the East coast compared to the West coast, a fact which has been attributed to migration patterns and ethnic differences (Chung and Myrianthopoulos 1968).

The work of Borman and Cryer (1990) has called into question the validity of many of the epidemiological studies concerning the estimation of incidence and prevalence of NTDs, citing a lack of scientific rigour in design, and a lack of a standardised nomenclature and classification for NTDs.

The work of Van Allen (1996) on the use of monogenic mice models suggested that NTDs may be better classified using a multisite closure model, rather than the

single-site closure method presently used. This study suggested that classification of NTDs by closure site, may be better in defining aetiologies and environmental susceptibilities, particularly in view of the heterogeneous nature of this disorder. Recurrence risk after an affected pregnancy is 1 in 25-33 and for second degree relatives the risk of recurrence is 1 in 70. After 2 or more affected pregnancies the recurrence risk is 1 in 10. (Connor and Ferguson-Smith 1993)

1.3.6 Prenatal diagnosis

An important advance in the secondary prevention of NTD births has been the introduction of large scale prenatal screening and diagnostic programmes used in conjunction with ultrasonography, such as the one in use at the present time in the west of Scotland. Since more than 95% of all women with a neural tube defect pregnancy have no previous family history, most affected pregnancies can be identified only by screening the entire pregnant population. In pregnancies where the fetus has a neural tube defect, maternal serum alphafetoprotein (MSAFP) and amniotic fluid AFP are elevated due to leakage from exposed fetal capillaries. MSAFP measurement is undertaken at between 15-20 weeks gestation, and if the level above the 95th centile, a second blood sample may be requested along with an ultrasound scan to check for fetal abnormality, confirm gestation or exclude other causes such as missed abortion or multiple pregnancy. If MSAFP level in a second sample also exceeds the 95th centile, further ultrasound examination and possibly prenatal diagnosis by amniocentesis are indicated. It is essential that a differential diagnosis be made to distinguish pregnancies at risk for NTDs and other types of fetal abnormalities, associated with raised MSAFP such as anterior abdominal wall defects (AAWDs) which may be surgically repaired. This may be acheived by examination of the pattern of cholinesterases in the amniotic fluid by polyacrylamide gel electrophoresis (PAGE). Normal amniotic fluid produces a single band of pseudocholinesterase, while open NTDs also produce a second faster band of acetylcholinesterase (ACHE), as do around 50% of abdominal wall defects, although the pattern of ACHE bands appears different between NTDs and AAWDs (Burton 1986 ; Rasmussen Loft *et al* (1990)).

When both AFP and ACHE determinations are combined, and abnormal results are obtained, it is stong evidence of open NTD and helps to minimise the false-positive results associated with the raised levels of AFP found in other conditions

(Aitken *et al* 1984; Amniotic fluid acetylcholinesterase collaborative study 1981). It is important to note that the biochemistry results should always be interpreted in conjunction with the data obtained from ultrasound findings, such that a differential diagnosis of these abnormalities may be made.

1.3.7 Aetiology

Epidemiological studies on the aetiology of human NTDs show that it is a multifactorial disorder where both genetic and environmental influences are implicated. While the examination of the potential teratogenicity in a number of environmental factors has been sought, none has been more extensively studied than those of nutritional factors. A number of studies in the UK showed that the NTD rate was higher in less privileged socio-economic groups Carter *et al* (1974), and that women from social classes III -V had lower intakes of several nutrients as compared to women in social classes I and II (Smithells *et al* 1977). Data both for and against the case of nutrition as a contributory factor have been cited. Al-Awadi *et al* (1984) quoted the dramatically falling prevalence of anencephaly in the Kuwaiti population, which was attributed to dietary improvement with an increase in both food consumption and food quality, in the absence of prenatal diagnosis or termination of pregnancy.

Paradoxically, within the African and Asian populations where poor nutrition or malnutrition is endemic, NTD rates are low. This may be due to the multifactorial nature of the disorder, where both the genetic and environmental factors must be present.

1.3.8 Homocysteine metabolism and cardiovascular disease

Kang *et al* (1988a; 1988b) examined a thermolabile variant of MTHFR shown to be different from that which caused the severe form of MTHFR deficiency. These workers showed that this variant exhibited in-vitro thermolability at 46°C which provided a clear distinction between the mutant and the normal enzyme, and subsequently compared two patients with an 8-15 fold increase in plasma homocysteine with normal controls. After heat treatment, the residual MTHFR activity for the controls was 37% , but only 15.2 and 15.1% in the patients. Further work (Kang *et al* 1991) to elucidate the association thermolabile MTHFR with the

development of coronary heart disease showed that in 212 patients with proven coronary artery disease and 202 controls with out clinical evidence of atherosclerotic vascular disease, 36 (17%) of the patients and 10 (5%) of the controls exhibited the thermolabile MTHFR. The results were consistent with autosomal recessive inheritance of the thermolabile trait.

The importance of the thermolabile allele as a contributory factor in homocysteinemia was subsequently confirmed by other workers (Engbersen *et al* 1995; Haworth *et al* 1993). The subsequent isolation of the cDNA for human MTHFR and mapping to the chromosomal region 1p36.3 (Goyette *et al* 1994) enabled Goyette *et al* (1995) to identify 9 mutations in this gene, in a severely deficient group of patients, using single-strand conformational polymorphism (SSCP) analysis and direct sequencing of polymerase chain reaction (PCR) fragments. The SSCP protocol identified a C to T (C→T) substitution at bp677 which resulted in the substitution of a valine codon for the evolutionarily conserved alanine residue. This alteration which created a *Hinf*1 restriction site was subsequently shown to encode the thermolabile variant of MTHFR (Frosst *et al* 1995). Studies from several populations have confirmed the association of this MTHFR mutation with hyperhomocysteinemia, as has the increased prevalence of the homozygous mutant genotype in cardiovascular cases compared to controls (Rozen 1997).

The recent work on the role of the thermolabile variant of MTHFR and the 677C→T mutation in cardiovascular disease by Frosst *et al* (1995), prompted Whitehead *et al* (1995) to examine the relationship of this genetic abnormality and NTDs since both appeared to have similar aetiologies, notably a disruption of homocysteine metabolism. Of the 82 people with NTDs studied, 15 (18.3%) were homozygous for the abnormal, thermolabile allele. This was significantly higher than the 6 (6.1%) controls found to have the mutation.

CHAPTER 2. AIMS

The aim of this work was to use biochemical genetic methods and procedures to examine the effectiveness of enzyme measurements for the diagnosis of a number of genetic disorders, notably LNS and gout (X-linked); ADA and PNP deficiencies (autosomal recessive) and NTDs (multifactorial), which are linked through their respective purine and homocysteine pathways. The work was divided into two phases:

Phase I :- Purines

The role of the purine enzymes was investigated by:

a) Establishment of reliable reference ranges for the purine enzymes HGPRT, APRT, ADA, PNP and PRPP-S in adult red cells, uncultured and cultured chorionic villi, amniotic fluid cells, fibroblasts, fetal red cells and cord red cells, by critical analysis of the parameters of the enzyme distributions.

b) Assessment of the usefulness of these reference ranges in the diagnosis or prenatal diagnosis of the X-linked disorders, LNS and gout and the autosomal recessive disorders, ADA and PNP deficiency.

Phase II :- Folate resistant NTDs

The role of the enzyme MTHFR in the development of NTDs was investigated by:

a) Assessment of the allele frequencies of the thermolabile variant of MTHFR in patients with NTD, their parents and other relatives and in a series of controls drawn from the Scottish population.

b) Assessment of MTHFR enzyme activity in lymphocytes, from each of these groups.

c) Relating the enzyme activity and nutritional status as assessed by folate and plasma vitamin B_{12} measurements to the genotype in the study populations.

CHAPTER 3. PATIENT AND CONTROL SAMPLES

3.1 Purine Study

3.1.1 Lesch-Nyhan Syndrome (LNS) Study

Prior to the analysis of samples from those individuals with a family history of LNS, it was necessary to establish reliable reference ranges for the purine enzymes under investigation. The term 'reference range' can be applied to the analytical values obtained for a specific analyte, from a population which exhibit no known occurrence of the diseases in question, in this case, no known family history of LNS. Control material for the study of the red cell purine enzymes was obtained by the voluntary donation of approximately 10mls of whole blood taken into plastic lithium heparin tubes (Sarstedt), from both male and female workers in Institute of Medical Genetics, Yorkhill Hospitals, Glasgow in 3 batches over the course of 2 years. Samples were separated and the constituents stored immediately after donation at -20°C until use.

Whole blood from affected individuals and their relatives, was obtained by collection of a 10ml lithium heparin sample, which was separated and the constuents stored immediately upon receipt with the department, usually within 48 hours of collection. In the cases of very young children, or where difficulty in obtaining blood was experienced, samples were typically smaller, usually between 1 and 3mls.

Cord blood was collected at parturation, by theatre staff from routine obstetric deliveries performed during the working week, at the Queen Mothers Hospital, Glasgow. Approximately 2mls of whole blood was collected into plastic lithium heparin tubes and stored at 4°C for not more than 24 hours prior to separation of the constituents.

Approximately 0.5-1ml of fetal blood was obtained from a number of pregnancies undergoing routine fetal blood sampling for chromosomal indications and with no family history of LNS, at the Fetal Medicine Unit of the Queen Mothers Hospital, Glasgow. These samples were collected into plastic lithium heparin tubes, separated and the constituents stored within 2 hours of the procedure.

Control amniotic fluid cells for the reference ranges were obtained from patients who had undergone routine karyotyping by the West of Scotland Prenatal Diagnosis Service at the Institute of Medical Genetics, between 15 and 21 weeks gestation. In addition to amniotic fluid cell preparations for the reference ranges,

individual diagnostic samples were used as intra-assay quality controls for the prenatal diagnoses of LNS, where amniocentesis had been performed. These cells underwent culture and harvesting in the same batch as the preparation for the prenatal diagnosis, and as such, were subjected to the same conditions as the diagnostic sample.

Amniotic fluid cells from pregnancies undergoing prenatal diagnosis for LNS were cultured and harvested identically to all other routine samples.

CV controls were obtained at between 8 and 11 weeks gestation from elective terminations performed at the Western Infirmary, Glasgow over a 2 year period. Where enough material was available, CV samples obtained for chromosomal diagnoses, were used as intra-assay controls for the specific LNS prenatal diagnoses. These were cultured in the same batch and under identical conditions as the test sample.

Control fibroblast material was obtained from tissues, received at Institute of Medical Genetics, Yorkhill Hospitals, Glasgow for chromosome analysis where there was no indication of congenital metabolic defect.

Samples of fibroblast tissue obtained from pregnancies deemed to be affected after prenatal diagnosis for LNS, were collected at termination and the fibroblast cells cultured and harvested prior to enzymic analysis.

As with amniotic fluid and CV cell preparations, routine fibroblast cultures were used as intra-assay controls from the same culture and harvesting regime as the diagnostic sample.

3.1.2 Gout Study

A random selection of the red cell preparations originally used to provide the LNS reference ranges, were selected to provide a reference range for comparison with patients under investigation in the gout study.

Whole blood was obtained from 54 individuals (45 males, 9 females), undergoing treatment for gout at the Department of Rheumatology, Glasgow Royal Infirmary over 2 years. A total of 20mls of blood was collected and divided equally between plastic sodium EDTA and lithium heparin tubes. The EDTA samples were used to extract DNA in the event that the enzyme results indicated the requirement for molecular analysis, which was then stored at -70°C until use. The heparin samples were separated and the constituents stored at -20°C until use.

3.1.3 ADA and PNP Studies

Controls for both the ADA and PNP investigations were those used for the Lesch-Nyhan Syndrome as previously described in Section 3.1.1.

3.2 Pteridine Study

3.2.1 MTHFR

Control blood for the NTD study was by voluntary donation from individuals with no known family history of neural tube defect. Where possible, samples from the control population were collected from friends of NTD couples, in an attempt to allow for partial matching of social class and diet. Since it was not always possible to obtain control material from this source, the reference range included a large number of random samples from individuals taken from the general population who had no known family history of neural tube defect. A whole blood sample was collected from both parents where a pregnancy had resulted in an NTD and from any affected or normal offspring and any other first or second degree relatives within the family willing to give a donation.

Control and at-risk samples were collected from families identified from 3 sources:

(i) The database of the West of Scotland Maternal Serum Screening program for NTDs, housed in the Institute of Medical Genetics, Yorkhill Hospitals, Glasgow.
(ii) The database of patients recruited from the West of Scotland to the MRC Vitamin Supplementation trial (MRC Vitamin Trial 1991) which contains comprehensive information of families who have undergone one or more NTD pregnancies.
(iii) The records of the Scottish Spina bifida Association to identify affected individuals and families not included in the previous 2 sources.

Consent forms (Appendix A) describing the proposed study were forwarded to subjects and controls, inviting them to donate a 20ml sample of blood. On return of the consent form, a box was dispatched containing the following items; a copy of the consent from and a covering letter to the subject's GP; a return stamped

addressed envelope and a 10ml EDTA and 10ml Lithium Heparin sample bottle. Individuals were invited to take these to their GPs where the blood would be collected and then to forward the samples to the Department of Medical Genetics where the blood constituents were separated and stored until use. The 10ml EDTA blood sample was used for DNA extraction, while the lithium heparin sample was used to obtain red cells, plasma and lymphocyte preparations. Blood samples obtained within the department were processed within 2 hours of being taken, however the time elapsing between donation and receipt for samples sent via normal mailing was in the order of between 2 and 5 days on average.

The method of sample collection was approved by the local Ethics Committee of Yorkhill Hospital, Glasgow. In summary there were 262 controls, 40 individuals with NTD, 85 mothers with NTD pregnancies, 35 fathers of affected pregnancies and 36 other family members.

CHAPTER 4. METHODS

4.1 Chemicals and reagents

All chemicals used were purchased from the Sigma Chemical Company unless otherwise specified in the text.

4.2 Blood samples for purine analysis

4.2.1 Separation of Constituents

The whole blood samples in the anticoagulent tubes were inspected for clotting, and mixed thoroughly by repeated inversion prior to being balanced and spun by an IEC Centra GP8 centrifuge at 900 x g for 10 minutes at room temperature. The upper straw coloured plasma layer was carefully aspirated using a 3ml plastic pasteur pipette into a labelled polystyrene LP3 tube (Sarstedt) (capacity ~2.5mls) and stoppered. The samples were stored at -20°C, pending analysis.

The intermediate layer containing plasma and buffy coat was removed to just below the beginning of the red cell layer and discarded.

The remaining red cell fraction was stored frozen using a suitable cryoprotective agent, in this case cold buffered citrate-glycerol, which prevents red cell lysis on freezing. This solution consisted of 200mls of glycerol (BDH) mixed with 300mls of a solution consisting of 3.25% Tripotassium citrate (BDH), 0.6% Dipotassium hydrogen phosphate (K_2HPO_4) (BDH), and 0.47% of Potassium dihydrogen phosphate (KH_2PO_4) (BDH) and was stored at 4°C until use. An equal volume of this solution was added to the remaining red cells, mixed thoroughly by repeated inversion and allowed to stand for a few minutes to ensure the complete diffusion of the glycerol into the cells. The samples were stored at -20°C until use.

4.2.2 Retrieval and Handling

Plasma, white cell and red cell samples were allowed to thaw at room temperature. The tubes were mixed by re-inversion to ensure complete homogeneity before use. This procedure was repeated after the analysis when the samples were returned to -20°C storage.

4.3 Amniotic fluid cells

4.3.1 Cell culture

Samples obtained from at risk and control pregnancies were spun in a MSE centrifuge at 200 x g for 10 minutes. Most of the supernatant was removed and forwarded for subsequent biochemical analysis , while the cell pellet was resuspended in the remainder (1-1.5 mls), prior to being dispensed into 3 Nunc tissue culture tubes (110 x 16mm) using a sterile cannula attached to a 2ml syringe. To each tube was added 2mls of culture media consisting of Hams F10 supplemented with 2% Fetal Calf serum (Gibco); 4% Ultroser serum substitute and 1% Penicillin/Streptomycin solution (10000 IU/ml Penicillin and 10000 UG/ml Streptomycin). The presence of cells was checked by observation under an inverted microscope and the tubes incubated at 37°C. After 7days, the tubes were examined for evidence of cell settlement. If sufficient active growth was observed, cultures were fed twice a week until judged suitable for harvesting.
Poor cell growth over the tube's surface required that subculturing by means of a trypinisation step be introduced into the procedure.

4.3.2 Trypsinisation

The trypsin/EDTA solution consisted of 800mls distilled water, 0.9 % phosphate buffered saline (PBS) (BDH) , 0.4g EDTA (Versene) (disodium salt, dihydrate) and 2g of Trypsin dispensed into 20 ml Universal containers and stored at -20°C. The solution was filter sterilised by a 0.2μm Acrodisk 32 filter prior to use.
Removal of the media was followed by addition of 1ml of the trypsin/versene solution, which was immediately discarded and replaced by a further 2 drops, and incubated at 37°C for 5 minutes, at which time the cells have detached from the surface of the tube. 2mls of fresh media is added and the tubes returned to the incubator until ready for harvest.

4.3.3 Pelleting and storage

Amniotic fluid cells were pelleted when sufficiently confluent. The existing media was poured off from the flask, replaced by 1.5mls of Trypsin/EDTA solution, and the flask swirled gently to facilitate coverage of all the cells, after which this was

poured off. A further 0.5mls of fresh Trypsin/EDTA solution was added and the cells incubated for 5mins at a temperature of 37°C. Detachment of the cells from the walls of the flask was facilitated by a gentle tap of the flask on a hard surface and confirmed by observation using a binocular microscope. Using a long, siliconised, glass Pasteur pipette, the cells were transferred to a 20ml polystyrene Universal container containing 10mls of Hams F10 medium supplemented with 20% fetal calf serum. After thorough mixing by inversion, the container was centrifuged in an MSE centrifuge at 400 x g for 10 minutes, the supernatant was removed and the pellets washed with 10mls of phosphate buffered saline , prior to a further centrifugation for 10 minutes at 400 x g . This was repeated twice more, before the remaining buffer was discarded, the pellet reconstituted and transferred to a large Eppendorff tube for centrifugation at 7,500 x g in a Microcentaur microcentrifuge for 5 minutes. The remaining pellet was air dried, and snap frozen in liquid nitrogen before storage at -70°C.

4.3.4 Retrieval and handling

After thawing at room temperature, the pellet was reconstituted with an appropriate amount of distilled water. The relative volume, was determined by the size of pellet obtained, but was typically between 60 and 120µl. After gently vortexing to ensure complete homogeneity, the cells were sonicated at 4µm for 10 seconds on an MSE Soniprep 150 sonicator and the resulting lysate spun for 10 minutes at 7,500 x g in a Microcentaur microcentrifuge. The supernatant was kept on ice until use.
The minimum volume of 60µl was determined by the limitation of the sonicator which, due to the type of probe used, would not adequately disrupt the cell preparation in a lesser volume.
After analysis, the remainder of the cell supernatants were returned to long term storage at -20°C.

4.4 Chorionic villus cells

4.4.1 Preparation

The entire aspirate was transferred to a glass petri dish and a small amount of RPMI 1640 media (Gibco) poured in to clarify the tissue from the accompanying blood. The CV was cut from the surrounding tissue using scissors, and with

siliconised glassware, was transferred to a clean petri dish containing fresh media where it was sorted to remove blood clots and fragments of maternal decidua from the villi. The most adequate method of performing this was found to be with 2 Microlance 19g2 gauge needles, allowing small pieces of the villi to be teased apart. After removal of this tissue to another petri dish and a further wash with RPMI 1640, a more detailed sort was performed under a phase microscope. If the samples proved to be of adequate size, they were divided into two equal parts, and an aliquot containing a minimum of 10-20mg tissue was pelleted and used immediately for biochemical analysis. The remainder of the tissue was cultured as described below.

Control CV was prepared identically and the material snap frozen in liquid nitrogen and stored at -70°C for up to 7 days before analysis.

4.4.2 Collagenase Dissociation and Culture

Approximately 10mg of the CV tissue was transferred to a culture tube containing 4ml sterile, Trypsin/EDTA and incubated for 1 hour at 37°C, ensuring that the tube was mixed after each 30 minute period. After centrifugation at 400 x g for 5 minutes on an MSE centrifuge, the supernatant was discarded and the pellet resuspended in 1ml of 3% collagenase culture medium (CCM) (1:1 RPMI and Hams F10 medium; 3% Ultroser serum substitute and 3% Penicillin/Streptomycin, filter sterilised through a 0.22 µm filter); and 1ml collagenase (20mg collagenase in 10ml CCM, filtered and stored in 1ml aliquots at -20°C). This was mixed thoroughly and incubated at 37°C for 2 hours, again mixing the tube after each 30 minute period. The cells were spun down for 5 minutes at 1500rpm and washed in 1ml of fresh CCM. This step was repeated and the cells transferred to a Nunc 25cm culture flask with 5ml of Tube media (consisting of Hams F10 with Hepes buffer and L-glutamine (Gibco); 20% Fetal calf serum and 1% Penicillin/Streptomycin) and incubated at 37°C , with refeeding every 3-4 days until confluence was reached.

The method for pelleting, storage, reconstitution and handling of CV was identical to that described previously for Amniotic Fluid cells (Section 4.3.3).

4.5 Fibroblast cells

Fibroblast cells were prepared and cultured by a method identical to that employed for CV cells. The only salient difference was that due to the nature of fibroblast tissue, it was necessary to chop the large pieces into much smaller ones. This was done using a small sterile plastic petri dish and sterile scissors.

4.6 Lymphocyte cells

4.6.1 Collection and Preparation.

Approximately 10mls of whole blood was collected from control patients and study cases in lithium heparin tubes as described previously and stored for not more than 2 days at 4°C before processing. A volume of 4mls Lymphoprep solution (Nycomed) was placed in a 15ml plastic tube and to this was added 5mls of whole blood. After centrifugation at 400 x g for 40mins, the constituents had separated into 3 fractions; the plasma, the white cells (buffy coat) and the red cell components. The plasma component was removed and discarded, while the white cells , which lay at the centre of the interface, were removed to a second 15ml plastic tube. Preparation of the remaining red cells was as described in section 3.1.1. , before storage of these aliquots at -20°C. The buffy coat containing the white cell fraction was washed with 12mls of RPMI medium containing 1% Penicillin/Streptomycin antibiotic and centrifuged at 400 x g for 20mins. The supernatant was disposed of into 10% Chloros and the cell pellet resuspended in a further 10mls of RPMI medium, with a further centrifugation at 400 x g for 20 minutes. Contamination of the cell pellet by significant amounts of red cells, was minimised by addition of 1ml of red cell lysis mix (155mM NH_4Cl (BDH); 10mM $KHCO_3$ (BDH) and 1.0mM EDTA (pH 8.0)), and allowed to stand for 20 minutes. Since a volume of approximately 10mls of whole blood was processed as 2 separate cell preparations, these were combined in a large Eppendorf microtube and centrifuged at 7,500 x g for 10 minutes before a further wash with 1ml of RPMI buffer and a further centrifugation step to pellet the washed cells . The pellet was then snap frozen in solid CO_2 , prior to storage at -70°C until use.

Control lymphocyte preparations used in the evaluation of the various assay parameters were obtained from the Scottish Blood Transfusion Service, as 2 batches of 'buffy coat'. Arrival was 4 weeks between each batch, preparation was

as described above for heparinised blood, with the exception that the final cell pellets were dispensed into 100µl aliquots, transferred to small Eppendorf tubes, spun at ·7,500 x g and the supernatant discarded, and the pellet stored at -70°C until use.

4.7 DNA extraction

DNA was extracted from fresh whole blood samples collected in di-sodium EDTA for both the purine and pteridine studies.

A maximum of 10mls of whole blood was transferred to a 50ml polyethene tube and 2 volumes of cold lysis mix added, The lysis mix consisted of 0.32 M sucrose, 10mM Tris, 5mM $MgCl_2$, 1% Triton X-100, and adjusted to pH 7.5 before storage at 4°C. Separation was achieved by spinning the solution at 4°C for 5mins at 1000 x g in a Sorvall RT 6000D refrigerated centrifuge. Supernatant was poured off into a beaker of 5% Chloros, with particular care taken for those blood samples which have been frozen prior to extraction. After addition of 2mls of nucleic acid mix (0.4M NaCl; 10mM Tris; 2mM EDTA; pH 8.2); 200µl 10%SDS and 100µl of a 10mg/ml preparation of Proteinase K, the mixture was vortexed briefly to resuspend the pellet. Incubation in a 37°C waterbath overnight was followed by addition of 700µl of a saturated NaCl solution with a vigorous shaking prior to a further centrifugation for 15 minutes at 200 x g at room temperature.

Using a disposable Pasteur pipette the supernatant was transferred to a 5ml centrifuge tube containing 600µl of a phenol/chloroform solution (water saturated phenol) (BDH) with 1M Tris to give pH 7.5 containing 0.1% 8-hydroxyquinoline mixed with an equal volume of chloroform (BDH) and allowed to settle) before mixing by gentle inversion for at least 1 minute to ensure complete mixing. Centrifugation for 15minutes for 1200rpm at room temperature, allowed the removal of the supernatant from the aqueous layer into a universal container. After addition of 2 volumes of ethanol, the DNA fibres were spooled out using a flame sealed glass Pasteur pipette, and allowed to dry thoroughly in air. The dried DNA was dissolved in a 1ml screwtop container containing TE buffer (10mM Tris; 1mM EDTA; pH to 7.5), using approximately 500µl of buffer for a typical 10ml blood sample yield of DNA, and left at room temperature overnight. The DNA was stored at -70°C until use.

All solutions with the exception of the phenol / chloroform solution were autoclaved prior to use.

4.7.1 Oligonucleotide primer preparation

Two stock preparations of oligonucleotide primers were constructed for analysis of the 677C→T mutation, corresponding to the base sequences :-

1) 5'-AGAAGGAGAAGGTTGTCTGCGGGA-3' (exonic)
2) 5'-AGGACGGTGCGGTGAGAGTG-3' (intronic)

The primers were synthesised on an ABI Model 391 Oligosynthesiser using a Cruachem 0.2μM column and stored at 4°C until deprotection.

4.7.1.1 Primer deprotection

Deprotection of the primers consisted of drawing 1ml of Ammonium hydroxide into a 1ml polypropylene syringe before connection to one end of the column. An empty syringe was attached to the other end and the column washed with back and forward flushing approximately 3 times to remove the primer preparations for the column bed. This was repeated at 30 minute intervals over a period of 3 hours, always ensuring that the column is left in contact with the solution. A decolourisation of the column bed from yellow to white was an assurance of the success of the removal procedure. The ammonium hydroxide solution containing the primer was withdrawn and expelled into a small round bottomed screw-top tube (Nunc), and the volume made up to 2.5 mls with more ammonium hydroxide flushed through the column. The tubes were then incubated at 55°C overnight and stored at -70°C until extraction of the oligonucleotide is performed.

4.7.1.2 Ethanol precipitation of the primer

A volume of 0.45mls of the ammonium hydroxide / primer solution was aliquotted into a large Eppendorf tube and to this was added 50μl of 3M sodium acetate and 1ml of 95% ethanol (BDH). After gentle agitation of the mix for approximately 30 seconds, it was incubated at -40°C for 2 hours prior to centrifugation at 10,000 x g for 10 minutes at 4°C. The supernatant was removed and the pellet washed with 85% ethanol before a further centrifugation at 7,500 x g for 2 minutes. The ethanol supernatant was removed and the pellet allowed to air dry before being resuspended in 200μl of TE buffer.

The resulting stock was measured at OD^{260} and the required dilution was calculated to give an appropriate concentration for the PCR assay as follows:-

Example of the calculation for primer stock dilution

Gram formula weight = number of base pairs x 300 (average weight of each base)

= 24 x 300 = 7200 g / l

(if 1M = 7200g/l , then 1μM = 7.2μg/ml)

Spectrophotometric measurement of primer at 260nm = 0.241

Therefore, 0.241 x 33 x 100 = 795.3 μg/ml

where: 33 is the extinction coefficient of DNA in μg/ml (1 OD^{260} = 33μg/ml)

100 is the dilution factor in the cuvette (5μl into 500μl distilled water).

The molarity of the stock primer is then $\frac{795.3\ \mu g / ml}{7.2 \mu g / ml}$ = 110.4 μmol

This is then diluted in distilled water to give a final stock preparation of 5μM, and stored at -20°C until use.

4.8 Purine enzyme assays

4.8.1 Sample Preparation

Since the activities of the enzymes under study for any specific tissue have to be compared, it was necessary to relate these to a common factor for the specific tissue under examination. Prior to the commencement of any of the purine enzyme assays, the concentration of either haemoglobin or total protein as appropriate was measured.

4.8.2 Haemoglobin Estimation

Haemoglobin concentration was measured by modification of the method described by van Kampen and Ziljstra (1961).

The activity of the red cell enzymes was expressed as a measure of the amount of haemoglobin present in the prepared haemolysate. This method measures haemoglobin concentration by the reaction of ferricyanide-cyanide on blood, which converts haemoglobin to cyanmethaemoglobin. The resulting optical density, determined at 540nm, is directly proportional to the concentration of cyanmethaemoglobin present.

The ferricyanide-cyanide reagent (colloquially known as Drabkins solution), contained 200mg potassium ferricyanide ($K_3Fe(CN)_6$) (BDH); 50mg potassium cyanide (KCN) (BDH) and 140mg potassium di-hydrogen phosphate (KH_2PO_4) (BDH) dissolved in 1 litre of distilled water. This solution is stable for several months when stored at 4°C.

4.8.2.1 Preparation of haemolysates

Using a plastic disposable pipette, approximately 2 drops of the citrate-glycerol red cell sample was added to 8 drops of distilled water in a large Eppendorf reaction tube, and vortexed thoroughly for 5 seconds. The resulting haemolysate was spun in a Microcentaur centrifuge at 7,500 x g for 10 minutes at room temperature to remove the stroma, and placed on ice.

A volume of 5µl of the haemolysate was added to 1ml of the Drabkins solution in a 1ml Sarstedt disposable plastic spectrophotometer cuvette, and mixed carefully avoiding the introduction of bubbles with a 1ml capacity Gilson pipette. The tubes were left at room temperature for 5 minutes to allow complete conversion of the haemoglobin to cyanmethaemoglobin, and read on a Pye Unicam 8800 UV/VIS double beam spectrophotometer at OD^{540}. The spectrophotometer was zeroed using 1ml of Drabkins solution alone as a sample blank, prior to measurement.

4.8.2.2 Calculation

The concentration of haemoglobin was calculated as follows:-

$$\frac{d \times OD^{540} \times M \times 10^{-4}}{\varepsilon_{CN}^{540}} = \text{grams / 100mls (g/\%)}$$

where:

d = dilution factor $= \frac{0.005 + 1.000}{0.005} = 20$

OD^{540} = Optical density of solution at 540nm.

ε_{CN}^{540} = millimolar coefficient of absorptivity for cyanmethaemoglobin.

M = molecular weight of haemoglobin = 16,520.

All estimations were performed in duplicate and the average of the readings for each sample used to calculate the final result.

The resulting preparations were further diluted to obtain working red cell samples for the radiolabelled assays with haemoglobin levels in the region of 1-2g% for HGPRT and APRT analysis, and 0.5-1g% for ADA analysis. For the spectrophotometric analysis of PNP a concentration of 0.5 - 1.0 g% haemoglobin was also used. Values were converted to units of mg/5µl to correspond to the sample volume used in the radiolabelled assay calculation .

4.8.3 Total Protein Estimation

This method was modified from that described by Lowry *et al* (1951).

In this widely used technique, the protein in the sample is pre-treated with an alkaline copper solution, before addition of a phenolic reagent. The colour produced results from the reduction of the phosphotungstic and phosphomolybdic acids to molybdenum blue and tungsten blue by the copper-peptide bond complex, the tyrosine and tryptophan moieties and to a lesser extent the cysteine and histidine groups of the protein.

Three solutions were combined into a working reagent for the assay. These consisted of 2% Na_2CO_3 (BDH) in 0.1M NaOH (solution A); 1% $CuSO_4$ (BDH) in distilled water (solution B1) and 2% Na K Tartrate (BDH) in distilled water. These were combined in the ratio of 98 : 1 : 1 of solutions A, B1 and B2 respectively, mixed and 800µl of this working reagent was added to each tube.

A solution of 500µg/ml bovine serum albumin (BSA) was prepared which was diluted down to 400,300,200,100,50 and 0µl in distilled water, to construct a standard curve. Dilutions of the sample material were judged to be appropriate at doubling dilutions of x20, x40 and x80 in distilled water.

A volume of 80µl of either the standard or sample dilution was added to the appropriate tube and vortexed thoroughly before being left at room temperature for 10 minutes. After addition of 80µl of a 1:1 dilution of Folins Reagent (BDH) and distilled water, the reaction tubes were placed in the dark at room temperature for 45 minutes to enable the reaction to reach completion.

Optical densities of standards and samples were read from a Pye Unicam 8800 UV/VIS double beam spectrophotometer and sample concentration calculated from the resulting standard curve using Genesis curve fitting software. (Genesis: Windows software for microplate based assays, Labsystems (UK) Limited). Values obtained for the protein concentration in µg/ml were adjusted to units of mg/5µl to correspond to the sample volume used in the radiolabelled assay activity calculation.

4.8.4 Radiolabelled Assays

The enzyme activities were determined by a modification of the method described by Galjaard (1980).

4.8.4.1 Hypoxanthine-Guanine Phoshoribosyl Transferase (HGPRT)

HGPRT in the presence of 5'-phosphorybosyl pyrophosphate (PRPP), acts upon the radiolabelled substrate [8-^{14}C] hypoxanthine, to produce the products inosine and inosinic acid (IMP). These radiolabelled products were then separated by thin layer chromatography, and subsequently measured and calculated to determine the activity of the enzyme.

4.8.4.1.(i) Liquid Scintillation Counting

One of the best methods of measuring a particular moiety is to use the radioactive form of the substance under investigation. A small quantity of the radioactive form (tracer) is usually mixed with a much larger amount of its non-radioactive counterpart, and the behaviour of the two is then assumed to be identical. The tracer can be easily detected and measured by its radioactivity and can readily provide information about the behaviour of the non-radioactive form. The effectiveness of radioactive tracers necessitates a sensitive method of detection, and to this end scintillation counting, specifically liquid scintillation counting provides this.

Scintillation counting is based on the principle that a charged particle emitted from the source material (either as an alpha or beta), is absorbed by a fluorescent material, the 'scintillator' (or 'fluor'), which convert the kinetic energy of an incoming charged particle to photons which are re-emitted and detected by a photomultiplier tube, converted to electrical energy and analysed.

Scintillation counters are of two main types, the solid scintillation counter and liquid scintillation counter. In the liquid scintillation counter, the penetration of β-particles is so short that they cannot pass through the walls of the sample container and so must be brought into close relationship with a suitable solvent which acts as a scintillator. Since some of the most important and convenient radioisotopes in biochemistry emit β-particles of low energy, such as ^{14}C and ^{3}H, only liquid scintillation counting is suitable.

4.8.4.1.(ii) Method

The HGPRT assay was performed in 200µl volume Eppendorff tube and consisted of 10µl 1.0M/l Tris-HCl buffer (pH 7.4) containing 5.0mM/Ll PRPP and 12.5mM $MgCl_2$; 5µl of 19.1mM hypoxanthine (dissolved in acidified distilled water and pH adjusted to between 4.0-4.4 with NaOH) and 5µl of 0.909mM [8-^{14}C] hypoxanthine (specific activity 54mCi/mmol (Amersham)). This was maintained on ice at all times. Following careful vortexing for 10 seconds, 5µl of the sample lysate was added to the reaction mixture and vortexed. A thin layer of paraffin oil was applied to the surface of the mixture and the tube returned to ice until all sample preparation was complete. Incubation was at 37°C for either 1 hour for red cells, or 2 hours for other tissues. The reaction was terminated by addition of 5µl of ice cold 20% trichloroacetic acid (TCA), and the resulting denatured proteins removed by centrifugation at 10,000 x g in a MSE Microcentaur microcentrifuge. Reaction products and unused substrate were separated by ascending chromatography on polyethylimine-F cellulose (PEI-F) (BDH) by spotting 2µl sample supernatant over 6µl of a carrier solution consisting of a 1:1:1 mix of 5mg/ml hypoxanthine : inosine :IMP, in 5% Na_2HPO_4 (BDH) as solvent for 1.5 - 2 hours. The completed chromatograms were dried gently in a warm air stream and the spots identified in a light box under short wave ultra-violet light, before being cut out and the radioactivity quantified by heterogeneous liquid scintillation counting in a Wallac 1407 counter with Ecoscint A scintillation fluid (National Diagnostics).

4.8.4.1.(iii) Calculation of results

Results were calculated using the formula:-

$$\frac{\text{CPM (product} - \text{blank)}}{\text{CPM (total counts)}} \times \frac{100}{T} \times \frac{1}{\text{Hb or protein} / 5\mu l} = \text{nmoles /hr/mg}$$

where:- CPM = Counts per minute

T = Time (hrs)

100 = substrate concentration (nmole/l)

Hb = Haemoglobin content of sample in mg.

4.8.4.2 Adenine Phosphoribosyl Transferase (APRT)

APRT in the presence of PRPP, acts upon the radiolabelled substrate [8-^{14}C] adenine, to produce the products adenosine, and AMP. These radiolabelled products are detected by thin layer chromatography, and subsequently measured and calculated to determine the activity of the enzyme.

4.8.4.2.(i) Method

The method for APRT was a modification of that described previously for HGPRT (Section 4.8.4.1.(ii)). The assay was performed in 200µl volume Eppendorff tube and consisted of 10µl 1.0M/l Tris-HCl buffer (pH 7.4) containing 5.0Mm/l PRPP and 12.5mM $MgCl_2$; 5µl of 19.1mM adenine (dissolved in acidified distilled water and pH adjusted to between 4.0-4.4 with NaOH) and 5µl of 0.909mM [8-^{14}C] adenine (specific activity 54mCi/mmol (Amersham)) and maintained on ice at all times. Following careful vortexing for 10 seconds, 5µl of the sample lysate was added to the reaction mixture and vortexed. A thin layer of paraffin oil (BDH) applied to the surface of the mixture and the tube returned to ice until all sample preparation was complete. Incubation was at 37°C for either 1 hour for red cells, or 2 hours for other tissues. The reaction was terminated by addition of 5µl of ice cold 20% TCA (BDH), and the resulting denatured proteins removed by centrifugation at 10,000 x g in a microcentrifuge. Reaction products and unused substrate were separated by spotting 2µl supernatant over 6µl of a carrier solution consisting of a 1:1:1 mix of 5mg/ml adenine : adenosine : AMP before ascending chromatography in 5% $NaHPO_4$ as solvent for 1.5 - 2 hours. The subsequent chromatograms were dried gently in a warm air stream and the spots identified in a light box under short wave ultra-violet light, before being cut out and the radioactivity quantified by heterogeneous liquid scintillation counting in a Wallac 1407 counter with Ecoscint A scintillation fluid.

4.8.4.2.(ii) Calculation of results

Calculation of results was as described for HGPRT (Section 4.8.4.1.(iii)).

4.8.4.3 Adenosine Deaminase (ADA)

ADA acts upon the radiolabelled substrate [8-^{14}C] adenosine, to produce the product, inosine. The radiolabelled product is detected by thin layer chromatography, and subsequently measured and calculated to determine the activity of the enzyme.

4.8.4.3.(i) Method

The method for adenosine deaminase was a modification of that described for HGPRT. The assay was performed in 200µl volume Eppendorff tube and consisted of 10µl 0.25M/l Tris-HCl buffer (pH 7.4): 5µl of 0.875 unlabelled Adenosine and 5µl of 0.91mM [8-^{14}C] adenosine (specific activity 46 mCi/mmol (Amersham)). This was maintained on ice at all times. Following careful vortexing for 10 seconds, 5µl of the sample lysate was added to the reaction mixture and vortexed. A thin layer of paraffin oil was applied to the surface of the mixture and the tube returned to ice until all sample preparation was complete. Incubation was at 37°C for either 1 hour for red cells, or 2 hours for other tissues. The reaction was terminated by addition of 5µl of ice cold 20% trichloroacetic acid, and the resulting denatured proteins removed by centrifugation at in a microcentrifuge. Reaction products and unused substrate were separated by spotting 2µl supernatant over 6µl of a carrier solution consisting of a 1:1 mix of 5mg/ml adenosine : inosine, before ascending chromatography on PEI-F cellulose in 5% Na_2HPO_4 as solvent for 1.5 - 2 hours. The subsequent chromatograms were dried gently in a warm air stream and the spots identified in a light box under short wave ultra-violet light, before being cut out and the radioactivity quantified by heterogeneous liquid scintillation counting in a Wallac Rackbeta 1407 counter with Ecoscint A scintillation fluid (National Diagnostics).

4.8.4.3.(ii) Calculation of results

Results were calculated using the formula:-

$$\frac{\mathrm{CPM(product - blank)}}{\mathrm{CPM(total\ counts)}} \times \frac{\text{substrate conc}}{1} \times \frac{1}{T} \times \frac{1}{\mathrm{Hb\ or\ protein} / 5\mu l}$$

= nmoles / hr/mg protein.

where:-

CPM = Counts per minute

Substrate concentration = 9 nmol/l

T = Time (hrs)

Hb = Haemoglobin (mg)

4.8.5 Spectrophometric assays

The Beer-Lambert law states that the concentration of a substance is directly proportional to the amount of radiant energy absorbed or inversely proportional to the logarithm of the transmitted radiant energy. This can be described by the formula:

$$A = abc$$

where A is the absorbance; a is the absorptivity; b is the light path and c is the concentration of the substance of interest. The absorptivity is a proportionality constant related to the chemical nature of the substance, and is called 'molar absorptivity' (denoted as ε) when the concentration is expressed in moles per litre and the light path in centimetres. Molar absorptivity is constant for a given compound at a given wavelength under specified conditions of solvent, pH and temperature and is a means of relating concentration and absorbance for any substance.

Thus, the equation becomes :-

$$A = \varepsilon bc$$

Absorbance measurements for these studies were performed using a Pye-Unicam 8800/03 UV/Visible Spectrophotometer. The instrument has the facility to perform a power-on self diagnostic, where both the optics and peripherals are tested prior to use, and as the name suggests is capable of measuring in both the ultra violet and visible spectra by virtue of having both a quartz-halogen and deuterium arc source. Glass and plastic disposable cuvettes can be used at wavelengths above 320nm, but due to their absorbance characteristics below this wavelength, were replaced by optically identical quartz cuvettes when samples were measured at ultra-violet wavelengths.

4.8.5.1 Purine Nucleoside Phosphorylase (PNP)

PNP acts upon the substrate inosine to produce hypoxanthine. The auxilliary enzyme, xanthine oxidase converts this via xanthine to uric acid. The uric acid concentration is measured spectrophotometrically to determine the activity of the enzyme.

4.8.5.1.(i) Method

A reaction volume of 0.5ml of inosine (1.5mM) in 0.05M sodium phosphate buffer (pH 7.5) and 6.25µl xanthine oxidase (0.72units/mg protein), was mixed thoroughly in a 0.5ml micro quartz cuvette using a 200µl Gilson pipette. This was placed into the thermostatted cuvette carrier compartment, of a Pye Unicam 8820 double beam UV / VIS spectrophotometer, the carrier having been pre-heated to a temperature of 37°C. To a second optically matched cuvette was added 0.5ml of 0.05M sodium phosphate buffer and 6.25µl xanthine oxidase. After thorough mixing, this cuvette was placed in the reference beam carrier of the spectrophotometer to act as the reagent blank. The cuvettes were allowed to incubate for 3-4 minutes before addition of 6.25µl of haemolysate to each, again ensuring complete homogeneity by careful mixing. Accumulation of uric acid was measured by an increase in the absorbance at 293nm. All measurements were performed in a heating block connected to a water bath controlled thermostatically and the result displayed as a change in optical density (ΔOD) on an integral thermal printer.

4.8.5.1.(ii) Calculation of Results

The results for the PNP assay were calculated using the following formula:-

$$\frac{\Delta OD / min}{\varepsilon} \times \frac{100}{Hb} \times \frac{V_c}{V_h} \times 60 = \mu moles / hr / gHb \text{ or protein}$$

where

V_C = final volume in cuvette.

ε = millimolar extinction coefficient of uric acid (12.6).

Hb = quantity of haemoglobin in original haemolysate (g %)

V_h = volume of haemolysate.

100 = conversion factor from mls to 100mls for Hb.

4.8.6 High Performance Chromatographic Assays

High Performance Liquid Chromatography (HPLC) is a chromatographic technique which yields highly efficient separations within a short period of time, in which the sample mixture is distributed between 2 phases in the chromatographic bed. One phase is stationary, consisting either of a solid porous surface active material in small particle form, or a thin film of liquid coated on a solid support or column, while the other phase is mobile and consists of a liquid which distributes itself throughout this stationary phase. The various components to be separated must have different distribution coefficients in the chromatographic system if the mixture is to be separated efficiently.

Reversed phase (RP) chromatography is the term used to describe the state in which the stationary phase is less polar than the mobile phase. Chemically bonded octadecylsilane (ODS) an alkane, with 18 carbon atoms is the most frequently used stationary phase. In normal phase adsorption chromatography, water interacts strongly with the active centres of silica and alumina bases of the stationary phase, so that absorption of sample molecules become highly restricted and as a consequence are rapidly eluted from the column. In RP the opposite applies, in that water cannot interact with the non-polar (hydrophobic) alkyl groups. Hence it is the weakest RP mobile phase, giving the slowest sample elution rate, since samples will tend to bind to the solid phase in preference to water. The more water in the eluent , the longer the retention time.

Ionic samples may be separated by reversed-phase chromatography provided they contain only weak acids or only weak bases present in undissociated form as determined by the chosen pH.

Ion-pair chromatography uses the technique of "ion-suppression", where an organic ionic substance is added to the mobile phase and forms an ion-pair with a sample

Ion-pair chromatography uses the technique of "ion-suppression", where an organic ionic substance is added to the mobile phase and forms an ion-pair with a sample component of the opposite charge. This salt has the chromatographic behaviour of a non-ionic molecule. The advantages of ion-pair chromatography for separating ionic molecules are that RP systems can be used for separation; mixtures of acids, bases and amphoteric molecules can be separated and the selectivity of the system may be influenced by the choice of the counter ion.

4.8.6.1 Phosphoribosyl pyrophosphate synthetase (PRPP-S)

The assay of PRPP-S was based on a modification of the method described by Sakuma *et al* (1991).

The enzyme PRPP-S catalyses the synthesis of PRPP from R5P and ATP in the presence of inorganic phosphate and magnesium. AMP is then reconverted back to ADP by adenylate kinase. The reaction sequence is as follows:

$$R5P + ATP \rightarrow PRPP + AMP$$
$$AMP + ATP \rightarrow 2ADP$$

Where the concentration of ATP present is in excess of AMP.

The ADP produced is followed by measuring the increase in the absorbance of ADP after separation of the ADP from ATP and AMP by an ion-pair reversed-phase HPLC, and is a direct measure of the reaction catalysed by PRPP-S.

All reagents were of the highest purity available, and the water used was purified to 18MΩ.cm by a Millipore Milli-Q RG Ultrapure Water System.

4.8.6.1.(i) Sample Preparation

The red cell samples prepared and stored as previously described were thawed at room temperature, and 1.0ml of the mixed homogenate added to a 1.5ml plastic Eppendorf tube. This was spun at 10,000 X g for 10 minutes in a microcentrifuge, pre-cooled to 4°C. After discarding all but a small amount of the supernatant (required to overcome the viscosity of the preparation), the remaining red cells were sonicated x2 at 2μm for 5 seconds and diluted with 5 volumes of a charcoal-dextran T70 solution consisting of 10g/L charcoal:1g/L Dextran T70 in cold distilled water. After a 10 second vortex and a 10 minute equilibration period on ice, the

Haemoglobin was estimated by the method of van Kampen and Ziljstra (1961) as previously described in Section 4.8.2.

4.8.6.1.(ii) Enzyme Assay

A reaction mix was prepared consisting of 100mls of a 40mM sodium phosphate buffer (pH 7.4) to which was added 1.0mM R5P; 1.4mM ATP; 6mM $MgCl_2$ and 1.0Mm reduced glutathione. The pH of the buffer was rechecked and readjusted to 7.4 if necessary, before use, either immediately or after storage at 4°C for not more than 5 days.

Immediately prior to use, the appropriate volume of reaction mix required for the number of samples to be processed was measured out and to this was added 1.8 IU/ml of adenylate kinase (AK; Myokinase) (Boehringer). Plastic 2.5ml LP2 tubes were labelled accordingly and using a Gilson pipette, 2.0mls of this reagent was added to each. All tubes were pre-incubated at 37°C for 5minutes before addition of 100µl of the sample. All tubes were then returned to the 37°C water bath. Volumes of 0.5mls were removed after 10 minutes and after 30 minutes incubation and transferred to Eppendorf tubes containing 0.5mls of 0.6M Perchloric acid. After a 10 second vortex mix and equilibration for a further 10 minutes at room temperature, all tubes were centrifuged at 10,000 x g in a microcentrifuge for 10 minutes before removal of 0.2mls of the mix into 0.4M sodium phosphate neutralising buffer (pH 7.4). A final vortex followed by centrifugation at 10,000 x g was sufficient to remove all particulate matter. The sample was stored at 4°C until determination of ADP levels by HPLC.

4.8.6.1.(iii) HPLC

A volume of 50µl was syringed into the injection port of an HPLC apparatus (a modular LKB system) consisted of a model 2152 controller, a model 2150 single pump and a Uvicord SD detection system with a flow cell. The gradient was generated and maintained by the controller via a switching valve which determined the appropriate concentrations of the two buffers throughout the run. The UV detector was set at 254 nm on 0.05 AUFS and the signal output was displayed on a 2210 potentiometric chart recorder running at a speed of 2mm/minute.

All separations were performed at ambient temperature on a Resource RPC 3 reversed phase column (100 x 6.4 mm: Pharmacia Biotech) with 15μm bead size and a Waters guard column. The mobile phase consisted of 2 separate buffers; Buffer A which comprised 0.03M/L potassium di-hydrogen phosphate / ammonium chloride (pH 3.9) containing 300μM/L of the ion-pair reagent, Tetrabutylammoniumhydrogen sulphate (TBAHS) and Buffer B which was 0.1M/L potassium di-hydrogen phosphate / ammonium chloride (pH 3.4) containing 50 mM/L TBAHS and 30% Methanol.

The buffers were degassed in-line using a Whatman Aqueous In-Line Filter / Degasser (IFD) which traps gas and prevents it entering the flow system.

The flow rate used was 1.0 ml/minute and to maintain adequate separation the gradient was 0-60%B for 20 minutes for the run followed by 60-0%B for 10 minutes as an re-equilibration step.

Prior to each batch of samples, 3 in-house standards comprising 140μM/L, 70μM/L and 35 μM/L ADP were run in order that the concentration of the samples could be calculated by reference to the size of standard peaks obtained.

Since the deleterious effects of the use of ion-paired reagents have been well documented, the column was cleaned at the end of the days run using the protocol specified on the data sheet for the column for contaminations which dissolve at acidic pH. This involved reversing the direction of the column flow and running 2 solutions; 1M/L HCL (Solution A) and 90% acetonitrile (Solution B) in the gradient 0f 0-100% B for 30 minutes followed by 100-0%B for 30 minutes at a flow rate of 1.0 ml/minute.

When not in use overnight or for any prolonged length of time, the system was left running with 50% ethanol at a low flow rate of 0.01ml / minute, to maintain column integrity and to prevent salt build up on pump seals and other components of the system.

Prior to the analysis of the samples the HPLC system was assessed for its suitability of application for determining the adenosine nucleotides. The column parameters were quantified using a reversed phase test mix (Sigma 251SA) to establish the retention parameters and the efficiency of the column. Periodic testing of the retention and efficiency properties was performed to assess the quality of the sample results obtained. The reversed phase test mixture used, consisted of six components in varying concentrations: uracil (0.005mg/ml), phenol (0.7mg/ml), benzaldehyde (0.05mg/ml), N,N-diethyl-m-toluamide (1mg/ml), and

toluidine and ethybenzene (4mg/ml each) dissolved in a 65% acetonitrile - 35% water and was stored at 4°C until use.. The components were resolved on the column by injection of 10µl of the test mixture at a sensitivity of 0.5 AUFS at 254nm wavelength, and the retention time of each component calculated.

4.9 Enzymes of the Folate pathway

4.9.1 PCR Method for thermolabile MTHFR Alleles

This method is a modification of that described by Frosst *et al* (1995).
A mutation which alters a highly conserved amino-acid in the homozygous and heterozygous state correlates with the presence of reduced enzyme activity and thermolability in lymphocyte extracts. This mutation (C to T substitution) at nucleotide 677 converts an alanine residue to valine and creates a *Hinf*1 site. This can be cleaved to give 175bp and 23bp fragments in the mutation, compared with a 198bp fragment in the wild type allele.

4.9.1.1 PCR Method

The primers used in the analysis of the thermolabile allele were as described in Section 4.7.1.
To facilitate consistency of the reagents, a working solution was prepared to accommodate the appropriate number of samples (plus 1 extra) and a total of 2.5µl primer 1; 2.5µl primer 2; 2.5µl of *Taq* buffer (supplied with the *Taq* polymerase enzyme) (Boehringer) and 2.5µl of deoxy nucleic triphosphates (dntp's) was prepared for each sample to be assayed. An Eppendorf tube was labelled for every sample to assayed, and 10µl of this working solution was added to each with thorough mixing, followed by 13µl of distilled water and 1µl of the appropriate individual DNA. After overlaying the mixture with mineral oil using a disposable plastic pipette (just enough to cover the preparation, ~1-2 drops), the samples were loaded into a Perkin Elmer 480 DNA Thermal Cycler, and a 'soak' file run to bring the temperature of the tubes up to 90°C. This step also prevents premature reaction at room temperature, prior to the addition 1µl of *Taq* polymerase (1 Unit) as soon as this initial temperature is reached. The PCR was then run until

completion of the pre-set program for 34 cycles (usually around 2.5 hours) utilising the following protocol:-

⇒ Heating to 95°C for 5 minutes
⇒ 34 cycles of 95°C for 30 seconds
55°C for 30 seconds
72°C for 45 seconds
⇒ 72°C for 10 minutes
⇒ 4°C until removal from PCR instrumentation.

On completion of the PCR, the tubes were removed from the heating block and either the *Hinf*1 digest performed immediately, or they were stored at 4°C overnight until the digest could be performed.

4.9.1.2 *Hinf*1 Digest

The *Hinf*1 enzyme (Boehringer) was removed from -20°C storage, allowed to thaw and pulsed on a microcentrifuge to ensure that all the preparation was at the bottom of the tube. A small Eppendorf tube was labelled for each sample and to this was added 1.5µl *Hinf*1 buffer (previously thawed from -20°C); 10µl of the appropriate sample PCR; 1µl *Hinf*1 enzyme and 2.5µl distilled water. This reaction mix was incubated overnight at 37°C. Using tris-acetate buffer, a 2% agarose solution was prepared and boiled until molten. To this was added 4µl ethidium bromide, and the solution mixed thoroughly prior to pouring into a gel mould containing a 2 x 10 tooth combs. The gel was allowed to solidify and moved to a 4°C environment for 30-60 minutes prior to use. A volume of 2µl of loading mix consisting 50% glycerol and 50% bromophenol blue dye of was added to each digest tube, which was then mixed. The gel comb was removed from the gel, which was placed in the mini-gel tank, followed by Tris-acetate tank buffer. Addition of samples and molecular weight ladder was added under the buffer and the apparatus run at 125V for 2 hours or until the upper series of samples had run almost to the lower comb. The gel was removed from the tank and visualised under UV light with a photographic record retained for comparison.

4.9.2 Lymphocyte MTHFR Assay

Lymphocyte preparations were made as described previously in Section 4.6.1 (Lymphocyte cells: Collection and preparation.)

The lymphocyte cell pellets were removed from -20°C storage and allowed to thaw at room temperature. After addition of 100µl distilled water, the preparation was thoroughly vortexed prior to sonication twice for 5 seconds at 2µm amplitude and a further centrifugation for 5 minutes at 7,500 x g . The supernatant was assayed for total protein using the method described in Section 4.8.3, and the protein concentration adjusted to 2mg/ml with distilled water for use in the assay.

Addition of 30µl of sample (or distilled water for the blanks) to each of two screwtop microtubes was followed by the incubation of one tube in a block heater for 5 minutes at 46°C while the other remained at room temperature. On completion of this step, both tubes were placed on ice for the remainder of the subsequent reagent additions.

To each tube was added 579.6µl of 0.217M potassium phosphate buffer (pH 6.8) containing 10.5M L (+) ascorbic acid (Fisons)(the addition of which was made just prior to use); 50µl of 16.1mM EDTA (pH 7.0); 5.4µl [Methyl -^{14}C] methylene tetrahydrofolate (50µCi/ml) (Amersham); 5µL of 7.56mM flavine adenine dinucleotide (FAD) and 50µl of either treated or untreated sample or distilled water for the blank. This gave a final volume in the Eppendorff tube of 690µl. All tubes were mixed thoroughly and placed on ice until the same stage of preparation, at which time 10µl of 7mM menadione was added to all tubes. The tubes were vortexed thoroughly and incubated in a waterbath at 37°C for 1.5 hours, after which time they were again removed to ice. Derivatisation of the reaction product was accomplished by a mixture of 10µl of 1.0M formaldehyde; 200µl of 50µM Dimedone in 50% ethanol and 100µl of 3.0M potassium acetate solution (pH 4.5 with acetic acid), combined with heating at 95°C on a solid heating block for 15 minutes. After cooling on ice for 10 minutes, the mixture was removed with a 1.0ml Gilson pipette to 3.0mls of toluene (BDH) in a 15ml screwtop tube. Refluxing the tube with toluene from the upper phase of the preparation ensured the complete transfer of the derivative. This toluene preparation was vortexed for 15 seconds and centrifuged for 10 minutes at 900 x g in an IEC Centra GP8, with 2.0mls of the upper phase removed into a 20ml plastic scintillation vial containing 5.0mls of Ecoscint A scintillation fluid. The remainder of the upper and lower phases were

also treated similarly in a second vial. All vials were counted on a Wallac 1407 liquid scintillation counter for 2 minutes and the activity calculated as follows.

$$\frac{DPM\,(product-blank)}{DPM\,(totalcounts)} \times \frac{20x10^{-6}M}{1} \times \frac{1}{T} \times \frac{1010}{50} \times \frac{3}{2} \times \frac{1}{P}$$

where : DPM = Disintegrations per minute

20×10^{-6} M = Label concentration in 1.0ml final volume

T = Time (hours)

P = Total protein (mg/ml)

1010 = final volume of stopped reaction (µl)

50 = sample volume (µl)

3/2 = Toluene fraction removed

FAD was prepared as a 7.56mM stock solution and dispensed into 100µl aliquots. Stored at -20°C until use and thawed at room temp. Menadione was prepared as a 7mM stock solution and dispensed into 200µl aliquots and stored at -20°C until use and thawed in the
dark at room temperature until use. Aliquots were kept out of the light at all times during assay preparation.

4.10 Red cell folate and plasma vitamin B_{12} Estimation

The nutritional status of individuals participating in the study was assessed by the measurement of Folate and vitamin B_{12} using an ICN Simultrac-SNB Radioassay kit. This method simultaneously determines levels of folate and B_{12} by competitive protein binding, where the unlabelled folate or B_{12} competes with its labelled species for the limited number of available binding sites on the specific binder. Therefore the level of radioactivity is inversely related to the concentration of in the patient sample. Both species are determined in a single assay tube since the two tracers [^{57}Co] for vitamin B_{12} and [^{125} I] for folate produce energies at levels which can be easily separated by a two channel gamma scintillation counter.

4.10.1 Sample and reagent Preparation

Plasma and red cells samples previously separated and stored as described in the procedure in Section 4.2 were thawed and mixed thoroughly by vortexing. The plasma samples were used neat in the assay. Red cell samples were diluted by adding 100µl to 2ml of freshly prepared 0.2% ascorbic acid solution (w/v). This 1 in 21 dilution was mixed thoroughly and left to stand at room temperature in the dark for 1.5 hours prior to assay. The haematocrit of the packed red cell samples was estimated using a Coulter ZM particle analyser, and the haemoglobin concentration was estimated by the cyanmethaemoglobin method described in Section 4.8.2. The results were noted for use in the later calculation of folate concentration. The reagent kit is stored at 4°C and brought to room temperature prior to use.

The tracer solution containing both of the isotopes was prepared by the addition of all of the contents of the dithiothreitol (DTT) reagent to the tracer solution. In order to maximise the number of samples analysed by the kit, the recommended volumes were scaled down by a factor of 2, after a small pilot study had confirmed that the quality of the results obtained and the method itself were not compromised by this modification.

4.10.2 Radiolabelled assay

All patient samples and standards for the radio-assay were assayed in duplicate, in batches of 120 tubes according to the following design:

Tubes 1,2 :- Total Counts
Tubes 3,4 :- Standard A (blank)
Tubes 5-16 :- Standards A-F (These corresponded to concentrations of 0-2000 pg/ml for Vitamin B_{12} and 0-20mg/ml for folic acid)
Tubes 17,18 :- Normal range control
Tubes 19-118 :- Patient samples
Tubes 119-120 :- Normal range control

A volume of 100µl of each standard, control, patient red cell or plasma sample was added to the appropriate polythene tubes (Sarstedt), followed by 100µl of the tracer to all tubes. The total counts were capped and no further reagent additions

were made. All tubes were vortexed thoroughly and left to stand at room temperature in the dark for 15 minutes, before the addition of 100µl of the extracting reagent, followed by vortexing and a further incubation at room temperature in the dark for 10 minutes. The bottle of Simultrac-SNB blank reagent was thoroughly mixed and 500µL added to tubes 3 and 4 only.

The bottle of Simultrac-SNB binding solution was shaken vigorously and 500µl added to the remainder of the tubes. After vortexing, incubation was at room temperature in the dark for 60 minutes from the time of the last addition of the binder.

All tubes were capped and centrifuged at 1000 x g for 10 minutes at room temperature and the supernatant decanted, assisted by gentle tapping onto absorbent paper to remove any remaining drops. The pellets were counted in a Packard Cobra 5010 gamma scintillation counter using proprietary software to perform a dual RIA, 4 parameter Log-Logistic plotting of the standard curve and calculation of the patient sample folic acid and vitamin B_{12} concentrations after automatic blank subtraction using the values from tubes 3 and 4.

To ensure that the assay meets its required specifications, the trace binding of the 0 standard (B_0) was calculated for each assay batch, by dividing the average of tubes 5 and 6 by the average total counts. Any assay failing to attain a B_0 of greater than 35% was repeated.

4.10.3 Calculation

It is not routinely necessary to correct for plasma folate since this value is very small compared to that of red cell folate, however occasionally elevated plasma folate levels will occur. In this assay, if the plasma folate concentration was greater than 10% of the calculated red cell folate concentration a plasma correction was made to the red cell result.

An example of the red cell folate calculation was as follows :-

Plasma folate concentration	= 32ng/ml
Red cell haemolysate folate concentration	= 3ng/ml
Haematocrit	= 30%
Red cell haemolysate dilution factor	= 21
Uncorrected red cell folate concentration	$= \frac{3.0\text{ng/ml} \times 21}{0.30} = 210\text{ng/ml}$

Since 32ng/ml is greater than 10% of 210ng/ml, the red cell folate concentration must be corrected as follows:-

$$\text{corrected ng/ml of red cell folate} = \frac{(3.\text{ng/ml} \times 21) - (32\text{ng/ml}\ (1-0.3))}{0.30}$$

$$= \underline{135\text{ng/ml}}$$

While the example quoted uses the haematocrit of the red cell sample to calculate the folate levels, assessment of the haemoglobin content of the sample as an alternative was also made.

In obtaining whole blood by postal methods for control and patient evaluation of red cell folate status, it is inevitable that a proportion of the samples may be haemolysed on receipt. The haemolysis renders the folate analysis questionable since the calculation requires that red cell integrity be maintained in assessment of the haematocrit by particle counting methods. In an attempt to avoid this the haemoglobin content was analysed and the value also assessed in the red cell folate calculation.

The accurate usage of the folate/ B_{12} dual labelled kit is highly dependent on the ability of the specified gamma scintillation counter to discriminate between disintegrations of the [^{125}I] and [^{57}Co] isotopes. The counting windows must be set such that there is minimal interference from the [^{125}I] in the [^{57}Co] channel and vice-versa (i.e. spillover).

Assessment of spillover between the two channels was performed using [^{125}I] and [^{57}Co] calibration standards. These were obtained from the West of Scotland Radionuclide Dispensary (Western Infirmary, Glasgow), as 0.2 and 0.02 Megabecquerels (MBq) in 5ml volumes respectively, and diluted with distilled water to give working standards of ~20,000 counts per minute for both, based on a recommended counting efficiency of 20% for [^{125}I] and 50-60% for [^{57}Co]

The method to determine the spillover was as follows:-

1) In the [^{125}I] channel of the gamma counter, count [^{125}I] source = A; count [^{57}Co] source = B;
2) In the [^{57}Co] channel of the gamma counter, count [^{57}Co] source = C; count [^{125}I] source = D;

Calculate spillover by [^{57}Co] in the [^{125}I] channel by $\frac{B}{C} \times 100$

Calculate spillover by [^{125}I] in the [^{57}Co] channel by $\frac{D}{A} \times 100$

Should spillover exceed 3% for either channel, the window setting for the channel must be narrowed or a mathematical method of correction applied.

4.11 Statistical Methods

All statistical calculations were performed on a Dell Dimension XPS P60 microcomputer using "SPSS for Windows" statistical software package.

4.11.1 Medians and Centiles

The median is defined as the value of that observation which, when the observations are arranged into ascending or descending order of magnitude, divides them into two equal sized groups. As a descriptive method, the median maintains the advantage of being unaffected by extreme observations. The median may be determined from a frequency distribution where the data has been divided into classes of equal width, using the formula:-

$$\text{Median} = \left[\frac{(P-f)}{(F-f)} \times (X-x)\right]$$

where
- X = upper boundary of median class
- x = lower boundary of median class
- F = upper frequency of median class
- f = lower frequency of median class
- P = median frequency i.e. (n+1) / 2

Centiles other than the median value (i.e. the 50th centile) may be derived form the same set of data. The *p* th centile can be found by calculation of the (n - *p*) / 100 th value.

4:11.2 Mean, Standard deviation and Coefficient of Variation

The mean (or 'arithmetic mean', $\bar{x}$) is a measure of central value for a series of ungrouped data and is defined as the total of the observations divided by the number of observations. It is calculated by the following formula:-

$$\bar{x} = \frac{\sum_{n=1}^{n} x}{n}$$

where x = individual observations

n = number of measurements

The standard deviation (sd) is a measure of the dispersion or variability of observations around the central value and is described by the formula:-

$$sd = \sqrt{\frac{\sum_{n=1}^{n} \left(x - \bar{x}\right)^2}{n}}$$

The coefficient of variation (cv), is a method whereby the relative amount of variation of a set of values in a distribution may be calculated. This is independent of the absolute values and is expressed as a percentage by the following formula:-

$$cv = \frac{sd}{\bar{x}} \times 100\%$$

where sd = standard deviation

$\bar{x}$ = mean

To avoid the influence of outlying values for normally distributed data, the standard deviation can be estimated as the difference between the 90th and 10th centiles divided by 2.56, and for $\log_{10}$ normally distributed data from from the difference between the $\log_{10}$(90th centile) and $\log_{10}$(10th centile) divided bt 2.56.

4.11.3 Gaussian Distribution

The Gaussian or normal distribution is defined as a symmetrical 'bell shaped' area with the formula:

$$y = \frac{1}{sd\sqrt{(2p)}} \cdot e^{-\frac{1}{2}\left[\frac{x-\bar{x}}{sd}\right]^2}$$

where $\bar{X}$ = mean

sd = standard deviation

4.11.4 Probability Plots

Prior to using the mean and standard deviation for comparative statistics, it is important to know whether the data can reasonably be regarded as being sampled from an underlying Gaussian distribution. Even a sampling from a population whose shape departs from the Gaussian distribution, may still provide the basis for statistical analysis, providing the data is transformed. One method of data transformation is the use of the logarithmic value of the observed values rather than the actual values themselves. The probability plot provides a graphical method of examining departures from normality in both the non-transformed and transformed data.

For a given data set, all values are sorted into ascending order and each value assigned its centile value and from these the standard deviation may be derived from the normal distribution tables which give areas under the normal distribution . A graph is subsequently plotted with theses standard deviations (x-axis) against the actual values or the log transformed values (y-axis). A continuous line of the appropriate Gaussian or log Gaussian distribution, derived from the mean and standard deviation is plotted and the graph inspected for departures from the expected distribution.

4.11.5 Kolmogorov - Smirnov (KS) Test

The KS test is a non-parametric test of the cumulative distribution of a set of values, and is used to determine whether the an observed frequency distribution deviates from the expected one. The KS value , D, is the absolute value of the largest difference between the observed cumulative distribution and the theoretical cumulative distribution. Each value once converted to a standard deviation is read against the statistical tables and the cumulative area under the curve derived. A comparison with the predicted cumulative frequency distribution then allows the individual difference to be calculated for each value. The maximum difference, D_{max}, is compared to the critical value from the statistical tables and the probability (p) derived for the appropriate sample size.

4.11.6 Log Transformation of data.

The term 'normal range' has certain assumptions implicit within it, notably that results obtained for a test conform to the 'bell-shaped' statistical distribution or 'Gaussian distribution'. There is no reason to assume that a particular analyte will exhibit this distribution, and so for those which do not conform , a number of methods are available for mathematically processing raw data in order to more closely match the Gaussian distribution. Since the Gaussian or normal distribution is central to the concept of statistical inference, where even a sampling from a population whose shape departs from this may still provide the basis for statistical analysis. With a normal distribution there are fewer restrictions in the application of statistical methods, hence it is sometimes extremely valuable to make adjustment of these raw values to accommodate this, and yield derived data which approximates that of the classical normal distribution. One of these is the use of logarithmic transformation of the observed values rather than the values themselves.

4.11.7 Correlation

The product - moment correlation coefficient (r), (also known as Pearson's coefficient of correlation), describes the relationship between two mutually dependent variables, *x* and *y* and is described by the formula:

$$r = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\sqrt{\left(\Sigma(x-\bar{x})^2 \cdot \Sigma(y-\bar{y})^2\right)}}$$

The correlation coefficient, r , may have a range of values from 0 to ± 1, with positive and negative r values representing positive and negative correlation respectively. If plotted on a scatter diagram, the points begin to deviate from a perfect straight line as the correlation coefficient departs from +1 or -1. Values of close to 0 indicate virtually no association between the variables, while the values closer to ± 1, describe the magnitude of the association. To determine the statistical significance of the correlation, the r value may be compared at n-2 degrees of freedom, to the appropriate statistical tables, the probability of significance obtained for the data-set under observation.

4.11.8 Students t-test

The parametric or Students t-test is used to compare two different populations, each with a known mean and standard deviation. Since this is a parametric test of significance, this test can only be used on populations which have been shown to be normally distributed. The t-test may be calculated using a number of varying formulae, depending on whether the data is paired or unpaired. The formula used in this instance is as follows:-

$$t = \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{(((n_1 \bullet sd_1^2) + (n_2 \bullet sd_2^2)) / (n_2 + n_2 - 2)) \bullet \sqrt{(1/n_1 + 1/n_2)}}}$$

where :- $(n_2 + n_2 - 2)$ = degrees of freedom.

sd_1 and sd_2 = the standard deviations from each population.

The null hypothesis states that there is no difference in the underlying populations for the two samples. The calculated t values were compared against the t-test statistical table using the appropriate degrees of freedom and the probability values (p) associated with the found value of t derived. On the basis of the probability, i.e. $p<0.05$, the difference of the two means is significant, and the null hypothesis rejected.

4.11.9 Chi-square (χ^2) significance test

The χ^2 test is used to compare differences between 2 independent proportions, with the test statistic based on a measure of the overall differences between observed and expected frequencies displayed in a tabular form. If the observed and expected frequencies in each cell are equal, the value of χ^2 will be zero, and the greater the relative differences between the observed and expected frequencies, the greater the value of χ^2 . Both the observed and expected data must be numerically equal, and this requires adjustment of the data on the expected side of the table prior to calculation of the χ^2 statistic. After calculation of the value of χ^2 a statistical table of distributions may be consulted to determine the significance of the comparison at the 5 or 1% levels, with the appropriate degrees of freedom. The significance can then be used to accept or reject the null hypothesis. The formula used to calculate χ^2 is as follows:

$$\chi^2 = \Sigma \frac{(O-E)^2}{E}$$

where: O = Observed numbers
E = Expected numbers

4.11.10 Odds Ratio

If a factor is associated with the particular di sease or trait , then it should appear more commonly in the cases than in the controls. An estimate of the relative risk may be obtained, providing the cases and controls are random samples from their corresponding populations and the incidence of the particular event under study in the population is small. This approximation of the relative risk is called the "odds

ratio" (OR) and is calculated by dividing the 'odds' of a case exhibiting the trait by the 'odds' of a control exhibiting the trait. The odds of the event occuring is related to its probability, but the odds are defined as the number of individuals who experience the event, divided by the number of individuals not experiencing the event.

CHAPTER 5. RESULTS

5.1 Reference ranges

Enzyme activities estimated in a randomly selected group from the normal population, are not identical and will fall within a defined range of values, which may described by the term 'normal range'. The use of the word 'normal' in this context may be misleading and the term 'reference range' should be used. A comparison of enzyme values between individuals of a given population depends on the use of a common unit of measurement. For example, in analyses where erythrocytes are used, the activity of the enzyme may be related to the concentration of haemoglobin within the haemolysate. In tissues, such as CV, amniotic fluid cells, and fibroblasts, comparisons may be made by expressing the activity against the protein concentration of the homogenised sample. Statistical evaluation of the parameters of the distribution of reference values is important to ensure that appropriate statistical tests are used for comparisons between patient samples and controls

5.2 Purine study

5.2.1 Control Reference Ranges

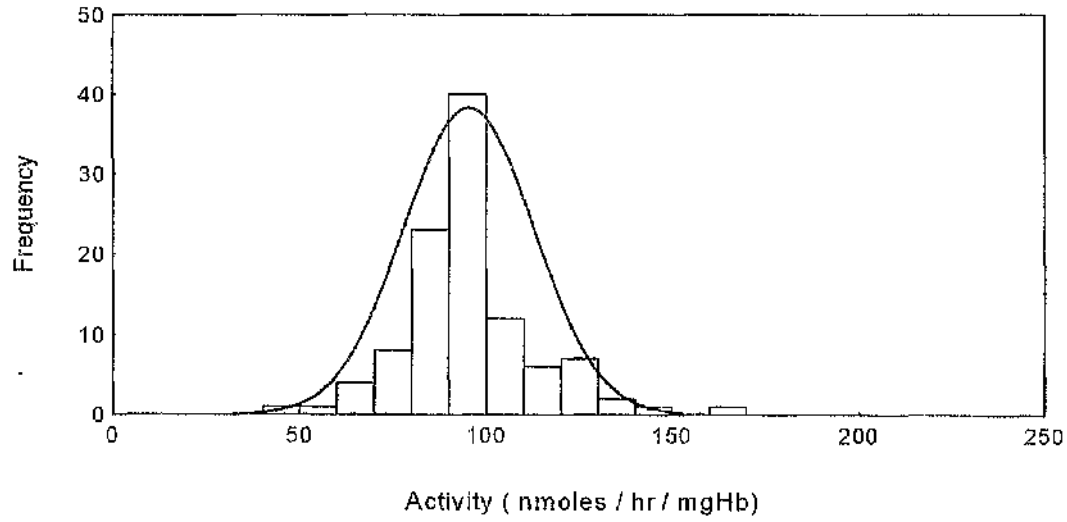
The results of the KS test on the distribution of values obtained for each enzyme in the various tissues are shown in Table 5 - 1. The results show that for all enzymes in each of the tissue types studied $\log_{10}$ transformation of values are gave a reasonable fit to Gaussian distributions. If untransformed values are considered, HGPRT and APRT in adult red cell samples show a significant departure (i.e. $p<0.05$) from a normal Gaussian distribution indicated that these enzyme activities were skewed to higher values. In order that that valid statistical comparisions of the red cell HGPRT and APRT activities could be made with the enzyme activities in the other tissue types, $\log_{10}$ transformation was applied to all data, and the transformed values used for parametric testing. The graphical representation of the effect of this transformation for HGPRT and APRT on red cells controls can be seen in Figures 5 - 1 and 5 - 2, with the corresponding probability plots in Figures 5 - 3 and 5 - 4.

Table 5 - 1

Kolmogorov-Smirnoff D-values and probabilities for Log_{10} transformed and non-transformed HGPRT and APRT values for all tissue types analysed. The symbol "***" denotes where significant probabilities (Prob.) were obtained.

			Adult Red cells	Amniotic Fluid cells	Cultured CV	Uncultured CV	Cultured Fibroblasts	Fetal Red Cells	Cord Red Cells
HGPRT	Non-Transformed	(D Value)	0.1363	0.1151	0.1653	0.0718	0.0989	0.1224	0.0772
		(Prob.)	0.039 ***	0.5890	0.0887	0.9436	0.9946	0.8968	0.9741
	Transformed	(D Value)	0.1014	0.0776	0.0768	0.1127	0.1737	0.1011	0.0759
		(Prob.)	0.2259	0.9490	0.8899	0.4990	0.6495	0.9781	0.978
			(n=106)	(n=45)	(n=57)	(n=54)	(n=18)	(n=22)	(n=39)
APRT	Non-Transformed	(D Value)	0.1477	0.1696	0.1700	0.1062	0.1774	0.1376	0.1042
		(Prob.)	0.0196 ***	0.1500	0.0935	0.6510	0.6228	0.7769	0.7913
	Transformed	(D Value)	0.0752	0.1297	0.1020	0.1121	0.1355	0.1996	0.1564
		(Prob.)	0.5869	0.4350	0.6398	0.5830	0.8955	0.3166	0.2957
			(n=106)	(n=48)	(n=43)	(n=48)	(n = 18)	(n=23)	(n=39)
ADA	Non-Transformed	(D Value)	0.0702	-	0.0780	-	-	-	
		(Prob.)	0.7957	-	0.6873	-	-	-	
	Transformed	(D Value)	0.1038	-	0.0973	-	-	-	
		(Prob.)	0.3324	-	0.4044	-	-	-	
			(n=83)		(n=37)				
PNP	Non-Transformed	(D Value)	0.1251	-	-	-	-	-	
		(Prob.)	0.1397	-	-	-	-	-	
	Transformed	(D Value)	0.0834	-	-	-	-	-	
		(Prob.)	0.5950	-	-	-	-	-	
			(n=85)						
PRPP-S	Non-Transformed	(D Value)	0.1922	-	-	-	-	-	
		(Prob.)	0.5328	-	-	-	-	-	
	Transformed	(D Value)	0.1547	-	-	-	-	-	
		(Prob.)	0.7820	-	-	-	-	-	
			(n=18)						

(a)



(b)

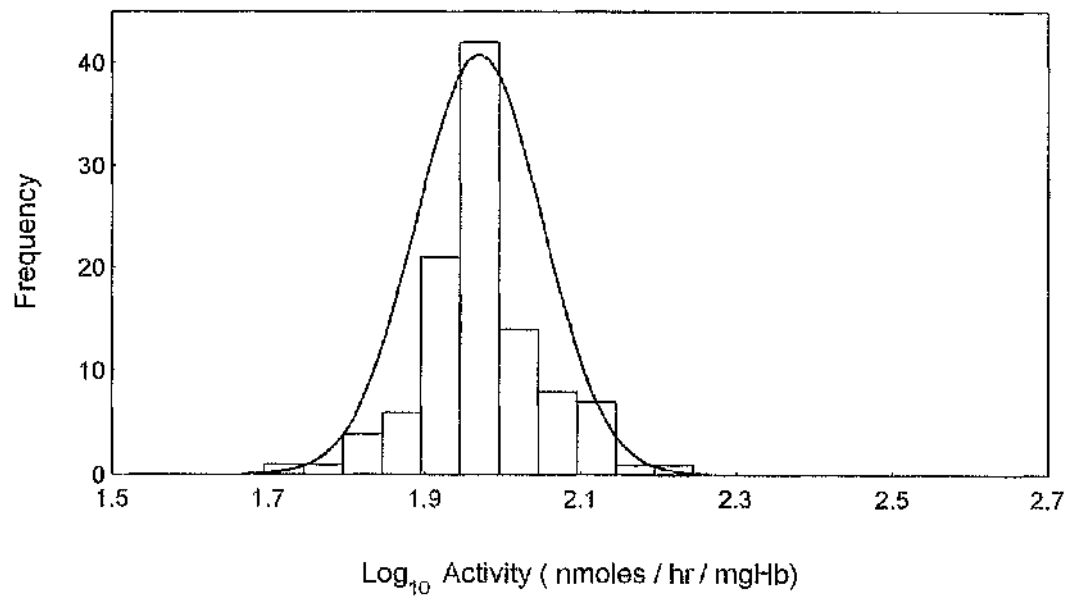
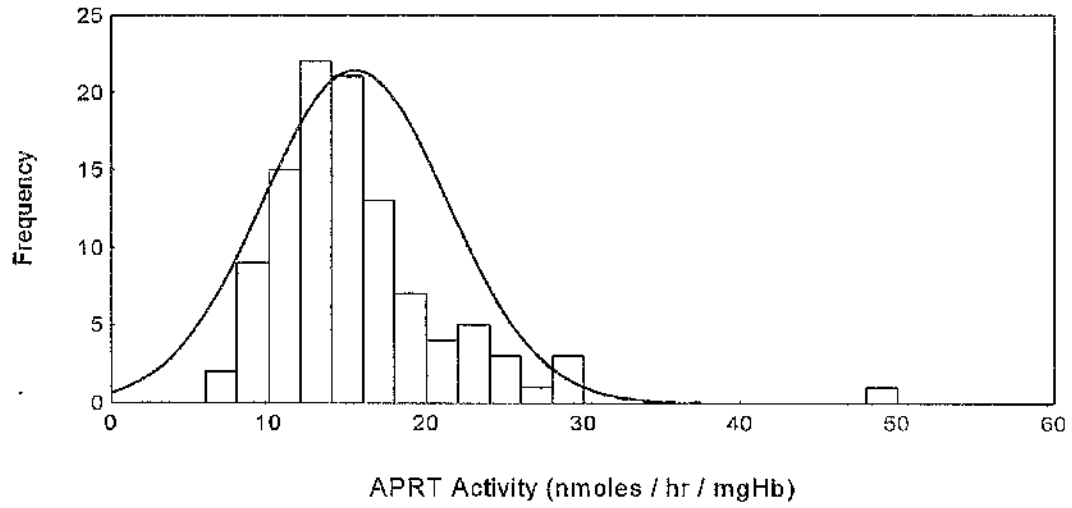


Figure 5 - 1

Frequency distributions of adult red cell control (a) non-transformed HGPRT activity and (b) $\log_{10}$ transformed HGPRT activity.

(a)



(b)

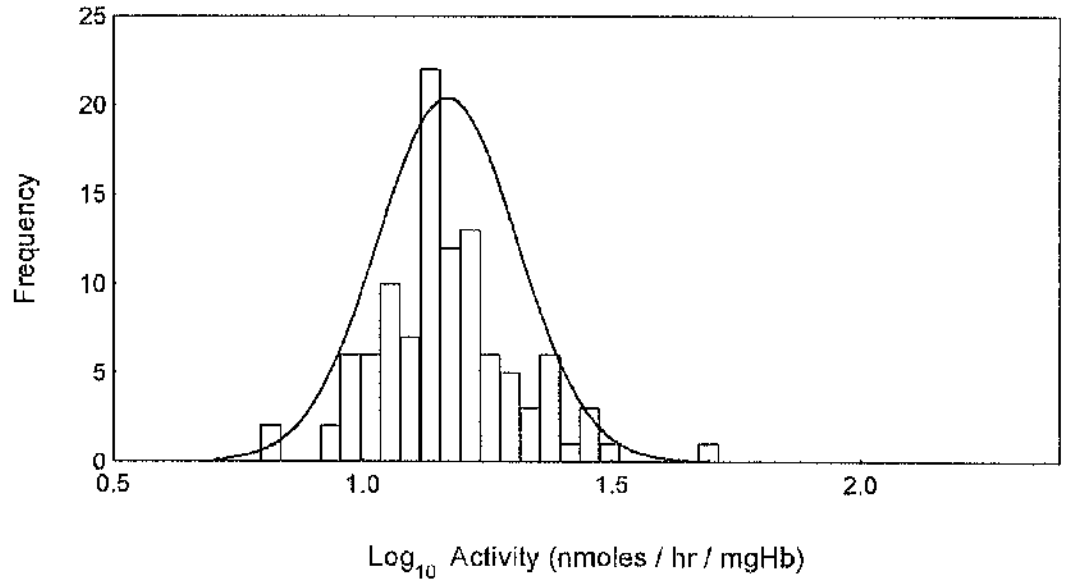
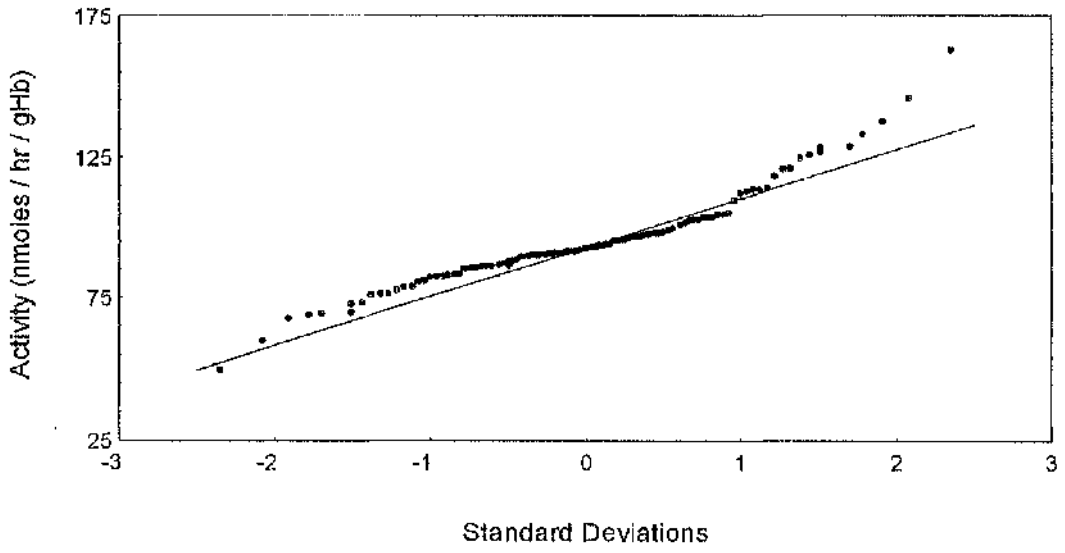


Figure 5 - 2

Frequency distributions of adult red cell control (a) non-transformed APRT activity and (b) $\log_{10}$ transformed APRT activity.

(a)



(b)

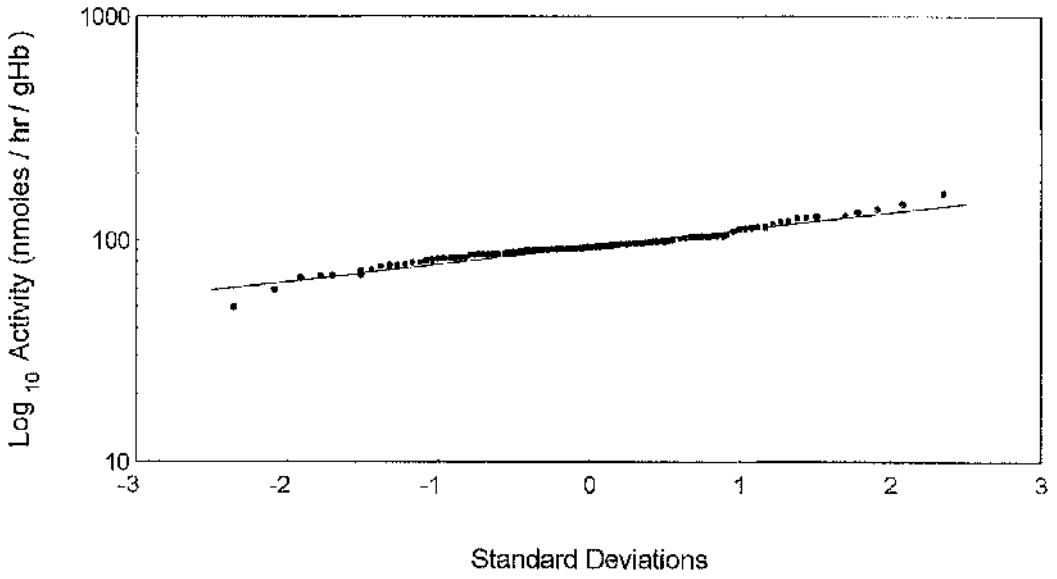
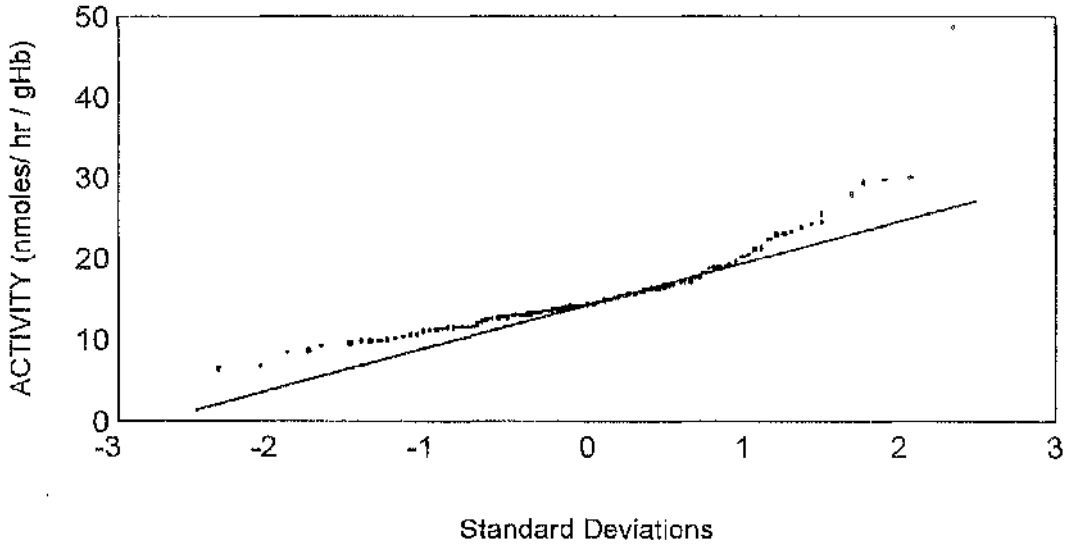


Figure 5 - 3

Probability plots of (a) non-transformed and (b) Log $_{10}$ transformed HGPRT activity for adult red cells. The continuous lines are defined by means and standard deviations from the Gaussian distributions of each population.

(a)



(b)

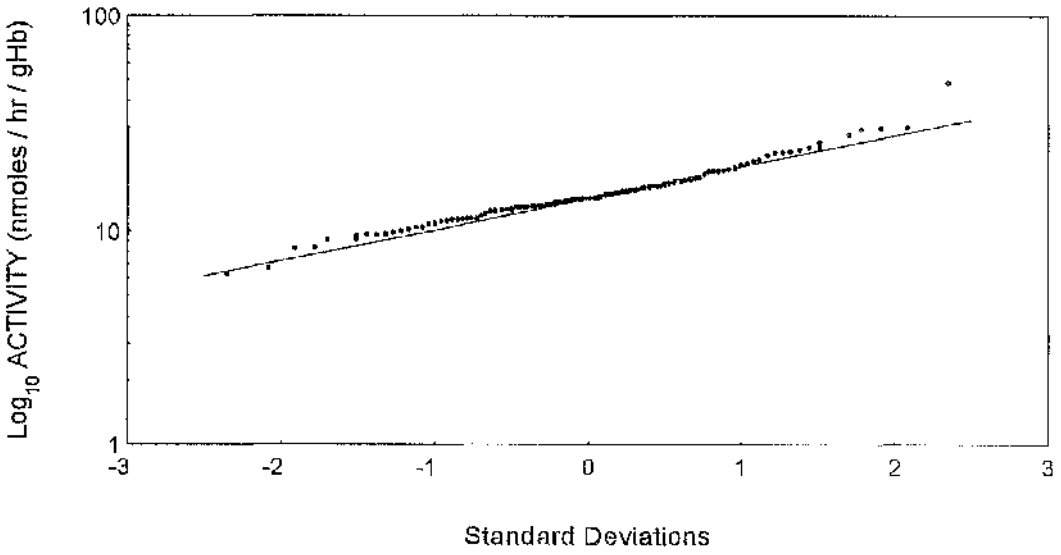


Figure 5 - 4

Probability plots of (a) non-transformed and (b) Log $_{10}$ transformed APRT activity for adult red cell controls. The continuous lines are defined by means and standard deviations from the Gaussian distributions of each population.

The enzymes ADA, PNP and PRPP-S exhibited normal distribution statistics when values were $\log_{10}$ transformed in all the tissues under examination, as did HGPRT and APRT for amniotic fluid cells, cultured and uncultured CV, fibroblasts, fetal red blood cells and cord blood red cells.

The construction of the reference ranges was based on the normally distributed data, for ease of comparison of the means and standard deviations of the control and patient populations under study. The subsequent data obtained is presented in Tables 5 - 2 to 5 - 8. Upper and lower limits of the reference range for each enzyme in each tissue can be set using the mean and standard deviation. For example, the mean ± 2 standard deviations indicate the 95% confidence limits for the given population.

Table 5 - 2

Adult red cell control reference range for HGPRT, APRT, ADA and PNP in nmoles / hr / mgHb , and the HGPRT / APRT ratio. Log_{10} transformed values were used to define the reference ranges, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

	HGPRT	APRT	HGPRT / APRT Ratio	ADA	PNP
Median	92.7	14.1	6.7	55.5	2887
Log_{10} Mean	1.973 (93.9)	1.166 (14.7)	0.807 (6.4)	1.732 (53.9)	3.471 (2958)
Log_{10} sd	0.077	0.142	0.163	0.143	0.130
Range (Mean ± 2sd)	65.9 - 133.9	7.6 - 28.2	3.0 - 13.6	27.9 - 104.2	1625 - 5383
n	106	106	106	83	85

Table 5 - 3

Uncultured CV control reference ranges for HGPRT and APRT, in nmoles / hr / mg protein and the HGPRT / APRT ratio. Log_{10} transformed values were used to defiine the reference ranges, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

	HGPRT	APRT	HGPRT / APRT Ratio
Median	63.1	48.0	1.26
Log_{10} Mean	1.751 (56.4)	1.638 (43.5)	0.110 (1.29)
Log_{10} sd	0.239	0.257	0.235
Range (Mean $\pm$ 2sd)	18.7 - 169.4	13.3 - 141.9	0.44 - 3.8
n	54	48	48

Table 5 - 4

Cultured CV control reference range for HGPRT and APRT in nmoles / hr / mg protein and the HGPRT / APRT ratio. Log_{10} transformed values were used to define the reference ranges, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

	HGPRT	APRT	HGPRT / APRT Ratio	ADA
Median	34.5	50.7	0.68	480.5
Log_{10} Mean	1.538 (34.5)	1.690 (48.9)	-0.201 (0.63)	2.66 (457.0)
Log_{10} sd	0.399	0.348	0.289	0.180
Range (Mean $\pm$ 2sd)	5.5 - 216.8	9.9 - 242.6	0.17 - 2.4	199.5 - 1047
n	57	53	51	37

Table 5 - 5

Amniotic fluid cell control reference range for HGPRT and APRT, in nmoles / hr / mg protein, and the HGPRT / APRT ratio. Log_{10} transformed values were used to define the reference ranges, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

	HGPRT	APRT	HGPRT /APRT Ratio
Median	164.9	139.1	1.190
Log_{10} Mean	2.207 (161.1)	2.157 (143.5)	0.050 (1.12)
Log_{10} sd	0.204	0.230	0.309
Range (Mean ± 2sd)	62.9 - 412.1	49.8 - 413.9	0.46 - 2.75
n	45	45	45

Table 5 - 6

Fibroblast cell control reference ranges for HGPRT and APRT in nmoles / hr / mg protein, and the HGPRT / APRT ratio. Log_{10} transformed values were used to define the reference ranges, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

	HGPRT	APRT	HGPRT / APRT Ratio
Median	52.4	60.8	0.760
Log_{10} Mean	1.631 (42.7)	1.719 (52.3)	-0.087 (0.818)
Log_{10} sd	0.385	0.428	0.349
Range (Mean ± 2sd)	7.3 - 251.8	8.6 - 443.6	0.16 - 4.1
n	18	18	18

Table 5 - 7

Fetal red cell control reference ranges for HGPRT and APRT in nmoles / hr / mg Hb, and the HGPRT / APRT ratio. Log_{10} transformed values were used to define the reference ranges, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

	HGPRT	APRT	HGPRT / APRT Ratio
Median	71.9	31.7	2.4
Log_{10} Mean	1.867 (73.6)	1.462 (28.9)	0.400 (2.51)
Log_{10} sd	0.078	0.150	0.138
Range (Mean ± 2sd)	51.4 - 105.4	14.5 - 57.8	1.3 - 4.7
n	22	23	22

Table 5 - 8

Cord blood red cell control reference ranges for purine enzymes in nmoles / hr / mg Hb, and the HGPRT / APRT ratio . Log_{10} transformed values were used to define the reference ranges, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

	HGPRT	APRT	HGPRT / APRT Ratio
Median	94.9	28.9	3.467
Log_{10} Mean	1.970 (93.3)	1.424 (26.5)	0.548 (3.53)
Log_{10} sd	0.085	0.208	0.184
Range (Mean ± 2sd)	63.1 - 138	17.7 - 69.2	1.5 - 8.2
n	39	39	39

The HGPRT / APRT ratio was estimated in all tissues and the distributions of the ratio in both the control and patient samples assessed as a potential discriminator between these populations. The median HGPRT / APRT ratio increased in the following order; Cultured CV (0.68) > Fibroblasts (0.76) > Amniotic fluid cells (1.19) > Uncultured CV (1.26) > Fetal red cells (2.40) > Cord red cells (3.47) > Adult red cells(6.70), indicating a difference in the specific requirements for purine moieties within different tissues with red cells giving higher HGPRT and lower APRT activities than other tissues.

The spread of the distributions in each tissue can be seen in the graphical representations of the populations in Figures 5 - 5 to 5 - 11.

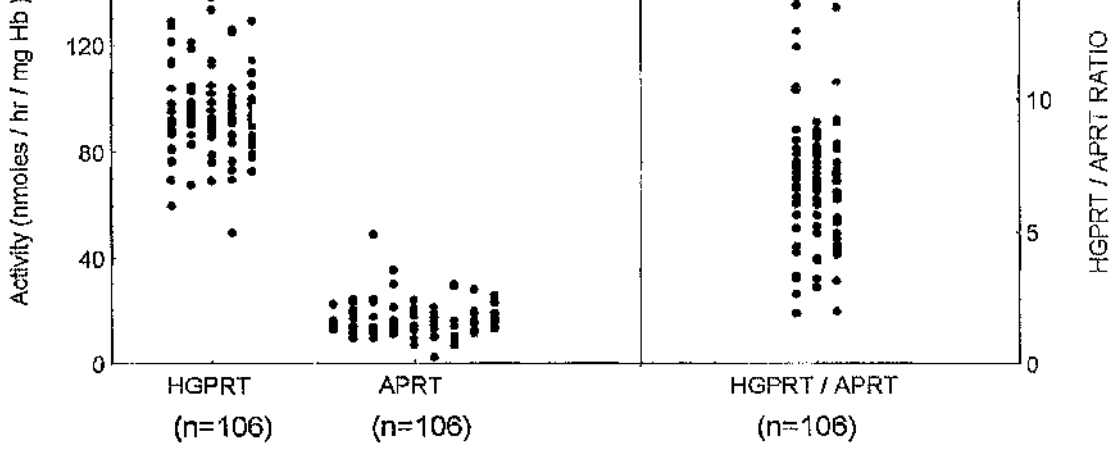


Figure 5 - 5

Distributions of HGPRT and APRT activity, and HGPRT / APRT ratio in adult red cells for individual control samples summarised in Table 5 - 2.

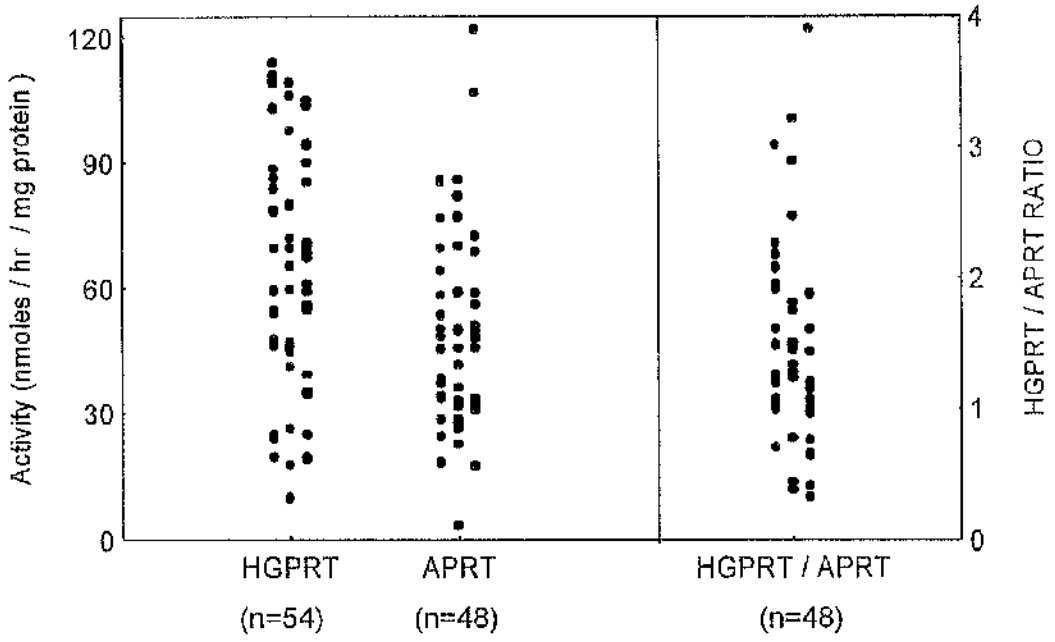


Figure 5 - 6

Distributions of HGPRT and APRT activity, and HGPRT / APRT ratio in uncultured CV for individual control samples summarised in Table 5 - 3.

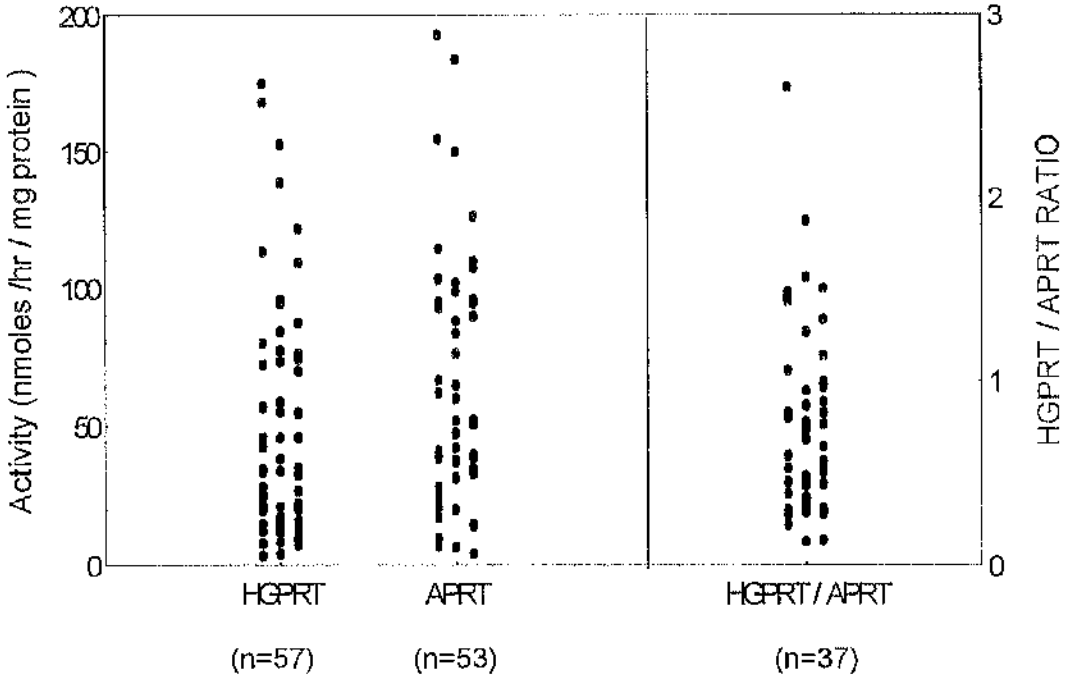


Figure 5 - 7

Distributions of HGPRT and APRT activity, and HGPRT / APRT ratio in cultured CV distributions for individual control samples summarised in Table 5 - 4.

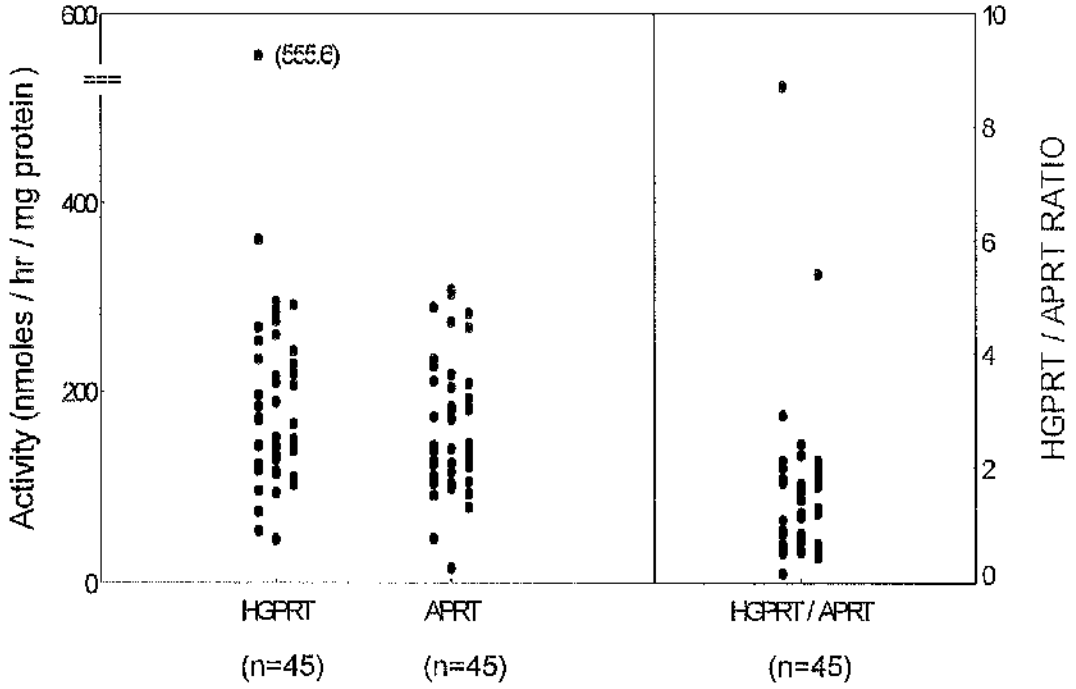


Figure 5 - 8

Distributions of HGPRT and APRT activity and HGPRT / APRT ratio in amniotic fluid cells for individual control samples summarised in Table 5 - 5.

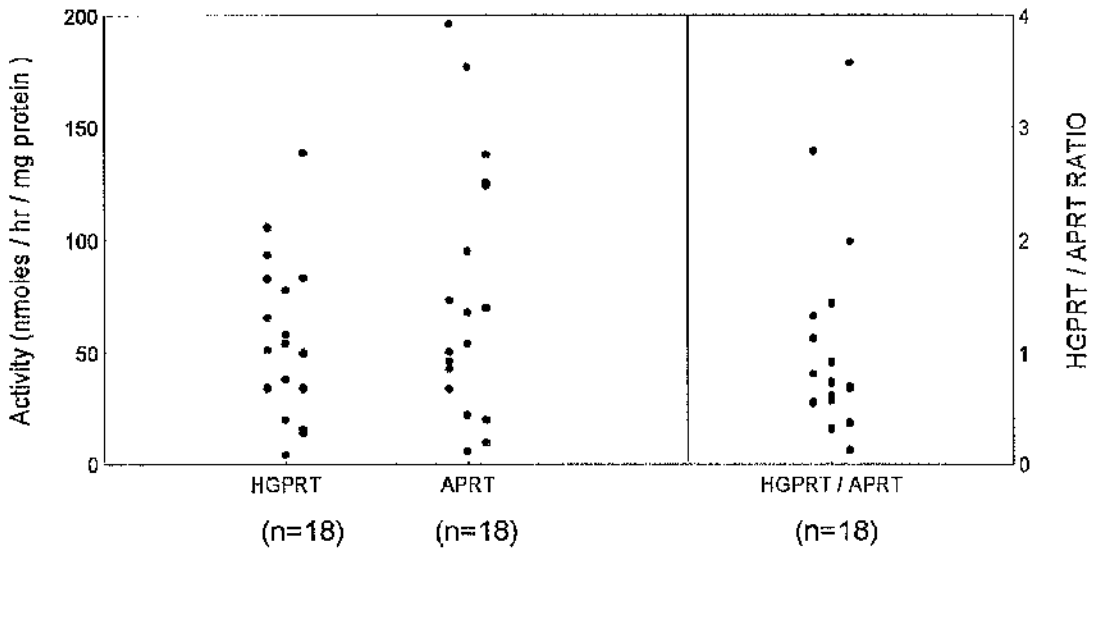


Figure 5 - 9

Distributions of HGPRT and APRT activity and HGPRT / APRT ratio in fibroblast cell for individual control samples summarised in Table 5 - 6.

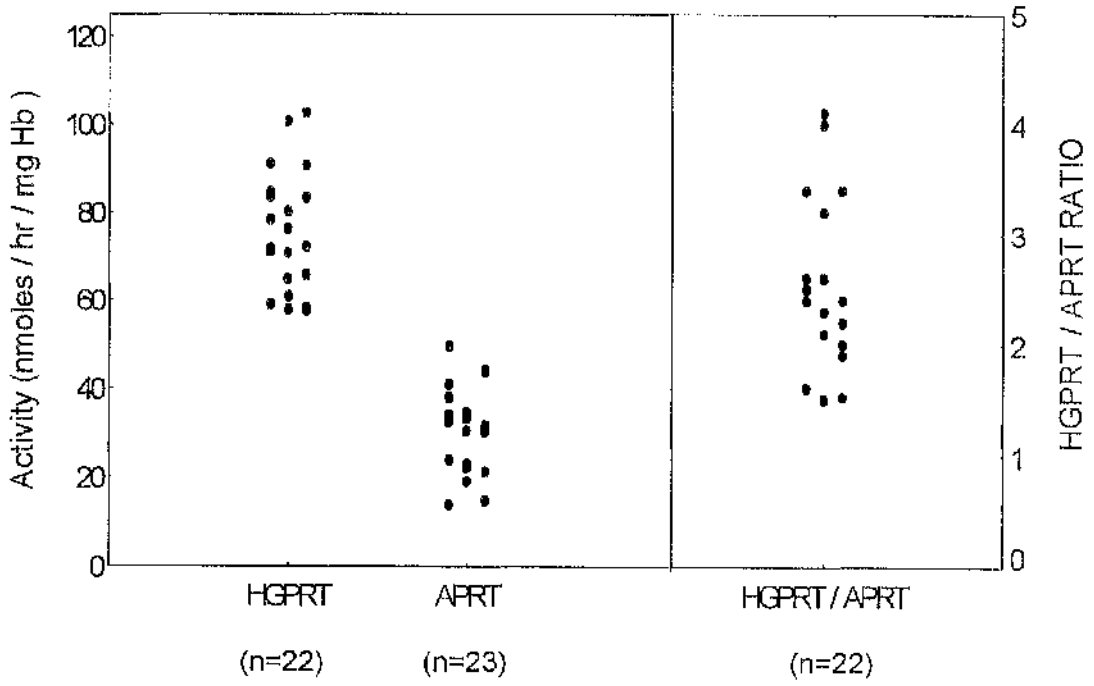


Figure 5 - 10

Distributions of HGPRT and APRT activity and HGPRT / APRT ratio in fetal red cells for individual control samples summarised in Table 5 - 7.

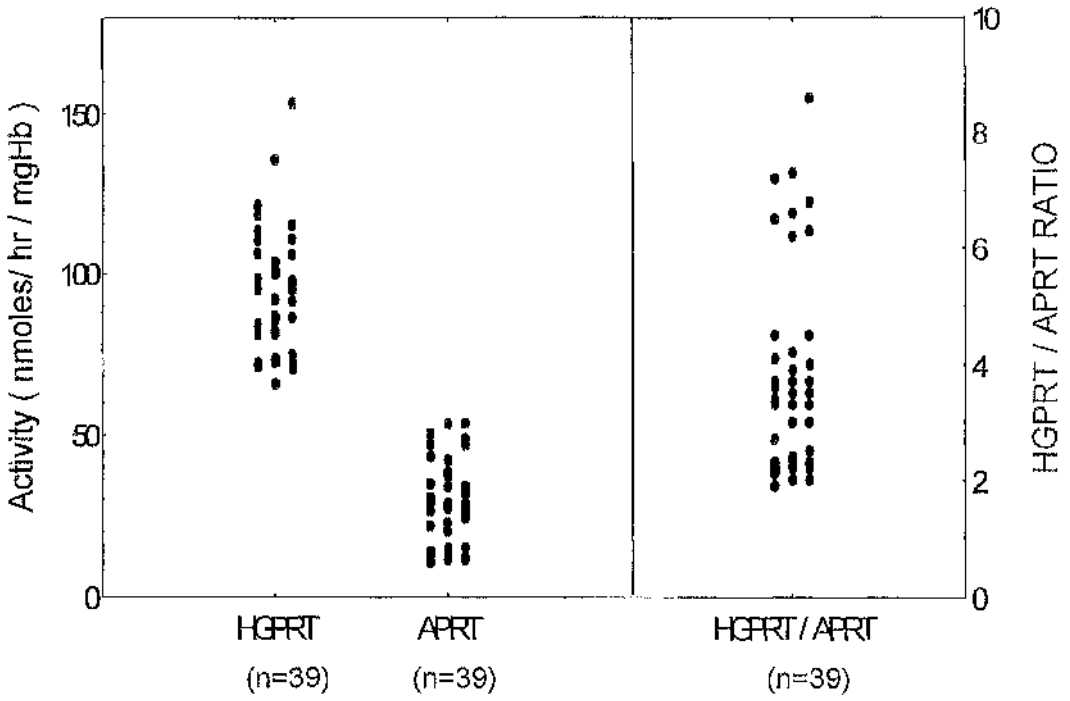


Figure 5 - 11

Distributions of HGPRT and APRT activity and HGPRT / APRT ratio in cord red cells from individual control samples summarised in Table 5 - 8.

In examining the relationships of the enzymes in each tissue studied, it was observed that for the adult red cells, a significant correlation ($p<0.004$) existed between ADA and PNP enzyme activities. No significant correlation ($p>0.05$) existed between the other enzymes in adult red cells, however significant correlation ($p<0.05$) was observed in cord erythrocytes for HGPRT and APRT. For the other tissues, significant correlation was observed in cultured CV ($p<0.02$), uncultured CV ($p<0.01$) and fibroblasts ($p<0.01$) between HGPRT and APRT, while for amniotic fluid cells and fetal erythrocytes, no significant correlation was found for HGPRT and APRT. (Table 5 – 9).

Table 5 - 9

Correlation coefficients and probabilities for log_{10} transformed HGPRT, APRT, ADA and PNP in all tissues under study for control population. CV denotes Chorionic Villi.

	Adult red cells	Uncultured . CV	Cultured. CV	Amniotic Fluid cells	Fibroblasts	Fetal red cells	Cord red cells
HGPRT / APRT	0.914 (p>0.05)	0.577 (p<0.01)	0.348 (p<0.02)	-0.008 (p>0.05)	0.635 (p<0.01)	0.411 (p>0.05)	0.452 P<0.05
HGPRT / ADA	-0.008 (p>0.05)	-	-	-	-	-	-
HGPRT / PNP	0.107 (p>0.05)	-	-	-	-	-	-
APRT / ADA	0.053 (p>0.05)	-	-	-	-	-	-
APRT / PNP	0.169 (p>0.05)	-	-	-	-	-	-
ADA / PNP	0.231 (p<0.04)	-	-	-	-	-	-

5.2.2 Quality Control

In order to assess and compare the results obtained from any test it is essential that all assay parameters are controlled both within and between different batches of samples under investigation. Prior to the beginning of investigation of control ranges, or any other test/ control comparisons, 3 separate erythrocyte quality control samples were run in 3 assays and the mean and standard deviation of the HGPRT and APRT activities used as interim QC values in order to assess the reliability of the subsequent assay batches, until a more reliable estimate of the QC sample could be obtained.

Using 18 values , the working QC sample had a mean of 87.6 ± 11.9 nmoles/ hr / mg protein for HGPRT and 12.5 ± 2.9 nmoles/ hr / mg protein for APRT and a combined HGPRT / APRT ratio of 7.0 . These gave coefficients of variation (cv) for HGPRT and APRT assays of 13.6% and 23.2% respectively. During the period of patient sample analysis, internal QC results were compared individually to this working mean and the results within it were accepted or rejected on the basis of a ± 2sd confidence limit. At the end of the study, a reappraisal of the internal QC for 70 results from HGPRT and APRT assays with common samples showed that the inter-assay mean for HGPRT was 87.8 ± 12.8 nmoles/ hr / mg protein, 16.1 ± 4.2 nmoles/ hr / mg protein for APRT and gave an HGPRT / APRT ratio of 5.9 ± 2.1 . Figure 5 - 12 shows the Levy - Jennings plot for both the HGPRT and APRT values over the course of the evaluation. While a small number of HGPRT values fall outwith the ±2 sd limits, the majority fall within the limits of the assay. With APRT a considerable number of results were above the mean and occasionally over the +2sd level. This may be explained by the initial working mean and standard deviations for APRT , being too low, possibly due to the small number of observations on which the means and sd's were based. This is borne out by comparing the mean of 16.1 nmoles/hr/mg protein obtained from 70 values, with that of 12.5 ± 2.9 nmoles/ hr / mg protein obtained during the initial estimation.

When the final mean and sd were applied retrospectively to the Levy-Jennings plot of the data it was clear that these new values were more appropriate, particularly for the APRT assay.

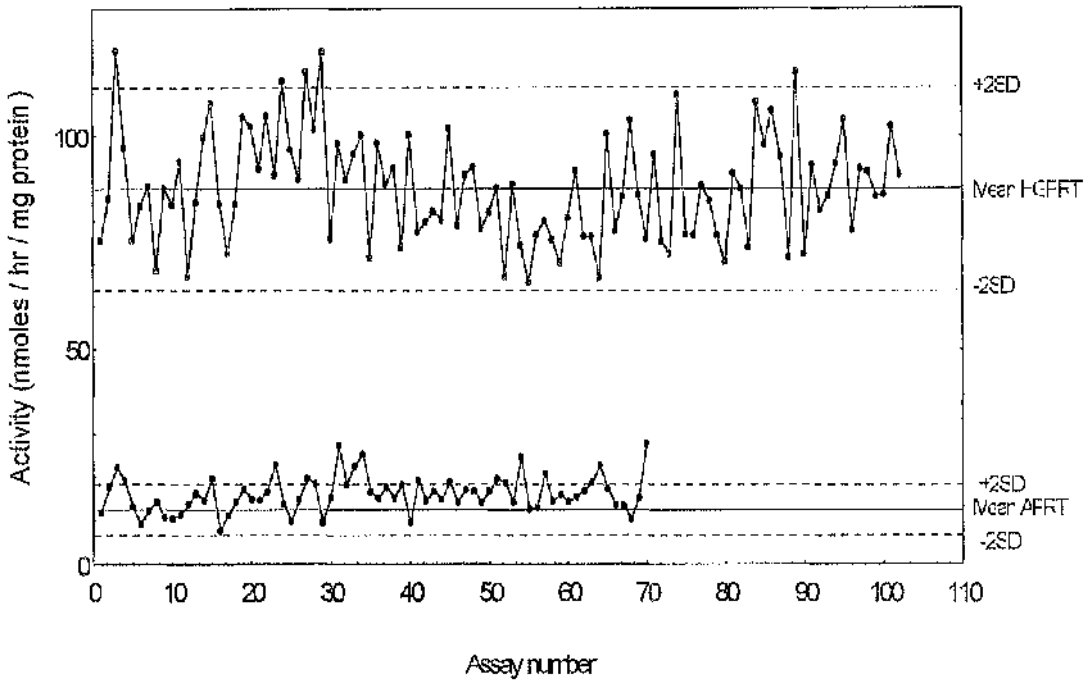


Figure 5 - 12

Levy-Jennings plot of red cell internal quality control sample for HGPRT and APRT values, over the course of the control range evaluation. Means and sds are retrospectively applied from the final data of 70 values.

5.2.3 Lesch-Nyhan Syndrome

5.2.3.1 Pre-natal Diagnosis

During the period of the study, a total of 23 pregnancies were investigated in 15 women known or suspected through family history to be carriers for the Lesch-Nyhan Syndrome. Three successive pregnancies were investigated in two patients, and two in each of four other obligate carriers, while nine other women had prenatal investigation in only one pregnancy. In 8 of these cases, CV was obtained from the first trimester of pregnancy and 3 from the second trimester while amniotic fluid samples were obtained second trimester from 10 cases. No gestation was specified in 2 cases (Table 5 - 10).

Enzyme activities (either HGPRT alone or HGPRT/APRT ratio) were assessed against the reference ranges derived in Tables 5 - 3 (uncultured CV), 5 - 4 (cultured CV) and 5 - 5 (amniotic fluid cells) as appropriate. Nineteen of these pregnancies gave normal enzyme activities, of which 13 had normal outcome verified by the referring clinician; SFS, LHN, JHR, JJN(a), JJN(b), PMD, DSN(a), DSN(b), GWS(b), GWS(c), NFN(a), NFN(b) and K-SN. Two pregnancies miscarried (GTR, MOH) and four (underlined in table) had abnormal enzyme results (JRL(a), JRL(b) and GWS(a), AGN(a)). The remaining 4 cases (DSN (c), AGN(b), CRE, and STA) all female, gave normal enzyme results with the outcome yet to be confirmed.

HGPRT activities in the affected pregnancies, ranged from 0.06 - 13.9% of the mean of the appropriate reference range, showing that in this series of affected cases, there was significant residual HGPRT activity. No post-mortem tissues were available for confirmatory assays from either of the terminations of patient JRL or from pateint AGN(a) , but cultured fibroblasts assayed from the index case gave a residual HGPRT activity of 9.9 per cent of normal fibroblast levels. The HGPRT/ APRT ratio in patients JRL(b) and AGN(a) showed a 31.5 and 112 fold decrease respectively, compared with the mean control ratios. Patient GWS(a) opted for a termination of pregnancy after enzyme results revealed a HGPRT activity of 9.0 % of normal. Post-mortem fibroblast tissue form this pregnancy was obtained, and gave an HGPRT activity of 13.8% , confirming the prenatal diagnosis results.

Table 5 - 10

Prenatal diagnoses for the Lesch-Nyhan Syndrome. Underlined cases denote affected males. Key:- AFC = amniotic fluid cells; CV (U) = uncultured CV; CV (C) = cultured CV; Fibro = fibroblasts; Cord = cord red cells

Case	Fetal Kary.	Gest. (wK-S)	Sample Type	HGPRT (nmols/hr/mg	APRT protein or Hb)	HGPRT/APRT Ratio	Outcome
SFS	46XX	17	CV (U)	94.0	ND	-	normal female
LHN	46XX	11	CV (C)	32.6	116.6	0.28	normal female
JHR		7	CV (U)	61.8	ND	-	
			CV (C)	247.5	348	0.71	normal female
JJN(a)	46XX	16	AFC	87.7	ND	-	normal female
JJN(b)	46XY	16	AFC	116.0	110.1	1.05	normal male
PMD	46XX	16	AFC	145.0	52	2.80	normal female
MOH	46 XY	11	CV(U)	49.0	ND	-	miscarriage
JRL(a)	46XY	10	CV (U)	4.7	ND	-	
			CV (C)	4.8	ND	-	affected male
JRL(b)	46XY	8	CV (U)	2.1	ND	-	
			CV (C)	2.9	142.6	0.02	affected male
DSN(a)	46XY	17	AFC	103.9	ND	-	normal male
DSN(b)	46XY	25	CV (U)	70.1	38.5	1.82	
			CV (C)	120.1	95.9	1.25	normal male
DSN(c)	46XX	-	CV (C)	73.6	105.8	0.69	? normal female
GTR	46XY	12	CV (U)	54.8	ND	-	
			CV (C)	76.8	ND	-	miscarriage
GWS(a)	46XY	10	CV (U)	5.1	ND	-	
			FIBRO.***	5.9	ND	-	affected male
GWS(b)	46XY	14	CV (U)	51.6	39.1	1.32	
			CV (C)	113.8	134.1	0.85	normal male
GWS(c)	46XX	11	CV (U)	35.4	30.1	1.18	
			CV (C)	117.3	ND	-	
			CORD.	73.8	14	5.30	normal female
NFN(a)	46XX	14	AFC	103.7	90.7	1.14	normal female

Table 5 - 10 (continued)

Prenatal diagnoses for the Lesch-Nyhan Syndrome.

Key:- AFC = amniotic fluid cells; CV (U) = uncultured CV; CV (C) = cultured CV; Fibro = fibroblasts; Cord = cord red cells

Case	Fetal Kary.	Gest. (wK-S)	Sample Type	HGPRT (nmols/hr/mg	APRT protein or Hb)	HGPRT/APRT Ratio	Outcome
NFN(b)	46XX	15	AFC	210.4	107.5	2.00	normal female
AGN(a)	46XY	16	AFC	1.0	184.3	0.01	affected male
AGN(b)	46XX	16	AFC	124.8	84.0	1.49	? normal female
CRE	46XX	17	AFC	115.5	51.2	2.2	? normal female
K-SN	46XY	16	AFC	152.7	84.1	1.81	
			Cord	115.1	15.0	7.4	normal male
STA	46XX	-	CV(C)	26.2	31.8	0.82	? normal female

5.2.3.2 Post-natal and Adult Diagnosis

Table 5 - 11 shows the comparison obtained for the HGPRT, APRT, HGPRT/APRT ADA, PNP data in red cells from postnatal diagnostic analyses involving 3 male Lesch-Nyhan index cases, 5 obligate carriers and 33 other relatives, compared to the control population, after $\log_{10}$ transformation.

The Lesch-Nyhan index cases displayed significantly altered levels of HGPRT (t-test: $p<0.001$), APRT ($p<0.001$), HGPRT/APRT ratio ($p<0.001$), ADA ($p<0.001$) and PNP ($p<0.05$). Enzyme activities for HGPRT ranged from 0.95 - 4.9% of normal. These residual levels emphasise the fact that in this condition a complete deficiency of the enzyme is not always evident and that residual levels of HGPRT are often found. APRT levels were found to be above the upper levels of the reference ranges for all three index cases. ADA activity for the index case JMD were observed to be 168% of the upper limit if normal while the remaining two cases (MBE and KHY) gave activities in the upper normal range. For PNP, cases MBE and KHY gave activities of between 170 and 175% above the upper limit of

normal, while JMD had activities in the upper part of the normal range. Statistical comparisons of the HGPRT, APRT and ADA and PNP activities between the control and obligate carriers using a t-test, showed only HGPRT in these cases to be significantly different ($p<0.02$) from the reference range mean, while for the other family members, both HGPRT and APRT were found to be significantly different ($p<0.01$ and $p<0.05$ respectively). Despite the significantly reduced activities of HGPRT in these groups, the degree of overlap between the reference and control ranges, prevent this from being diagnostically useful. However this data was based on a small number of only 5 samples, further data may clarify this. Activities for ADA and PNP in both of these groups was found not to be significantly different from normal ($p>0.05$). Since only 5 obligate carrier cases were again available for this comparison, a larger data-set may be required to show whether the activities of these salvage pathway enzymes are altered in LNS. While all Lesch-Nyhan affected cases were distinguishable by HGPRT activity alone, the elevated APRT levels found in these patients allowed the HGPRT/APRT ratio to be used to better differentiate between the affected and control populations.
Figures 5 - 13 to 5 - 17 show the graphical distributions of these populations.

Table 5 - 11

Red cell enzyme activities in nmoles / hr / mg Hb for HGPRT, APRT ADA and PNP for samples in individuals at risk for the Lesch-Nyhan Syndrome. Log_{10} transformed values were used to define the reference ranges for the controls, obligate carriers and other family members, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

		HGPRT	APRT	HGPRT / APRT ratio	ADA	PNP
Controls (Data from table 5 - 2)	Log_{10} Mean	1.973 (93.7)	1.166 (14.7)	0.807 (6.4)	1.732 (53.9)	3.471 (2958)
	Log_{10} sd	0.077	0.142	0.163	0.143	0.130
	Range (Mean ± 2sd)	65.9 - 133.9	7.6 - 28.2	3.0 - 13.6	27.9 - 104.2	1625 - 5383
	n	106	106	106	83	85
Lesch-Nyhan index cases	Patient JMD	4.6	34.0	0.14	79.3	2724
	Patient MBE	1.7	42.0	0.04	175.9	5925
	Patient KHY	0.9	56.0	0.02	75.1	5875
Obligate Carriers	Log_{10} Mean	1.888 (77.2)	1.228 (16.9)	0.66 (4.6)	1.655 (45.1)	3.456 (2857)
	Log_{10} sd	0.023	0.116	0.122	0.135	0.115
	Range (Mean ± 2sd)	69.5 - 85.9	9.9 - 28.8	2.6 - 8.0	24.3 - 84.1	1683 - 4853
	n	5	5	5	5	5
Other Relatives	Log_{10} Mean	1.930 (85.1)	1.092 (12.4)	0.839 (6.9)	1.745 (55.5)	3.483 (3041)
	Log_{10} sd	0.077	0.173	0.196	0.159	0.110
	Range (Mean ± 2sd)	60.3 - 121.3	5.6 - 27.4	2.8 - 17.0	26.7 - 115.6	1832 - 5047
	n	33	33	33	31	31

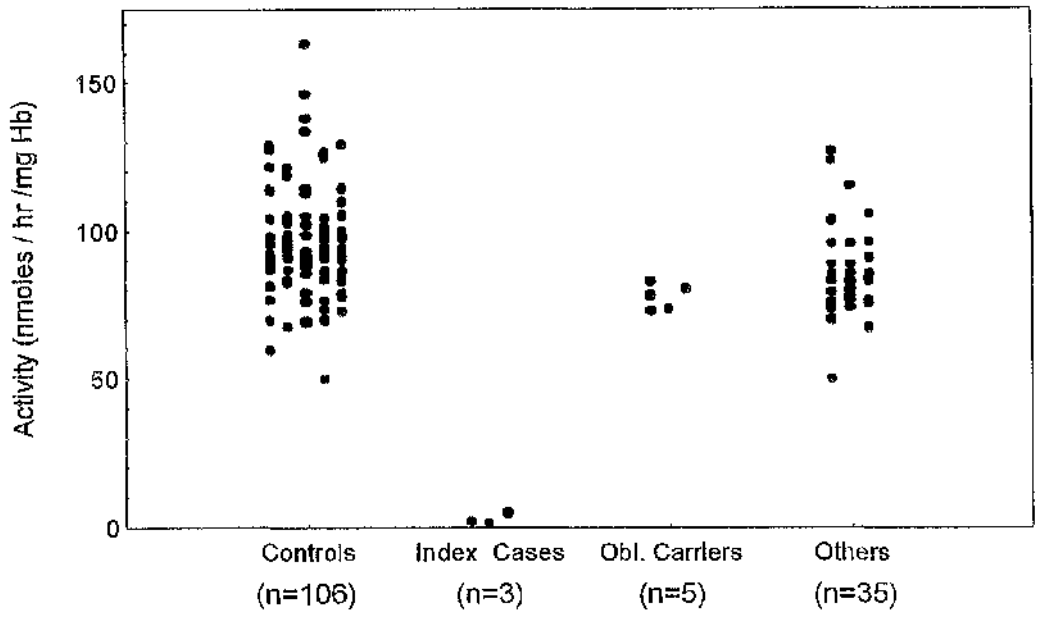


Figure 5 - 13

Distribution of red cell HGPRT activity for Lesch-Nyhan index cases, obligate carriers and other family members

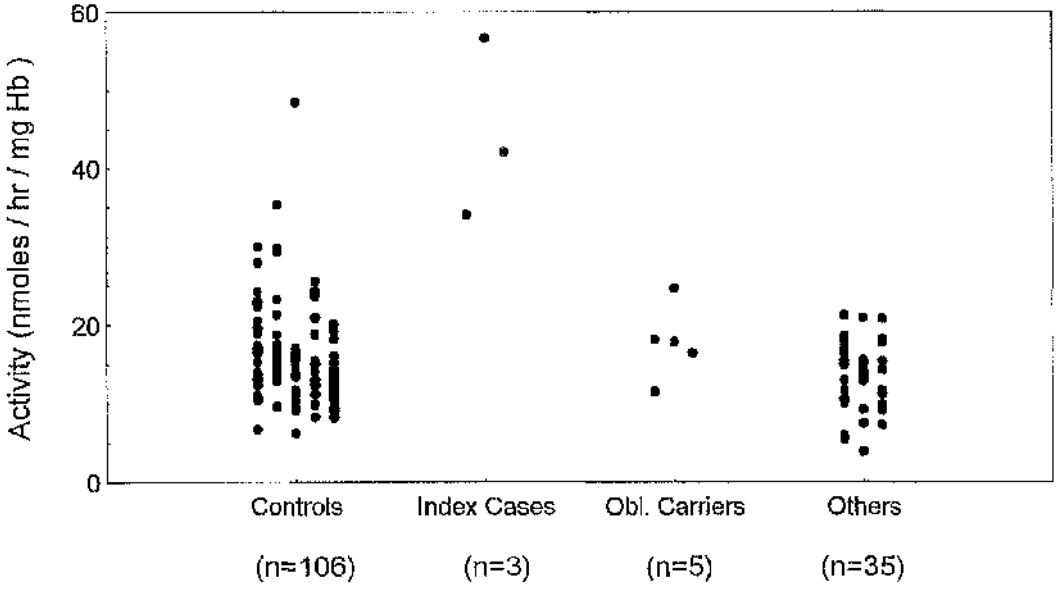


Figure 5 -14

Distribution of red cell APRT activity for Lesch-Nyhan index cases, obligate carriers and other family members

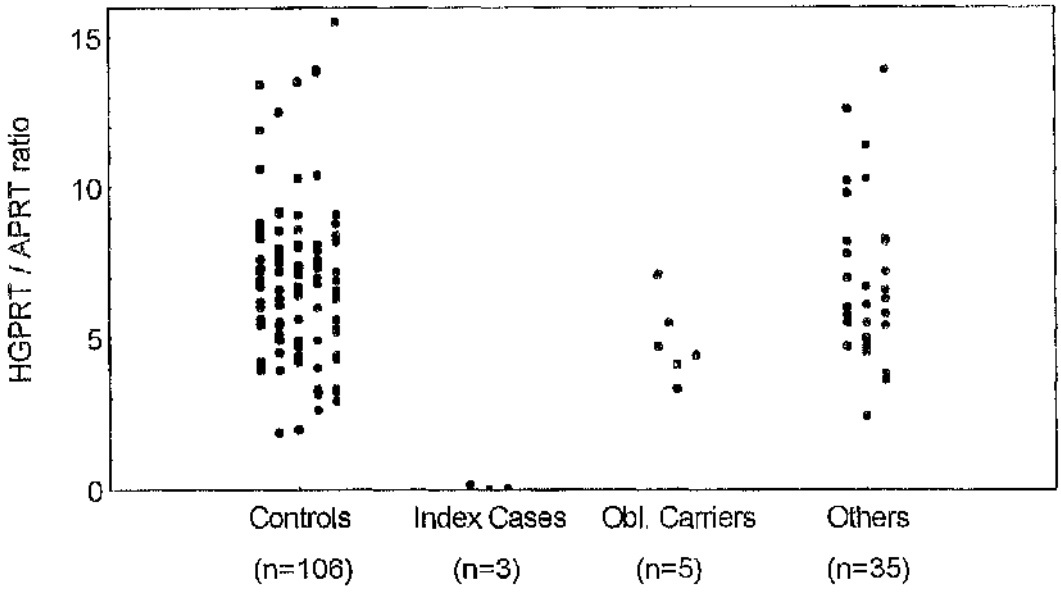


Figure 5 - 15

Distribution of red cell HGPRT / APRT ratio for Lesch-Nyhan cases, obligate carriers and other family members.

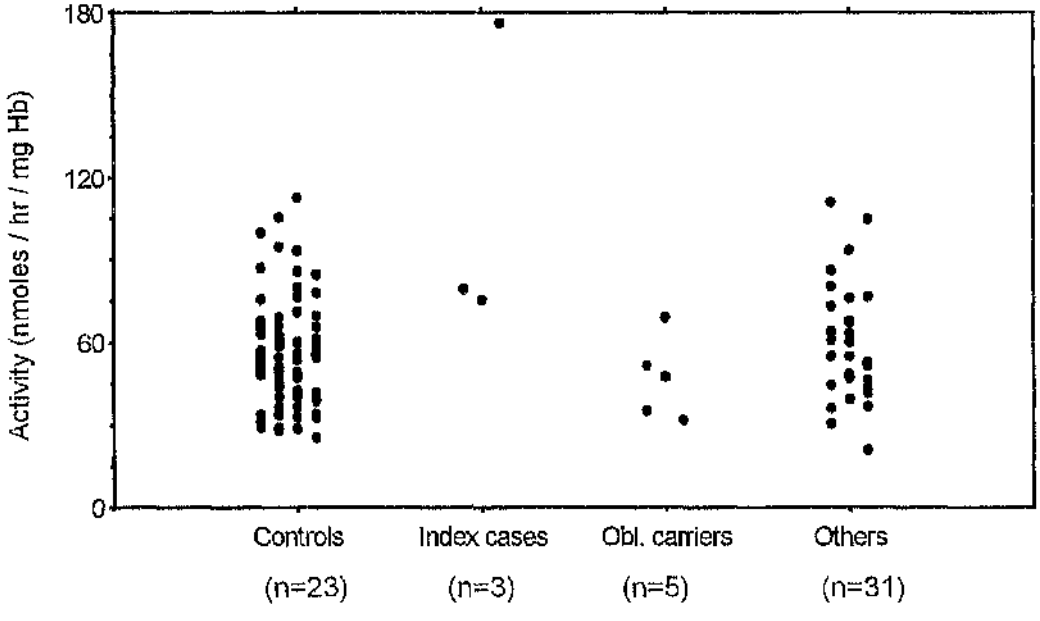


Figure 5 -16

Distribution of red cell ADA for Lesch-Nyhan cases, obligate carriers and other family members.

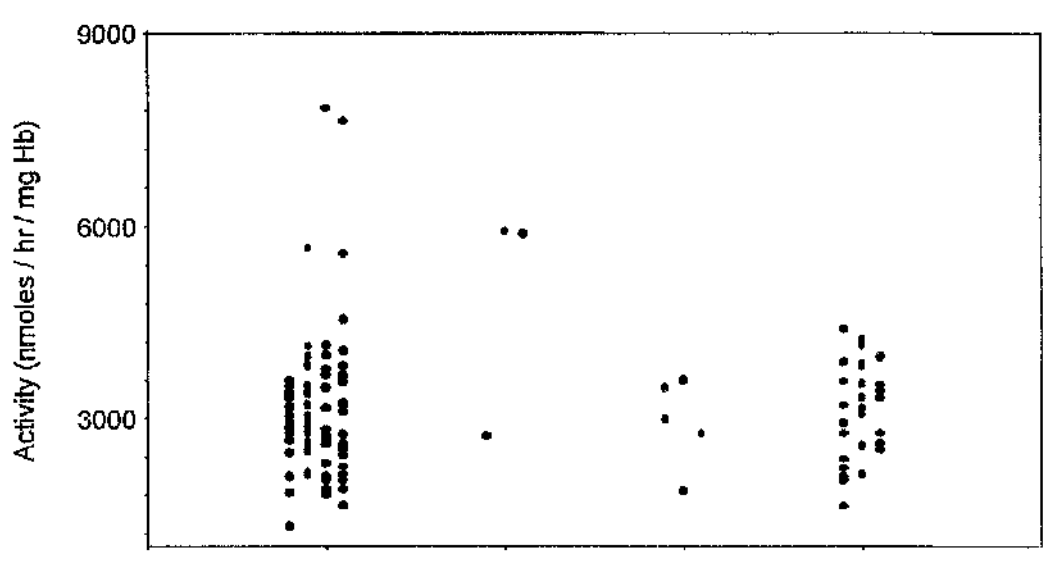


Figure 5 -17

Distribution of red cell PNP for Lesch-Nyhan cases, obligate carriers and other family members.

5.2.4 Gout Study

HGPRT, APRT and PRPP-S activities in the series of all controls and gout patients is summarised in Table 5 -12.

This data was subsequently broken down and analysed by sex to determine whether any differences could be discerned between males and females in both the control and patient populations. This data is summarised in Table 5 - 13, but does not include 5 samples which appear in Table 12 due to lack of information regarding the gender for these patients. Application of the KS test to the range of enzyme activities found in the 54 patients analysed, indicated a reasonable fit to $\log_{10}$ Gaussian distributions for HGPRT, APRT, HGPRT/APRT ratio and PRPP-S with p values of 0.83, 0.27, 0.81 and 0.91 respectively. No significant differences were found for the KS test when applied to the data after separation with regard to gender. Comparison of the means of the control and patient ranges (Table 5 - 12) for each enzyme (t-test) showed a significant difference for HGPRT ($p<0.002$) and HGPRT / APRT ratio ($p<0.003$), while APRT and PRPP-S showed no significant difference. The reference ranges for the control and patient groups show a marked

degree of overlap, which indicates that these red cell enzymes cannot be used with any confidence in distinguishing these populations.

When the data was broken down by gender, the statistical difference for HGPRT found in the total population was associated with males only ($p<0.05$), with no corresponding significant difference in APRT or PRPP-S activities. For female gout patients, only APRT was found to be statistically significantly different ($p<0.05$). While the HGPRT activity for females was not found to be significantly altered ($p=0.068$), it contributed to a significant difference for the HGPRT / APRT ratio in this population. These gender differences may be due in part to the number of values used in the calculations (45 for males and 9 for females) and more data are required to verify this result (Table 5 – 13).

Uric acid data on these patients was not included in this evaluation since most of the subjects under study were on medication for hyperuricacidemia at the time of sampling.

From the entire series of 54 gout patients, two male individuals FFR and TGR gave results of around 50% of the mean of the red cell HGPRT control range. Although these patients gave HGPRT activities of 49.3 and 42.7 nmoles / hr / mg Hb, respectively (i.e. below the lower limit of the HGPRT reference range), their HGPRT / APRT ratios of 4.7 and 6.4 were within the normal range. Of the series of 54 patients with gout, these two had polyarticular gout, whereas the remaining 52 presented with gout of an idiopathic nature. These results suggest that HGPRT activity alone may be useful in classifying these patients whereas the HGPRT/APRT ratio is not. The distributions of HGPRT and APRT associated with these populations are presented in Figures 18 and 19, while Appendix B shows the entire dataset for these patients.

Table 5 - 12

Summary of results for HGPRT, APRT, and PRPP-S for all controls and gout patients studied. Activities of HGPRT and APRT are in nmoles / hr / mg Haemoglobin, while those of PRPP-S are in μmoles / min / mg Haemoglobin. Log_{10} transformed values were used to define the reference ranges for the controls and gout patients, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

	HGPRT		AP RT		PRPP-S		HGPRT / APRT Ratio	
	Controls	Patients.	Controls.	Patients.	Controls.	Patients.	Controls.	Patients.
Log_{10} Mean	1.973 (93.7)	1.930 (85.1)	1.166 (14.7)	1.208 (16.1)	-0.467 (0.341)	-0.512 (0.307)	0.807 (6.4)	0.723 (5.3)
Log_{10} sd	0.077	0.082	0.142	0.190	0.111	0.153	0.163	0.179
Range (Mean ± 2sd)	65.9 - 133.9	58.2 - 124.2	7.6 - 28.2	6.7 - 38.7	0.205 - 0.569	0.152 - 0.622	3.0 - 13.6	1.43 - 12.1
n	106	54	106	54	18	54	106	54

Table 5 - 13

Summary of HGPRT, APRT, and PRPP-S activities and HGPRT / APRT ratio for male (♂) and female (♀) controls and gout patients . Activities of HGPRT and APRT are in nmoles / hr / mg Haemoglobin, while those of PRPP-S are in μmoles / min / mg Haemoglobin. Log_{10} transformed values were used in the assessment of the reference ranges for the controls and gout patients, while the brackets indicate the antilogarithmic conversion back to concentration units. (n = number of samples in distribution).

		HGPRT		APRT		PRPP-S		HGPRT / APRT Ratio	
		Controls	Patients.	Controls.	Patients.	Controls.	Patients.	Controls.	Patients.
Mean	♂	1.970(93.3)	1.929(84.9)	1.163(14.6)	1.191(15.5)	-0.523(0.299)	-0.518(0.303)	0.808(6.4)	0.737(5.5)
	♀	1.98(95.4)	1.937(86.5)	1.166(14.7)	1.288(19.4)	-0.431(0.370)	-0.481(0.330)	0.813(6.5)	0.649
sd	♂	0.092	0.085	0.144	0.181	0.104	0.158	0.173	0.163
	♀	0.063	0.070	0.140	0.222	0.105	0.131	0.147	0.242
Range (Mean ± 2sd)	♂	61.7 - 141.2	81.7 - 125.6	10.5 - 20.3	10.2 - 23.6	0.236 - 0.381	0.210 - 0.436	4.3- 9.6	3.7 - 7.9
	♀	70.8 - 128.8	62.7 - 95.7	10.6 - 20.2	11.7 - 32.3	0.291 - 472	0.244 - 0.445	4.6 - 9.1	2.6 - 7.8
n	♂	47	45	47	45	7	45	47	45
	♀	54	9	54	9	11	9	54	9

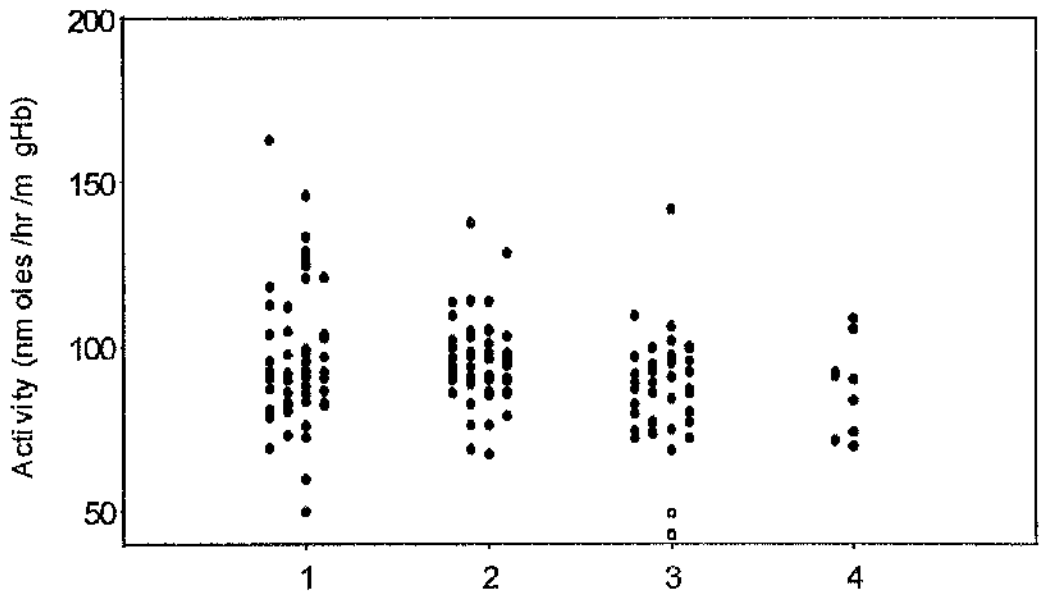


Figure 5 - 18

Distribution of HGPRT activity in nmoles/ hr/ mg Hb for controls and gout patients, where 1 = Controls (Male), 2 = Controls (Female), 3 = Gout (Male), 4 = Gout (Female). The symbol o indicates the position of the two polyarticular patients FFR and TGR .

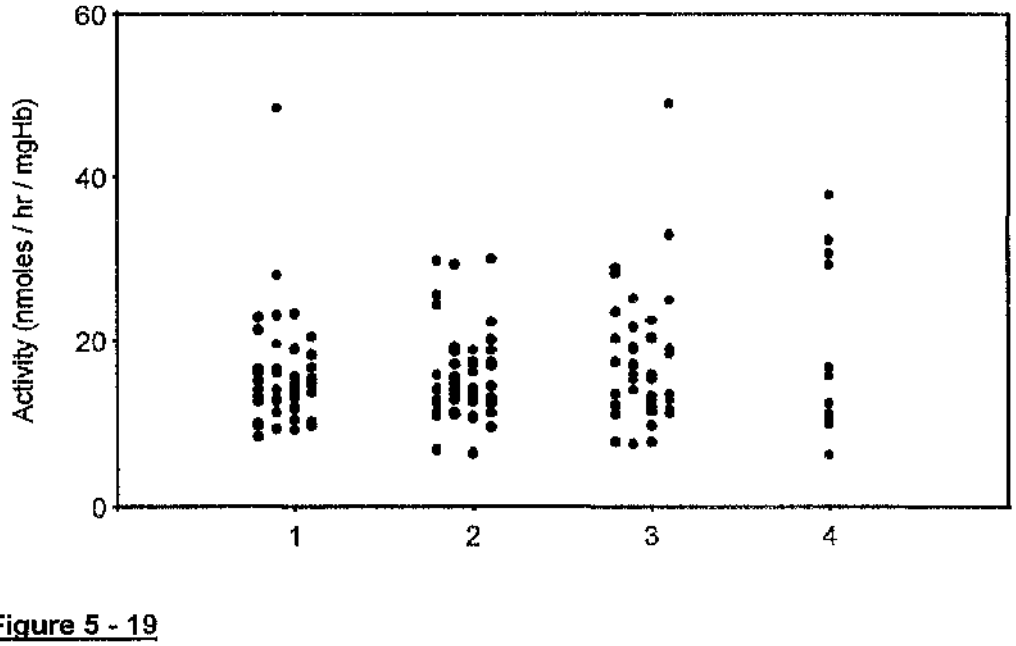
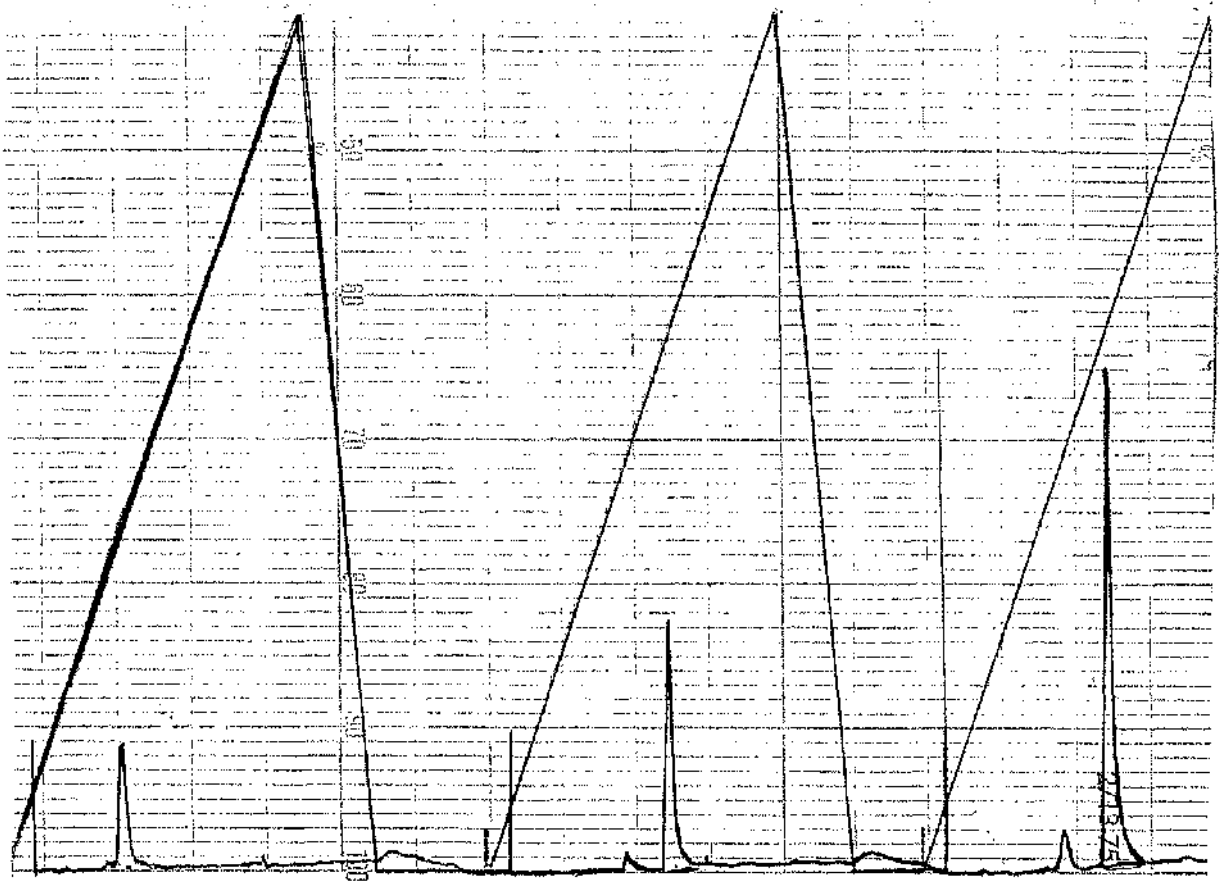


Figure 5 - 19

Distribution of APRT activity in nmoles/ hr/ mg Hb for controls and gout patients, where 1 = Controls (Male), 2 = Controls (Female), 3 = Gout (Male), 4 = Gout (Female)

One factor which was not tested in this study, was the effect of the medication which most of the patients were on at the time of the blood donation (data not shown). The pharmacological regimes varied from individual to individual and included a wide spectrum of drugs, the most common being allopurinol, related to the specific symptoms of each patient. Due to this fact, it is unclear whether the data obtained here in terms of the purine enzyme activities for these individuals is modified by the specific medication being taken.

The enzyme PRPP-S was analysed in all gout patients and compared with a subset of the same adult red cell control population used for HGPRT and APRT estimations to determine if PRPP-S superactivity could be implicated as a cause of gout in these patients. Examples of the chromatograms obtained from the HPLC run of the standards and samples used to calculate PRPP-S levels in the control and patient populations are shown in Figures 5 - 20 and 5 –21, and the range of enzyme activities in patients and controls in tables 5 –12 and 5 – 13.



35μM ADP 70μM ADP 140μM ADP

Figure 5 - 20

HPLC chromatograms and buffer gradients of 35, 70 and 140μM ADP standards.

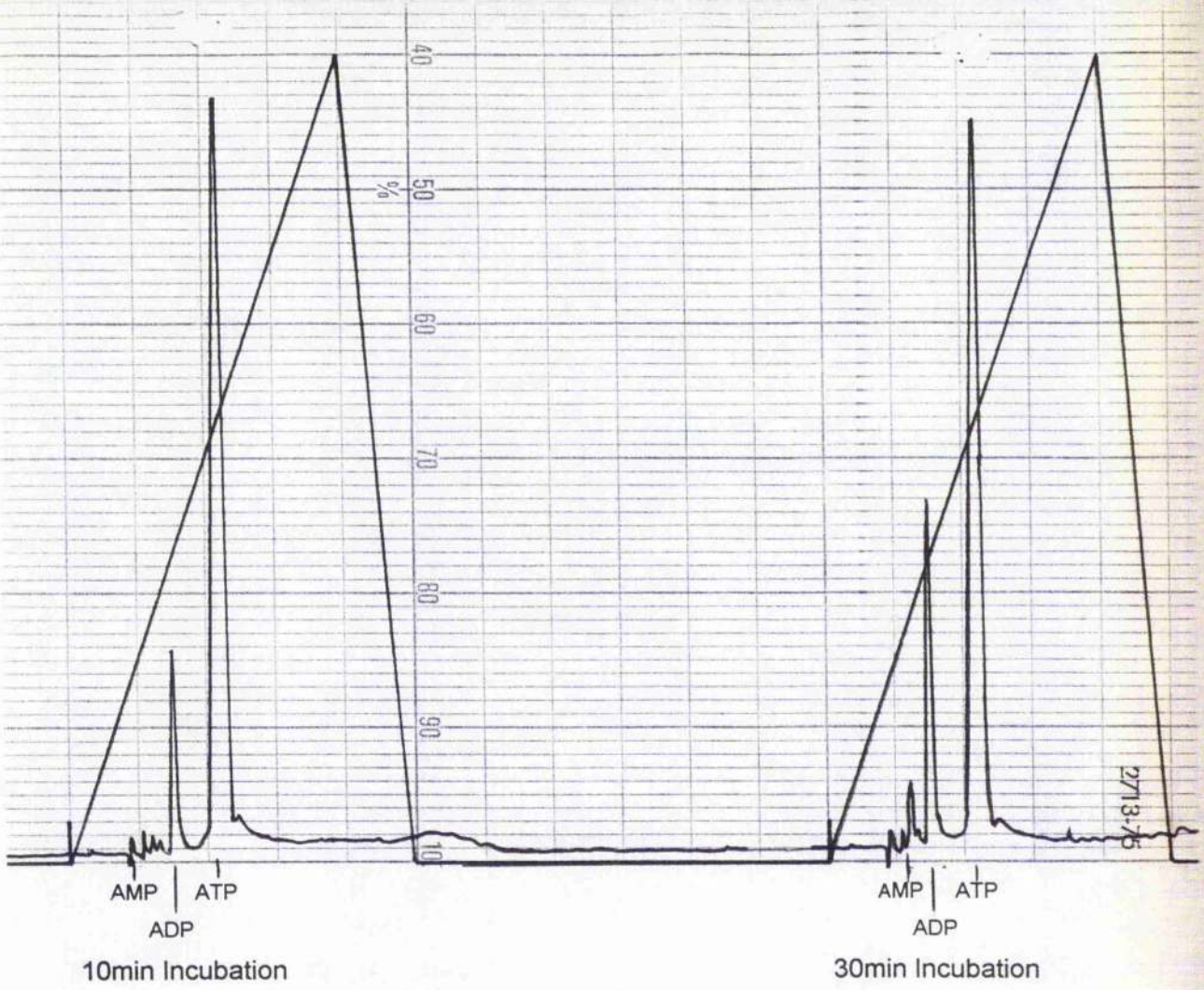


Figure 5 -21

HPLC chromatograms and buffer gradients of AMP, ADP, ATP production by PRPP-S, after 10 minutes and 30 minutes incubation of the red cell sample.

As with HGPRT and APRT, $\log_{10}$ transformed data was used and this gave a reasonable fit to a Gaussian distribution. No statistical significance was observed for PRPP-S activity ($p>0.05$), either using the entire patient group or when individual male and female groups were analysed, indicating that the cause of the condition was unlikely to be due to PRPP-S superactivity.

Patient MHD, a 2 year old male who presented with elevated uric acid and dyskinetic cerebral palsy, was investigated regarding a possible diagnosis of Lesch-Nyhan syndrome by HGPRT and APRT analysis. There was no evidence of an HGPRT deficiency with red cell HGPRT and APRT activities of 114.9 and 16.6 nmoles / hr /mg Hb respectively, translating to an HGPRT / APRT ratio of 6.9. However, when PRPP-S was investigated, the enzyme activity was 1.26 μmoles / hr / mg Hb, which was 3.7 times the mean of the red cell reference range suggesting PRPP-S superactivity. Unfortunately, due to non-compliance, no further blood samples could be obtained and further attempts to investigate this patient were unsuccessful. The results obtained for this study suggest that while HGPRT may be useful in determining certain types of gout, PRPP-S superactivity should be considered as a cause in those cases where the diagnosis is less obvious. The graphical representation of the distributions of PRPP-S activities is shown in Figure 5 - 22

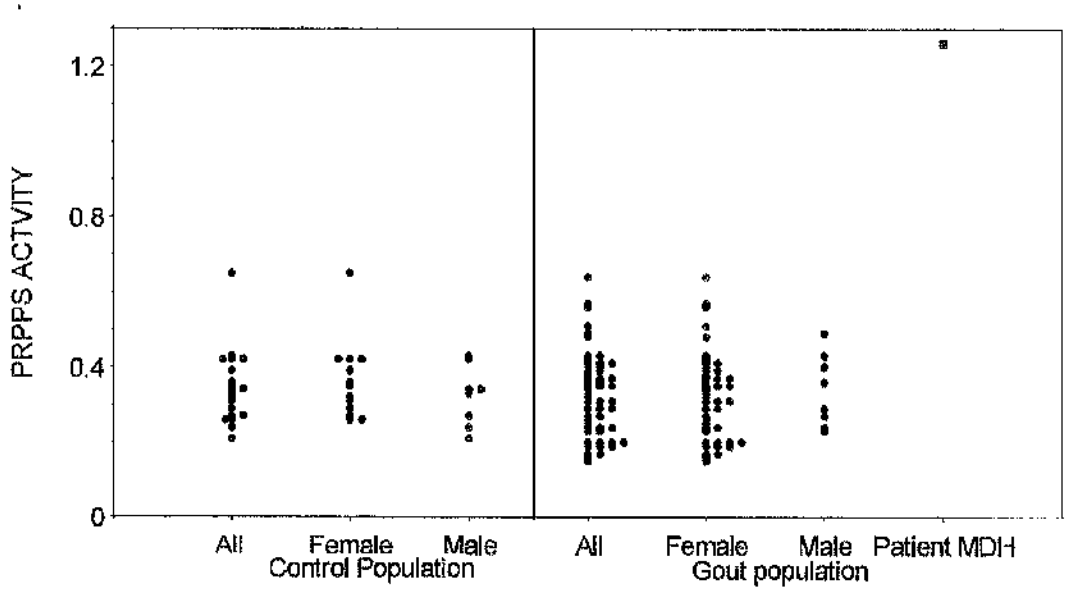


Figure 5 - 22

Distribution of PRPP-S activity in μmoles / hr/ mg Hb for controls, gout patients and a diagnostic case (Patient MHD).

5.2.5 ADA and PNP

5.2.5.1 Controls and Patients

The results of the assessment of enzyme activity in individuals at risk for ADA and PNP disorders are shown in Tables 5 - 14 and 5 – 15 with the graphical representations of the distributions shown in Figures 5 - 23 and 5 - 24. Of the patients initially presenting for ADA assessment; 17 had long standing fungal or viral infections (GST, JGN, RHS(a) and (b), ASD, CBN, JLW, AWR, RML, SGD, DRE, JSH, SBN, ABN, KMT, UIL, MMC; DGN) ; 2 had ataxia telangectasia (GFS, RCS); 1 had Graft versus Host disease (DME (a) and (b)); 6 individuals from 4 families had a history of ADA deficiency (TGG, JRS, RMN, JTD, SRN, MMH); 3 individuals from one family initially suspected of ADA deficency, were subsequently found to have PNP deficiency (DOD, LOD, KOD(a) and (b)) and 1 patient had an uncertain diagnosis (SBN). For those initially presenting for PNP investigation, 5 had recurrent infections (LID, CFR, CCY, CCR, CRE); 5 had ataxia (LFY, NPE, SBT, DRD, GST); 2 had unexplained diplegia (LDN, IMD); 1had athetosis (AMN); 1 patient with cerebral palsy. Also, 3 members of a family with PNP deficiency were investigated (PFK, FFK, IFK) as were 4 members of the family who initially presented with ADA deficiency (MIL, ELB, MOL, KO (c) and (d)). The red cell and CV reference ranges were constructed using the same samples as those used in the evaluation of normal ranges for the Lesch-Nyhan study. Where possible, ADA and PNP analyses were performed in tandem on any samples obtained for investigation of a suspected immunodeficiency disorder. Of the patients referred for ADA investigation (Table 5 - 14), 3 (TGG, JTD, MMC) had a complete deficiency of ADA activity confirming a diagnosis of severe combined immunodeficiency disease. Child TGG presented at 14 months with recurring chest infections, neutropenia, failure to thrive and hepatosplenomegaly. Investigations showed a complete deficiency of ADA activity with normal levels of PNP, and the child subsequently died aged 2 years. The sister of this index case, JTD , born at 36 weeks gestation, initially showed good APGAR scores, but when tested was shown to have a complete deficiency of red cell ADA activity with normal levels of PNP. The pedigree of family GG is shown in Figure 5 – 25. All other patients had ADA activities above the lower end of the appropriate reference range (Tables 5 - 2, 5 - 3, 5 - 4), while several had ADA activities greater than +2

SDs beyond the mean. There was one prenatal diagnosis at 15 weeks gestation based on an analysis of ADA activity in cultured amniotic fluid cells. The use of intra-assay AF cell controls confirmed that an ADA deficiency could be excluded in the fetus. There was no evidence of ADA carrier status in any of the patients tested.

Table 5 -14

Summary of affected and at-risk individuals referred for ADA investigation. Activities for red cells are expressed as nmoles / hr / mg Hb, and nmoles / hr / mg protein for Amniotic fluid cells, CV and fibroblasts. Affected cases are underlined.
Key:- RBC = Red cells; AFC = Amniotic fluid cells; FIBRO. = Fibroblast cells.

NAME	SEX	AGE.	CELL TYPE	ADA	PNP	COMMENTS
TGG	M	14 months	RBC	0	4085	ADA deficient
JTD	F	< 1 month	RBC	0	2292	ADA deficient
JRS	M	31 yrs	RBC	65.0	1995	Normal ADA/PNP
RMN	F	21 yrs	RBC	51.6	2340	Normal ADA/PNP
GST	M	8 yrs	RBC	72.5	2292	Normal ADA/PNP
JGN	M	7 months	RBC	101.6	3957	Normal ADA/PNP
SRN	F	gest 16wks	AFC	282	-	PND: Normal activity in fetus
RHS(a)	M	< 1 month	RBC	82.9		Normal
RHS(b)	M	3 months	RBC	103.8	3048	Normal
ASD	F	18 months	RBC	122.6	2402	High Normal ADA
CBN	F	2 yrs	RBC	134.9	-	High Normal
JLW	F	1 month	RBC	59.9	2141	Normal
AWR	F	9 months	RBC	81.6	3517	Normal
RML	M	8 yrs	RBC	64.8	4629	Normal
GFS	M	12 yrs	RBC	106.6	4356	High Normal ADA
SGD	F	-	RBC	117.2	2526	High Normal ADA
DRE	M	3 yrs	RBC	47.3	2104	Normal
RCS	M	5 yrs	RBC	105.2	3867	High Normal
JSH	F	6 months	RBC	52.2	2337	Normal
SBN	F	3 months	RBC	69.9	-	Normal
ABN	F	2 months	RBC	66.4	3174	Normal
KMT	F	< 1month	RBC	62.8	4154	Normal
MMH	F	22 yrs	RBC	46.0	-	Normal
UIL	M	4 months	RBC	97.3	2734	Normal
DME(a)	M	2 months	RBC	117.3	-	High Normal
DME(b)	M	4 months	FIBRO.	668.9	-	High Normal
MMC	M	-	RBC	0	4612	ADA deficient
DGN	F	9 yrs	RBC	66.0	2444	Normal
SBN	F	-	RBC	106.8	-	High Normal

Table 5 - 15

Summary of affected and at-risk individuals referred for PNP investigation. Activities for red cells are expressed as nmoles / hr / mg Hb, and nmoles / hr / mg protein for Amniotic fluid cells, CV and fibroblasts.

Key:- RBC = Red cells; CCV = cultured chorionic villi; FIBRO = Fibroblast cells.

NAME	SEX	AGE	CELL TYPE	ADA	PNP	COMMENTS
Family OD						
KOD (a)	F	27 yrs	Red Cells	40.9	1739	PNP carrier
KOD (b)	F	gest tours	CCV	381.2	0	PNP deficiency
KOD (c)	F	gest tours	CCV	284.4	743	Normal PNP/ADA
KOD (d)	F	gest tours	CCV	-	2804	Normal PNP
DOD	M	-	RBC	66.8	1803	PNP carrier
LOD	F	3 yrs	RBC	257.1	0	PNP deficiency
MIL	M	3 months	RBC	-	1495	PNP carrier
ELB	F	39 yrs	RBC	-	3727	Normal PNP
MOL	M	30 yrs	RBC	-	1626	PNP carrier
Family FK						
PFK	M	-	FIBRO	646	6.5	PNP deficiency
FFK	F	42 yrs	RBC	-	1316	PNP carrier
IFK	M	39 yrs	RBC	-	1319	PNP carrier
Others						
LDN	F	2 months	RBC	-	2000	Normal PNP
LID	F	5 yrs	RBC	-	2046	Normal PNP
CFR	F	1 month	RBC	-	2606	Normal PNP
AMN	M	1 month	RBC	-	3300	Normal PNP
LFY	F	5 yrs	RBC	-	2471	Normal PNP
NPE	F	2yrs	RBC	-	2028	Normal PNP
IMD	M	4 yrs	RBC	-	3450	Normal PNP
ZPY	F	2 yrs	RBC	-	1486	?PNP carrier
CCY	M	2 yrs	RBC		4088	Normal PNP
CCR	M	3 yrs	RBC		3277	Normal PNP
SBT	M	2 yrs	RBC		3567	Normal PNP
CRE	M	< 1 month	RBC	91.4	4800	Normal PNP
DRD	F	1 yr	RBC		2100	Normal PNP
GST	M	1 yr	RBC		3500	Normal PNP

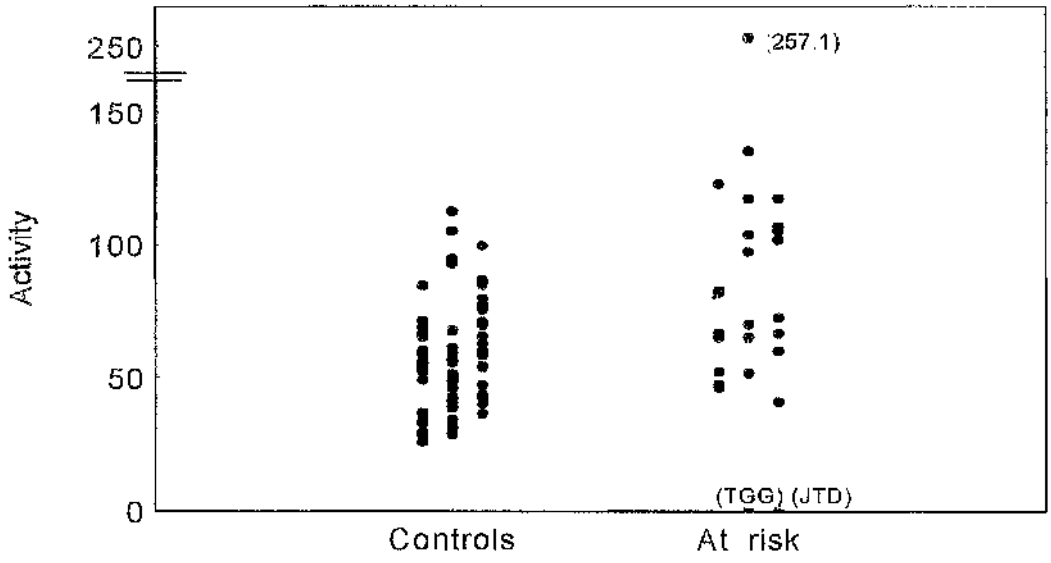


Figure 5 - 23

Distributions of red cell ADA activity in control and patient samples. Activity in nmoles / hr / mg Hb. The annotations, JTD and TGG indicate those patients found to have a complete deficiency of ADA.

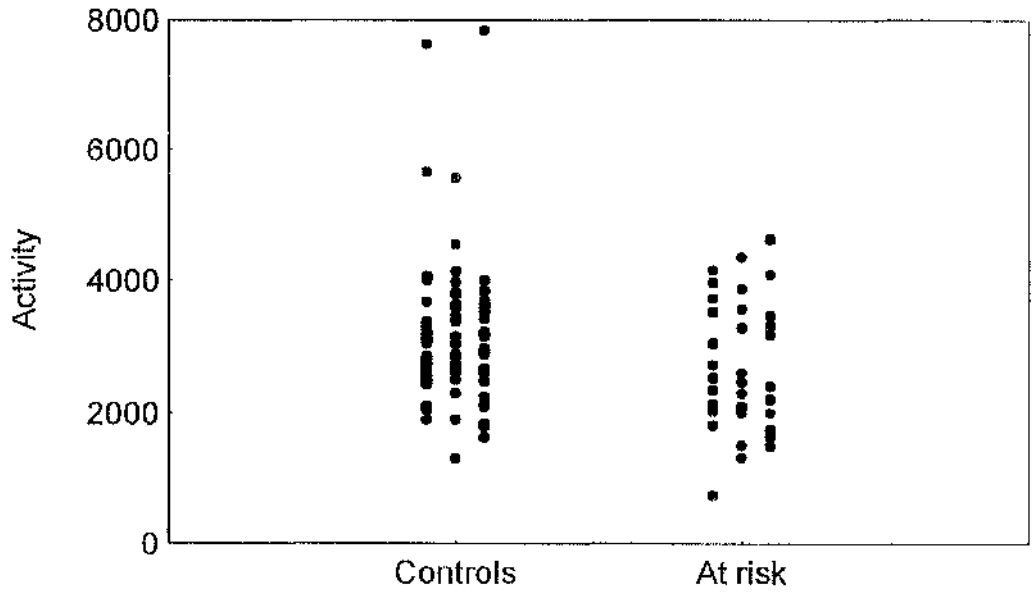


Figure 5 - 24

Distributions of red cell PNP activity in control and at-risk samples. Activity in nmoles / hr / mg Hb.

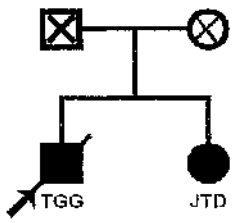


Figure 5 - 25

Pedigree for Family GG

Key: ⊠ = untested male; ⊗ = untested female; ● = affected female; ■ = affected male (deceased). The arrow denotes the proband.

Of the patients referred for PNP investigation (Table 5 – 15) around half of the cases originated from two families in which PNP deficiency was later identified.

Family OD

The pedigree of Family OD is shown in Figure 5 – 26. Child LOD presented with neutropenia, lymphopenia and an absence of cell cluster designation markers, CD4 and CD8. ADA activity was very high at 257.1 nmoles / hr /mgHb, but there was a complete deficiency of PNP. Blood samples from the mother and the father of this child (KOD(a) and DOD) tested within the red cell reference range for ADA, but were found to have levels of 59% and 61% of the mean of the PNP controls, placing them in the extreme lower end of the red cell reference range, and confirming their obligate carrier status. Further testing of red cell PNP activity in two siblings of the affected child (MIL and MOL) gave activities of 51% and 53 % respectively, and these levels are consistent with carrier status. Prenatal diagnosis was carried out on CV in 3 further pregnancies for the mother (KOD (b), KOD (c), and KOD (d)). The first of these showed a complete deficiency of PNP indicating an affected fetus and the parents opted for termination. In the second pregnancy, PNP activity was found to be low at 743nmoles / hr / mg protein (equivalent to 25% of the control mean). This was interpreted as possible carrier levels thus excluding a deficiency of PNP activity and the pregnancy proceeded to term. The third pregnancy exhibited normal PNP activity and also proceeded to term. The ADA results where performed on these samples, showed normal levels. No further samples could be obtained in these prenatal diagnostic cases to allow follow up and confirmation of the diagnosis, however karyotype analysis showed them to be male. Patient ELB, an aunt of the index case had normal red cell PNP activity.

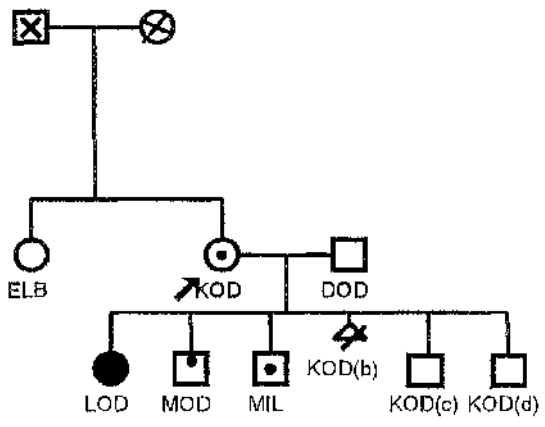


Figure 5 – 26

Pedigree for family OD.

Key:- ⊠ = untested male; ⊗ = untested female; ○ = normal female; ⊙ = carrier female (arrow denotes proband); □ = normal male; ◘ = carrier male; ⚡ = termination (male);

Family FK

The pedigree of family FK is shown in Figure 5 – 27. In family FK a diagnosis of PNP deficiency immunodeficiency disease was suspected only after the index case (PFK) was deceased. A fibroblast cell line had been stored in liquid nitrogen. Blood from the parents gave red cell PNP activities in the carrier range, and the cell line was reconstituted for investigation of PNP activity in the index case.

Due to the lack of fibroblast control material, no formal reference range for PNP in cultured cells was available. Therefore, intra-assay fibroblast controls were used to verify whether the patient sample was deficient for ADA and PNP activities. The cell line from subject PFK gave a result of 6.5 nmoles / hr / mg protein for PNP activity and 646 nmoles / hr /mg protein for ADA activity with the corresponding control values of 353.8, 179.1 and 286.6 for PNP and 623, 543 and 903 for ADA, confirming a diagnosis of PNP deficiency.

All other patients investigated for PNP deficiency had levels within the normal reference (Table 5 – 2) except for 1 case (ZPY), where carrier levels were found.

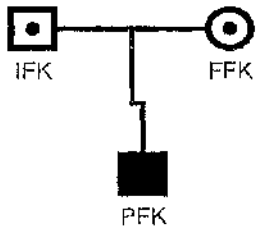


Figure 5 – 27

Pedigree for family FK.

Key:-; ⊙ = carrier female ; ⊡ = carrier male; ■ = affected male

5.3 Pteridine study

5.3.1 Results of molecular analysis of the 677C→T mutation

The resulting 198 and 175 base pair fragments obtained from the PCR and digest of the MTHFR gene were examined for all control and at risk individuals for the 677C→T mutation. Figure 5 - 28 shows the relative positions of each band for each group studied, while Table 5 -16 gives the genotype frequencies for each study group.

The results indicate that within the study population of NTD affected individuals, the frequency of the MTHFR thermolabile (+/+) allele (10.0%) is not significantly different from that of the controls (11.8%). However, the mothers of affected individuals had a significantly higher frequency of the +/+ genotype (15.3%) than the controls ($p<0.01$), suggesting that possession of this MTHFR genotype in the maternal environment may be a risk factor in the development of some NTDs. Neither the fathers (8.3%), nor other family members (11.1%) had significantly different frequencies of the MTHFR +/+ genotype compared to the controls. Relative to the controls, the odds ratio for the presence of the +/+ genotype was higher only in the mothers group (OR 1.3). Similar results are obtaind when the allele frequencies in each study group are compared. The relative proportions of the MTHFR + and MTHFR - alleles in the controls, NTD affected, mothers, fathers and other groups were: 0.36, 0.44, 0.31, 0.28 respectively. Only in the mothers

group was there a statistical significant increase (χ^2 $p<0.01$) in the frequency of the MTHFR allele.

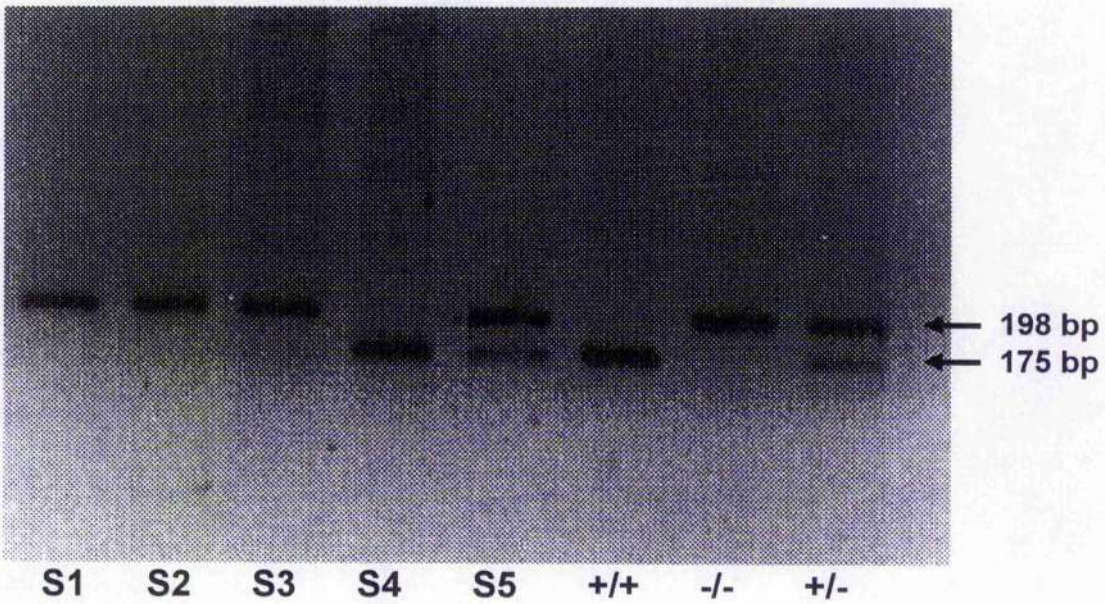


Figure 5 - 28

Ethidum bromide stained 2% agarose gel showing PCR products corresponding to the fragments of MTHFR gene after H*inf1* digestion (198 and 175 base pair). Lanes S1-S5 correspond to subject samples, while +/+, -/- and +/- are homozygous thermolabile, homozygous normal and heterozygote control samples respectively.

Table 5 – 16

Genotype frequencies for the MTHFR allele in the study population. Values in brackets indicate the relative percentages of each genotype.

MTHFR Genotype	n	Homozygous Normal (-/-)	Heterozygous (+/-)	Homozygous Thermolabile (+/+)	Odds Ratio (OR)	(+) Allele frequency
Controls	262	126 (48.1)	105 (40.0)	31 (11.8)		
NTD Affected	40	15 (37.5)	21 (52.5)	4 (10.0)	0.9	0.36
Mothers of NTD pregnancies	85	24 (28.2)	48 (56.4)	13 (15.3)	1.3	0.44
Fathers of NTD pregnancies	35	16 (44.4)	16 (47.2)	3 (8.3)	0.7	0.31
Other family members	36	20 (55.5)	12 (33.3)	4 (11.1)	0.9	0.28

5.3.2 Lymphocyte MTHFR activities

Observation of raw values showed that the MTHFR activities were positively skewed to higher values. Log_{10} transformation gave a reasonable fit to Gaussian distributions as confirmed by KS testing (data not shown), with the subsequent means, sds and reference ranges defined from this log_{10} transformed data. The lymphocyte MTHFR activities and % residual activity after heating for the various populations are summarised in Table 5 - 17, while graphical representations of the % residual activities are shown in Figures 5 - 29 to 5 - 31.

From Table 5 - 17 it can be seen that there is a marked reduction in MTHFR activity associated with the thermolabile allele (+) . However the extent of the reduction in MTHFR activity in the individual patient study groups must be interpreted with

caution as the number of individuals in each +/+ group is small. Inspection of the combined (overall) data suggests that specific activity in heterozygous (+/-) individuals is around 75% of the homozygous normal (-/-) levels, and in the homozygous thermolabile (+/+) group, 55% of -/- levels suggests that one thermolabile allele has approximately half the activity of one normal (-) allele.

If the data are considered within each of the individual study groups compared to the normal -/- control levels, the lowest MTHFR activities were found associated with the +/+ NTD affected group (21%). The +/- NTD cases had a mean activity of 52% of controls while the normal -/- NTD cases had 75% of -/- MTHFR activity. The MTHFR total activity in both the +/+ and +/- individuals in the affected group were significantly different from the -/- control group (t test: $p<0.005$ and $p<0.02$ respectively). A similar trend was found when the activities in each of the maternal MTHFR genotypic groups were compared with the -/- control activity. These varied from 25.6% for the +/+ mothers ($p<0.001$) , through 53% for the +/- mothers ($p=0.001$), to 54% for the -/- mothers. The +/+ fathers had 38.5% of normal -/- activity and the +/+ other relatives, 40.2%. The significantly higher proportion of homozygosity for the +/+ MTHFR allele amongst the mothers of NTD children (OR 1.3, Table 5 - 16) and the significantly reduced activity associated with this allele in both mothers and affected individuals support the hypothesis that at least some cases of NTD may be due to a reduced utilisation of folate caused by a low activity enzyme variant.

Heating serum samples at 46°C for 5minutes before measurement of MTHFR activities showed little diference in the extent of the reduc tion in activity between each genotypic group. Overall , -/- individuals retained 38.4% of the starting activity, +/- individuals 37.8% and +/+ individuals 34.0%. Therefore under the conditions used in the study, the MTHFR + allele does not appear to be significantly more heat labile than the normal - allele.

Table 5 - 17

Total lymphocyte MTHFR activity in μmoles formaldehyde / hr / mg protein and residual activity after heating for each genotypic group of controls, affected individuals, mothers of affected, fathers of affected and other family members. Values in brackets denote conversion of log_{10} transformed data back to concentration units. % Residual activity is the percentage residual MTHFR activity after heating.

		Homozygous Normal (-/-)			Heterozygous. (+/-)			Homozygous Thermolabile (+/+)		
		Total Activity	Residual Activity	% Residual Activity	Total Activity	Residual Activity	% Residual Activity	Total Activity	Residual Activity	% Residual Activity
Controls	Log_{10} Mean	1.067 (11.7)	0.666 (4.6)	39.3%	0.916 (8.2)	0.497 (3.1)	37.8%	0.962 (9.16)	0.358 (2.3)	25.0%
	Log_{10} sd	0.369	0.326		0.339	0.413		0.359	0.274	
	Range (Mean ± 2sd)	2.1 - 63.8	1.0 - 20.8		1.7 - 39.3	0.5 - 21.0		1.8 - 47.4	0.7 - 8.1	
	n	38	38	38	39	39	39	9	9	9
Affected	Log_{10} Mean	0.944 (8.8)	0.424 (2.7)	30.7%	0.788 (6.1)	0.344 (2.2)	36.0%	0.384 (2.4)	0.141 (1.4)	58.3%
	Log_{10} sd	0.292	0.419		0.392	0.364		0.182	0.222	
	Range (Mean ± 2sd)	2.3 - 33.7	0.39 - 6.9		1 - 37.3	0.4 - 11.8		1.1 - 5.6	0.5 - 3.8	
	n	13	13	13	17	17	17	3	3	3
Mothers	Log_{10} Mean	0.801 (6.3)	0.412 (2.6)	38.8%	0.795 (6.2)	0.367 (2.3)	37.0%	0.481 (3.0)	0.047 (1.1)	36.6%
	Log_{10} sd	0.419	0.36		0.357	0.322		0.284	0.313	
	Range (Mean ± 2sd)	0.9 - 43.6	0.5 - 13.6		1.2 - 32.2	0.5 - 10.3		0.82 - 11.2	0.5 - 2.3	
	n	21	21	21	43	43	43	9	9	9
Fathers	Log_{10} Mean	0.931 (8.5)	0.514 (3.3)	40.3%	0.754 (5.7)	0.369 (2.3)	40.0%	0.658 (4.5)	0.189 (1.5)	33.3%
	Log_{10} sd	0.306	0.350		0.592	0.584		0.081	0.137	
	Range (Mean ± 2sd)	2.1 - 34.9	0.65 - 16.4		0.4 - 67.1	0.1 - 34.4		3.1 - 6.6	0.8 - 2.9	
	n	13	13	13	15	15	15	3	3	3
Others	Log_{10} Mean	0.793 (6.2)	0.404 (2.5)	38.4%	0.689 (4.8)	0.245 (1.8)	37.5%	0.675 (4.7)	0.267 (1.8)	38.2%
	Log_{10} sd	0.420	0.402		0.32	0.39		0.041	0.113	
	Range (Mean ± 2sd)	0.9 - 43.0	0.4 - 16.1		1.1 - 21.3	0.3 - 10.6		3.9 - 5.7	1.1 - 3.1	
	n	17	17	17	10	10	10	3	3	3
Overall	Log_{10} Mean	0.934 (8.6)	0.520 (3.3)	38.5%	0.819 (6.6)	0.395 (2.5)	37.9%	0.672 (4.7)	0.201 (1.6)	34.0%
	Log_{10} sd	0.384	0.373		0.39	0.401		0.345	0.276	
	Range (Mean ± 2sd)	1.5 - 50.4	0.2 - 18.5		1.1 - 39.7	0.39 - 15.7		0.9 - 23.0	0.4 - 5.7	
	n	102	102	102	124	124	124	27	27	27

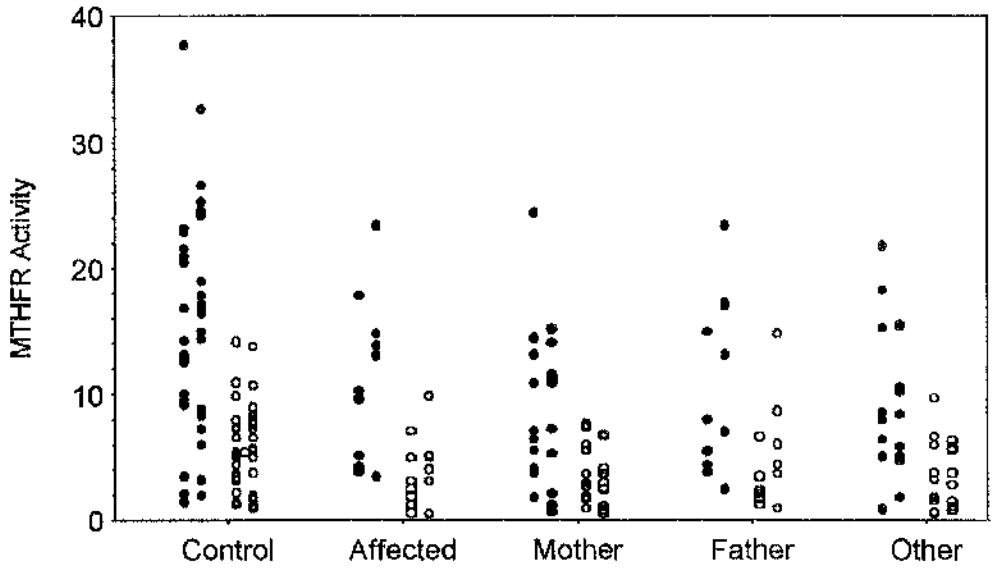


Figure 5 - 29

Residual MTHFR activity after heating for 5 minutes at 46^0 C for individuals with homozygous normal genotype. MTHFR activity in μmoles formaldehyde / hr/ g protein

Key:- ● = Total MTHFR activity O = Residual MTHFR after heating

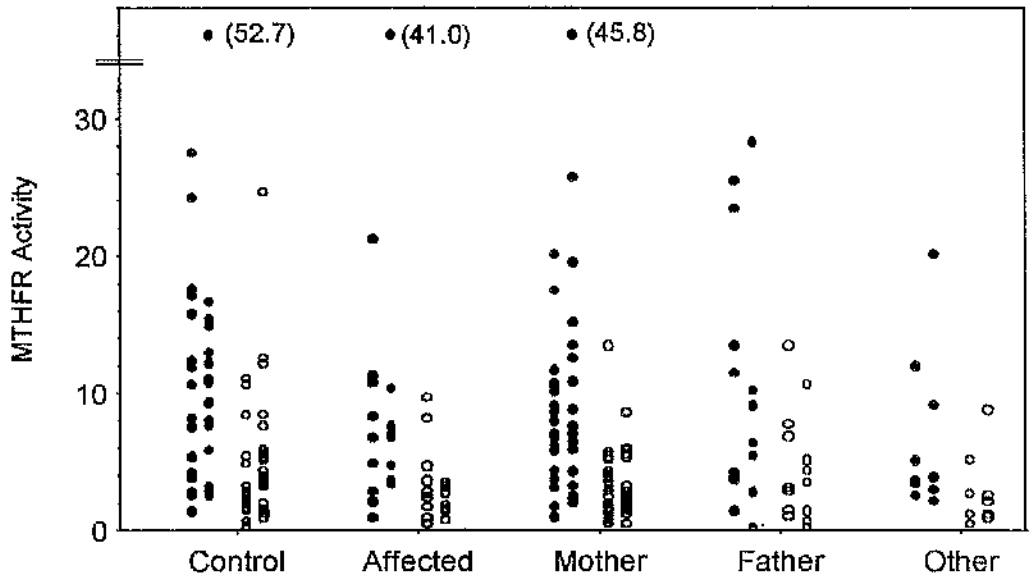


Figure 5 - 30

Residual MTHFR activity after heating for 5 minutes at 46^0 C for individuals with heterozygous genotype. MTHFR activity in μmoles formaldehyde / hr / g protein. The annotated data in brackets indicates outlying values.

Key:- ● = Total MTHFR activity O = Residual MTHFR after heating

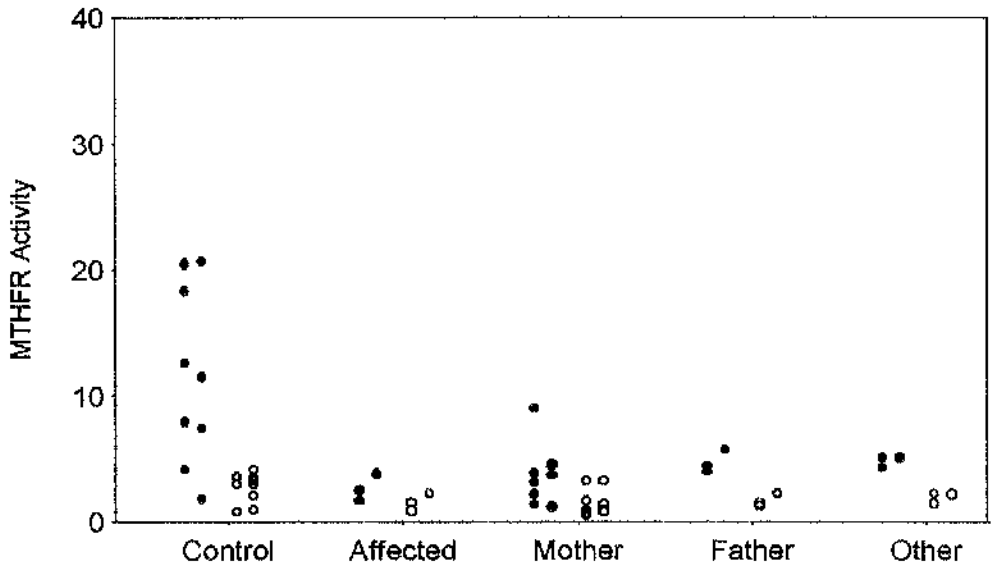


Figure 5 - 31

Residual MTHFR activity after heating for 5 minutes at 46^0 C for individuals with homozygous thermolabile genotype. MTHFR activity in µmoles formaldehyde/ hr / g protein

Key:- ● = Total MTHFR activity O = Residual MTHFR after heating

5.3.3 Enzymatic analysis of Lymphocyte MTHFR

The method used in the evaluation of the lymphocyte MTHFR activity (Section 4.9.2) was a modification of that described by Engbersen *et al* (1995). Prior to its application in the analysis of samples from the MTHFR study, a number of assay parameters were assessed and optimised where necessary. In addition, to the estimation of the MTHFR activity in lymphocytes, the plasma component of the whole blood sample obtained was also examined for MTHFR activity.

5.3.3.1 Optimisation of the MTHFR assay

5.3.3.1.(i) Concentration of [^{14}C] Methylenetetrahydrofolate substrate

The original radiolabelled substrate concentration was assessed to suit the conditions of the modified assay protocol. A volume of 5.4µl was calculated to give around 600,000 counts in the final assay mix and a Km of at least 18µm/l as specified in the original method. Results for the Lineweaver-Burk equation showed that the Michaelis constant (K_m), defined as the activity of an enzyme at half maximal velocity, was 26.5µm/l. (Figures 5 – 32 and 5 - 33).

Evidence of significant hydrolysis of the radiolabelled substrate caused by a working menadione concentration of 3.5mM, prompted further evaluation of the optimum concentration of menadione require to maintain a suitable level of activity of the MTHFR assay.

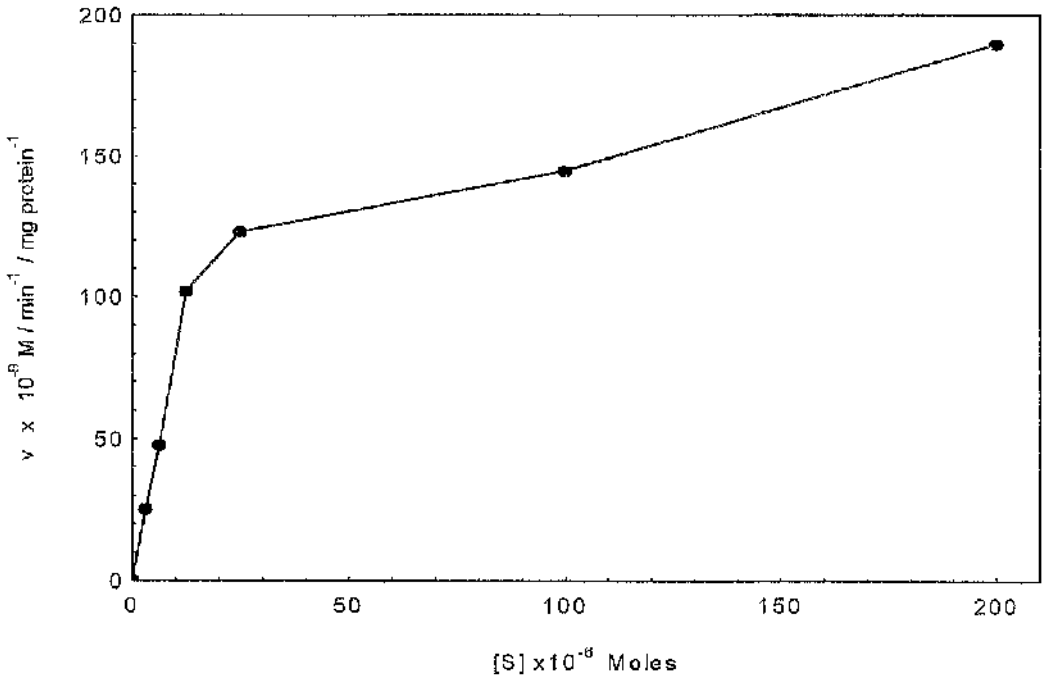


Figure 5 - 32

Velocity (V) versus substrate concentration [S] plot for the [^{14}C] Methylenetetrahydrofolate radiolabelled substrate.

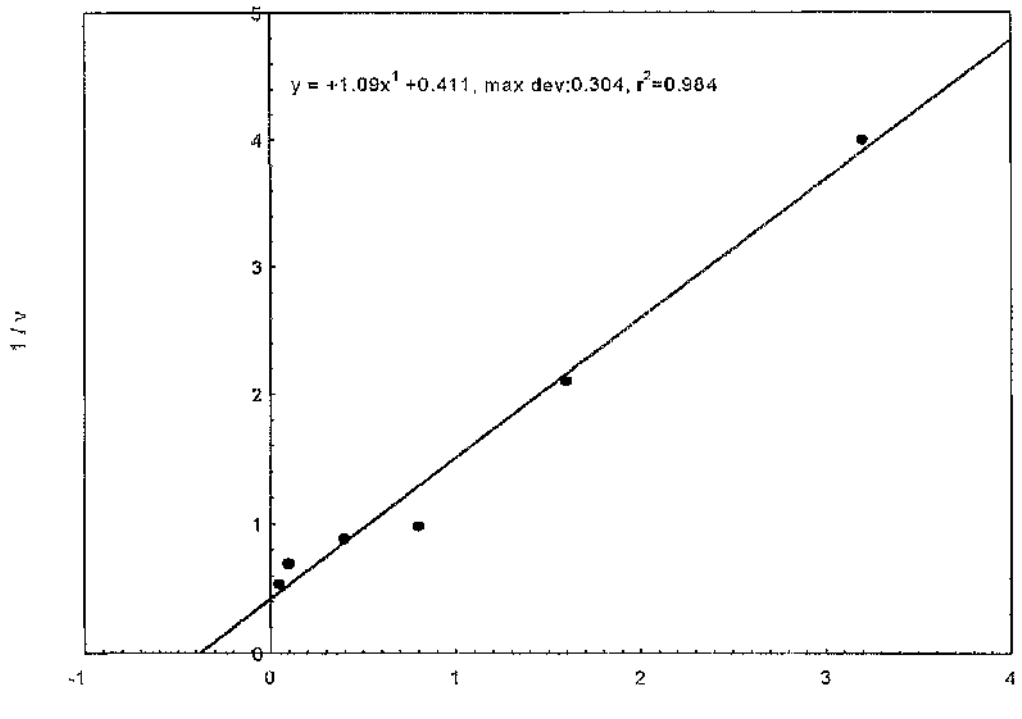


Figure 5 - 33

Lineweaver-Burk plot for data based on the reciprocal velocity (v) and substrate concentration ([s]) data from Figure 31 for [^{14}C] Methylenetetrahdrofolate radiolabelled substrate. The Michaelis constant (K_m) extrapolated from the graph was 0.3906 which was calculated to be 25.6 μmoles/l.

5.3.3.1.(ii) Menadione Concentration

Assessment of the optimal menadione concentration required for use in the modified lymphocyte MTHFR assay, indicates that the maximum enzymic activity occurs at a final assay concentration of 0.01mM (10μm), giving a 40% turnover of product compared to the initial substrate. However, since this value is very close to inflection point of the curve, a higher concentration, of 0.1mM (corresponding to an original stock concentration of 7mM) , was used in the assay .

Figure 5 - 34 shows that this modification still gives a significant difference between the blank and the sample levels of activity for this lymphocyte control. This is in marked contrast with the menadione concentration of 3.5mM specified in the original method.

The re-assessed menadione concentration of 0.1mM was used in a routine MTHFR assay to evaluate the enzyme activity of the lymphocyte control under the thermolabile conditions specified in Section 4.9.2. The results show that MTHFR residual activity for separate aliquots of the lymphocyte control gave a total activity of 39.6 μm formaldehyde / hr / mg protein respectively corresponding to 66% residual activity after heating.

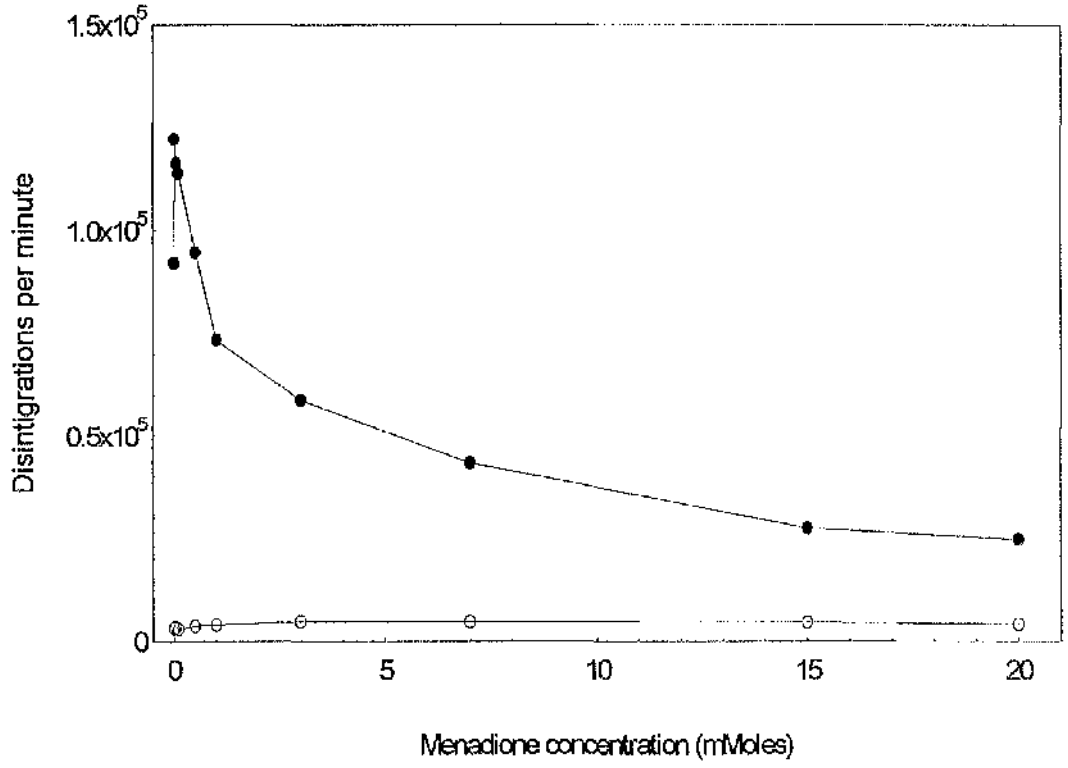


Figure 5 - 34

Graph for the lymphocyte control sample and the sample blank over a range of menadione concentrations. Key:- ● = sample; ○ = blank.

5.3.3.1.(iii) Protein concentration of sample

The concentration of total protein in the sample used for MTHFR enzyme assay is of importance, since the least protein required for measurable and reproducable activity compared to the blank, the more sample is available for subsequent analysis. This also has implications for the optimal sample volume. The method described by Rosenblatt *et al* (1977) specified an optimal protein concentration of between 0.3 - 0.6 mg/ml, albeit in a fibroblast sample. Using dilutions of the original lymphocyte control preparation, results suggest that a sample consisting of a total protein concentration of 6.4mg/ml diluted down to 0.2mg/ml in distilled water, gave a good signal to noise ratio at about 2.1 mg/ml, appropriate to a x3 dilution factor respectively. Concentration of protein at these levels was considered appropriate, as a compromise of maximal dilution and maximal product produced to give an adequate signal for scintillation counting (i.e around 35,000 dpm). (Figure 5 - 35).

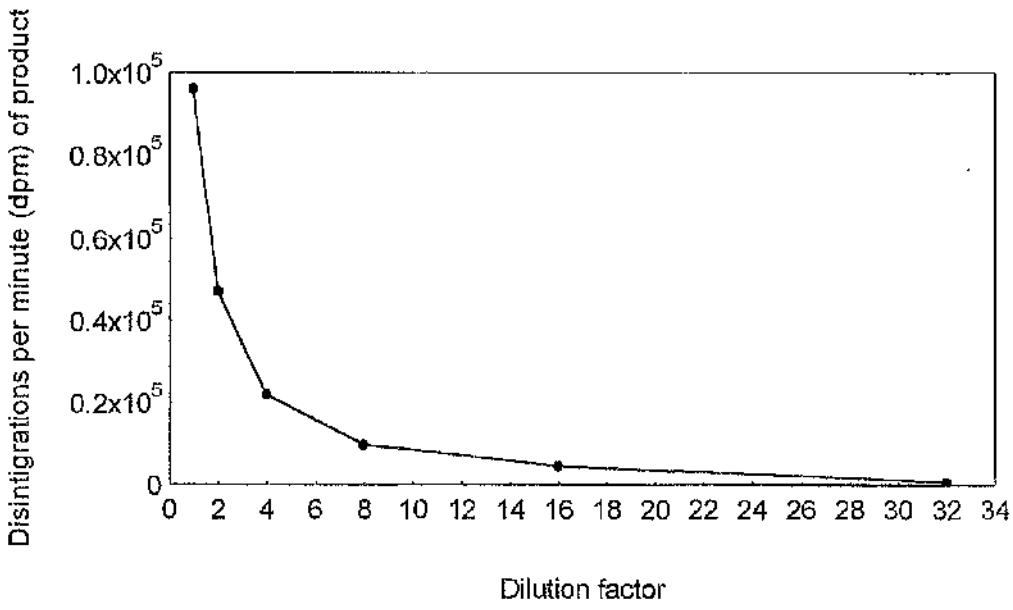


Figure 5 - 35

Disintigrations per minute of lymphocyte control sample product for varying dilutions of the total protein concentration.

5.3.3.1.(iv) Sample volume

From previous experiments it is apparent that lymphocytes can be used in estimation of MTHFR estimation. While Engbersen at al (1995) suggest a volume of sample of 250µl and an incubation time of 20 minutes. Due to the nature of the blood collection process in this study and the inherent delays in receipt and separation of the sample constituents, yields of lymphocytes tend to be somewhat reduced compared to those obtained from fresh blood. Consequently, the protein concentration and hence the volume of sample used is a primary concern in assessment of this assay. A sample volume of 250µl as specified in the original report, is greater than would be wished for this assay given the lymphocyte purification protocols. Figure 5 - 36 shows the effect of a decrease in the volume of sample to a proposed working volume of 50µl while increasing the incubation time to 1.5hours. This provides adequate turnover of the MTHFR enzyme, while at the same time maintaining a low background count for the blank.

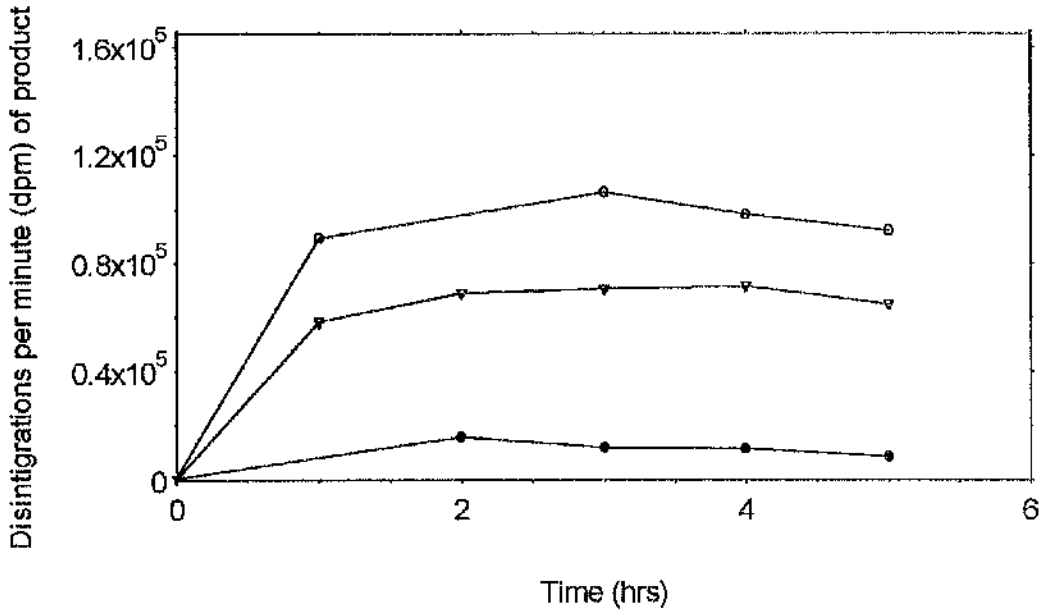


Figure 5 - 36

Comparison of sample volume for use in the lymphocyte MTHFR assay during 5 hour incubation.

Key:- O - 100µl sample ; Δ - 50µl sample; ● - 10µl sample ;

5.3.3.1.(v) Thermal stability

Thermal stability is a crucial element in the evaluation of the lymphocyte MTHFR enzyme assay. The original method by Engbersen *et al* (1995) stipulates a sample pre-incubation of 5 minutes at a temperature of 46°C for a residual activity of 40% for the control population. There is however no specification as to whether this was for sample alone or in pre-incubation with the auxilliary reagents. This aspect of the protocol was evaluated over a range of temperatures involving sample in a total reaction volume (minus FAD and menadione, which were considered too labile) and with sample alone. There appears to be no difference in the resulting residual enzyme activities, with both protocols indicating that pre-incubation for 5 minutes at 46°C was suitable for the prospective enzyme assay (Figure 5 - 37). For ease of assay preparation, pre-incubation of the sample prior to addition of the auxilliary reagents was the preferred option.

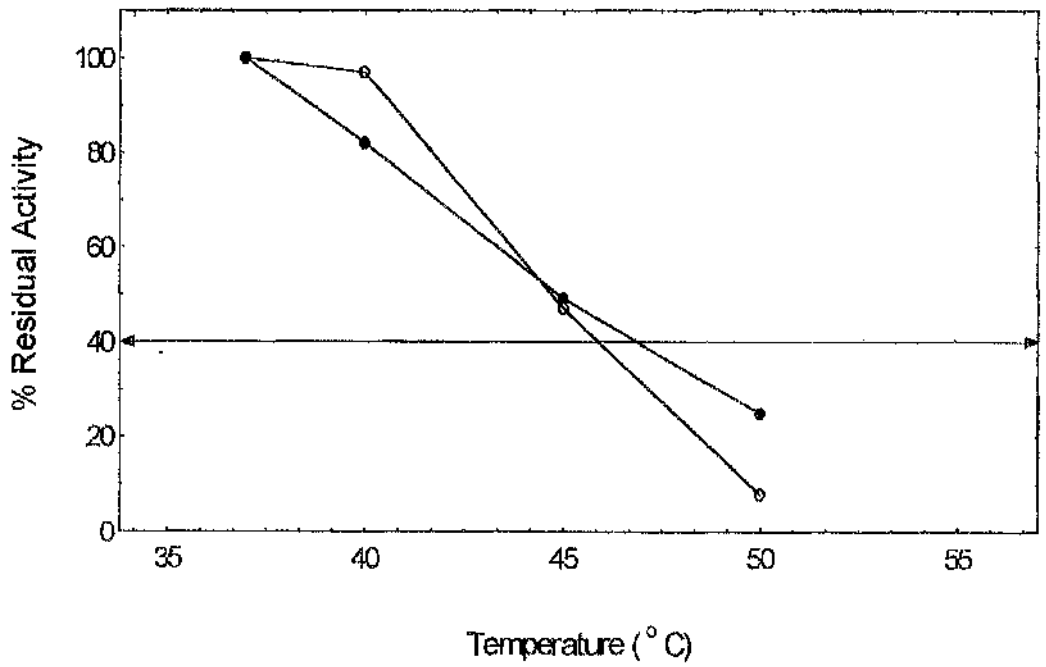


Figure 5 - 37

Thermal stability of MTHFR after pre-incubation with and without other assay reagents. Key:- ● - sample pre-incubation with other reagents; o - sample pre-incubation with no other reagents.

5.3.3.1.(vi) Quality Control

The pooled lymphocyte preparation was assayed 6 times in 2 batches of the MTHFR enzyme anlysis, to give a preliminary consensus value with which to assess the quality control of each assay. This pooled sample gave results of 29.3 ± 2.45 with a coefficient of variation of 7.3%. The residual activity after heating was 53.4 ± 6.7 % with a coefficent of variation of 12.4%.

5.3.3.1.(vii) Comparison of plasma and lymphocyte enzyme activities

The original assay method specifies lymphocytes as the preferred material for the measurement of MTHFR enzyme activities. An evaluation of MTHFR assay using plasma was performed with the object of assessing the potential of this as an alternative to using the lymphocyte method, which is both labour intensive and provides only a small amount of material for analysis. The results indicate that over a 24 hour incubation period , plasma activities were very much reduced compared with those of lymphocyte. There appears to be a significant amount of spontaneous hydrolysis of the label as evidenced by the blank, where the radioactive product is equal or, in some cases greater than that seen in the tubes containing the plasma samples (Figure 5 - 38).

Further investigation suggested that the high dpm in the blank was due to the menadione in a concentration of 3.5mM, as specified in the method by Engbersen *et al* (1995) which was responsible for the hydrolysis of the label, since absence of menadione in the assay gave no appreciable radioactivity in the product compared to that of the sample blank. Removal of the FAD cofactor still gave increased levels of radioactivity in the product, which supports the conclusion that the menadione appears to be the causitive factor (Figure 5 - 39).

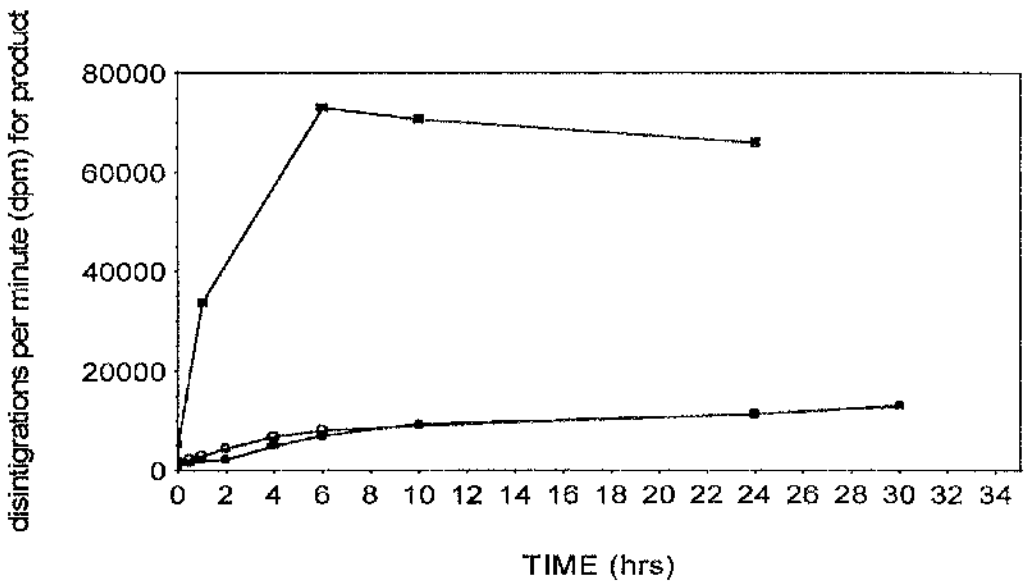


Figure 5 - 38

Comparison of MTHFR activity for lymphocyte and plasma samples over 30 hour incubation time. Key:- ■ - lymphocytes; ● - plasma; o - blank

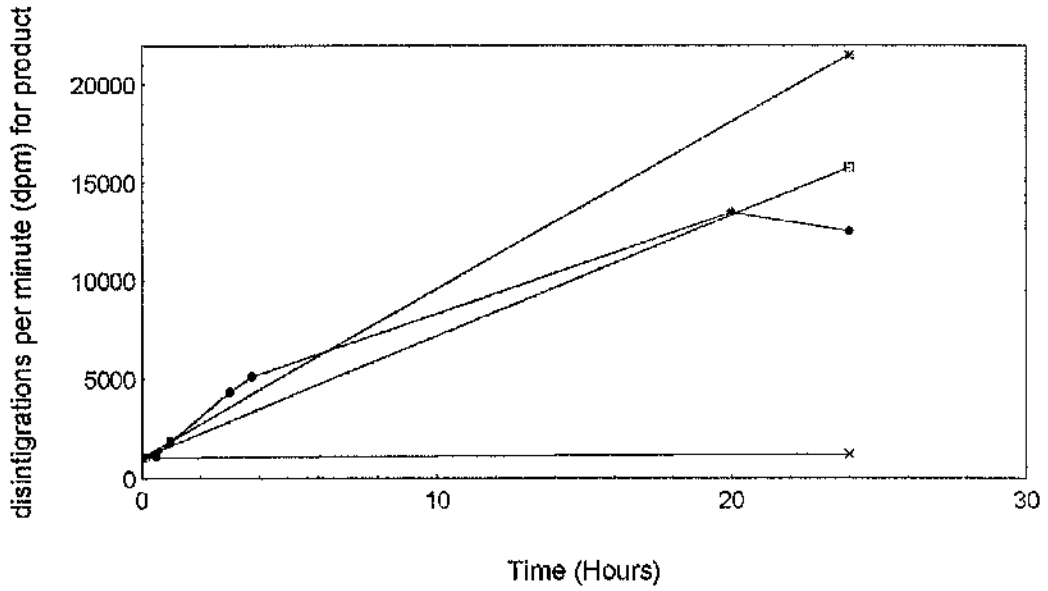


Figure 5 - 39

Comparison of radioactive product produced in a 24 hour incubation for plasma MTHFR, with and without FAD and Menadione.

Key:- * - plasma blank ; □ - plasma (no FAD) ; ● - plasma ; x - plasma (no menadione)

The results for estimation of plasma MTHFR seem to suggest that due to the very low levels of expression in plasma compared to those in lymphocyte preparations, that plasma can not be used in determination of MTHFR activities with any degree of accuracy using this assay method, and that lymphocyte preparations should be used in MTHFR estimations.

5.3.4 Nutritional status

The nutritional status of individuals in the populations under study was assessed using red cell folate, and plasma vitamin B_{12} . While the method for analysis of red cell stipulated the use of the haematocrit in the calculation of the final value, this was not a viable option since due to transportation and storage problems, a number of samples were haemolysed on receipt. In light of this, the haemoglobin content of the sample was also evaluated as a comparitive index in red cell folate assessment. A comparision of haematocrit and haemoglobin gave a correlation coefficient of 0.79, indicating that haemoglobin would be a good substitute for haematocrit in the calculation of red cell folate.

The red cell folate and plasma vitamin B_{12} results were assessed with relation to the genotype of each population. Examination of the distribution data using the Kolmogorov-Smirnoff statistical test showed a non-Gaussian distribution for red cell measurements in the +/- controls ($p<0.01$), mothers ($p<0.05$) and other family members ($p<0.04$). Normal distributions were obtained for red cell folate and plasma vitamin B_{12} in all genotypic groups after $\log_{10}$ transformation and this was used in the statistical assessments of results and establishment of the reference ranges.

Inspection of the combined red cell folate data in Table 5 - 18 (overall), shows that red cell folate levels are reduced (81%) in +/+ individuals compared with -/- controls. When the data within each of the respective study groups is compared to the -/- controls, +/+ affected, mothers and fathers show reduced folate concentrations of 75%, 68% and 70% respectively. The corresponding +/- individuals in these groups showed the same or marginally increased red cell folate levels ranging from 98% to 120% of the mean -/- control group. It should be noted however, that these differences may be due in part to the low numbers of +/+ samples obtained and must be interpreted with caution.

Examination of the plasma B_{12} data for each of the study groups (Table 5 – 18), indicated higher plasma B_{12} concentrations in +/+ individuals in all of the study groups , with increases of 32%, 64%, 69% and 89% for the mothers, affected, others and fathers respectively. The +/- in these groups failed to show the correspondingly high values for plasma B_{12}, where, with the exception of the affected individuals, who had reduced B_{12} levels, all groups showed modest increases of between 15% and 24% compared to the -/- controls.

There was no significant difference in the red cell folate levels between any of the patient groups with the +/+ thermolabile genotype and the controls, however plasma vitamin B_{12} levels were consistently higher in all +/+ groups, with levels in the affected, group significantly so ($p<0.05$).

Table 5 - 18

Red cell folate and plasma vitamin B_{12} concentrations for each genotypic group of controls, affected individuals, mothers of affected, fathers of affected and other family members. Red cell folate units are ng/ml, while plasma B_{12} units are pg/ml . Values in brackets denote conversion of log_{10} transformed data back to concentration units.

		Homozygous Normal (-/-)		Heterozygous. (+/-)		Homozygous Thermolabile (+/+)	
		Red cell folate	plasma B_{12}	Red cell folate	plasma B_{12}	Red cell folate	plasma B_{12}
Controls	Log10 Mean	0.728 (5.3)	2.498 (314.7)	0.731 (5.4)	2.454 (284.4)	0.587 (3.9)	2.491 (309.7)
	Log10 sd	0.366	0.205	0.319	0.241	0.348	0.265
	Range	1.0 - 28.6	123 - 809	1.2 - 23.4	94 - 863	0.8 - 19.2	132 - 1049
	n	46	47	42	42	12	12
Affected	Log10 Mean	0.683 (4.8)	2.551 (355.6)	0.714 (5.2)	2.430 (269.2)	0.598 (4.0)	2.712 (515.2)
	Log10 sd	0.455	0.284	0.244	0.572	0.175	0.096
	Range	0.6 - 39.2	96 - 1340	1.7 - 15.9	19 - 3749	1.8 - 8.7	331 - 802
	n	15	15	18	18	3	4
Mothers	Log10 Mean	0.682 (4.8)	2.487 (306.9)	0.721 (5.3)	2.559 (362.2)	0.558 (3.6)	2.618 (414.9)
	Log10 sd	0.383	0.194	0.345	0.224	0.484	0.181
	Range	0.8 - 28.1	125 - 753	1.1 - 25.7	129 - 1016	0.4 - 33.6	180 - 955
	n	23	23	43	43	11	12
Fathers	Log10 Mean	0.497 (3.1)	2.602 (399.9)	0.805 (6.4)	2.590 (389.0)	0.564 (3.7)	2.775 (595.7)
	Log10 sd	0.22	0.172	0.158	0.191	0.345	0.157
	Range	1.1 - 8.6	181 - 887	3.1 - 13.3	161 - 937	0.78 - 17.9	289 - 1227
	n	15	15	16	17	3	3
Others	Log10 Mean	0.713 (5.2)	2.484 (304.8)	0.725 (5.3)	2.563 (365.6)	0.722 (5.3)	2.725 (530.9)
	Log10 sd	0.209	0.339	0.621	0.205	0.139	0.198
	Range	2.0 - 13.5	64 - 1452	0.3 - 92.7	142 - 940	2.8 - 10.0	212 - 1327
	n	20	20	11	12	4	4
Overall	Log10 Mean	0.680 (4.8)	2.510 (323.6)	0.730 (5.4)	2.510 (323.6)	0.590 (3.9)	2.61 (407.4)
	Log10 sd	0.35	0.24	0.34	0.30	0.36	0.22
	Range	2.1 - 10.7	165.2 - 562.3	2.5 - 11.7	162.2 - 645.7	1.7 - 8.9	245.5 - 676.1
	n	119	120	130	132	33	35

CHAPTER 6. DISCUSSION

6.1 General Discussion

In this study, the metabolic pathways of purine and pteridine synthesis and catabolism, and the devastating disorders associated with them, have by necessity, been treated as separate processes. However, the links between these two pathways are evident in many diverse cellular reactions such as the role played by tetrahydrofolate as 5' 10- methenyl and 10 formyltetrahydrofolate as the cofactor for enzymes in the de novo synthesis of the purines, and conversion of 5-methylenetetrahydrofolate to methionine as a source of S-adenosyl methionine (SAM) in the methylation of proteins, DNA, phospholipids and DOPA. Perturbations in the metabolism of folate may also have widespread and related effects in the synthesis of the purine ring structure.

This study has examined a spectrum of genetic disorders associated with purine and pteridine metabolism and attempted to evaluate the diagnostic approaches used in their diagnosis. These disorders cover a number of inheritance patterns including X-linked (LNS and gout), autosomal (ADA and PNP) and multifactorial (folate resistant NTD's) and their investigation has required various biochemical and molecular techniques used including radiolabelled enzyme assay, kinetic enzyme assay, HPLC, radioimmunoassay and PCR.

For purine metabolic disorders, the target of the analysis has been specific enzyme defects which are not affected by environmental factors. For investigation of NTDs, which have a multifactorial aetiology, a single candidate gene, MTHFR, was chosen. Here, environmental factors (i.e. folate levels) are implicated resulting in a more difficult interpretation of results, through the need to rationalise nutritional status with the functional level of enzyme activity.

6.2 Purine metabolism

6.2.1 Distribution of purine enzyme activity in various tissues.

Analyte levels measured in selected (control) individuals cannot be assumed to be "normally" distributed. Unless distributions can be shown to be Gaussian in nature, statistical comparisons between different groups must be based on non-parametric analysis. Analyte levels from substances found to be asymetrically distributed in

the healthy population may be treated in the same manner as symetrically distributed data if a suitable mathematical conversion or transformation can be made to bring them into an underlying Gaussian form. In this study, KS testing confirmed that $\log_{10}$ transformation of all ranges gave a normal Gaussian distribution.

Examination of the results obtained from the HGPRT, APRT, ADA, PNP and PRPP-S in the present study, indicated that results tended to be skewed to higher values. Log_{10} transformation was carried out on all data which resulted in a reasonable fit to Gaussian distribution. The probability plots for HGPRT and APRT in adult red cells (Figures 5 - 3 and 5 - 4) illustrate the effect that $\log_{10}$ transformation has on the individual data, and shows the value of applying the KS test to data, before statistical comparisons are carried out. Remarkably, little attention appears to have been paid to the distribution of purine enzyme activities in other studies. Burgemeister and Gutensohn (1989) reported data from a large study examining controls ranges for a number of enzymes including HGPRT, ADA and PNP in homogenates of chorionic villi from induced abortions. They examined enzyme activities in homogenates obtained from both uncultured and cultured sources. They found wide ranges for these enzymes in the all tissues, and also reported that HGPRT and PNP activities did not correspond to the normal distribution. This study confirms that trend, and extends the findings to other enzymes and other tissues. One interesting finding in these CV homogenates was the significant correlation of activities between HGPRT and ADA ($0.001>p<0.05$) , and HGPRT and PNP ($P<0.001$), while ADA and PNP showed no significant correlation ($p>0.05$).These workers found that villi taken from various sites of the same placenta, showed markedly different enzyme activities, particularly with HGPRT. They postulated that this may have contributed to the wide ranges of activities found. Accurate description of enzyme reference ranges, derived from the parameters of the distribution, are important for the correct interpretation of individual results. While many of the purine metabolic disorders examined in this study are associated with zero enzyme activity and are therfore readily distinguishable from normal, some cases have been described where substantial residual activity associated with an affected individual have been found. In the present study, analysis of HGPRT for both pre-natal and post-natal diagnoses, have found affected cases with residual activities ranging from 0.6% - 13.9% in CV and amniotic fluid cells (Table 5 - 10) and 0.95% - 4.9% in red cells (Table 5 - 11).

In these cases, an accurately defined lower limit of normal may allow an affected individual with significant residual enzyme activity to be distinguished from an unaffected individual with activity at the lower end of the normal range.
More critical analysis of normal and carrier reference ranges for autosomal recessive conditions may also allow a clearer distinction to be made between heterozygotes and homozygous normal individuals.

6.2.1.1 Assay performance and quality control

The control ranges obtained for all tissues (Tables 5 - 2 to 5 - 8) clearly show that better precision can be achieved for HGPRT and APRT in erythrocytes, at 19.4% and 39.5% respectively, than in any other tissue investigated, with the poorest precision being obtained in cultured CV for HGPRT and in fibroblasts for APRT. The coefficients of variation for these assays in the respective tissues are relatively high, reflecting the manual nature of the assays. In addition, while the HGPRT and APRT coefficients in red cells were based on the analysis of 106 samples, the other ranges were based on fewer samples, with fibroblast coefficents being calculated on only 18 samples. Quality control is an essential part of all analytical assessments. The purpose of quality control is to assess the analytical data obtained to ensure the reliability of each measurement performed and therefore to clarify any clinical decision making process.
Inter-assay controls provide verification of batch to batch reliability with regard to the higher coefficients of variation which can be obtained in manual compared to automated assays, particularly where reagents, such as substrates are prepared fresh for each analysis. In this study, the internal QC sample used was obtained from an individual with no family history of metabolic disorders and analysed in each batch of the HGPRT and APRT assay. The Levy-Jennings graph in Figure 5 - 12 shows benefit of good quality control procedures where the batch to batch variations of the assays can be tracked and any problems rectified immediately. This was of particular relevance to the HGPRT assay, where the substrate could only be maintained in solution below a pH about 4.4, with any precipitation of the substrate having adverse effects on the assay kinetics, leading to erroneous results. Thus the QC values reflect any assay variability both of the short and long term variablity of this reagent. In addition to a well defined reference range , intra-assay controls are also vital in ascertaining the reliability of results. Where

possible, in addition to an internal QC, red cell samples from both normal and affected individuals also used in the assay provide valuable infomation of the performance of the assay, in both the normal ranges and in the area of enzyme activity associated with enzyme deficiency.

Ideally, tissues from both normal and affected cases should be analysed along side the sample being tested for the condition. For assays involving CV, fibroblasts or amniotic fluid cells, samples from pregnancies with normal karyotype and no histroy of metabolic disorders can harvested and pelleted from the same batch of cultures, to minimise procedural differences in the preparation of the at-risk sample. In practice, particularly when fresh CV from the affected individuals is used direct in the assay, the small size of the sample leaves very little remaining for further analyses. Consequently, CV should be cultured not only for confirmatory purposes, but also, in the case of affected preganancies, as a ready source of control material for future assays.

With diagnostic samples where red cells are the tissue of choice, samples from affected individuals may be successfully stored after cryopreservation in buffered citrate glycerol (Section 4.2.1) and used as intra-assay controls in subsequent assays.

6.2.1.2 Substrate

As previously indicated in Section 4.8.4.1 (ii), the radiolabelled assay system used for the analysis of the purine enzymes in this study, utilised a radioactive substrate tracer to provide a detectable signal for the assessment for enzyme activity. In each assay procedure, the tracer was mixed with a higher concentration of the unlabelled substrate to provide the optimum substrate concentration to fulfil the kinetic requirements of the assay. When assaying HGPRT, the unlabelled hypoxanthine is added as a 19.1mM stock solution dissolved in distilled water. Since the disssociation constant (pK_a) of hypoxanthine is 2.0 (Joule and Smith 1972), it was necessary to acidify it to around pH 2.0 in order to dissolve it entirely within the stock solution. However, since the pH of the Tris-HCl buffer system for the enzyme assay was 7.4, it was essential to bring the pH of the stock substrate solution as close as possible to this pH in order that the Tris-HCl may buffer it sufficiently during the assay. Experimentation showed that at this concentration, the maximal pH which could be obtained before the hypoxanthine came out of solution was pH 4.2 - 4.4. This proved to be a time consuming and precise

requirement of the assay preparation, since a pH above this value, resulted in precipitation of the hypoxanthine with the risk that a lower than optimal substrate concentration might be used in the assay yielding inaccurate results. With the substrate for the other enzyme analysis, Adenine, the preparation of substrate was staightforward, since the pK_a for this substance was 4.2 (Joule and Smith 1972), and as such it displayed better solubility at higher pH values.

6.2.1.3 Red cell sample preparations

A potential drawback in using lysed red cells for the diagnosis of LNS by HGPRT assay, has been highlighted by Fairbanks *et al* (1987). They proposed that the assessment of purine (and pyrimidine) enzyme activities may be misleading when using lysed red cells and that intact red cells may provide a better indication of the functional capacity of the enzyme in vivo, in addition to showing a closer correlation with the clinical phenotype. They studied 8 patients exhibiting virtually undetectable HGPRT activity in lysed red cells. Only 2 patients failed to convert some [8-^{14}C] hypoxanthine into [8-^{14}C] IMP when the HGPRT activity was assessed using intact red cells. These patients were subsequently shown to have LNS while the remaining 6 who presented with gout and/or renal failure or LNS without self-mutilation converted up to 25% [8-^{14}C] hypoxanthine into [8-^{14}C] IMP. In addition, these workers reported that when investigating two patients who appeared to be homozygous for a complete absence of ADA activity in red cell lysates, intact red cell studies indicated a 60% normal conversion of the substrate.

Since Fairbanks *et al* (1987) also demonstrated lower residual levels of HGPRT (<0.1nmoles / hr / mg Hb) than was evident in 2 out of the 3 patients presented here, it may be that both the use of intact red cell preparations and an ultra-sensitive detection system such as HPLC are required to obtain this level of correlation with enzyme activity and phenotype, and that TLC as a separation mechanism is not sensitive enough. While it is clear from the results presented in this current study, that lysed red cell preparations can distinguish between those patients affected with purine enzyme deficiencies and controls, further work remains to be done to determine whether intact red cells can clarify the relationship between the clinical phenotype and the level of this red cell enzyme activity.

6.2.2 Pre-natal and Post-natal Diagnosis

6.2.2.1 Technical observations

The enzyme micromethod described here for the prenatal diagnosis of HGPRT and APRT in Lesch-Nyhan Syndrome, provides a rapid means of identifying affected fetuses without the requirement for detailed mutation analysis. For the most part, the turnover time of the assay is within 1 working day from receipt of sample to reporting of the results to the clinician. In this study, a microassay using thin-layer chromatography as a separation step to determine the amount of nucleotide turnover was used, however other more precise diagnostic systems such as HPLC, may be used here, by determining the concentration of intermediate or aberrant nucleotides. (Morris and Simmonds 1985; Simmonds *et al* 1991; Simmonds *et al* 1997; Duran *et al* 1997) . While HPLC undoubtedly provides a rapid and sensitive method for the separation of the various purine species, its drawback is that its expense and complexity which limits its use to specialised laboratories and centres, where metabolic profiles are used to obtain a diagnosis. The microassay method described here, involving TLC as a separation step is simple, cost effective and much less labour intensive, and although limited in its application, still provides the means of detecting specific enzyme deficiencies in purine disorders such as LNS.

6.2.2.2 Index cases

Detection of specific inborn errors of metabolism in the index case is an absolute requiremant if prenatal diagnosis in any future pregnancy is to be undertaken. Where the defect can be detected in red cells, this would be the tissue of choice as it provides the opportunity for rapid diagnosis, however other cell types such as skin fibroblasts may also be used. The storage of these tissues also provides a source of long term reference material, irrespective of the survival of the patient, upon which subsequent biochemical or molecular testing can be performed, a fact which may be of great benefit in the elucidation of the defect in other family members.

In the specific prenatal diagnostic cases for LNS cited here, where low levels of residual activity are found, the security of the result is greatly enhanced by the information available from the levels of enzyme activity expected in an index case.

One caveat however is that enzyme activities may vary from tissue to tissue and even from each family due to the heterogeneous nature of LNS.

6.2.2.3 Chorionic villi

CV sampling has become an increasingly important means of prenatal diagnosis of LNS. In theory, material may be obtained as early as the eighth week of gestation onwards, (although in practice, sampling is more often performed around the tenth week), and can be used uncultured, therefore obtaining results earlier than by amniocentesis. The primary disadvantage of CV sampling is the increased risk of contamination by maternal cells, the small size of sample and cells with a more limited life span in culture than amniotic fluid cells. The results obtained in this study for the control ranges of HGPRT and APRT are similar to a number of studies where prenatal diagnosis of LNS was assessed using amniotic fluid cells and CV tissue. At least two other studies have reported HGPRT and APRT activities in amniotic fluid cells and CV tissue (Zoref-Shani *et al* 1989; Page and Broock 1990). These data are shown with comparison to the results from the present study in Table 6 - 1. During prenatal diagnosis in in 3 fetuses at risk for LNS, Zoref-Shani *et al* (1989) reported residual HGPRT activities 5.4% of normal in cultured amniocytes, 16% and 9-10% in CV and cultured CV extracts respectively, 6.7% - 16 % in skin fibroblasts and APRT/HGPRT ratios 4 - 10 fold higher than the normal ratio. This compares with the 0.06%-13.9% residual HGPRT activity found in the affected pregnancies from the present study. The HGPRT / APRT ratio used here provides a reciprocal figure to that of Zoref-Shani *et al* (1989) but in either case the differentiation between the control and affected cases is clear.

Page and Broock (1990) have drawn attention to a possible analytical problem with the measurement of HGPRT activity in frozen and in cultured chorionic villi. In these types of cells, rapid catabolism of the products of the HGPRT reaction may cause normal cells to appear HGPRT-deficient and these investigators advocate the use of a 5' nucleotidase inhibitor (α,β-methyleneadenosine diphosphate; AOPCP) in assays to block the removal of the HGPRT reaction products. These workers stressed the important differences in composition of CV compared to amniotic fluid when assessing these tissues for use in prenatal diagnosis, a

difference reflected in the levels of activity for the three studies indicated in Table 6 - 1.

CV is optimally suited to the transport of small molecules whereas the fetal amniocytes are adapted to perform rapid cell division. Consequently the observed enzyme levels may change as the CV cells grow and divide in culture. The rate of nucleotide catabolism is relatively higher in CV than in cultured amniotic fluid cells, and may outstrip the synthetic capacity of the cells and cause normal cells to appear HGPRT deficient.

In the present study, none of the CV samples from at-risk pregnancies was frozen before HGPRT analysis and little difference was evident between cultured and uncultured CV controls, although APRT specific activities were higher in the cultured CV. However within the series of at-risk pregnancies, both enzymes consistently gave higher activities in cultured cells than in uncultured material. The low levels of HGPRT activity found in three pregnancies examined, can be interpreted as due to the presence of an affected fetus and not attributed to the increased catabolism of the HGPRT reaction products, as similar residual activities were found in non-placental tissues from the probands (two cases) and the abortus (one case). In the affected pregnancies, the highest residual activity was found to be 12.3% of normal, while in the unaffected pregnancies, the lowest HGPRT activity was 52 % of normal, suggesting minimal loss of HGPRT reaction products in CV due to intracellular catabolism. From these data it appears that provided CV material is not frozen, the use of AOPCP in the assay is unnecessary.

A number of workers have reported on the experience of using CV for first trimester diagnosis of inherited metabolic defects . Besley *et al* (1991) collected data from a total of 339 diagnoses from 4 centres in the UK, and while none of the centres quoted data specifically for purine metabolic disorders, the data provide useful insights into the advantages and disadvantages of using CV as a first trimester prenatal diagnostic tissue and highlighted the problems of using small or poor quality CV samples for enzyme analysis as a cause of equivocal results. In subsequent work, Besley (1992) recommended that samples of between 10 and 50 mg wet weight are optimal amounts for biochemical assays.

For prenatal diagnosis of the purine enzyme disorders in this study, only fresh CV samples of 10mg or greater were analysed directly, with cultures also being set up and analysed after confluence for confirmation of the direct result. Samples of less than this amount were considered unsuitable and were cultured prior to the

analysis. Fortunately, most CV samples obtained for prenatal dagnosis were adequate to allow analysis both fresh and after culture.

Where sufficient material for analysis of cultured and uncultured CV can be obtained , and where cells from an index case are available, enzyme analysis in CV a reliable method for prenatal diagnosis of purine metabolic disorders.

Table 6 - 1

Comparison of HGPRT and APRT enzyme activies for amniotic fluid cells, cultured and uncultured CV, in 3 studies. Activities are in nmoles / hr / mg protein. The ranges in brackets indicates results obtained using $\log_{10}$ transformed data converted back to concentration units.

		Amniotic Fluid	Cultured CV	Uncultured CV
Zoref-Shani (1989)	HGPRT	154.6 ± 42.2 (n=8)	52 (n=1)	103.8 ± 60.4 (n=5)
	APRT	406.6 ± 77.0 (n=8)	103 (n=1)	202.4 ± 114.0 (n=5)
	APRT/HGPRT ratio	2.7 ± 0.68 (n=8)	2.0 (n=1)	2.04 ± 0.37 (n=5)
Page and Broock (1990)	HGPRT	105 ± 48.6 (n=8)	59.7 ± 56 (n=6)	14.1 ± 5.1 (n=4)
	APRT	250.0 ± 155.0 (n=8)	78.2 ± 63.0 (n=6)	2.5 ± 3.4 (n=6)
	HGPRT/APRT Ratio	n/a	n/a	n/a
This study	HGPRT	161.1	34.5	56.4
	(Range)	(62.9 - 412.9) (n=45)	(5.5 - 216.8) (n=57)	(18.7 - 169.4) (n=54)
	APRT	143.5	48.9	43.5
	(Range)	(49.8 – 413.9) (n=45)	(9.9 - 243.6) (n=53)	13.3 - 141.9) (n=48)
	HGPRT/ APRT	1.12	0.63	1.29
	(Range)	(0.46 - 2.75) (n=45)	(0.17 - 2.4) (n=51)	(0.44 - 3.8) (n=48)

This present study indicated that when carrying out prenatal diagnosis for LNS, amniotic fluid cells still have a useful place, and may even have some advantages over uncultured or cultured CV. Apart from the benefits that second trimester diagnosis is still available should the first trimester analysis fail to obtain a result, the reduced levels of HGPRT in affected pregnancies may be very close to the lower limits of the normal control ranges in cultured and uncultured CV and as such may cloud the distinctions between the affected and reference populations. Since the overall mean and therefore the lower limits of the reference ranges of HGPRT and APRT are markedly greater in amniotic fluid cells, there is a greater difference between normal levels of HGPRT and APRT and the residual levels found in individuals affected with LNS. It is for this reason that the HGPRT/APRT ratio in determining affected individuals for the normal population is used since this affords a more precise method of separating normal and affected individuals.

The confirmation of a prenatal diagnosis by the analysis of fetal material such as fibroblast tissue taken at post-mortem or cord blood at delivery is essential to assess the accuracy of the prenatal diagnostic results. Despite requests to referring clinicians, very few tissues for confirmatory analyses were obtained either after termination or delivery, with only two pregnancies (WS (a) and WS (c)) providing fibroblast and cord red cell samples respectively. Cell cultures from affected pregnancies also provide an important source of analytical controls for prenatal diagnosis in any future pregnancies.

6.2.2.4 Fetal blood sampling

While prenatal diagnosis of Lesch-Nyhan has, concentrated on the use of fetal tissues such as CV, amniocytes and fibroblasts, assessment of the HGPRT and APRT levels in fetal erythrocytes was made in this study to assess their suitability for PND (Table 5 - 7). Despite the fact that the technique of fetal blood sampling has been used for greater than 20 years, it is seldom used for the prenatal diagnosis of enzyme disorders. Fetal blood sampling is generally carried out late in gestation, due to the small size of the feto-placental unit and increased risks of sampling before 21 weeks gestation (Ghidini *et al* 1993). While maternal contamination of the sample may also be a contributing factor the lack of use of this procedure for prenatal diagnosis, this may become less important as experience of this technique becomes more widespread. The establishment in this

study of fetal red cell reference ranges indicate that mean HGPRT, APRT and HGPRT/APRT of 71.9 and 31.7 nmoles/ hr/ mg Hb and 2.4 respectively, while lower than the adult values, would provide the basis for fetal red cells to be used in prenatal diagnosis. While CV and amniotic fluid cells would still be the tissue of choice for prenatal diagnosis of purine disorders, fetal sampling may provide a useful alternative, should these two approaches fail to provide a definitive result.

6.2.2.5 Carrier detection

A drawback in the use of biochemical methods for prenatal diagnosis of Lesch-Nyhan Syndrome, is their inability to predict the carrier status of a female fetus due to the effect of non-random X-inactivation. Lyonisation is the process of inactivation of one of the two X chromosomes in every female cell around 12 days for trophoblast tissues and 16 days for the embryo. Inactivation only occurs in somatic cells and is random as to whether the maternal or paternal X chromosome is inactivated. As only one X chromosome is active in the female, the product levels for most genes on the X chromosome are similar in females and males where the single X chromosome remains active. The difficulties of performing carrier detection by enzymic methods in tissues such as red cells, due to X-inactivation have been well documented. Kelley (1968) attempted to diagnose heterozygote status by HGPRT analysis in red cells in 6 test subjects using guanine as a substrate, but found that HGPRT activities fell within ± 2 sd of the mean of the control range. This work was substantiated by Nyhan *et al* (1970) who in addition to finding normal HGPRT activities in the red cells of female heterozygotes, found no evidence of mosaicism in autoradiographs of hypoxanthine uptake in lymphocytes of these subjects. These workers postulated that X-inactivation in the marrow of obligate carriers is not random or that following random inactivation there was selection against the cells with the deficient HGPRT gene. The results shown in Table 5 - 11 confirm the findings that obligate carriers of LNS cannot be detected by HGPRT analysis in red cells, due to the overlap in the ranges between the carriers an the controls. This overlap is reflected not only in HGPRT but also in APRT, thereby rendering the HGPRT / APRT ratio unusable in differentiating these subjects from the controls. Some success however has been achieved using hair roots for carrier detection (Gartler *et al* 1971; Silvers *et al* 1972). Hair roots constitute a readily available enzyme source for the detection of heterozygotes in Lesch-Nyhan Syndrome. Hairs are taken from sevaral areas of the scalp, and the

visible intact root put into an appropriate buffer. Lysates are prepared by repeatedly freezing and thawing at -20°C and room temperature and an aliqout of the preparation taken for enzyme activity. Hair roots provide a good material for X chromosome studies, as the probability of obtaining a follicle with only one of the X-linked alleles being expressed is high, due to the fact that the cells on the scalp are not distributed at random, but exhibit clonal growth. Thus a heterozygous female will exhibit enzyme levels in a bimodal distribution. Each mode of the distribution compaing with the control female and hemizygous affected male distributions.

Alford *et al* (1995) demonstrated that the use of molecular methods in the identification of female carriers provides families with accurate and reliable information regarding risk and family planning information. In a recent study where they reported their experience with prenatal diagnosis in 26 pregnancies, 14 were investigated by HGPRT assay only, four by HGPRT and molecular analysis and eight by mutation analysis alone. For those cases investigated biochemically, six affected pregnancies were identified and all had low but detectable HGPRT activities. APRT in these pregnancies was not measured. Techniques such as linkage analysis and quantitative PCR and densitometry, rely on the acquisition of DNA from the proband to identify the familial mutation, thus allowing effective carrier detection.

6.2.3 Gout Study

The aim of this part of the purine study was to investigate the HGPRT and APRT activities in patients who had presented at a Rheumatology clinic with chronic gout to assess whether these enzymes helped to identify the severe form of gout showing the X-linked pattern of inheritance. The clinical information suggested the familial nature of the disorder in a number of patients, however, no evidence of HGPRT deficiency at the residual levels of enzyme activity expected in partial deficiencies was found. The finding however of lower levels of HGPRT in two male patients presenting with polyarticular gout raises the question of the role played by low levels of HGPRT activity in the differentiation of the various presentations of gout. Lawry *et al* (1988) reported that of 106 men presenting with gout at one hospital, 11 had polyarticular involvement, and these cases tended to be less abrupt in onset, less severely painful and more likely to be associated with

onset , a longer history and more previous attacks. The raised serum uric acid in these patients may not aid diagnosis since symptom-less hyperuricaemia may accompany other forms of arthritis. Although gout in older patients tends to be sub-acute to chronic and often polyarticular in nature, it is important to note that in the present study population, the patients were the only ones to exhibit polyarticular symptoms. Since this older population of patients do not exhibit the X-linked form of gout to any discernable degree, it would be perhaps more rewarding to investigate a population where the incidence is higher, for example in cases of juvenile gout.

Many of the current techniques for PRPP-S estimation are radiochemical techniques and are difficult to apply in clinical labs, while those based on enzyme-linked systems lack the required sensitivity. The original method of Sakuma *et al* (1991) translated well to the HPLC system used in this study, but gave higher results than those obtained here. Sakuma *et al* (1991) quoted PRPP-S values of 1.28 $\pm$ 0.11 μmo l/ min / gHb (n=28) for gout patients and 1.110 $\pm$ 0.11 μmol / min / gHb for normal controls (n=27), while this study gave a mean of 0.307 μmol / min / gHb (Range = 0.152 - 0.622) and 0.341 μmol / min / gHb (Range = 0.205 - 0.569) for gout patients (n = 54) and normal controls (n=18) respectively.

Patient MHD presented with increased uric acid and cerebral palsy (CP) and the request for HGPRT estimation was intended to exclude the diagnosis of LNS where a number of clinical symptoms are common. CP is caused by the non-progressive defects of lesions of the immature brain and is a persistent disorder of movement and posture, often complicated by neurological and mental problems and characterised by delayed motor development and choreoathetosis (Hull and Johnstone 1981).

The finding of elevated PRPP-S in this patient is an indication for further investigation, but no further samples could be obtained. Roessler *et al* (1993) and Becker *et al* (1995) demonstrated the value of using point mutations of the genes PRS1 and PRS2 in the examination of the molecular pathology underlying PRPP-S superactivity, while a study by Bory *et al* (1995) showed the validity of using the altered concentrations of NAD, UDPG, GTP and IMP in red cells associated with a rise in uric acid and hypoxanthine levels as a biochemical characteristic of PRPP-S superactivity.

6.2.4 ADA / PNP

Around 20% of patients with SCID have a deficiency of ADA activity (Hershfield *et al* 1997). Also, in 20% of ADA deficient patients, immune deficiency is initially less severe and as a result often present later in childhood or even may not be diagnosed until 15-40 years of age with chronic pulmonary insufficiency due to recurrent respiratory infections, or other manifestations of immune deficiency and dysregulation (Santisteban *et al* 1993). Enzyme analysis allows the distinction to be made between the ADA and non ADA types of SCID, and also between immunodeficiency syndromes associated with ADA and those with PNP. While some clinical indices, such as neurological involvement, give clues as to the type of immunodeficiency, the only certain method of determination, remains the demonstration of the specific enzyme defect, in the appropriate tissue.

In this study, it is clear that the ADA activities can differentiate between those individuals with ADA type of SCID and those with other forms of immunodeficiency. Patients TGG, MMC who presented with recurrent infections and patient JTD who was the sister of TGG, all had immunodefciencies of the ADA type as seen by the lack of any detectable ADA activity. It can be seen from the results obtained from the ADA study however, that the majority of those patients presenting with recurring infections and therefore possible immunodeficiencies gave ADA activities in the normal reference range, and therefore may have had SCID of the non ADA type. A number of patients gave ADA activities above 104.2 nmoles / hr / mgHb , the upper level of the normal reference range. Three of these (ASD, CBN, SGD) presented with recurrent infection and gave red cell ADA concentrations of 122.6, 134.9 and 117.2; while patient DME (a) presented with Graft versus Host disease and gave red cell ADA activity of 117.3. It is not clear whether these high values simply represent outliers beyond the upper end of the normal range or whether they indicate disruption of the salvage pathway. One patient with a family history of PNP deficiency, and immunodeficiency (LOD) was observed to have ADA of 257.1 nmoles / hr / mg Hb. The results in Table 5 - 9 show correlation between ADA and PNP in adult red cells and suggests that a disruption in one of these enzymes affects the other. The results here suggest that both ADA and PNP may give valuable information in these cases.

While enzymatic analysis has traditionally been the most common method of analysis of ADA and PNP deficiencies, more recently molecular methods have become more widespread. The most common cause of mutations in the ADA gene

are single base pair changes (around 75%), followed by splicing mutations then small and a few large deletions. One missense mutation identified in a healthy child with partial ADA deficiency, was later found 2 sisters with adult onset of immunodeficiency (Shovelin 1994). A report by Hershfeld *et al* (1997) on studies with 25 DNA samples carrying missense mutations suggests that patients with 2 alleles providing <0.01% of wild type ADA activity have SCID, while those with 0.1-3% activity have a delayed or late onset SCID and those inheriting alleles that provide 5% or greater of wild type activity can be regarded as healthy idividuals, and this is consistent with the finding that there is good correlation between clinical severity and red cell dATP (or total deoxy-adenosine (dADO) nucleotide content) an index of overall capacity to eliminate ADA substrates.

Stone and Simmonds (1991) highlighted a problem of stability when analysing ADA. As ADA is reportedly unstable at -20°C, they recommended that blood transportation should be at room temperature with storage at -70°C. These recommendations were made in light of the fact that at least 11 heathy individuals with undetectable ADA in lysed red cells have been found, and was attributed to the instability of the erythrocyte enzyme on lysis since some ADA activity was detected in the intact cells of obligate carriers. In this study, transport of red cells for ADA and PNP deficiency was at room temperature, while storage was at -20°C as unlysed cells in a suitable cryoprotective preservative. Both at-risk and control samples were subjected to identical transportation and storage conditions and while the controls gave levels in the normal range, the affected patients gave undetectable levels of ADA and were considered deficient individuals. It is possible however, that where levels of ADA and PNP activity fall at the lower limits of the normal range, suggesting possible carrier status, intact red cell analysis may be of use in clarifying these results. This raises the question of whether red cell ADA and PNP analysis should be analysed in both lysed and intact red cell in order that the reference ranges for affected, heterozygotes and normal individuals are as stringent as possible. The solution β-mercaptoethanol-EDTA has been used in previously reported methods of haemolysate preparation, as a stabilizing agent to prevent the oxidation of the enzyme disulphide bonds and thus maintain catalytic activity (Beutler 1984). It may be that addition of β-mercaptoethanol-EDTA to haemolysates, provides a comparable effect to that reported with intact red cell preparations. Further work is required on this question.

6.3 Pteridines

6.3.1 MTHFR allele frequencies

Whitehead *et al* (1995) postulated that folic acid supplementation does not act to correct a simple nutritional deficiency since most pregnant women carrying NTD affected fetuses have levels of folate above the deficient range. Instead they suggested that an increased folate concentration may prevent some cases of neural tube defects by overcoming a partial block in the conversion of 5,10 methyltetrahydrofolate to 5,10 methylenetetrahydrofolate caused by reduced catalytic activity of the enzyme MTHFR. In a study of the thermolabile variant of MTHFR in the Irish population, they found only 13% of all NTDs could be attributed to this genetic variant while 50-75% of NTDs in the Irish population are folate related. While they considered that this was good evidence for a genetic role for MTHFR variants in the development of some NTDs, they speculated that other causitive factors in folate metabolism remain to be ellucidated. This is in keeping with other studies where a combination of the genotype frequencies of both the mother and child accounted for only 27.4% of the observed protective effects of folate (van der Put *et al* 1996).

Subsequent studies by other groups reported these investigations of MTHFR variants in NTD and control populations. This data was summarised by van der Put *et al* (1997) who carried out a meta-analysis which combined the observed frequencies of the 677C→T mutation in a prospective Dutch study with data reported from a series of 13 other studies. This analysis combined control data from both cardiovascular and NTD studies and compared the frequencies in relation to the NTD cases and their parents. (Table 6 - 2). The frequency of 11.8% obtained for the 677C→T variant of the Scottish control population in this study compares with a mean of 9.2% obtained for the combined studies which ranged from 2.3% (Papapetrou *et al*) to 16.3% (de Franchis *et al* 1995). The frequency of the homozygous variant in the Scottish NTD affected population was 10.0% which was considerably lower than the mean for the meta-analysis of 16.4% and not significantly different from the control group. The meta-analysis suggested that homozygosity for this mutation was relevant not only in the affected patients but also in the mothers of affected pregnancies. The results of the present study in the Scottish population suggested that the maternal MTHFR genotype may be important, as the mothers of affected individuals homozygous for the variant allele

showed a variant frequency significantlly different to the control group. The frequency of 15.3% for these homozygous mothers compared with the 14.5% observed in the pooled data. The claims by van der Put *et al* (1997) that the homozygosity is relevant in the fathers of affected individuals, as well as the mothers, is not borne out by the Scottish data where the fathers were found to have a low incidence of homozygosity of 8.3%. Other relatives of these affected individuals gave a frequency of 11.1%, which was comparable to that of 11.8% obtained for the control data. Van der Put *et al* (1997) quote odds ratios of 1.5-1.8 in patients and 1.6-1.9 in mothers, compared to 0.9 and 1.3 respectively in this study.

This would suggest that there is a population difference in the 677C→T mutation frequency between some European countries, and the Scottish population, and raises the possibility that other affected loci are involved, perhaps in conjunction with environmental factors in the development of an NTD.

A recent study by Shields *et al* (1999) using the transmission dysequilibrium test (TDT) presented data on 271 NTD cases in the Irish population, to support the contention that the +/+ allele was a significant genetic risk factor for NTD. They hypothesised that since the vast majority of +/+ mothers do not have NTD pregnancies, that the +/+ genotype is a risk factor with a very low penetrance and subsequently large numbers of individuals (and their parents) are needed to provide evidence that the +/+ genotype is indeed, a risk factor for NTDs. On the basis of the results obtained by Shields *et al* (1999), they hypothesised that the +/+ status of the developing embryo rather than the +/+ status of its mother was the critical genetic determining factor. Their data did not exclude the possibility, however that the maternal genotype may confer an addition risk to a +/+ embryo over and above the risk attributed to the genotype of the embyo itself, and that the maternal +/+ genotype might become a modest risk factor by further depressing maternal folate levels which are already below a nominal threshold, thus enhancing the already present risk of NTD in the fetus. The results obtained this study, where significantly higher frequency of the +/+ allele was found in mothers of affected cases, lend support to this contention.

Table 6 - 2

Prevalence of thermolabile MTHFR due to 677C →T mutation among Dutch and international controls, NTD patients and their parents. Data after van der Put *et al* (1997).

Control Groups	Group size	+/+ Frequency (n)
Dutch controls		
Unpublished	833	8.9% (74)
Put *et al* (1995)	318	7.9% (25)
Verhoef *et al*	99	7.1% (7)
Engbertsen *et al*	23	4.3% (1)
Total Dutch Controls	1273	8.4% (107)
Reported International controls		
Whitehead *et al* (1995)	99	6.1% (6)
Ou *et al* (1995)	109	4.6% (5)
Frosst *et al* (1995)	57	12.3% (7)
Franchis *et al* (1995)	289	16.3% (47)
Wilcken and Wang (1995)	225	10.7% (24)
Papapetrou *et al* (1996)	199	12.1% (24)
Papapetrou *et al* (1996)	44	2.3% (1)
Papapetrou *et al* (1996)	45	8.9% (4)
Kang *et al*	202	5.0% (10)
Total all Controls	2542	9.2% (235)
This study	262	11.8% (31)
Reported NTD cases		
Put *et al* (1995)	55	12.7% (7)
Whitehead *et al* (1995)	82	17.6% (15)
Ou *et al* (1995)	41	22.0 (9)
Papapetrou *et al* (1996)	41	12.2% (9)
Total all NTD cases	219	16.4% (36)
This study	40	10.0% (4)
Reported mothers		
Put *et al* (1995)	70	15.7% (11)
Whitehead *et al* (1995)	32	18.8% (6)
Franchis *et al* (1995)	28	7.1% (2)
Papapetrou *et al* (1996)	36	13.9% (5)
Total all mothers	166	14.5% (24)
This study	85	15.3% (13)

Table 6 - 2 (continued)

Control Groups	Group size	+/+ Frequency (n)
Reported fathers		
Put *et al* (1995)	60	10.0% (6)
Whitehead *et al* (1995)	24	29.2% (7)
Papapetrou *et al* (1996)	26	15.4% (4)
Total fathers	110	15.4% (17)
This study	35	8.3% (3)

The differing frequencies obtained by all these studies has raised questions about the validity of the selection criteria for controls on which MTHFR variant population frequencies are based. This study attempted to establish control ranges for the frequency of the MTHFR variant by genotype frequency and by MTHFR enzyme activity using control samples obtained from individuals in the same social class as the test individuals. The rationale was that by obtaining a blood sample from a friend or neighbour of the test subject there would be some matching of ethnicity, social class and a likelyhood that diet would be reasonably similar. This was only partially successful, and it became necessary to obtain blood samples from unselected members of the general population in order to provide adequate sample numbers. Posey *et al* (1996) raised a number of questions concerning the methodological aspects of studies investigating the 677C→T mutation as a risk factor for NTDs. These concerns centred around the lack of an appropriate control group in the studies of Ou *et al* (1995), van der Put *et al* (1995) and Whitehead *et al* (1995). The "convenience controls" used in these studies took no account of the race or ethnicity of the control populations or any account of differing social status and consequently any dietary anomalies. One approach to this problem was proposed by Papapetrou *et al* (1996) who advocated the use of within-family genetics studies of the variant MTHFR allele , which are not affected by population differences in the allele frequency. This approach is being used at present in a study of the effects of folate metabolism in the epidemiology of orofacial clefting in the Scottish population, where family controls are used through the technique of transmission dysequilibrium (which uses the non-transmitted parental alleles to remove the element of genetic stratification between case / controls populations),

and sib pair analysis as an alternative to the classical case / control methods of comparison (Dr. P. Mossey, Dundee: personal communication).

6.3.2 MTHFR enzyme activity

The hypothesis that the development of at least some causes of folate NTD are due to impaired utilisation of folate due to the presence of a defective enzyme, has been tested in various studies by investigating the frequency of the MTHFR 677C→T variant as a risk factor in NTD patients and their parents. It follows therefore that if this is the underlying cause, the functional capacity of the enzyme to catalyze this key step in the pathway must be reduced and this should be demonstrable by measuring MTHFR activity in patients and controls. While the the majority of work on defining the relationship of the genotype to the enzyme activities of the thermolabile MTHFR allele has been reported using subjects with cardiovascular indications (Frosst *et al* 1995 ; Engbersen *et al* 1995), few studies have applied this to individuals with a family history of NTD. One such study, (van der Put *et al* 1995) reported that the thermolabile mutation was associated with decreased MTHFR activity, high homocysteine and high plasma folate concentrations, and concluded that the 677C→T mutation should be regarded as a genetic risk factor for spina bifida. However, since no specific data was available from the report on the MTHFR enzyme activites for each group of patients , it was impossible to assess that particular study in light of the results obtained here. The study by Frosst *et al* (1995) using patients with premature vascular disease, reported lymphocyte MTHFR activities of 22.9 ± 1.7 (Range 11.8-33.8); 15.0 ± 0.8 (Range 10.2-18.8) and 6.9 ± 0.6 (Range 2.6 - 10.2) nmol formaldehyde / mg protein / hr for the homozygous normal, heterozygous and homozygous thermolabile genotypes respectively. After heating at 46°C for 5 minutes these samples gave a corresponding residual activity of 66.8 ± 1.5, 56.2 ± 2.8 and 21.8 ± 2.8 % respectively. In a comparison of control and hyperhomocysteinemic vascular patients,

Engbersen *et al* (1995) reported similar results of 15.6 ± 4.7 nmol formaldehyde / mg protein / hr and residual activity of 55.3 ± 12.0 % for controls and 8.7 ± 2.1 nmol formaldehyde /mg protein/hr and residual activity of 0-33% for the patients exhibiting the themolabile variant of MTHFR.

The results of this NTD study indicated that when the mean overall MTHFR activity of each particular genotype was examined, this gave 8.6, 6.6 and 4.7 nmol formaldehyde /mg protein / hr for the homozygous normal, heterozygous and homozygous thermolabile alleles for the combined study groups. While these total activities were lower than those quoted by Frosst *et al* (1995) and Engbersen *et al* (1995), they compare favourably with those Kang *et al* (1991) who observed MTHFR specific activities in controls and patients with coronary artery disease of 10.33 ± 2.9 and 5.58 ± 0.91 nmol formaldehyde / mg protein / hr respectively.

The expectation, based on the findings of other workers, of a residual MTHFR activity after heating at 46°C for 5 minutes, of ~50% for controls and <30% for those individuals exhibiting thermolabile allele was not borne out by the results of this study for the overall residual actitivities where 39.7, 37.7 and 38.8 % residual activity was found for the -/- , +/- and +/+ groups respectively. The result here suggests that when overall values for each genotype are considered, residual activity does not provide a indicator for discrimination of the genotypes. The explanation for these results is unclear at present. One obvious reason would be the failure of heating phase at 46°C to produce the residual activities expected, however this is not the case , since the internal QC results obtained (Section 5.3.3.1.(vi) show that the expected residual activity of ~50% was achieved, The batch processing of samples may have provided a reason for the variation of results, particularly in the case of the higher residual activities of the thermolabile affected individuals, had they been asayed together. However, since all samples were assayed in the order with which they were received, this ensured that no bias was encountered in assaying large numbers of one particular group in one batch. This was particularly relevant to the 3 affected thermolabile patients who gave high residual activies despite being assayed in 3 distinct batches. Whether these diffences in thermolabile characteristics of the MTHFR enzyme is a factor specific to the Scottish population as is suggested by the allele frequency data, ramains to be elucidated.

The significantly reduced MTHFR activity found in +/+ affected cases in this study (21%), indicates that a potential association may exist between the genotype and an impaired level of folate metabolism. However, as the frequency of the +/+ genotype in affected cases is not significantly different from the -/- controls, it seems likely that other factors must be contributing to the etiology of NTDs in these individuals, since it wiould be expected that a significantly greater number of these affected individuals would be found if the +/+ genotype were a causal factor. A

significantly reduced MTHFR activity in the +/+ mothers (25.6%) combined with a significant difference in the frequency of the +/+ genotype compared to the -/- controls, lends support the hypothesis that the maternal genotype may play some part in the occurance of NTDs. The mechanism by which this is acheived is as yet unclear, however the hypothesis that a reduced folate turnover caused by the presence of the +/+ genotype in the mother, places additional stress on a +/+ fetus which already has an impaired folate metabolism, may be a reasonable. While this may suggest a way in which the genetic factors of NTD formation may interact, there remains the crucial question of the environmental component to be considered, notably that of folate and vitamin B_{12}.

6.3.3 Folate and vitamin B_{12}

It is still unclear as to how folic acid affords the developing embryo protection from birth defects, and a number of suggestions have been put forward with regard to the mechanisms involved. It is possible that the level of folic acid available to the developing embryo compromises its ability to perform single-carbon transfer reactions, subsequently interfering with purine biosynthesis, or equally likely, that insufficient concentration of folic acid may adversely affect the normal functions of proteins or gene expression, essential to the closure of the neural tube. While there is overall reduction in red cell folate in +/+ individuals compared to the -/- controls of 81% this result is not significant, therefore it would appear on the basis of these results, that there is no evidence for implication of significantly reduced levels of red cell folate, in mothers of affected individuals or the affected individuals themselves, associated with the thermolabile MTHFR allele.

The relationship between circulating levels of folate in indiividuals and the aetiology of NTDs has been closely examined in a number of studies. A report by Molloy *et al* (1985) examined 32 samples from mothers with affected pregnancies found low concentrations of folic acid, vitamin C and vitamin B_{12}, but no significant difference in serum folate and vitamin B_{12} concentrations between mothers of affected and controls. Unfortunately no red cell folate results were obtained in this study . They postulated that if maternal nutritional status plays a part in the aetiology of NTDs, this may be reflected in the low serum status of that particular nutrient before or during the pregnancy. Smithells *et al* (1976) found lower RBC folate in the first trimester in six mothers with affected pregnancies compared with the control group,

while the study by Laurence *et al* (1981) showed the mean red cell folate concentration during the first trimester in women who had recurrent NTD pregnancies was lower, although not significantly so, compared to the controls. A systematic review of the literature by Wald *et al* (1996) showed that on average during the first trimester of pregnancy, serum folic acid was 0.6 mg/ml lower ($p<0.01$), red cell folate was 77ng/ml lower ($p<0.001$) and that serum vitamin B_{12} was 38ng.ml lower ($p<0.001$) in NTD affected pregnancies compared to the controls. Yates *et al* (1987) found the mean concentration of red cell folate in mothers with affected pregnancies was significantly lower than the mean for the control groups, and that these levels showed a linear relationship with the number of NTD affected pregnancies where levels were lowest in women who had had 3 or 4 NTD affected offspring. They concluded that since there was no significant difference in serum folate, B_{12} or trace elements e.g. magnesium, copper or zinc and dietary intakes of total folates were lower, although not significantly so, that these NTDs were not due entirely to a lower folate intake, and that a specific inborn error of metabolism may be implicated.

One major problem in assessing the effect of folate on the developing neural tube is that much of the works retrospective in nature and since none of these women were pregnant when the samples were taken (as in this study) the data may not represent a true picture of the state of folate metabolism during the formation of the neural tube where folate requirements may be substantially different, in order to cope with the increased demands of the fetus.

In addition to folate, these studies have reported that vitamin B_{12}, and consequently the enzyme methionine synthase, as having a potential role in the determining susceptibility to NTDs. Reduced levels of vitamin B_{12} could compromise the function of methionine synthetase leading to to an increase in homocysteine which may have a deleterious effect on the developing neural tube. The results from this study differ from other workers, in that there appears to be no evidence of a reduction in vitamin B_{12} levels between the studied groups and the controls.However it is interesting to note that in both the mothers and fathers of affected individuals significant differences were observed in the B_{12} levels, these differences were higher and not lower as might be expected. The reasons for this are as yet unclear, however a re-evaluation of the assay method for both folate and vitamin B_{12} may shed light on this result.

Alterations of dietary habits to high or low folate intake are reflected initially in changing serum folate values. Day to day variations in serum folate may occur in a

particular subject while on a reasonably similar diet over a period of time, and serum folate may fluctuate dramatically between deficiency and sufficiency ranges. It was considered that since serum folate is an unreliable marker of nutritional status when a random sample is taken, in this study the value was used solely in the calculation of the erythrocyte folate which was considered to be a more useful index of nutritional status. Traditionally, serum and red cell folate was measured by microbiological assay but these were difficult to set up , maintain and notoriously slow in obtaining results. In recent years, these assay have been somewhat superceded by the competitive binding radio-assays such as the Simultrac method quoted in this study. The microbiological method remains the 'gold standard' method for folate analysis by most investigators (Molloy and Scott 1997).

Molloy and Scott (1998) reported differing results for red cell folate values for the subjects with the homozygous thermolabile MTHFR variant when the microbiological method was compared to three other studies using the radioassay. While the former study showed lower erythrocyte folates in the homozygous variant population compared to the controls and heterozygotes, the latter showed the converse. This has implications for this study's folate results where the hypothesis that NTD affected and at risk individuals have lower erythrocyte folate levels due to the thermolabile allele was not observed. Current European studies into gene-nutrition interaction for orofacial clefting, have utilised the microbiological method for folate analysis as the method of choice (Welcome-Sparks study - Dr. P.Mossey, personal communication), and so it may be useful at a future date to re-assess the folate levels in this study using this microbiological method.

The relative folate deficiency may not be the main contributory factor in the aetiology of NTDs. Deficient red cell folate in association with normal serum values imply diminished uptake of folate into tissues, either due to Vitamin B_{12} deficiency or some other transport mechanism. Folic acid supplements taken periconceptually can greatly reduce a womans risk of having child with an NTD, however, despite adequate amounts of folate in their diet, a significant number of women still have affected pregnancies, where this folic acid supplementation in some women fails to prevent the NTD. The data obtained from this study suggests that reduced levels of folate already present in some mothers, may be exacerbated by the demands of pregnancy, which combined with a genetic predisposition in both mothers and the fetus, to reduced activities of the folate processing enzymes, contribute to the malformation of the NTD. Supplementary folate in the diet, by overcoming a metabolic block may prevent NTD in the majority of pregnancies, whereas in those

pregnancies where folate supplementation fails to prevent such an abnormality, other mechanisms, as yet unclear may be at work.

As Yates *et al* (1987) so aptly pointed out:

> "from the point of view of of preventing human malformations it may be necessary to look for causes which are in themselves, not abnormal at all but which are unfavourable in combination with one another. The maternal blood level of some chemical substance, hormone or vitamin, may be quite normal , according to ordinary clinical standards but below (or above) a value which is critical from the point of view of a developing fetus with a given genetic constitution"

6.3.4 Folate and diet

The results from this and other studies have raised a number of questions by the scientific and health communities regarding the appropriate levels of folate intake within different, populations, on both the amounts and methods of supplementation of folate (Wald 1994).

Surveys have shown that the wide distribution of folate intake within adult populations across Europe may partly reflect differences in dietary habits among European countries, where France, Spain and Portugal have a higher proportion of folate rich foods such as vegetables, fruits and whole grains compared to their northern counterparts (Kushi *et al* 1995). This combined with methodological differences in collection of dietary information and the unreliability of estimating folate content in food has contributed to the variation in reported recommended dietary intake (RDI) (Black *et al* 1985).

Because of the critical timing of folic acid supplementation, the general advice to women has been to take folic acid supplements from the time they decide to become pregnant. (Wald 1993) reported that data from the United States indicates that a large proportion (perhaps the majority) of pregnancies are unplanned, and so advising women planning a pregnancy to take extra folate supplements is unlikely to target the large numbers of preventable NTDs in the population. Based on a survey of British adults which showed the median intake of folate was 200μg/day, Wald theorised that even folate supplementation by a factor of 3 was unlikely to attain the levels recommended for occurrent NTD prevention, and that

the only practical public health strategy was folic acid fortification of a limited range of commonly consumed or staple foods such as flour.

The ethical question of fortification of staple foods with folic acid has attracted a spectrum of views from both proponents and critics alike. Wald (1995) advocated this measure as a simple, safe and economical method of reducing the incidence of birth defects in the pregnant population, other workers have warned of the risks involved to the general population as a whole. Following a proposal by the US Food and Drug Administration, Gaull *et al* (1996) have questioned the wisdom of fortifying food given a variety of uncertainties. These reservations included, the multifactorial and polygenic nature of NTDs; the demographic nature and decreasing prevalence of NTDs; the lack of a dose-relationship between folate and NTDs and launching of a nutritional strategy without assurances that the health risks would not shift from one group to another, notably from developing embryos to adults with pernicious anaemia.

One powerful argument likely to sway opinion in favour of folic acid fortification has come from studies in the United States on the economic effect on health care costs with regard to birth defects, premature birth and coronary heart disease (Bendich *et al* 1997) . This study projected annual cost reductions in US hospitalisation charges, derived from data on the percentage of at-risk Americans with daily vitamin intake levels lower than those associated with a reduction in disease risk, and found that annual hospital charges for birth defects, low-weight premature births and coronary heart disease could be reduced by 40, 60 and 38% respectively. This equated to a Figure of nearly $20 billion dollars in hospital charges, avoidable by the daily use of folic acid and zinc containing multivitamins by all women of childbearing age and daily Vitamin E supplementation of individuals over 50 years of age.

6.4 Conclusions

It is clear from the study of the distribution of purine enzyme activities that the range of observable activities does not always fall within the limits of normal or Gaussian distributions and that data transformation is required to allow meaningful statistisical analysis to be performed. In this instance the transformation technique chosen was that of conversion to a $\log_{10}$ value although there are other methods of transformation which may also be suitable to obtain reference ranges for comparison of patient values. This should optimise the possibility of distinguishing

normal and pathological values, although for many inborn errors where affected individuals have zero activity, interpretation of results is usually straight forward. However for some inborn errors of metabolism, e.g. Lesch-Nyhan syndrome, significant residual activity may be present and the approach used in this study allows the lower limit of normal to be clearly defined. This also helps in the interpretation of carrier levels, as reported for ADA and PNP individuals in this work.

For prenatal diagnosis of the purine disorders, CV is the tissue of choice, due to the fact that first trimester enzymic analysis is a well established procedure both in the method of sampling and of obtaining reliable results for the analysis. Where CV tissue fails to give unequivocal results , perhaps due to small quantities of tissue or a failure of culture, amniotic fluid cell cultures are a suitable alternative. Amniotic fluid cells give considerably higher enzyme specific activities for the 3 purine enzymes studied here, thus enhancing the differentiation between the lower normal limits of the control range and residual activities of affected pregnancies. However, second trimester analysis is less desirable in light of the potentially distressing nature of a termination later into the pregnancy. The study has also shown that in extreme cases where the CV and amniocentesis failed to give an unequivocal result, these enzymes can be measured accurately in fetal blood, although obtaining a sample of this nature is restricted specialist centres.

It is important that confirmation of the prenatal diagnosis be made in tissue from the index case, whether in red cells for a liveborn infant or in the case of selective termination in fibroblast tissue, since only by this procedure can the accuracy of the diagnostic process be assessed. During the time of this study, it was clear from the very few postnatal samples obtained subsequent to a prenatal diagnosis that this message requires to be constantly reinforced.

The study confirms the work reported from other sources that while biochemical methods are appropriate for use in the prenatal and postnatal diagnosis of affected individuals, the lack of differentiation between the normal ranges and carrier females in Lesch-Nyhan syndrome dictates we must look toward molecular genetics techniques for a reliable assessment of carrier status.

The assessment of HGPRT appears to be unhelpful in the determination of the causes of idiopathic gout. However, low levels of HGPRT observed in cases of polyarticular gout may help in the differentiation of the types of gout presenting

and aid in the long term management of the condition. The study has shown that enzymes other than those present in the salvage pathway may play a vital part in certain cases of gout and this should be borne in mind when a diagnosis is being made or request for enzyme analysis is being made.

Results for the analysis of ADA and PNP suggest when investigating the type of immunodeficiency present in a patient, it is of value to analyse both ADA and PNP in tandem where a diagnosis of immunodeficiency is suspected. Evidence is also presented that carrier detection by careful enzyme analysis is possible for ADA and PNP.

Clear evidence from other studies of a role for folate in the development of fetal neural tube defects, raises questions about the mechanism and biochemical pathway involvement of this co-factor. In this study a folate-dependent enzyme, MTHFR, was investigated to establish the importance of a common, genetically determined variant of the enzyme in the aetiology of NTDs. Comparison of the gene frequencies of the thermolabile MTHFR allele indicate that between the European data and that obtained in the Scottish study, an underlying population effect may exist, in that, the NTD affected patients for the latter do not exhibit a higher frequency of the 677C→T genotype than the control group. This suggests that factors other than this particular MTHFR mutation may be influencing the aetiology of this disorder in the NTD affected individuals, a contention supported by the increased frequncy of the 677C→T mutation, low MTHFR enzyme activities and low red cell folate levels compared to the controls in mothers who have had affected pregnancies. This raises the possibility, that a pre-existing reduced folate level and a reduced MTHFR processing capacity, perhaps exacerbated by the increased demands of the fetus during pregnancy, may be a potential risk factor in the development of some NTDs. In view of the contradictory findings between this and other reported studies, it is unlikely that this is the only factor associated with the formation of NTDs in humans, and it remains to be ellucidated whether other folate enzyme of transport defects contribute to this multifactorial disorder.

6.5 Future prospects

The association between the neurological dysfunction and the biochemical deficiencies in purine metabolism have been well documented, the mechanisms connecting the cause and effect are still unclear. There have been a number of hypotheses proposed as to the mechanisms involved as they relate to disorders such as Lesch-Nyhan Syndrome and gout, but these hypotheses are not mutually exclusive, and in some cases are consequences of one another. With the advent of the ever increasing computer power now available, the field of bio-mathematical analysis is proving a useful tool for screening these mechanisms and for detecting patterns relating the biochemical abnormalities to the physiological and neurological functions observed in patients. This analysis provides the opportunity of simulating experiments that would be impossible or perhaps unethical in animals or humans. Bio-mathematical modelling allows an analytical system to be set up in such a way that corresponds to the physiological or disease states and by manipulating the parameters of the model, insight can be gained into the importance of individual intermediates or analytes within the system, which can then be translated into biochemical and clinical terms. Curto *et al* (1998a; 1998b), using this modelling method have shown that the metabolic dysfunctions that lead to hyperuricaemia and gout can be ranked in order of importance as: changes in the effect of uric acid on its own excretion; changes in the inhibition of PRPP-s by adenylates and guanylates; changes in PRPP-S activity; changes in HGPRT activity and changes in the inhibition of AMPD by guanylates and phosphate. In addition, they reported that in Lesch-Nyhan Syndrome, the processes leading to the neurological dysfunctions are strongly affected by the guanylate pool depletion and that the ratio of flux of GPRT to HPRT is correlated with these perturbations.

As these methods of modelling become more sophisticated and the parameters of the dysfunction are better understood, a clearer understanding of the underlying mechanisms of this disorder may be obtained and may be applied to other metabolic systems.

Given that the mechanism for NTD risk reduction by folic acid supplementation remains unknown and the maternal supplementation in the periconceptional period does not reduce the risk in susceptible individuals, there remains the possibility that there is deficiency in the transport or metabolism of folic acid as the cause of fetal

abnormality. This study has examined only one aspect of the multifactorial nature of this disorder, namely the role played by the enzyme MTHFR in the aetiology of neural tube defects. Results here and elsewhere indicate that potential population differences on the risk association of MTHFR to neural tube defects and while the majority of the data suggests an association, it has been limited to studies where there is a relatively low risk population. Scotland and in particular the West of the country is considered to have one of the highest incidences of NTD in the world, and yet the data reported in this study indicates no significant risk factor of NTD with MTHFR. One possible reason may lie in the method of data reduction. Recent studies on disorders with similar aetiologies, such as orofacial cleft defects, suggest that in order to identify the genetic and enviromental factors which may underlie these disorders, several approaches may be necessary to analyse family and population based data. This rationale has its basis in the fact that aetiological factors in multifactorial disorders are diverse in their mode of contribution with monogenic factors being best identified by segregation analyses such as linkage analysis, where as association studies can take into account environmental factors. The limitations of both of these approaches prompted Spielman *et al* (1993) to use the the transmission/dysequilibrium test (TDT) as an alternative to testing other genetic markers for linkage markers when a population difference has been found. The data obtained in this and future studies by the analysis of data by the TDT approach rather than the traditional and somewhat limited case-control study, may provide a better basis for the genotypic study of multifactorial disorders.

In order to accommodate DNA synthesis, large amounts of nucleotides are required by the rapidly dividing cells of the developing neural tube. If the supply of the nucleotides to these cells is reduced, the rate of cellular replication will be slowed and the development of the neural tube retarded. Folate receptors which are less efficient at binding and transporting 5-methyltetrahydrofolate into the cell have been implicated in this process, with the periconceptional folate supplementation increasing maternal blood folate levels and therefore driving these reactions across a defective receptor. Studies of the folate receptor which consists of 3 forms, FR-α , FR-β AND FR-γ, have been disappointing with only 2 mutations detected in the coding region of FR-α gene in nearly 2000 individuals, and of these two mutations only one occurred in an NTD individual (Barber et al 1999). Although there appears from this data that no association exists between

the receptor and the NTD risk, the nature of the multifactorial aetiology of NTD's, suggests that a combination of other gene variations may be required with the receptor status for NTD risk to be increased. Whether it is the maternal, fetal or even the paternal genotype plays an part in this aetiology is as yet unclear, however the results obtained in this study suggest that if MTHFR is one of the factors implicated , that the maternal genotype may be a vital component. What is clear is that much further work in this area is subsequently required to elucidate these mechanisms.

As the intricate nature of folate metabolism implicates a large number of potential candidate genes, it is certain that many other loci will be examined as risk factors involved for NTDs. Recently a number gene families notably those of *Msx* and PAX, have been implicated in the development of the neural tube. While these candidate genes have ostensibly provided insight into the mechanisms of formation of the neural tube in animal models, the question remains whether this data can be translated into the elucidation of the aetiology of NTD's in humans (Davidson 1995; Hol *et al* 1996; Trembath *et al* 1999) .

APPENDIX A

Copy of the consent form used in the neural tube defect study.

A Study of a Possible Genetic Cause of Neural Tube Defects in Scotland

There is good evidence that folic acid in the diet at the time of conception and in the earliest stages of pregnancy can prevent most cases of neural tube defect (NTD). We are trying to find out why not all cases of NTD can be prevented by folic acid, and if there is a difference in the benefit of folic acid between different families.
Recently, it has been found that some people in the population have an unusual form of an enzyme called MTHFR which prevents folic acid from being used so effectively by the body. Although such people are perfectly healthy, some women in this group may have an increased risk of having an affected pregnancy. Researchers have found that people with NTD and their parents had the non-standard form of MTHFR more often than people with no family history of NTD.
As this form of MTHFR is inherited, it is possible that this may be a genetic cause of at least some cases of NTD. To learn more about this, a study is about to begin at the Institute of Medical Genetics in Glasgow of families with NTD to see how common the non-standard form of MTHFR is in Scotland.

To carry out the study we require to collect blood samples from people who have a history, and from others who do not have a history of NTD in their family. **Taking part in the study is entirely voluntary.** Only one sample (about 20 mls taken from your arm) is required. It is not essential that all members of the family donate blood. All samples will be most valuable contributions to the study.

If you wish to volunteer please fill in your details below and return the whole form in the envelope provided to the Institute of Medical Genetics. Someone from the Institute will then contact you and arrange for the blood sample to be taken, either by your GP or at a convenient clinic.

Thank you for taking time to read this leaflet. If you have further questions please telephone 0141 201 0365 and ask for Dr. Margo Whiteford or Mr Gordon Graham.

✂ ---

CONSENT FORM

I wish to help the study of a possible genetic cause of NTD in Scotland by donating a sample of my blood

Name_____ dob____/____/____

Address_____

_____Telephone:_____

Postcode._____ Signature:_____

(Signature of Parent/Guardian_____if under 16 years)

Family Details (please tick):

☐ I have spina bifida

☐ I have had a spina bifida pregnancy and prenatal diagnosis

☐ I have a son/daughter/other relative with spina bifida (Name: .....)
(.....dob)

☐ There is no history of spina bifida in my family

Name and Address of GP: _____

_____

APPENDIX B

Summary of the patient data obtained for the gout study.

LABNO	DOB	HGPRT	APRT	PRPP-S	SEX	HGPRT / APRT RATIO	AGE OF ONSET	PRE-TEST URATE	FAMILY HISTORY
JME	10/17/45	97.1	28.1	0.64	M	3.5	28	626	N
MSH	12/20/28	71.6	30.6	0.36	F	2.3	55	633	N
AGS	04/09/19	69.8	29.2	0.24	F	2.4	47	622	N
RMA	08/02/35	73.7	15.2	0.34	M	4.8		460	N
TGR	03/20/36	68.6	11.4	0.36	M	6.0	54	390	N
MRE	02/04/34	74.2	16.7	0.43	F	4.4	53	510	N
TDS	12/06/52	95.8	49.0	0.39	M	1.96	33	640	Y
TTN		109.5	23.3	0.36	M	4.7			
JDM	12/02/36	74.2	21.6	0.39	M	3.4	52		N
WMR	03/28/42	84.2	7.6	0.32	M	11.1		528	N
CHS	04/26/45	77.2	11.5	0.40	M	6.7	24	688	N
TME	08/16/57	79.9	11.0	0.51	M	7.3	33	592	N
AWS	06/04/20	86.2	19.2	0.37	M	4.5	68	710	N
WBH	07/05/33	74.9	11.9	0.35	M	6.3	39	550	Y
FFR	07/10/50	49.3	10.4	0.56	M	4.7		481	N
WMA	10/14/41	72.5	11.7	0.42	M	6.2	50	820	Y
TWN	01/15/60	82.5	12.2	0.31	M	6.8	20	972	
TMA	05/16/37	94.8	15.9	0.43	M	5.9	42	627	N
DJN	12/27/23	91.0	15.4	0.57	M	5.9	50		N
MBE	03/01/21	92.2	9.9	0.40	F	9.3	68	680	Y
PCK	12/02/39	80.2	18.9	0.31	M	4.2	36	712	N
SMY	04/30/54	74.4	7.6	0.29	M	9.8	38	182	Y
RMN	11/10/44	92.9	19.2	0.41	M	4.8	42	500	N
JGN	12/14/83	68.8	22.4	0.26	M	3.1	43	>500	N
ASE	11/27/29	90.2	11.2	0.41	F	8.1	58	640	N
GET	01/20/34	87.2	18.4	0.41	M	4.7	55	460	N
WMN	04/28/42	72.5	12.3	0.37	M	5.9	42	470	N
JHE	04/24/29	76.9	17.1	0.16	M	4.5	46	690	N
JSH	08/11/20	101.9	20.3	0.20	M	5.0	64		N
EME	01/24/20	105.4	15.7	0.49	F	6.7	39		N
GIN	02/09/50	141.6	24.9	0.27	M	5.7			N
JTO	06/17/21	89.4	13.6	0.48	M	6.6	71	709	N
FMF	10/02/24	99.6	7.5	0.33	M	13.3	60	610	N
ESD		91.4	37.7	0.27	F	2.4			
JTR		96.3	15.8	0.31	M	6.1			
JST		100.1	13.6	0.35	M	7.4			
WCM	07/30/43	82.6	28.8	0.19	M	2.9	31	251	N
JCL	11/05/35	106.2	25.1	0.35	M	4.3	20	656	Y
WSE	07/12/54	74.9	12.4	0.25	M	6.0	30	519	Y
JWE	06/19/29	85.9	32.9	0.19	M	2.6	60	620	N

Appendix B (continued)

Continuation of summary in Appendix B of the patient data obtained for the gout study.

LABNO	DOB	HGPRT	APRT	PRPP-S	SEX	HGPRT / APRT RATIO	AGE OF ONSET	PRE-TEST URATE	FAMILY HISTORY
ACD	12/11/41	83.7	12.4	0.20	F	6.8	12	515	N
RHY	08/07/50	87.3	17.3	0.20	M	5.0	40	553	N
HWN	03/06/34	92.7	18.9	0.24	M	5	56	465	N
JKN	03/20/35	95.1	9.7	0.23	M	9.8		615	
JCK	07/18/47	92.5	11.2	0.17	M	8.3	37	706	
ASL	06/10/50	91.7	23.4	0.15	M	4	27		N
WWH	12/24/44	77.5	14.1	0.20	M	5.5	45		N
RMY	11/08/42	97.4	13.3	0.19	M	7.3	42	700	Y
PBE	09/08/58	99.5	12.9	0.29	M	7.7			
ASN	09/26/32	106.7	32.2	0.29	F	3.4	46	HIGH	Y
AFR	11/12/48	82.5	20.2	0.17	M	4.1	31	456	Y
ABL	07/08/31	69.2	16.9	0.24	M	5.3	53	460	Y
AFN	11/04/41	95.7	12.9	0.23	M	7.4			Y
TGR	08/27/36	42.7	6.2	0.37	M	6.9	27		N

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