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BIOCHEMICAL STUDIES OF HUMAN HYPERURICAEMIA

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BIOCHEMICAL STUDIES OF HUMAN HYPERURICAEMIA

A

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by

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Sponsoring establishment: Plymouth Polytechnic

Collaborating establishment: Dept. of Chemical Pathology
Torbay Hospital

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Podagra

Biochemical Studies of Human Hyperuricaemia.

Trevor R. Hardwell.

Summary.

Some of the enzymes involved in the biosynthesis of uric acid have been investigated in two groups of patients: hyperuricaemic and gouty individuals. A group of normal controls has been used for comparison. Human erythrocyte phosphoribosylpyrophosphate synthetase (2.7.6.1.) was found to be much more stable in all samples than has been reported. Kinetic parameters for this enzyme, using a modified assay, were identical in individuals from the three clinical groups. Inhibition studies using a range of inhibitors of widely differing chemical structure showed uniformity in characteristics amongst gouty or healthy individuals. Some inhibitors revealed possible genetic variants of the enzyme which are linked neither to hyperuricaemia nor to gout.

Statistical analyses have shown a significant increase in the enzymic activity of erythrocyte phosphoribosylpyrophosphate synthetase in both the hyperuricaemic groups compared with the controls. No correlation of age with enzymic activity occurs in any of the three clinical groups. A significant decrease has been found for the enzymic activity of erythrocyte glutathione reductase (1.6.4.2.) of the gout group compared with the other two groups. The activities of these two enzymes do not appear to be linked. A significant increase in serum adenosine deaminase (3.5.4.4.) activities has been found in a heterogeneous hyperuricaemic group compared with controls.

Electrophoretic studies of erythrocyte phosphoribosylpyrophosphate synthetase, adenosine deaminase, hypoxanthineguanine phosphoribosyltransferase (2.4.2.8.) and glutathione reductase have shown probable enzyme polymorphisms which appear to be unrelated to hyperuricaemia or gout. Modified staining techniques have been developed for the detection of isoenzymes in all systems.

There is no evidence for urate binding to plasma proteins in either healthy or hyperuricaemic individuals from occlusion, ultrafiltration, electrophoresis or gel filtration experiments. Urate appears to be present in human leucocytes combined to protein or other component of the cell. There is no evidence of the presence of urate in human erythrocytes.

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Declaration

I certify that I have not been a registered candidate for any other award of the Council for National Academic Awards or University during this research programme. All material in this thesis which is not my own work has been identified and no material is included for which a degree has previously been conferred upon me.

That I have attended the prescribed course of lectures in biochemistry at Plymouth Polytechnic, and seminars in medical biochemistry at Torbay Hospital.

Signed.

Trevor R. Hardwell

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* European Commission Number

ABBREVIATIONS

Å	Angstrom units
ΔA	Absorbance change
ACTH	Adrenocorticotropic hormone
ADA	Adenosine deaminase (E.C.3.5.4.4.)
ADP	Adenosine-5-diphosphate
AIR	5-aminoimidazole ribonucleotide
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AK	Adenylate kinase
AMP	Adenosine-5-monophosphate
APRT	Adeninime phosphoribosyltransferase (E.C.2.4.2.7.)
ATP	Adenosine-5-triphosphate
C-AIR	5-aminoimidazole-4-carboxylic acid ribonucleotide
DPG	2.3-Diphosphoglycerate
EDTA	Ethylene-diamine-tetra-acetic acid
FAD	Flavin adenine dinucleotide
FGAM	∝-N-formylglycinamidine ribonucleotide
FGAR	∝-N-formylglycinamide ribonucleotide
GAR	Glycinamide ribonucleotide
GDP	Guanosine-5-diphosphate
GK	Guanosine-5-monophosphate kinase
GMP	Guanosine-5-monophosphate
G-6-P	Glucose-6-phosphate
G-6-PD	Glucose-6-phosphate dehydrogenase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethansulfonic acid
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase (E.C.2.4.2.8.)
HK	Hexokinase
IDP	Inosine 5 diphosphate
IEF	Isoelectrofocusing
IMP	Inosine-5-monophosphate
ITP	Inosine-5-triphosphate
Km	Michaelis constant
LDH	Lactate dehydrogenase
NAD^+	Nicotinamide-adenine dinucleotide
$NADP^+$	Nicotinamide-adenine dinucleotide phosphate
NADH	Nicotinamide-adenine dinucleotide (reduced)

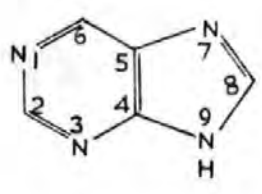
NADPH	Nicotinamide-adenine dinucleotide phosphate (reduced)
NBT	Nitro blue tetrazolium salt
NP	Nucleoside phosphorylase
PEP	Phosphoenol pyruvate
pH	minus log of hydrogen ion activity
Pi	Orthophosphate (inorganic)
PK	Pyruvate kinase
PMS	Phenazine methasulphate
PRA	5-phosphoribosyl-1-amine
PRPP	Phosphoribosylpyrophosphate
PRPP-AT	Phosphoribosylpyrophosphate amino transferase (E.C.2.4.2.14.)
PRPP synthetase	Phosphoribosylpyrophosphate synthetase (E.C.2.7.6.1.)
R-5-P	Ribose-5-phosphate
rpm	Revolutions per minute
SAICAR	5-aminoimidazole-4-N-succinocarboxamide ribonucleotide
SDS	Sodium dodecyl sulphate
T4	Thyroxine
TDP	Thymidine-5-diphosphate
TPTZ	2,4,6-tripyridyl s-triazine
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
u/l	Units per litre
UMP	Uridine-5-monophosphate
u.v.	Ultra violet
Vmax	Maximum velocity
XO	Xanthine oxidase

Introduction.

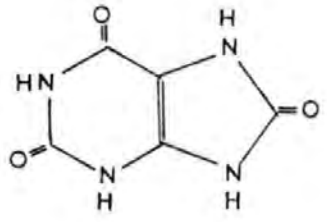
Uric acid is the principal end product of nucleic acid and purine metabolism in men and women and the anthropoid apes. Other mammals are able to oxidise uric acid to allantoin by the action of the hepatic enzyme uricase which is absent in man. The normal uric acid level in plasma throughout adult life lies in the range 1.5-6.0 mg/100ml (90-359 μmol/l) and in most individuals the level increases slightly with age. On average plasma uric acid levels are higher in males than in females. An increase in plasma uric acid level, or hyperuricaemia, occurs in many pathological conditions arising from an increase in the rate of purine catabolism de novo or a decrease in renal clearance of uric acid or a combination of both processes. The clinical conditions associated with hyperuricaemia will be discussed later.

Chemistry of Uric Acid.

Uric acid was first synthesised by Emil Fischer and Ach in 1895. The Fischer school demonstrated the chemical interconversions of the purines and thereby established the correct structural formula of uric acid.



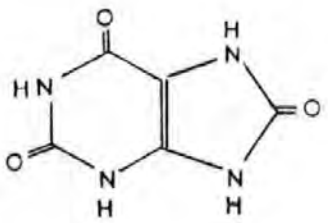
a) Purine ring



b) Uric acid

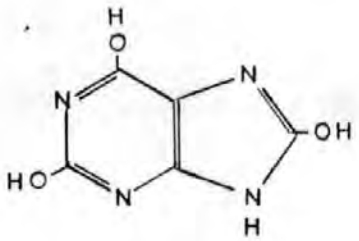
Diag.1 Structural formula of a) purine ring b) Uric Acid.

The hydroxyl structure of uric acid or lactim is formed by enolization of the tautomeric lactam or keto structure. (Diag.2) The tautomeric forms are in an equilibrium which is pH dependent and for example in aqueous solution at pH~7, the lactim is the predominant tautomer.



Lactam

2,6,8 Trioxypurine



Lactim

2,6,8 Trihydroxypurine

Diag.2 Tautomeric structures of Uric Acid.

It has been shown by spectrophotometric evidence that the lactam form predominates in aqueous solution at pH < 7.

Uric acid is a weak dibasic acid characterised by two dissociation constants at pK_a = 5.75 and 10.3 which Wyngaardan and Kelley(1976) explained by the ionisation of hydrogen atoms from positions 9 and 3 respectively (Diag.1). There is no significant ionisation of the hydrogen ions from position 1 or 7. Uric acid readily forms salts such as mono-or di-sodium or potassium urates. The crystals found in synovial fluid or tophi of gouty patients are monosodium urate monohydrate and these are deposited when the solubility is exceeded. Urate and uric acid show strong negative birefringence when viewed

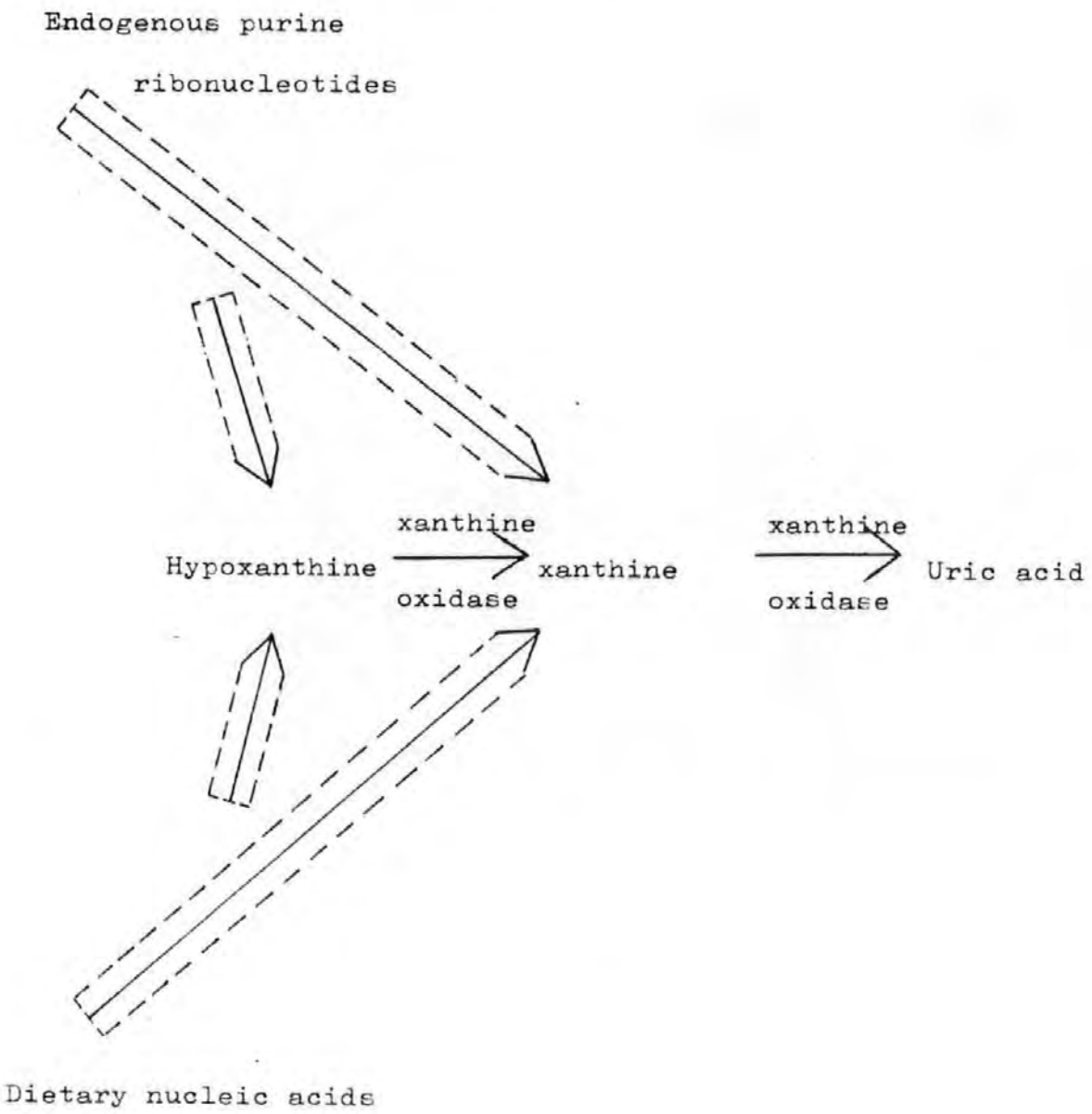
under polarised light . With a red compensator, urate crystals are yellow when orientrated parallel to the axis of polarisation and blue when perpendicular. These properties are diagnostic for the identification of urate crystals in synovial fluid and tissue deposits.

Monosodium urate is much more soluble than uric acid in aqueous solution but both are pH dependent. Monosodium urate solubility is dependent on sodium ion concentration in particular but it also increases with ionic strength. The solubility of monosodium urate in plasma is 7 mg/100 ml (419 µmol/l) and this exceeds the expected saturation value of 6·4mg/100ml (383 µmol/l) as calculated from the solubility product of the urate in the presence of the accepted sodium ion content of plasma (130×10^3 µmol/l). The increased solubility of the urate in plasma has been attributed to urate binding to proteins and other plasma components. Indeed supersaturated solutions of 400mg/100ml ($23{\cdot}92 \times 10^3$ µmol/l) can be prepared in vitro by dissolving uric acid in plasma and stable supersaturated solutions of urate in the range 40-90 mg/100 ml ($2{\cdot}392 \times 10^3$-$5{\cdot}382 \times 10^3$ µmol/l) plasma have been reported in patients with leukaemia (Gold and Fritz 1957) and lymphoma (Kjellstrand et al 1974) after extensive chemotherapy with cytotoxic drugs.

The pH of urine decreases as it passes along the renal tubule and in consequence a proportion of urinary urate is converted into uric acid. Urine is saturated with urates at 15mg/100ml (897 µmol/l) at pH5 whereas pH7 urine can accomadate 158-200mg/100ml ($9{\cdot}448 \times 10^3$-$11{\cdot}960 \times 10^3$ µmol/l). Patients with gout tend to excrete unusually acid urine and the limited solubility of urate at pH5 becomes clinically significant. (Wyngaarden and Kelley 1976 b).

Biosynthesis de novo of uric acid.

Uric acid is formed from the oxypurine bases, xanthine and hypoxanthine, in reactions catalysed by the enzyme xanthine oxidase. The catabolism of dietary nucleic acids and of endogenous purine ribonucleotides are the principal sources of the two purine bases. The whole process is represented in the following diagram:-

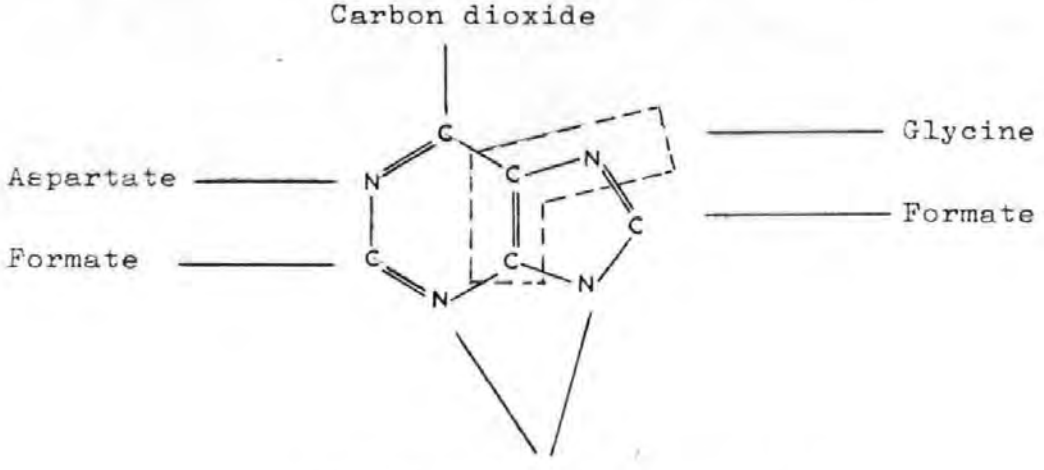


Diag.3 Formation of uric acid from oxypurine bases.

Endogenous purine ribonucleotides are formed by catabolism of nucleic acids and by de novosynthesis from phosphoribosyl pyrophosphate. Adenine and guanine are the two purine bases found in all nucleic acids and these undergo deamination to the corresponding oxypurines, hypoxanthine and xanthine, in reactions catalysed by the deaminases shown in Fig. 1.

Biosynthesis de novo of Purines.

Nearly all living matter, including humans, are able to synthesise the purine ring de novo. The use of labelled isotopes provided the first major contribution toward the determination of the metabolic pathway by which purine biosynthesis is accomplished. Radioactive labels indicated the origin of each of the atoms of the purine ring. Buchanan et al (1948 a and b) demonstrated that carbons 2 and 8 are derived from formate, carbon 6 is derived from CO_2 and that carbons 4 and 5 come from the carbonyl and methylene carbons of glycine respectively. Additionally, nitrogen 7 is also derived from glycine. This information is shown schematically below:-



Diag. 4 The precursors of the atoms of the purine ring.

Subsequent studies showed that nitrogen 1 is derived from aspartic acid and that nitrogen at positions 3 and 9 come from the amide nitrogen of glutamine. (Sonne 1953-1956).

A major contribution to the resolution of the biosynthesis of purine nucleotides was the recognition by Greenberg (1951) that the nucleotide, inosinic acid, was the first product with a complete purine ring formed in the pathway.

It is now accepted that the initial step in purine biosynthesis is the ATP dependent pyrophosphorylation of ribose-5-phosphate by the enzyme phosphoribosylpyrophosphate synthetase. ATP labelled with ^{32}P indicated that the formation of 5-phosphoribosyl-1-pyrophosphate (PRPP) involves one of the few reactions in which a pyrophosphate group is transferred intact from ATP (Khorana et al 1958). The second step in this pathway involves a glutamine dependent amination of PRPP which yield 5 phosphoribosyl-1-amine (PRA) by the action of enzyme pyrophosphoribosyl phosphoamidotransferase. The subsequent steps in the biosynthesis of inosinic acid are shown in Fig.2. The conversion of inosinic acid to either adenylic acid or guanylic acid involves the two stage processes illustrated in Fig.2.

It has been suggested that PRA formation is the rate limiting stage for the regulation of purine biosynthesis. Support for such an hypothesis has grown from the observations presented below:-

1) PRA is the product of the first reaction unique to purine biosynthesis and there are no branch points in the pathway between PRA and inosinic acid (IMP).

The reaction which controls the formation of PRA is therefore ideal to control the complete biosynthetic pathway.

2) Brockman and Anderson (1963) have reported that there is no accumulation of intermediates between PRA and IMP provided that there is neither chemical inhibition nor genetic variants of any of the enzymic reactions. In support of this evidence Rosenbloom et al (1968) have shown that in the absence of formylglycinamide ribonucleotide amidotransferase inhibitors there is no increase of formylglycinamide ribonucleotide (FGAR) in human cells.

3) In cells with an increased demand for purine metabolism such as erythroleukaemic cells, the activity of glutamine PRPP amidotransferase, which catalyses PRA formation, is increased (Reem and Friend 1975). It has been demonstrated by Nierlich and Magasanick (1963), albeit in bacteria, that PRPP amidotransferase is derepressed when cells are grown in media with a limited availability of purines.

4) PRPP, the substrate for PRPP amidotransferase appears to control purine biosynthesis (Fig. 2).

5) Purine biosynthesis is dependent on glutamine availability and this is a cofactor for PRPP amidotransferase (Fig 2).

6) PRA synthesis is controlled by the end products, purine ribonucleotides e.g. GMP, IMP and AMP, of the biosynthetic pathway.

The above evidence provides convincing support of the hypothesis that PRA synthesis is regulated and

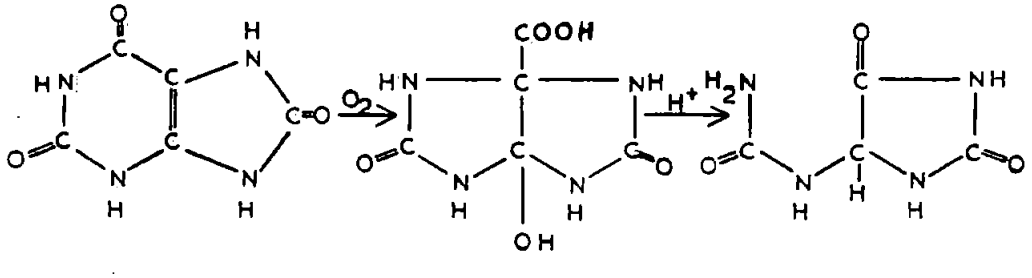
that its rate of formation is important in purine biosynthesis. But an increase in concentration of PRPP does boost the formation of PRA and consequently the rate of purine biosynthesis. It would therefore seem worthwhile, in a study of human hyperuricaemia to investigate the enzyme responsible for the biosynthesis of PRPP in vivo.

Measurement of Uric Acid.

The 'uric acid thread test' devised by Sir Alfred Garrod (1819-1907) is probably the first qualitative test reported for the detection of uric acid. It was performed by addition of acetic acid to serum into which were placed one or two threads from linen fabrics. The container was left in a cool room for 36-60 hours. Crystals of uric acid were deposited on the threads from sera containing large amounts of uric acid.

The murexide test depends on the oxidation of uric acid in acid solution to form alloxan. The latter on addition of ammonia forms ammonium purpurate, a purplish red substance. The test is not specific for uric acid and gives a positive result, albeit more brownish in colour, with xanthine (Wyngaarden, 1960). The qualitative murexide test has been superseded by quantitative colorimetric, enzymatic, fluorimetric, coulometric as well as by chromatographic methods.

Assay methods currently in use for the measurement of uric acid depend either on the chemical or enzymic oxidation of uric acid to allantoin. (Diag 5).



Uric Acid — Hydroxyacetylene diureide carboxylic acid — Allantoin

Diag. 5 Formation of allantoin from uric acid.

For chemical analysis a chromogen is reduced concurrently with the oxidation process and the resulting chromophore measured spectrophotometrically. Uricase is used to catalyse the enzymic reaction which can be followed by measurement either of the oxygen consumed or of the hydrogen peroxide formed. Measurement of the latter involves a coupled peroxidase or catalase reaction. Alternatively the enzymic reaction can be assayed using chemical oxidising agents and colorimetric measurements made before and after treatment with uricase.

The reduction of sodium tungstate by uric acid in alkaline solution is the most commonly used colorimetric method of assay, (Folin 1933). The reaction is subject to interference by other reducing agents commonly present in either urine or plasma and these include ascorbic acid, free thiols, salicylates, homogentisic acid, caffeine, theophyline, theobromine, L-dopa and high concentrations of glucose (Lum and Gambino 1973). Archibald (1957) has modified the phosphotungstate method (Kern and Stransky 1937) by replacement of the cyanide reagent by polyanethol sodium sulphonate to eliminate the turbidity which often develops with cyanide reagent. The use of diagnostic kits has been investigated by Logan et al (1970) but it is a sobering thought to find that many kits were found to be unacceptable with no correlation between cost and performance (Kim et al 1971).

Uric acid, an oxy-purine base, is capable of ligand formation with metallic ions and the reduction of the metallic complexes provide a more specific assay for uric acid than the reduction of phosphototungstate. (Bittner et al (1963) used a cupric sulphate-neocuproine indicator

in which the cupric ion is reduced by uric acid in a weakly acid medium. Morin and Prox (1973) investigated several reactions capable of reducing Fe^{3+} to Fe^{2+} over a wide range of pH and finally selected 1, 10-phenanthroline for its solubility, molar absorption, optimum wavelength and stability. Morin (1974) replaced 1, 10 phenanthroline by 2,4,6-tripyridyl s-triazine and found that the sensitivity was doubled and interference by bilirubin was decreased fourfold. The reaction is colorimetric and does not require deproteinisation.

Automated colorimetric methods usually require dialysates and they depend on either the reduction of phosphotungstate or arsenotungstate or on the reduction of a metal complex (e.g.cupric phenanthroline, neocuproine or bathocuproine). All appear to overestimate urate by 0·4-1·0 mg/100 ml (Logan et al, 1970 Lum and Gambrino 1973).

Uric acid shows a maximum at 292 nm with a molar absorption coefficient of 12,500 $cm^2 mol^{-1}$ at pH 9·4 in its ultra violet absorption spectrum (Cavalieri and Bendich, 1950 Bergmann and Dikstein, 1955) whereas allantoin has no ultra-violet absorption at 292nm. In the presence of uricase, the decrease in optical density at 292 nm indicates the amount of uric acid present in a buffered assay mixture (Praetorius 1949) but Haeckel (1979) reports that albumin can interfere at this wavelength. Other substances present in biological fluids absorb u.v. in this region and such absorbance can be eliminated by the introduction of a suitable blank (Liddle et al 1959) or by differential spectrophotometry. The high purity and specificity of uricase now available make this potentially one of the most reliable methods currently in use.

Kageyama (1971) coupled catalase to the uricase reaction, oxidised methanol to formaldehyde and measured colorimetrically the dehydrolutidine formed by condensation of formaldehyde with acetylacetone and ammonia. The reactions are as follows:-

$$\text{Uric Acid} + 2H_2O + O_2 \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + CH_3OH \xrightarrow{\text{catalase}} HCHO + 2H_2O$$

$$HCHO + \text{Acetylacetone} + NH_3 \longrightarrow \text{3,5-diacetyl-1,4-dehydro-lutidine} + 3H_2O$$

The uricase reaction has also been coupled with the hydrogen peroxide-peroxidase oxidation of a chromogen such as e.g. O-dianisidine hydrochloride (Lorenz and Berndt 1967) or tribromophenol-aminoantipyrine (Kabasakalian et al 1973). Serum can be used without deproteinisation and there is no significant interference by haemolysis, bilirubin (< 10mg/100ml) ascorbic acid or glucose.

Block and Lata (1970) have developed a fluorescent method using scopoletin (7-hydroxy-6-methoxycoumarin) capable of detecting uric acid in the picomole range. Fluorescence of scopoletin (excitation max 348nm; emission max. 465nm) is abolished by oxidation with hydrogen peroxide catalysed by horseradish peroxidase. The method combines the specificity of the uricase method with the simplicity of fluorimetric detection.

A coulometric determination of uric acid in serum and urine has been reported by Troy and Purdy (1970). This method gives comparable values to the uricase methods for plasma but higher values for uric acid in urine.

Separation of uric acid from protein can be accomplished by passage of serum or other biological fluid through a strong anion-exchange resin in the acetate form (Carr and Pressman 1962). Extraneous U.V. absorbing material can be

removed by washing with water and the uric acid eluted from the column with NaCl in buffer and estimated by enzymic assay. An alternative chromatographic method involves the use of high pressure liquid chromatography. The uric acid is separated from the purine bases by a strong anion exchange resin with an acetate mobile phase. The uric acid in the eluant may be measured directly by monitoring spectrophotometrically at 293nm or by electrochemical detection. (Kissinger et al 1975).

During the past few years many modifications -usually minor- of the accepted methods of assay for uric acid have appeared and immobilised enzyme systems are being used for routine determination of serum uric acid (Sundaram et al 1978). It is not practical to consider all modifications in detail in the current work but some of the relevant information concerning the different methods is shown in Table 1 .

Several comparisons have been made in attempts to recommend an assay of choice.Itiaba et al (1975) found that the uricase -U.V. method gave significantly lower values of serum uric acid than the autoanalyser colorimetric phosphotungstate method using hydroxylamine for colour intensification. Slaunwhite et al (1975) have compared the colorimetric, enzymatic and liquid chromatographic methods and claim that the latter technique is more accurate than the other two. Three quantitative clinical chemical routine methods have been evaluated using as reference a mass fragmentation method (Ohman 1979). The latter involves the addition of (1,3-$^{15}N_2$) uric acid to serum followed by ion exchange chromatography. After conversion to the tetra-trimethylsilyl derivative

the purified mixture is analysed by combined gas chromatography-mass spectrometry using a multiple ion detector. A significant difference was observed with the reference only when using a uricase-oxygen consumption method. The Kageyama (1971) uricase/catalase method as well as the uricase/U V spectrophotometric method gave linear correlations.

Immobilised enzyme reactors will certainly become the method of choice for routine determination of serum uric acid in clinical laboratories. Analysis costs can be dramatically reduced ten fold as compared to the corresponding solution methods. Sundaram et al (1978) have immobilised uricase onto the inside surface of a thin nylon tube and have found the reactors are stable for $\sim$ 3 months or 4,000 tests. Similar stability of the enzyme electrodehas been found using immobilised columns (Endo et al 1979) and membrane sandwich reactors (Kamoun and Donay 1978). The immobilised enzymes are readily prepared and some are commercially available.

In a Technical Bulletin, Watts (1974) revues the assay of uric acid but no firm recommendation is reported and although the use of uricase is most widely favoured, the final selection is a personal choice dependent on laboratory facilities.

Distribution of Uric Acid.

In healthy individuals under conditions of normal production and removal, the body contains a readily miscible uric acid pool of about 1.2g. and between 50-75% of this is turned over daily. This fraction represents the rate of formation and excretion of uric acid in normal subjects but it is independent of the amount entering the lumen of the intestine and undergoing becterial destruction. The distribution of uric acid amongst different body fluids for a healthy individual is given below as quoted in Geigy. (1962)

Body fluid	Normal Value
Whole blood	1.0-3.0mg/100ml
Serum	3.0-6.0mg/100ml
Gastric juice	0.8-2.0mg/100ml
Breast milk	1.3-4.1mg/100ml
Pancreatic juice	0.2mg/100ml
Urine	80-976 mg/24hr
C.S.F.	0.3-0.6mg/100ml
Saliva Male	3.1mg/100ml
Saliva Female	1.9mg/100ml

Urate binding to plasma proteins.

The subject of urate binding by plasma proteins has remained controversial over many years. A loose binding of urate both to albumin (Campion et al 1973) and to a specific α_2 globulin has been reported and such interactions have been postulated to be of importance in the pathogenesis of gout (Alvsaker 1965; Alvsaker and Seegmiller, 1972). Many investigators have claimed substantial binding of urate to nondiffusable elements of plasma whilst others have concluded that all urate is in true solution because it is readily filterable and dialysable. Even electrophoretic studies of urate localisation in serum have yielded conflicting data.

Yu and Gutman (1953) used ultrafiltration and found no difference in urate content with the corresponding sera. Their data indicated that the plasma urate in man is virtually 100% filtrable at the glomeruli and that $\sim$ 90% of the filtered urate is normally resorbed in the tubules. They also postulated that some urate may be present in plasma in aggregates which are filtrable or readily depolymerised. It seems probable that such polymers would be more likely to occur at the higher plasma urate levels found in gout or nephritis.

Alvsaker (1965, 1966 and 1968) has studied urate binding by several methods which include a) equilibrium gel filtration b) immunoelectrophoresis and c) radio-autography. From these studies Alvsaker quotes binding levels of 30% in normal sera and reduced binding $\sim$ 13-30% in gouty patients. However, Klinenberg and Kipper (1970) using equilibrium dialysis experiments, found that the urate bound to plasma proteins was related to

free urate concentration, protein concentrations, ionic strength, pH and temperature. They also reported a decreased binding in their patients with severe tophaceous gout and in patients taking large doses of aspirin.

Sheikh and Moller (1968) examined urate binding to plasma proteins in human and rabbit by equilibrium dialysis, ultrafiltration and gel filtration at $\sim 40^{\circ}$ and 20° and found that urate binding decreases as the temperature increases. In contrast to Alvsaker they found only 5% binding at 20° and these findings support the hypothesis that the binding of urate in vivo is very small and consequently will have only minor physiological implications.

The work of Farrell et al (1971) indicated that the addition of lithium urate to plasma artificially increases the apparent binding of urate. Other extensive studies by Farrell et al (1971 and 1975) by ultrafiltration showed only negligible binding of urate to plasma protein under physiological conditions of temperature and medium composition. Conflicting results have been reported by Campion et al (1973 and 1974) who showed that 10% of urate in solution could bind to albumin at physiological temperatures. The experiments were done in an ultrafiltration cell that included a magnetic stirrer and Steele (1976) reinvestigated urate ultrafiltration using a similar apparatus. His result not only showed a disparity in urate ultrafiltrability between stirred and unstirred samples but other hazards in the complex nature of urate ultrafiltration.

A relationship between free urate, protein bound urate, hyperuricaemia and gout in Caucasians and Maoris

has been reported (Klinenberg et al 1977). Although Maoris have high serum uric acid concentrations and show an increased incidence of gout as compared to Caucasians living in the same environment (Prior et al 1964) it has been found that Maoris living on a remote island show a low incidence of gout in spite of hyperuricaemia. Free and protein bound urate has been measured in gouty patients, subjects with asymptomatic hyperuricaemia and normal control subjects in both Caucasian and Maori populations. The studies of urate binding to serum proteins showed no difference between gouty and asymptomatic hyperuricaemia subjects, thus both have similar free urate concentrations. This suggests that the clinical manifestations of gout are not directly related to the free urate concentration. The Maoris have not only markedly increased binding of urate to protein as compared to Caucasians but a urate binding globulin has been demonstrated which is not found in Caucasians. (Klinenberg et al 1977).

Further work on urate binding not only in healthy individuals but in patients with hyperuricaemia and especially gout is clearly desirable.

Clinical conditions of hyperuricaemia.

Hyperuricaemia is difficult to define and recent progress in the understanding of purine metabolism and urate deposition has revealed the necessity for some modifications of the traditional classification of hyperuricaemia and gout. Boss and Seegmiller (1979) have suggested that a rational definition of hyperuricaemia should be based on the physicochemical saturation of serum with urate, which at 37° is about 7mg/100ml (419 µmol/l).

Distribution curves of sodium urate in the population are not Gaussian but are slightly skewed toward the higher values. The absence of a bimodal distribution indicates the probable absence of two distinct groups, normal and hyperuricaemic individuals in the community. Hyperuricaemia has therefore been defined as a serum urate level in excess of the mean population value by at least two standard deviations. It would be clinically convenient if one could define the upper limit of normal as that sodium urate level above which complications are bound to occur and below which they never occur but in practice there is no critical cut-off value. Although the patient with a serum urate level of 9mg/100ml (538µmol/l) is at much greater risk to an attack of gout than one with a level of 7mg/100ml (419µmol/l), only 10% of gouty patients have serum urate levels exceeding 9mg/100ml (538µmol/l) and by no means all individuals with high uric acid levels present a clinical picture of gout. Although hyperuricaemia is a prerequisite for the development of gout, it may not be present at the time of diagnosis. Some degree of elevation usually persists

between attacks of gout but it can be intermittent and therefore missed. Asymptomatic hyperuricaemia has been found in blood relatives of gouty patients and female relatives tend to have lower serum urate levels than male relatives.

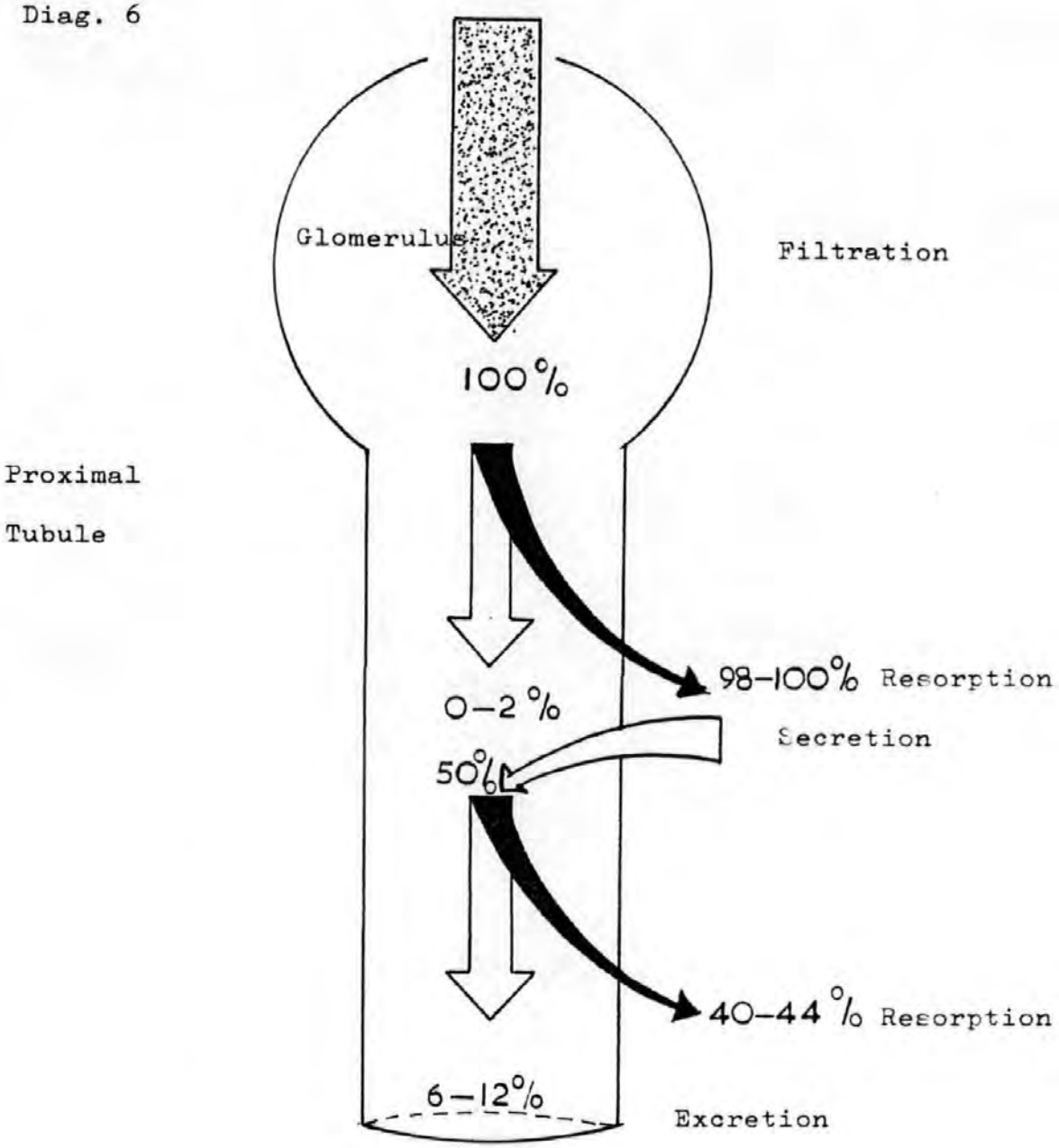
Increase in serum uric acid may be theoretically due to either overproduction or decreased destruction or decreased renal excretion of uric acid. Decreased destruction is not really relevant because uric acid is not metabolised by human tissues although an appreciable amount is degraded by intestinal bacteria in the digestive tract. In practice it is usually a combination of the other two factors which operate in many clinical conditions, but for simplification the clinical conditions associated with hyperuricaemia have been segregated into three categories which are summarised in Table 2 . Some of these maladies will now be discussed.

Decreased Renal Excretion.

Serum uric acid is freely filtered across the glomerulus and in the early part of the proximal tubule 98-100% of the filtered urate is resorbed (Steele 1971). A variable amount of the filtered load is secreted back into the proximal lumen in a more distal part of the proximal tubule. This classical three component hypothesis of filtration, resorption and secretion for renal handling of urate proposed by Gutman and Yu (1961) has been confirmed and extended by the introduction of a post secretary absorption component (Diamond and Paolino, 1973) which occurs in the late part of the proximal tubule or the distal tubule (Rieselbach and Steele 1974). The final concentration of uric acid in the urine is a function

of the relative amounts of tubular secretion and post secretary resorption since uric acid is almost completely resorbed in the proximal tubule. In healthy individuals the tubular secretary capacity for uric acid is high ($> 50\%$) and because only $\sim$ 10% of the filtered urate load appears in urine, it follows that the post secretary resorption of uric acid must also be high. Normal renal handling of urate has been depicted by Rieselbach and Steele (1974) as shown in Diag. 6.

Diag. 6



Diag. 6. Urate transfer in the human kidney

An impairment of the ability of the kidneys to handle any of the four component system for urate clearance will alter serum uric acid levels in an individual. Patients with chronic renal failure due to diseases other than gout initially show an enhanced clearance of urate as compared with creatinine but progressive deterioration in renal function eventually gives rise to hyperuricaemia. Increased serum uric acid may occur in eclampsia in the absence of retention of urea. In this condition there may be a decrease in uric acid clearance with no associated abnormality of other function of the kidney. On this basis the simultaneous presence of decreased uric acid clearance and usual urea clearance may aid in differentiating eclampsia from glomerulonephritis complicating pregnancy.

Hyperuricaemia may occur, in association with retention of other urinary constituents, in conditions associated with urinary obstruction (prostatic, bilateral, uretheral calculus etc), urinary suppression (acute tubular necrosis, shock, intestinal obstruction etc), destructive renal lesions (tuberculosis, pyelonephritis, polycystic kidney etc), congestive heart failure and adreno-cortical insufficiency (Addisons disease).

Renal disease is the most serious extra-articular consequence of hyperuricaemia and three types of renal disease have been reported by Klinenberg et al (1975), gouty nephropathy, acute intratubular crystal deposition and nephrolithiasis. The presence of monosodium urate monohydrate crystals in renal parenchyma is definitive for gouty nephropathy. Barlow and Beilin (1968) have found that diagnosis by means of renal biopsy is difficult

since crystals rarely form in the cortex but are usually present in the medulla. The high sodium concentration present in the medulla, which is higher than that of plasma, lowers the solubility of monosodium urate and thus favours deposition of urate crystals. Decreasing the medullary sodium concentration by generous hydration will decrease the tendency for intramedullary deposition of monosodium urate. Urate crystals are fairly soluble in formalin which is commonly used for fixation in histochemical investigations of renal autopsy and biopsy specimens and this fact is probably responsible for many reports of the absence of intrarenal urate crystals in patients with gout. However Talbott and Terplan (1960) reported that 176 of 191 gouty patients had urate crystals in renal parenchyma. The precise pathophysiological consequences, if any, of intramedullary deposition of urate crystals are unknown but Klinenberg et al (1975) report the decline in the kidney's ability to form concentrated urine as the earliest physiological disturbance. Renal insufficiency is certainly common in gout, Barlow and Beilin, (1963), but it appears to correlate better with the age of the patient than with the extent of the gout. Long-standing hperuricaemia does not inevitably produce gouty nephropathy and chronic renal insufficiency. Berger and Yu (1975) reported that after a 12 year study, no appreciable loss of renal function was found in 112 patients with gout as compared to normo uricaemic subjects. Additionally Rosenfeld (1974) found that the drug allopurinol, an inhibitor of the enzyme xanthine oxidase, had no value in the prevention or development of renal insufficiency in

asymptomatic hyperuricaemic subjects.

Nephrolithiasis associated with hyperuricaemia may be of three types: pure uric acid stones, mixed uric acid-calcium oxalate stones and calcium oxalate stones. The cause of mixed uric acid-calcium oxalate stones and pure calcium oxalate stones in the presence of hperuricosuria is unknown but the incidence of calcium oxalate stones is decreased by treatment of hyperuricosuric patients with allopurinol (Coe, 1977).

The Framingham Heart Study (Hall et al 1967), in 1949, represents an in-depth epidemiological survey of 5127 men and women aged 30-62 in Framingham, Massachusetts. The study showed that all cases of renal failure secondary to hyperuricaemia were preceeded by multiple attacks of arthritis or stones.

Hyperuricaemia may also be associated with an increased incidence of arteriosclerotic cardio vascular disease. The Framingham study showed that the incidence of coronary artery disease in patients with gout is twice as high as those individuals without gout. Other studies by Viozzi et al (1972) and by Bluhm and Riddle (1973) have demonstrated a statistically significant correlation between hyperuricaemia and arteriosclerosis but the Tecumseh Community Health Study showed no relationship between hyperuricaemia and coronary heart disease. (Myers et al 1968). The oversight of factors such as ethnic considerations, diet etc may cause erroneous interpretations in population studies. It must be remembered that the contribution of two major risk factors for coronary artery disease-hypertension and obesity- is difficult to eliminate since both appear to

be independently associated with hyperuricaemia (Hall 1965; Hall et al 1967). In vitro studies by Ginsberg et al (1977) have indicated that urate crystals may play a part in atherogenesis. Incubation of monosodium urate crystals with platelets results in the rapid release of serotonin, ATP and ADP. The latter is a potent stimulator of platelet aggregation and effects a further release reaction and aggregation. A local interaction of platelets and urate crystals at the blood vessel wall could result in platelet aggregation and consequent endothelial-cell damage (Mustard et al 1963). Such a progress could be an important initiating event in atherosclerosis.

Pathological conditions associated with increased cellular turnover lead to marked overproduction of uric acid. Hyperuricaemia is common in patients with myeloproliferation (such as polycythemia vera and myeloid metaplasia) or lymphoproliferative disorders. (Kjellstrand et al 1974). Acute intratubular precipitation of free uric acid leading to renal failure is an acknowledged complication in these patients undergoing cytotoxic treatment in the form of chemotherapy or radiotherapy (Talbott and Yu 1976 a)

Gout.

The acute gouty attack and crippling effects of chronic gouty arthritis have been described as early as the fifth century B.C. An updated version of the acute attack was described by Sydenham in 1683 as follows:-

'The victim goes to bed and sleeps in good health. About two o'clock in the morning he is awakened by a severe pain in the great toe: more rarely in the heel, ankle or instep. The pain is like that of a dislocation, and yet the parts feel as if cold water was poured over them.

Then follows chills and shivers and little fever. The pain, which was first moderate, becomes more intense. With its intensity the chills and shivers increase. After a time this comes to its height, accomodating itself to the bones and ligaments of the tarsus and metatarsus. Now it is a violent stretching and tearing of the ligaments, now it is a gnawing pain and now a pressure and tightening. So exquisite and lively meanwhile is the feeling of the part affected that it cannot bear the weight of bedclothes nor the jar of a person walking in the room. The night is passed in torture, sleeplessness, and turning of the part affected and perpetual change of posture' (Sydenham, 1850).

However it was not until the 19th century that elevated uric acid was demonstrated in gouty patients and not until the 20th century that urate crystals were definately implicated as the inciting cause of gout.

Hyperuricaemia and gout can either be primary disorders or secondary to other clinical conditions or occasionally to drug therapy. In patients with primary gout, elevated uric acid levels are caused by increased production of uric acid, impaired renal clearance of uric acid or a combination of both. Although the clinical expression of primary gout appears to be genetically controlled, specific metabolic defects have been identified in only a small proportion of patients and these will be discussed later. Epidemiological studies (e.g. Healy, 1975; Talbott and Yu 1976b) suggest that a number of environmental and social factors may not only contribute to the clinical manifestations of primary gout but may mask the underlying genetic defects in uric acid metabolism and/or excretion (Wyngaarden and Kelley 1976 c).

Prevalence and incidence of the diagnosis of gout.

A study of the prevalence of gout and of the incidence of diagnosis from 1971-1975 was carried out in a representative general practice sample comprising 64 practices and a population numbering 1 in 145 of the total population of Great Britain has been reported by Currie (1979). The results show an annual incidence of 0.25 to 0.35 per 1000 and an overall prevalence at 25th. December 1975 of 2.6 per 1000. The prevalence in England was greater than that in Wales which was greater than that in Scotland. 'In Scotland gout is much less frequently met with than in England.....and when it does occur is generally in the upper classes.....'(Garrod 1876). In 10% of all the cases the gout was believed to be secondary, with induction by diuretics being the most frequent cause. The prevalence of primary gout was estimated as 2.3 per 1000, which ~~is~~ in keeping with estimates covering Europe and North America (Lawrence 1960, Wyngaarden and Fredrickson 1960 Kellgren 1964). Scott et al (1976) showed that serum uric acid levels in England and Glasgow had the same frequency distribution and mean levels. It is as yet not possible to ascertain the effect of dietary alcohol on plasma uric acid levels. The average dietary consumption of alcohol in Europe amounts to between 10 and 15% of the total calorie intake and in the past women have not taken as much alcohol as men. However in this age of sex equality women are drinking more and so it will be of interest to observe whether the incidence of gout remains higher in males than in females. It should be noted that the consumption of alcohol in some countries is very low and

yet gout is not uncommon. Gout is not associated with alcoholics but it could be that high alcohol intake is a contributary factor in gout.

The clinical course of primary gout can be divided into three phases:-a) the asymptomatic stage b) the acute intermittent stage and c) the chronic stage. The main objective of treatment for all of these stages is prompt treatment and prevention of symptomatic gout, avoidance or reversal of any of the possible complications of hyperuricaemia and treatment of any associated conditions such as hypertension.

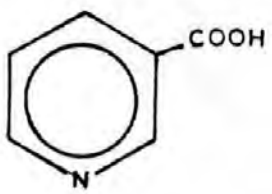
Drug induced hyperuricaemia.

Several drugs may induce hyperuricaemia by increasing production and/or decreasing excretion of uric acid. Diuretic induced hyperuricaemia is of some concern. About 50% of the patients with essential hypertension treated with diuretics become hyperuricaemic although fortunately many of them remain asymptomatic. Paulus et al (1970) routinely screened blood urate in patients in a large hospital in the U.S.A. and found that diuretics could be implicated as the cause of 20% of the cases of hyperuricaemia found in this survey. The mechanism of diuretic induced hyperuricaemia, although not resolved, possibly involves an increase in resorption of uric acid in the proximal tubule (Holmes et al 1972).

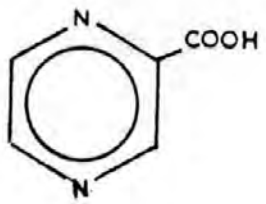
The popular concept between overingestion of alcohol and acute gout maybe due to lactic acidosis induced by ethanol but Luber et al (1962) have reported that there is a high purine content in wine and beer. Lactic acid, like other weak organic acids, interferes with the tubular secretons of urate. Alcohol ingestion may raise the serum

urate concentration as much as 1 to 3mg per 100ml.(60-179μmol/l).

The antituberculous agent pyrazinamide and its deaminated form pyrazinoic acid are potent inhibitors of renal-tubular urate secretion (Meisel and Diamond, 1977). Ethambutol, an extensively used antituberculous drug also decreases renal clearance of uric acid (Postlethwaite et al 1972). Nicotinic acid also decreases renal excretion of uric acid (Gaut et al 1971) Nicotinic and pyrazinoic acids are structurally similar.



Nicotinic acid



Pyrazinoic acid

Diag. 7 Structure Nicotinic and Pyrazinoic acids.

Aspirin exhibits a dual action in the excretion of uric acid. There is a "threshold" level whereby doses of less than 2 g. daily increases serum urate concentration due to inhibitors of tubular secretion; higher doses are generally uricosuric since resorption of uric acid is preferentially inhibited (Grayzel et al 1961). The drugs known to be inducers of hyperuricaemia are shown in Table3 which includes their known mechanism of action.

Hyperlipidaemia in Gout.

Elevations of plasma triglyceride and free fatty acid levels have been reported in patients with primary gout but the mechanism of this hyperlipidaemia is by no means established. A positive correlation ($r = 0.249$ $P < 0.05$) has been found between plasma triglycerides and free fatty acid levels in gouty patients by Nishida et al (1975). Hypertriglyceridaemia and normal serum cholesterol levels have been reported by Feldman et al (1964) and by Berkowitz (1964) in patients with gout. It has been suggested that the high levels of serum triglyceride found in association with gout may be explained on the basis of high alcohol consumption and the body weight problem in many countries. It would appear worthwhile to investigate hyperuricaemia and hyperlipidaemia using Fredrickson's classification for the latter condition (see Appendix I).

Despite the rather consistent presence of hyperuricaemia in acute gout it is important to realise that this is apparently not the cause of the acute symptoms. No such attacks occur in patients with renal failure with high blood uric acid concentrations. Moreover there is no consistent quantitative correlation between the clinical response to such agents as ACTH, cortisone, cinchophen, salicylates, probenecid etc. and their uricosuric or blood urate-lowering effects. Administration of uric acid, orally or intravenously, does not precipitate an attack even in gouty patients. Observations such as these have led to the hypothesis that some purine metabolite, probably a precursor of uric acid is the agent directly responsible for the acute attack. However attacks of acute gout, accompanied by hyperuricaemia, have been

precipitated by administration of chlorothiazide and other benzothiol compounds for purposes of inducing diuresis. The mechanism of this process has yet to be completely resolved. It does however become apparent that any systematic study of hyperuricaemia should include two groups of patients, additional to a group of healthy controls, one group with the diagnosis of gout and the other to have asymptomatic hyperuricaemia and absence of gout in their family history.

Enzymology of Hyperuricaemia and Gout.

The excessive production of uric acid may be primary, that is due to a pathological or a genetically determined abnormality or secondary as a complication of an acquired disorder. A number of specific enzyme defects presented in Table 4 have been reported and such mutations can account for some of the genetic basis of primary gout.

Phosphoribosylpyrophosphate synthetase (E.C.2.7.6.1.)

PRPP is an essential substrate for several pathways involved in the biosynthesis of pyrimidines, purines and nucleotides and in the salvage of purines. In fact PRPP serves as a phosphoribosyl donor in many reactions which are listed in Table 5. Any of these reactions are capable of competing with the reaction of the pathway for purine biosynthesis de novo for available PRPP.

The synthesis of PRPP from ATP and ribose-5-phosphate is catalysed by PRPP synthetase and the reaction involves direct transfer of the terminal pyrophosphate of ATP to the 1-carbon of ribose-5-phosphate (Khorana et al 1958). The enzyme is stereospecific for a pentose sugar with a hydroxyl group at position 2 and a phosphate at position 5 (Fox and Kelley 1972). Magnesium ATP is another

substrate for the enzyme and d-ATP can be substituted for ATP. But the lack of enzymic activity with ADP and nucleoside triphosphates other than ATP and d-ATP suggest that a triphosphate structure, the purine ring and the 6-amino-purine (Fox and Kelley 1971 a) are necessary for any compound to be an effective substrate. The enzyme has an absolute requirement for inorganic phosphate and the enzyme is inactive in the absence of phosphate (Fox and Kelley 1972).

The kinetic mechanism of the enzyme from human erythrocytes has been studied in detail and appears to be an ordered bi-bi mechanism. Fox and Kelley (1972) indicated by product inhibition studies that ribose-5-phosphate is bound first to the enzyme and PRPP released last. The regulation of PRPP synthetase activity is quite complex and there appears to be three different active sites for the binding of such diverse inhibitors of the enzyme as e.g. ADP which competes with Mg ATP and PRPP and 2,3 diphosphoglycerate which compete with R-5-P (Fox and Kelley 1972). There is no evidence that PRPP inhibits the enzyme under normal conditions (Fox and Kelley 1971 b) since the Ki for PRPP is 0.05 mM which is about 10 times its intracellular concentration in human cells. In marked contrast the Ki value of the enzyme for 2,3 diphosphoglycerate is 5.3mM which approximates to its concentration in human erythrocytes. A large number of compounds have been found to inhibit PRPP synthetase by a third mechanism which is non-competitive with respect to both ribose-5-phosphate and magnesium ATP. These include AMP,GDP,GTP,IDP,ITP,TDP, NAD^+, $NADP^+$ and FAD (Switzer 1971, Fox and Kelley 1972,

Green and Martin 1974). In general the di- and tri-phosphates are more potent inhibitors than the mono-phosphates (Fox and Kelley 1972) and it has been proposed that the enzyme is regulated by heterogeneous metabolic pool inhibitors with binding at a third site on the enzyme (Fox and Kelley 1972, Switzer 1971). This group of inhibitors have a low affinity for the regulatory active site.

Human PRPP synthetase is composed of a repeated single subunit of ~ 60,000 daltons (Fox and Kelley 1971 a, Becker et al 1977). The enzyme reversibly assume aggregated forms of molecular weights varying from ~ 60,000 to greater than 10^6. Smaller aggregates of the enzyme appear to be inactive whereas aggregates composed of 16 and 32 subunits are active (Fox and Kelley 1971 a). The effect of small molecule activators and inhibitors appears to be correlated with their effect on the state of aggregation of the enzyme (Becker et al 1977). It is now accepted that the subunit measured by Fox and Kelley (1971 a) is a dimer and the evidence for a subunit of molecular weight 33,000 is summarised in Table6 . The properties of the aggregated forms of PRPP synthetase are presented in Table7 . Structural alterations in PRPP synthetase could alter either the affinities of the binding sites for different inhibitors or the subunit aggregation. Such changes could alter activity with subsequent overproduction of PRPP, purine and uric acid.

Specific genetic alterations of PRPP synthetase appeared to be important in gout when Jones et al (1962) reported an increased turnover of PRPP with overproduction

of uric acid in three gouty patients. An increased rate of PRPP formation in erythrocytes in several gout patients was reported by Hershko et al (1968) and although the mechanism was not established this observation was confirmed by Sperling et al (1971). A markedly accelerated rate of PRPP synthesis with overproduction of urate was demonstrated in two brothers with gout (Sperling et al 1972) and this was later shown to be consequential to altered kinetic properties of PRPP synthetase (Sperling et al 1973). Two additional families have been reported in which excessive rates of PRPP synthesis can be attributed to abnormalities of the enzyme but the enzyme defect appears to be different in each of the three families (De Vries et al 1973, Becker et al 1974). The enzyme from a patient of the gouty family reported by Sperling et al (1972) was partially purified and kinetic analysis showed that although its affinities for ribose-5-phosphate, ATP and inorganic phosphate were normal, its sensitivity to inhibition by nucleotides and 2.3 diphosphoglycerate was markedly reduced (Sperling et al 1973). The results indicated that the gouty patient had a mutant enzyme structually altered in some way which rendered it relatively resistant to control by nucleotides.

The second variant form of PRPP synthetase was found in two brothers with gout in whom the rate of purine synthesis de novo as assessed by ^{14}C glycine incorporation into urinary uric acid was 3-5 fold greater than normal (Becker et al 1973). The enzymic activity was threefold the normal activity in erythrocytes over a range of inorganic phosphate concentrations. Increased thermal lability

and altered electrophoretic mobility (i.e. a lower isoelectric point) was found in the enzyme purified from gout patients in this family (Becker et al 1975). By means of antibody inactivation and immunodiffusion using rabbit antiserum developed to purified normal PRPP synthetase, the increased catalytic activity per molecule was shown by Becker et al (1975 and 1980) to account for the increased activity of the mutant enzyme.

Another structural variant of PRPP synthetase associated with excessive intracellular PRPP concentration was identified by Becker (1976) in the erythrocytes of two affected brothers. Although PRPP synthetase from these individuals was normal at saturating substrate concentration partially purified preparations of the erythrocyte enzyme had a three or four fold higher affinity for ribose-5-phosphate than did comparable preparations from healthy controls. This variant enzyme had a slower electrophoretic mobility than the usual enzyme.

Lejeune et al (1979) report abnormalities of PRPP synthetase in two cases of gout beginning at an early age. The kinetic abnormalities are manifest in an increase affinity for inorganic phosphate in the absence of inhibitors and a decrease inhibition by low concentrations of ADP in the presence of phosphate. No other abnormality was apparent. The two cases of gout were characterised clinically by their early occurence (one patient a premenopausal woman), their family character and their normal sensitivity to allopurinol.

The differences in the enzyme defects described above exemplify the heterogeneity found in some patients with

gout and suggest that additional mutations of PRPP synthetase may well account for purine overproduction in primary gout. The abnormalities in some of the enzyme variants are subtle and would be missed by routine mass screening of enzymic activities. The application of more refined techniques and the use of isoelectric focusing could more readily expose other enzyme variants present in gouty individuals. Kelley and Wyngaarden (1974) report that 'there is no question now that increased PRPP synthetase activity represents a specific genetic type of gout' but that the relative frequency of the disease within the gouty population remains to be determined but 'it would appear to be a relativily unusual, if not a rare, cause of gout'. This problem to our knowledge, has yet to be resolved.

Phosphoribosylpyrophosphate amidotransferase. (E.C.2.4.2.14)

Although this enzyme dominates the regulation of de novo biosynthesis of purines there is meagre evidence to date of its role in the pathogenesis of gout. Fibroblasts cultured from two patients with gout, who showed an increased production of uric acid, were found to be relatively insensitive to the effect of adenine and hypoxanthine on the early stages of purine biosynthesis. Henderson et al (1968) suggested that this insensitivity could be due to a mutation of one of the regulatory subunits of PRPP amidotransferase. Subsequently it was found that one of the patients had an increased PRPP synthetase activity (Becker et al 1973) which accounted for the increased PRPP in the fibroblasts. The second subject had normal PRPP synthetase and her lack of response to feedback inhibition has yet to be evaluated.

(Kelley and Wyngaarden 1974).

Hypoxanthine-guanine phosphoribosyltransferase HGPRT(E.C.2.4.2.8)

HGPRT catalysis the transfer of the phosphoribosyl moiety of PRPP to the 9 position of hypoxanthine and guanine to form IMP or GMP respectivily. Its affinity for different substrates has been reported by Krenetsky et al (1969). Xanthine as well as several purine analogues such as 6-mercaptopurinol and allopurinol act as substrates but PRPP is the only compound to serve as a donor of the phosphoribosyl moiety for HGPRT (McDonald and Kelley 1971).

A deficiency of the enzyme in man was found in patients with an unusual neurological disorder (Seegmiller et al 1967). Each of the patients had Lesch-Nyhan syndrome, an X linked disorder characterised by self-mutilation, spasticity, mental and growth retardation and hyperuricaemia due to an excessive production of uric acid (Lesch and Nyhan 1964). Shortly after (Kelley et al 1967) found some patients with gout also had a deficiency of this enzyme. But these gouty patients do not have the devastating neurological and behavioral features characteristic of Lesch-Nyhan syndrome. They generally had partial deficiency of the enzyme. The deficiency has been demonstrated in all tissues from affected subjects obtained at autopsy (Rosenbloom et al 1967),in fibroblasts and amniotic cells cultured at the time of amniocentesis. (Fujinoto et al 1968). From a consideration of enzymic activities alone, there is no clear cut off which will segregate the Lesch-Nyhan syndrome from gout. A partial deficiency of HGPRT has been reported in five members of two families having gout associated with overproduction of uric acid. The mutant enzymes of the two families

differ in their relative activity for the purine substrates hypoxanthine, guanine and adenine as well as in their heat stability (Kelley et al 1967). Other families with gout associated with a partial deficiency of HGPRT have been reported (e.g. Yu et al 1972, Fox et al 1975 Gutensohn and John 1979 and Edwards et al 1979) but the kinetic properties of the mutant enzyme indicates many variants of the enzyme with no single specific characteristic for the clinical condition. Edwards et al (1979) suggest that purine salvage is a major contributor to increased purine excretion and that the purine catabolic pathway responds differently to an increased substrate load in HGPRT deficiency. The purine salvage pathway is normally an important mechanism of the reutilization of hypoxanthine in man (Murray 1971). A deficiency of HGPRT was the first specific genetic variant demonstrated with primary gout and to date has been the most extensively studied.

Adenosine monophosphate-pyrophosphate phosphoribosyl transferase (APRT, E.C.2.4.2.7).

The activities of several enzymes are elevated in erythrocytes from patients with a deficiency of HGPRT. Many patients with a partial deficiency of HGPRT show increased activity of APRT (Kelley et al 1969). APRT deficiency has been reported in two families presenting with supposed 'uric acid' stones (Dean et al 1978, Simmonds et al 1978). The 'uric acid' stones were found to be 2,8-dehydroxyadenine and not associated with hyperuricaemia.

Glucose-6-phosphatase (E.C.3.1.3.9).

Patients with Type 1 glycogen storage disease or Von Gierke's disease cannot metabolise glucose-6-phosphate to glucose and inorganic phosphate because of glucose-6-

phosphatase deficiency. An associated complication of this disease is hyperuricaemia which frequently results in gout. The acidosis of serum lactate and ketone bodies resulting from hypoglycaemia interfere with renal secretion of uric acid (Yu et al 1957, Goldfinger et al 1965). Furthermore Alepa et al 1967 and Jacoveic and Sorensen 1967 have demonstrated an increased rate of uric acid synthesis de novo in these patients. Gout becomes significant in early adult life. The PRPP content of erythrocytes of the patients is normal (Greene and Seegmiller 1969) but this is not surprising since glucose-6-phosphatase is normally restricted to liver, kidneys and gastrointestinal tract.

Glutathione Reductase. (E.C.1.6.4.2.)

Glutathione reductase catalyses the reduction of glutathione with $NADPH+H^+$ or $NADH + H^+$ serving as hydrogen donors. Long (1967) has reported an increased activity of the enzyme in erythrocytes from 23/28 negro patients with gout and also in a group of Caucasians with untreated gout (Long 1962). Control screening of a large negro population showed that 31% had an increased enzymic activity and moreover the mean serum urate concentration was higher in the individuals with the increased glutathione reductase activity than in others with a "normal" enzymic activity (Long 1970). Beutler (1969) has reported that this enzyme activity is dependent on diet and it is accepted that not only pathology e.g. diabetes mellitus but the vitamins nicotinamide and riboflavin increase the activity of glutathione reductase (Beutler 1966). Changes in enzymic activity must be interpreted with reservation and not automatically be attributed to a pathological condition.

Increased glutathione reductase activity would increase $NADP^+$ production from the reduced coenzyme and an increased $NADP^+$/NADPH ratio would accelerate the pentose monophosphate shunt pathway with increased production of ribose-5-phosphate and subsequently PRPP by the following reactions.

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

$$G\text{-}6\text{-}P + NADP^+ \rightarrow D\text{ gluconø-5-lactone-6-phosphate} + NADPH$$

$$\xrightarrow{H_2O}$$

6-phospho-D-gluconate

$$\xrightarrow{+\ NADP^+}$$

D-ribulose-5-phosphate

D-ribose-5-phophate

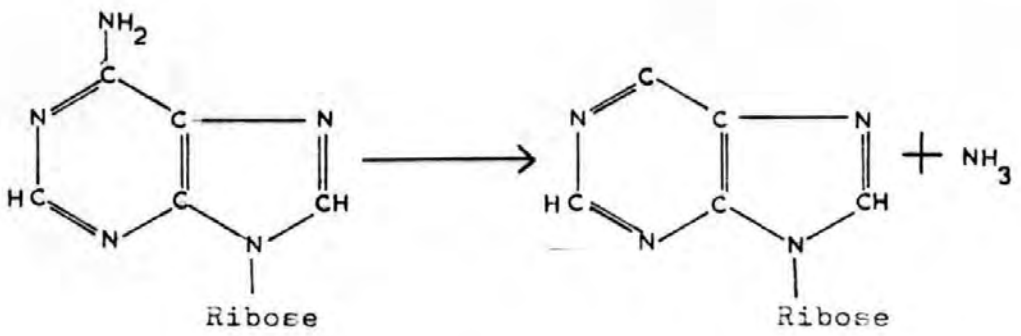
$$\xrightarrow{+\ ATP}$$

PRPP + AMP

The increased availability of PRPP would again increase purine biosynthesis de novo which may be a partial cause of the hyperuricaemia observed in gout.

Adenosine deaminase (E.C.3.5.4.4.)

This is a widely distributed enzyme which catalyses the deamination of adenosine to inosine and ammonia.



Adenosine Inosine

Diag.8 Deamination of adenosine.

Nishizawa et al (1975) studied ADA in patients with hyperuricaemia in primary gout and found increased enzymic activity in untreated cases. They did not indicate whether the increased enzymic activity was associated with primary or secondary gout.

Isoenzymes.

It is of historical interest that medical applications of multiple forms of an enzyme preceeded any biological understanding and the routine investigations of LDH isoenzymes in the clinical chemistry laboratory reflects the important role that these isoenzymes have assumed as an ancillary aid to diagnosis.

There is no particular isoenzyme that has been associated with either hyperuricaemia or gout but several electrophoretic variants have been reported in a few patients. These are summarised in Table 8.

Drug Therapy.

Agents currently used in the treatment of hyperuricaemia and gout can be conveniently divided into those used to treat an acute attack of gout by alleviating pain and inflammation and those used to control hyperuricaemia.

Treatment of an acute gouty attack.

Anti-inflammatory drug treatment should be started as soon as possible following the diagnosis of acute gout for the most effective response (Smyth 1972). Colchicine is the traditional drug of choice and it is relatively specific for acute gout (e.g.Wallace, 1977, Yu 1974) although Tabatabai (1978) reports that the drug is effective in pseudo gout (characterized by deposit of calcium pyrophosphate crystals in synovial fluid). Wallace (1972) has found that more than 90% of the

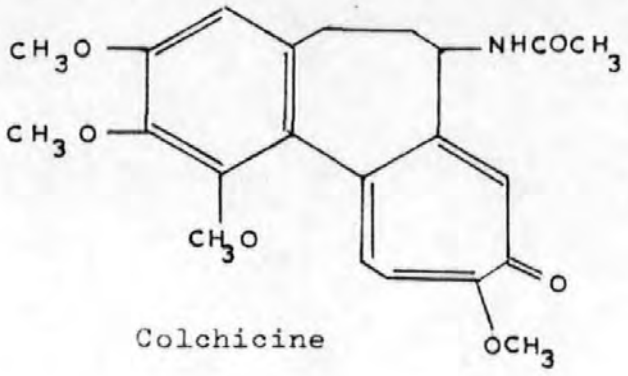
patients with gouty arthritis respond if colchicine is given orally within a few hours following the onset of an attack. There are however unpleasant side effects associated with colchicine: these are primarily gastro intestinal and include nausea, vomiting, cramps and diarrhoea. Gastrointestinal toxicity occurs in nearly 80% of patients having oral colchicine (Primer Rheumatic Diseases 1973). Sometimes these adverse effects occur before the beneficial effect of colchicine. Otherwise adverse effects include alopecia, neurotoxicity, myopathy, transient bone marrow depression and hepatotoxicity but these effects are usually associated with excessive parenteral colchicine or reduced hepatic and/or renal clearance of colchicine. (Golfinger, 1971, Wallace, 1974, Wyngaarden and Kelley, 1976).

Phenylbutazone is as effective as colchicine (Freyberg 1962, Gutman 1965) and untoward reactions occuring during the treatment of acute gout are rare (Smyth and Percy 1973) although gastro intestinal intolerance and fluid retention dominate the side effects that sometimes limit its usefulness. Relief is usually apparent within 6-24 hours and complete resolution of inflammation usually takes three to five days (Wyngaarden and Kelley 1976). Indomethacin has been shown to be as effective as phenylbutazone (Smyth and Percy 1973) but unpleasant side effects are not infrequent (Boardman and Hart 1965). A multi centre trial of naproxen and phenylbutazone in acute gout by Sturge et al (1977) showed that naproxen is a useful alternative agent to phenylbutazone for the treatment of acute gout and is of real value to patients with gastro intestinal intolerance to aspirin or indomethacin (Hill et al 1973; Kogstad 1973).

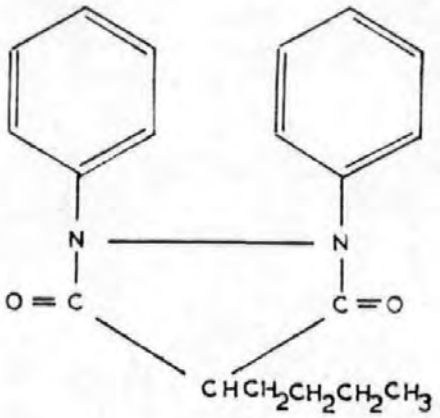
The mechanism of the intensely painful inflammation of the acute gouty joint is unknown. Activation of the Hagemann factor, complement and the kinin system have been implemented but subsequently rejected (Spilberg 1974, Phelps and McCarty 1969 Phelps et al 1966). The drugs are all potent inhibitors of prostaglandin synthetase (Taheguchi and Sih 1972, Flower et al 1973) suggesting that prostaglandins play a major part in the inflammation of acute gout. Colchicine does not inhibit prostaglandin synthesis and presumably acts by a different mechanism, colchicine therapy does appear to control serum glycosaminoglycan levels and the patient becomes asymptomatic when serum glycosaminoglycans fall to approximately normal values (Katz 1979). An inspection of the chemical formulae of these drugs would concur with different mechanistic action of colchicine and the other anti inflammatory agents. (Diag 9 shows the chemical structure of these drugs and allopurinol).
Fenoprofen and ibuprofen, recently marketed anti-inflammatory drugs, also appear to be effective in relieving the inflammation of acute gout with minimal adverse reactions (Wanasukapunt 1976, Schweitz et al 1978).

ACTH and corticosteroids may be used to treat acute gout but are primarily reserved for the 5% resistant cases (Wyngaarden and Kelley 1976). Rebound of attack may occur on steroid withdrawal but this can be overcome by colchicine therapy (Wyngaarden and Kelley 1976).

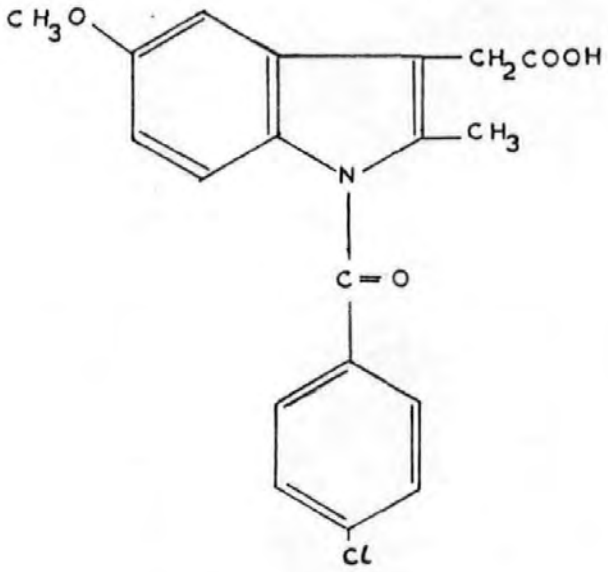
Smyth (1972) reports that prophylactic colchicine is useful in reducing the number of gouty attacks and that daily administration of 0·5-1·5 mg of the drug decreases the incidence of gouty attacks in as many as 75% of patients.



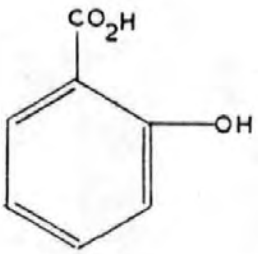
Colchicine



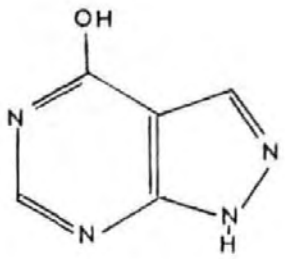
Phenylbutazone



Indomethicine



Salicylic acid



Allopurinol

Diag. 9. Chemical formulae of some anti-inflammatory drugs and allopurinol.

Control of hyperuricaemia.

Hyperuricaemia can be controlled with either uricosuric drugs or with the xanthine oxidase inhibitor allopurinol. Patients with moderate hyperuricaemia and serum urate concentrations less than 9.0mg per 100ml with just aches and pains or low back pain or other ill defined symptoms but without evidence of gout do not require treatment (Kelley 1977). In support of this, Fessel et al (1973) reported that in a group of 124 asymptomatic hyperuricaemic subjects followed over a four year period, the risk of gouty arthritis or nephrolithiasis was not higher than in 211 normouricaemic individuals.

The indications for urate lowering therapy according to Mangini (1979) are: a) frequent gouty attacks despite conservative treatment; b) chronic joint change with or without tophaceous deposits; c) uric acid nephrolithiasis; d) evidence of urate-induced renal damage e) uric acid level above 9mg/100ml and f) overproduction and hypoexcretion of uric acid. The aim being to maintain the serum uric acid level below 5.0-5.5mg/100ml.

Drug selection depends primarily on the mechanism of hyperuricaemia. In the overproducer/hypoexcreter allopurinol is the drug of choice while in the patient with normal renal excretion who excretes less than 600mg/day of uric acid on a purine restricted diet either class of drug can be used (Smyth, 1972). Urate lowering therapy should not be initiated until after an acute gouty attack has completely subsided (Wyngaarden and Kelley 1976).

Uricosuric Agents.

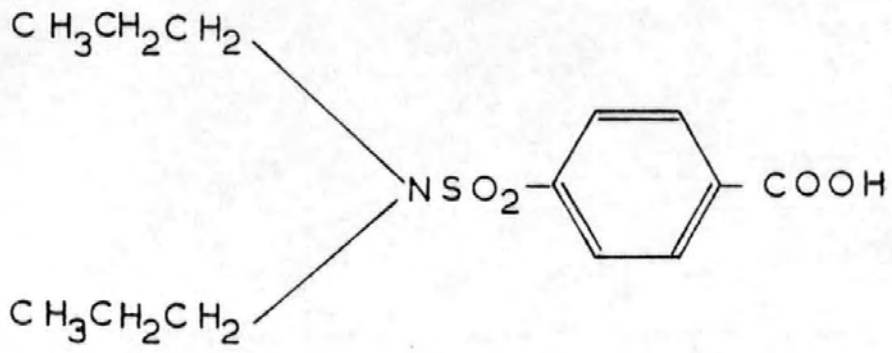
Uricosuric drugs inhibit both the presecretory and post secretary components of renal tubular resorption and increase the renal clearance of uric acid (Diamond and

Paoline 1973, Kelly 1975, Yu 1974, Fox 1977, Fanelli 1975). The pharmacological action of uricosurics is impaired in patients with renal insufficiency: increasing doses are required as renal function diminishes. When creatinine clearance falls below 25ml/min uricosurics are essentially ineffective regardless of the doses (Yu 1974, Fox 1977). Drug intolerance, impaired renal failure and aspirin therapy are the most common causes of treatment failure (Fox 1977). Reduction of serum uric acid by uricosuric s not only decreases the number of gouty attacks but prevents further tophaceous deposits. Preexisting tophi may be partially or completely resorbed (Smyth 1972, Fox 1977, Fanelli 1975).

The uricosuric agent of choice is probenecid. (Diag 10). It is readily absorbed from the gastrointestinal tract and becomes 90% protein bound. It is metabolised in the liver to active hydroxylated and carboxylated metabolites as well as inactive glucuronides (Fox 1977, Wyngaarden and Kelley 1976). Probenecid and its metabolites are excreted by the kidney but renal clearance can be markedly effected by urinary pH since the drug is a weak acid (Fox 1977, Wyngaarden and Kelley 1976). Excessive dosage must be avoided on commencing therapy to prevent marked uricosuria and the possibility of renal calculus formation (Fox 1977, Goldfinger 1971).

Approximately 60% of patients respond to probenecid (Wyngaarden and Kelley 1976). In patients allergic to probenecid, sulphinpyrazone is an effective alternative. It has a relatively short half life of three hours compared to probenecid (six to twelve hours).

Stone formation is a major complication of uricosuric treatment with about 10% of patients so treated likely to develop uric acid stones (Kelley 1975). Patients



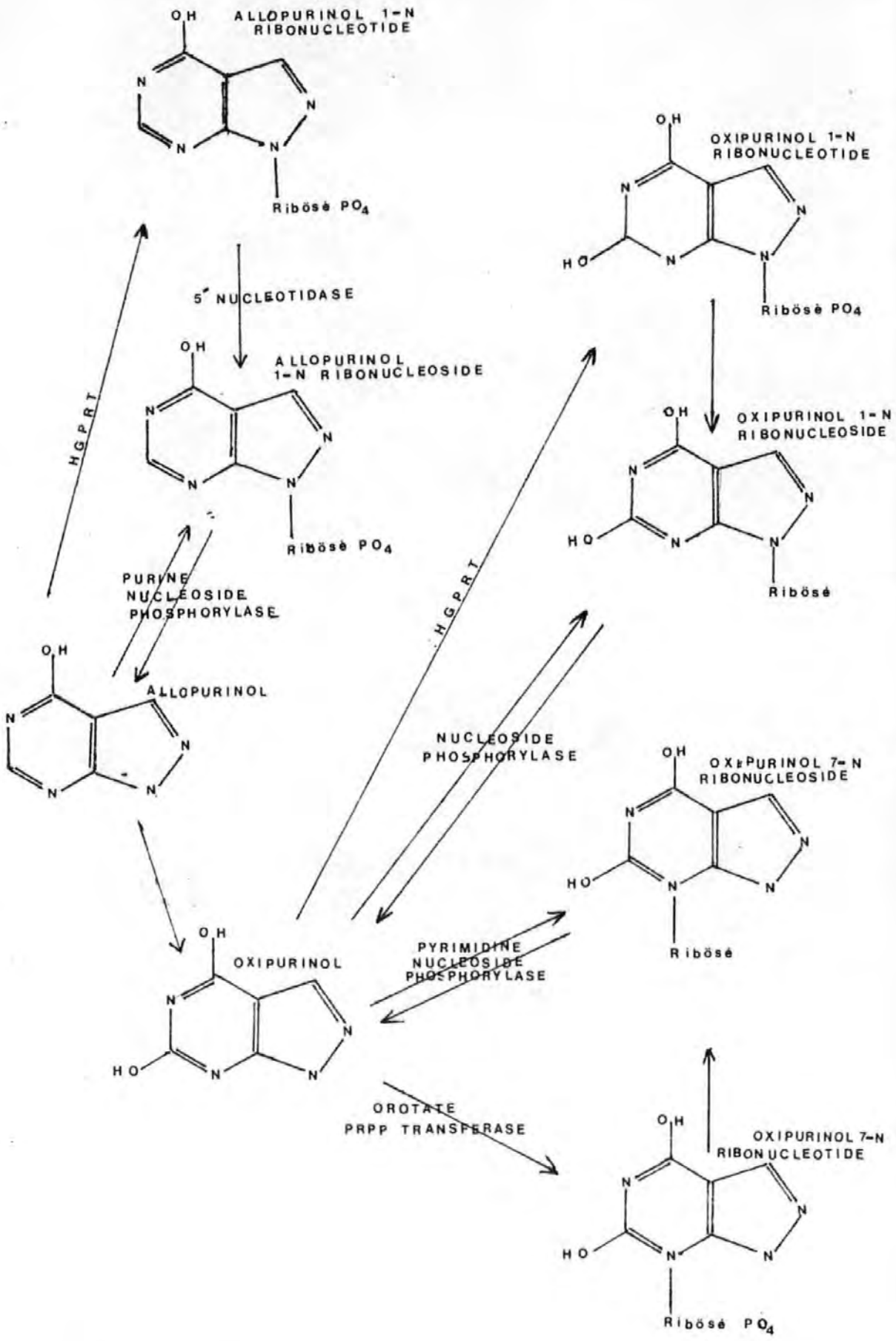
Diag. 10 Chemical structure of Probenicid

should be well hydrated and alkaline urine should be maintained during initiation of the therapy (Smyth 1972, Fox 1977). Other minor adverse effects to probenecid include gastrointestinal irritation (8-18% of recipients) and hypersensitivity reactions such as fever and rash (5%) but the drug has no cumulative toxicity and for this reason is often preferred to allopurinol for patients in whom either drug can be used (Fox 1977, Wyngaarden and Kelley 1976). Gastrointestinal complaints and hypersensitivity reactions are the major side effects of sulphinpyrazone but additionally the drug has antiplatelet activity and should be used with caution for patients with bleeding disorders or receiving anticoagulants (Kelley 1975).

A new class of uricosuric compounds, the benzofurans, have been discovered and tested, and benzbromarone has been found useful in the clinical control of hyperuricaemia (Sinclair and Fox 1975). This drug significantly increases the clearance of uric acid by a renal tubular effect and it has only a minor inhibitory effect on urate binding protein and xanthine oxidase. Only the renal tubular activity of the drug is relevant to its hyperuricaemic effects in man under normal circumstances. (Fox and Sinclair, 1977, Sorenson and Levison 1976).

Allopurinol

Allopurinol is the most effective drug for lowering serum uric acid. Both allopurinol and its long-acting metabolite, oxypurinol are xanthine oxidase inhibitors (Kelley 1975) thus preventing the formation of uric acid from xanthine. (Diag 11.) Inhibition of xanthine oxidase also reduces uric acid production by decreasing de novo



Diag. 11 Metabolism of Allopurinol.

purine synthesis as is evidenced by an overall reduction in the 24 hour excretion of uric acid, xanthine and hypoxanthine (Hitchings 1975). Allopurinol lowers the intracellular concentration of PRPP. It is a structural anologue of hypoxanthine and is converted to the ribonucleotide form by the action of HGPRT with the consumtion of PRPP (Fox et al 1970). Allopurinol has a relatively short half life of two or three hours but its metabolite oxypurinol has a half life of 28 hours and is slowly excreted by the kidney (Fox 1977).

Allopurinol is useful in treating all forms of hyperuricaemia and is the drug of choice when a) urinary uric acid exceeds 600 mg per day on a purine restricted diet where uricosurics may be hazardous because of the danger of uric acid stone formation b) there is a history of uric acid stones or calculi and c) hyperuricaemia is due to or is complicated by renal insufficiency (Kelley 1975, Fox 1977). Reduction in serum uric acid is dose dependent but low doses must be given in renal failure since elimination of oxypurinol depends on renal clearance.

Uric acid levels usually fall one or two days after treatment starts (Fox 1977) and the frequency of acute gouty attacks generally decreases within 3-6 months of allopurinol-induced normouricaemia. Tophi, if present, begin to resolve in size by six months (Fox 1977).

Side effects are usually minimal but initiation of treatment may induce exacerbations of acute gouty arthritis in 10% of the patients Kelley (1975). Gastrointestinal disturbances or minor skin rashes are common complaints in patients receiving allopurinol (Wyngaarden and Kelley 1976). However serious toxicity can occur and includes granulomatous hepatitis, vasculitis, agranulocytosis and toxic epidermal necrolysis (Jayobski et al 1970, Simmons et al 1972, Ulsinger and Yount 1976).

Nature of the problem undertaken.

The study of hyperuricaemia requires a rigid definition of the level of serum urate above which any patient will be classified as hyperuricaemic. In order to do this one must have confidence in the ability to measure uric acid in serum and do so accurately with reproduceable results. The large variety of assay methods at present available presents problems of selection. It therefore seems desirable to assess 3 or 4 different methods before deciding on our standard assay for uric acid measurement.

We have seen that there is much confusion regarding the nature, if any, of urate binding in plasma and it would seem profitable to ascertain whether we can find any evidence for urate binding not only in healthy control individuals but in hyperuricaemic individuals.

An analysis of previous work which has been condensed into the preceeding pages appears to indicate that it would be advisable to separate the known cases of gout from other hyperuricaemic individuals. Furthermore one must be prepared if necessary to subdivide the heterogenous collection of hyperuricaemics into further subgroups (see Table4). At this stage it seems desirable to confine our investigations to 3 main groups of individuals. The normal controls will consist of $>$ 20 healthy individuals with serum uric levels $<$ 7.0mg/100ml (420 µmol/l). Gout patients who have been refered to us with this clinical diagnosis will comprise the second group to be investigated. It may be necessary to subdivide this group into subclasses. e.g. those with tophi and those without tophi or to segregate the patients according to their drug therapy etc. The third group will be individuals who are hyperuricaemic according

to our definition. Heparinised blood samples will be collected from the three groups of individuals.

When one is considering overproduction states of a metabolite it is sensible to have regard to the biosynthetic pathways for the production or destruction of the matabolite. Uric acid is the end product of purine biosynthesis de novo and so we must investigate the production of uric acid. PRPP synthetase is not only required for purine biosynthesis de novo but it appears to be for rate limiting for purine synthesis and this pinpoints the enzyme for being of particular interest in purine overproduction states. But PRPP synthetase is reported to be an unstable enzyme which requires appropriate buffer conditions to maintain its activity during storage and manipulation and moreover the human enzyme is far less stable than that of rodent enzymes (Lebo and Martin 1976). In consequence little work has been done with this enzyme from human cells or tissues and most enzyme work on gout or hyperuricaemia has centred on HGPRT and other enzymes in the purine biosynthetic pathway. We have found in preliminary experiments that the human erythrocyte enzyme is more stable than we deduced from previous reports. We therefore decided to investigate the enzyme from erythrocytes of our three groups of individuals. It was proposed to study the enzyme kinetically and to examine the effect of inhibitors on all blood samples. By such means we hope to establish tests which would be useful in a clinical laboratory to not only differentiate between the three groups of individuals but to further segregate the apparent genetic heterogeneity which apparently occurs in gouty arthritis (Seegmiller 1975). Other

enzymes will be investigated as time permits so that each sample from patients with gout can be used to maximum effect.

Analysis of isoenzymes confers a greater specificity in diagnosis in the search for pathogenesis of various disease states than does analysis of total unseparated serum activity of the enzyme. Diagnostic specificity can be further increased by analysing several different isoenzyme systems especially when studied under a wide variety of different conditions not too remote to those prevailing in vivo. It is therefore proposed to investigate several isoenzymic systems. PRPP synthetase, HGPRT, glutathione reductase and ADA in the first instance to ascertain the frequency of any variants in our blood samples from the three groups of individuals. The genetic nature of such variants will be established by family studies.

It is hoped that the integration of our studies will shed some light on the complex nature of hyperuricaemia and gout and indicate the direction that future work should take.

Experimental Methods and Materials

1) Collection of blood samples.

10 ml venepuncture samples were collected in lithium heparin and were processed, in most cases, within 4 hours of the blood being taken. The samples were centrifuged at 3000 rpm for 10 minutes, the plasma separated, divided into small aliquots and kept frozen until required. The packed cells were washed three times with physiological saline and centrifuged at 3000 rpm for 10 minutes. At each separation care was taken to remove all traces of the buffy layer of cells. After the final wash, the saline was removed and the original volume of blood was restored by addition of physiological saline. A 1ml aliquot of the homogenous suspension of red cells in saline was added to 10ml ice cold water and mixed for 10 minutes on a rotor mixer. 8ml of phosphate buffer, (90 mmol/l, pH 7·4 containing 4 mmol/l EDTA) were added to the 'laked' cells and again mixed for 10 minutes on the rotor mixer prior to centrifugation at 3000 rpm for 10 minutes. The supernatant was aliquoted into plastic tubes and stored frozen until required; the remaining reconstituted cells were also stored frozen.

Preliminary experiments, over a period of time, had indicated that the activities of several enzymes, in particular PRPP synthetase, were best maintained in the presence of EDTA and high phosphate concentration. The presence of mercaptoethanol, reduced glutathione or no additive were unsatisfactory for maintaining the enzymic activity of PRPP synthetase.

a) Control samples.

Healthy volunteers from the technical staff were bled. Other controls were selected initially from O.P. samples after consultation of the relevant clinical summaries and ultimately by their plasma uric acid analysis. Any sample having a uric acid level greater than 420 μ mols/l was automatically transferred to the hyperuricaemic group of patients.

b) Hyperuricaemic samples.

This group of individuals consisted of two sub-groups: people with a metabolic high serum uric acid and hospital patients with uremia. All samples were checked for plasma uric acid content in excess of 420 μ mols/l. This criterion was breached by one individual. This was a lady with a long history of hyperuricaemia but whose serum uric acid was currently controlled chemotherapeutically with allopurinol. It seemed sensible to include this sample in this group in order to ascertain whether an unusual finding in her case might be attributed to the drug therapy and thereby justify further study with other individuals having similar chemotherapy.

c) Gout samples.

The letter shown in Diagram 12 was sent out to the G.Ps in the Torbay Hospital District, relevant O.P. clinics and hospital wards. The diagnosis of gout was the decision, based on clinical symptoms, of the patient's doctor. All specimens received from G. Ps were processed on arrival at the laboratory and it was assumed that they were despatched to Torbay Hospital within the 4 hours specified above. Our sample includes one case of secondary gout.

DR. BARRIE MURPHY, F.R.C. PATH.
DR. GERALD MANLEY, M.D., D.PHIL., M.R.C. PATH.
DR. LAURENCE HAYEK, M.A., M.R.C. PATH.
DR. CLIVE HAY, M.R.C. PATH.

TELEPHONE | 63112 DAY
64367 NIGHT

DEPARTMENT OF PATHOLOGY
TORBAY HOSPITAL
TORQUAY
TQ2 7AA

12th June 1980.

Diagram 12

Dear Colleague,

GOUT

PLEASE CAN YOU HELP US TO STUDY THE BIOCHEMICAL LESION ?

Gout appears to be a massively common diagnosis in the Torbay Health district. Every day in our laboratory we identify about a dozen cases of hyperuricaemia, and every week the Torbay Hospital Pharmacy issues about 400 Tablets of allopurinol. The cause of gout is, in the great majority of cases, unknown. Though there appears to be a genetic component; and suspicion is beginning to fall on enzyme variants in the purine pathway. The trouble is that many other conditions are associated with high uric acid levels – renal failure, dietary excess of purines or alcohol, certain forms of hyperlipoproteinaemia, etc. – and it is not at all easy to identify cases of true gout on the biochemical evidence we can obtain at present.

Mr Hardwell, chief MLSO in our Department, is actively studying the enzymes of the purine pathway in suspected cases of gout, with the aim of clarifying our understanding of the biochemical picture. The trouble is, we do need fresh blood samples from known & suspected cases, together with certain clinical data. We need to know the patient's name, age & sex, as always, also time & date of sample. But we also need to know in which clinical category the patient falls: (a) Undoubted gout with Tophi (b) Clinical gout without Tophi (c) hyperuricaemia of uncertain origin. It is also most important for us to know if the patient is on any drugs. Samples from new cases of undoubted gout, before treatment, will be especially valuable.

We require 10 ml + of venous blood for this study, in a lithium heparin (orange) tube, sent to the Chemical pathology Laboratory as soon as possible (Courier service should suffice) and marked FOR THE ATTENTION OF Mr HARDWELL.

We would be extremely grateful for any samples and data you can provide for us in this study, which we hope may help us to improve the biochemical diagnosis of gout.

Yours sincerely,

Gerald Manley.

To all Doctors
Torbay Health District.

2) Measurement of Uric Acid.

It was decided to compare several different assays for plasma uric acid and from a comparison of the results obtained, the method of choice would be selected for future analyses.

Assays were performed with a Technicon Autoanalyser I or a Gilford Stasar III spectrophotometer.

The four methods selected were (i) Technicon AAI phosphotungstate method monitored at 664 nm incorporating Crowley's modification with the addition of sodium carbonate; (Crowley,1964) (ii) an acid ferric reduction of urate with 2,4,6-tripyridyl-s-triazine measured at 593 nm (Morin, 1974); (iii) absorbance at 293 nm following uricase action (Haeckel 1979) and (iv) the Boehringer Mannheim Diagnostica Urica-quant enzymic colorimetric method involving a linked reaction of uricase with catalase in the presence of methanol and acetylacetone, monitored at 410 nm. Standard calibration curves from fixed concentrations of uric acid have been used throughout the analysis together with suitable 'blank' reactions in all cases.

It is appropriate at this stage to consider the outcome of these preliminary experiments in order to justify our ultimate method of assay. Sera analysed for uric acid content by the four methods gave a mean value per method as follows:-

Phosphotungstate	398·8 µmol/l
2,4,6-Tripyridyl-s-triazine	285·7 µmol/l
Uricase	333·3 µmol/l
Urica Quant	327·4 µmol/l

Correlation diagrams of these results for about 30 different sera are shown in figures 3, 4, and 5 . It is apparent that the phosphotungstate method overestimates the uric acid present by inclusion of miscellaneous nonurate reducing agents present in sera. Interference by such nonurate reducing agents may account for the random error found in the T P T Z method. By far the best correlation and precision was found with U V monitoring following the action of uricase and the colorimetric uricase assays. The colorimetric uricase method was adopted as the method of choice since it not only correlates closely with the uricase differential spectrophotometric method but it can be modified for the analysis of smaller aliquots of sera (0·2 ml instead of 1 ml) and is more convenient for batch analysis.

Urica-quant method for assay of serum uric acid.

This assay is based on the method of Kageyama (1971) and involves the following reactions:-

$$\text{Uric acid} + H_2O + O_2 \xrightarrow{\text{uricase}} \text{Allantoin} + H_2O_2 + CO_2$$

$$H_2O_2 + CH_3OH \xrightarrow{\text{catalase}} HCHO + 2H_2O$$

$$HCHO + 2\ \text{acetylacetone} + NH_3 \longrightarrow 3,5\text{-diacetyl-1,4-dihydro-lutidine} + 3H_2O$$

Reagents.

Solution 1 Ammonium phosphate buffer 0·6 mol/l pH 7·0, methanol 1·7 mol/l, catalase 700 u/ml.

Solution 2 Acetylacetone 0·42 mol/l, methanol 2·5 mol/l.

Standard uric acid. 357 μ mol/l.

Uricase 5 u/ml in 50% aqueous glycerol.

Uric acid reagent prepared by adding 5ml solution 2 to 100ml of solution 1. This reagent is stable for 1 month when stored at + 4°.

Procedure.

The sample blank is prepared by adding 0·2 ml plasma or serum to 2 ml of the uric acid reagent and mixed. 1 ml of this solution is added to 10 μl of uricase and mixed. The two solutions are incubated for 1 hour at 37° in the absence of sunlight. The absorbance of the sample is measured against the sample blank at 410 nm. A uric acid standard is read against a reagent blank in the absence of serum. All assays were at least duplicated.

Uric acid content of plasma = $357 \cdot \frac{A \text{ sample}}{A \text{ standard}}$ μ mol/l

where A_{sample} is absorbance of sample against sample blank at 410 nm and $A_{standard}$ is absorbance of standard against reagent blank at 410 nm. A reference sera is also included in each batch as a routine precaution.

3) Measurement of Protein content of haemolysates.

Biuret reagent.

Sodium potassium tartrate 14g, cupric sulphate 3g, potassium iodide 5g, sodium hydroxide 8g, surfactant in 1 litre aqueous solution.

Total protein blank solution.

Potassium iodide 4·0g, sodium hydroxide 6·4g,

surfactant in 1 litre aqueous solution.

Instrument- Gilford Stasar III with manual cuvette.

Method- 1 ml Biuret reagent is added to 20 µl haemolysate and allowed to stand for 15 minutes at room temperature after mixing. The blank solution is treated similarly after the addition of 1 ml of the protein blank solution to 20 µl of haemolysate. The absorbance of the test solution is read at 550 nm against a human protein standard solution using the blank reaction. Technicon human reference serum diluted to contain 14·4 g/l is used to prepare the standard which is treated as described above after the addition of 1 ml of biuret reagent to 20 µl of reference serum.

4) Urate binding to plasma proteins.

(a) Occlusion at diastolic pressure.

Haemostasis was produced in healthy "volunteers" by means of a sphygmomanometer cuff applied to the upper arm and maintained at diastolic pressure for 10 minutes. Blood samples were obtained after timed intervals by venepuncture from a canula with a tap. All samples were left to retract at room temperature for 2 hours, unless otherwise stated, prior to centrifugation at 3000 r p m for 10 minutes. The serum was collected and either stored at 4^{o} for not more than 6 hours before analysis or incubated to 37^{o} prior to assay. γ - globulins, uric acid and T_4 estimations were performed manually whilst other assays were automated. These methods together with the appropriate flow diagrams are included in the Appendix I.

(b) Ultrafiltration

Membrane ultrafiltration has been used to prepare protein-free filtrates of serum for the assay of free uric acid and protein-bound uric acid in the residium. 5 ml serum was placed in a Centriflo C F 25 cone (presoaked in distilled water for 1 hour) inserted in a centrifuge tube and spun for 20 minutes at 3000 r p m at room temperature. The uric acid content of the filtrate and untreated serum was estimated by the uricase method. All samples were analysed on the day of collection.

(c) Electrophoresis

A Shandon electrophoresis tank U77 was used with a Vokam power pack.

Gel buffer: Barbitone 0·07 M, pH 7·6. Prepared by dissolving 1·66g barbitone and 12·76 sodium diethyl barbitone in distilled water to 1 litre.

Bridge buffer: Barbitone 0·058M, pH 7·6. Prepared by diluting 100 ml gel buffer with 20 ml distilled water.

Cellogel cellulose acetate blocks (0·25 x 6 x 17 cms) were immersed in gel buffer for 2 hours prior to electrophoresis at 140 volts, 25-30 milliamps for 4½ hours at room temperature. The gels were subsequently stained for uric acid and counter stained for protein.

The visual detection of uric acid was a modification of the method of Haeckel (1976) involving the following reactions:-

$$\text{uric acid} \xrightarrow{\text{uricase}} H_2O_2 + \text{allantoin}$$

$$H_2O_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde}$$

$$\text{acetaldehyde} + NAD^{\pm} \xrightarrow[\text{dehydrogenase}]{\text{aldehyde}} \text{acetate} + NADPH + \overset{\pm}{H}$$

$$NADPH + \overset{\pm}{H} + NBT \xrightarrow{PMS} NADP + \text{Formazan}$$

The reagent was prepared by reconstituting lyophilized reagent obtained from Smith Kline Instruments Ltd with a supplied diluent. Each gel was immersed in 20 ml of the reconstituted reagent, containing 150 ul uricase, 3 mg NBT and 3 mg PMS, for 30 minutes. The fortified reagent was used within 10 minutes of preparation.

The cellulose acetate block was counterstained for protein by soaking in 3% aqueous trichloracetic acid containing 0·2% Ponceau S for 30 minutes. 5% aqueous acetic acid was used to remove excess stain and after washing, the cellulose acetate was dried between sheets of blotting paper.

d) Column chromatography.

0·2 ml serum was applied to a 50 x 2 cm diameter column packed with Sephadex G200 to separate the serum into molecular size fractions. Physiological saline was used as eluant and 0·5 ml fractions were collected throughout the elution. The protein content of the eluant was monitored continuously by recording the absorbance at 280 nm on a LKB Uvicord 254. Each fraction was evaporated to dryness in order to concentrate the amount of uric acid present prior to assay. The presence of uric acid was measured colorimetrically at 664 nm using phosphotungstate in the presence of sodium carbonate.

5) Hyperlipidaemia

10 ml venepuncture samples were taken from patients after a 10 hour period of fasting. No anticoagulant was added to the samples which were allowed to retract for 1 hour prior to centrifugation at 3000 rpm for 10 minutes. The serum was separated and kept at 4^{o}

until analysed for cholesterol and triglyceride content. Abnormal levels of either constituent indicated further analysis by lipid electrophoresis and nephelometry. All patients showing a chylomicron fraction after lipid electrophoresis had the rigidity of the fasting period queried but if a repeat specimen showed the same distribution of lipid electrophoretic bands the sample was designated Type I. The analytical methods for these investigations, which are routine procedure in a clinical laboratory, are outlined in the Appendix I.

All sera showing hyperlipidaemia were segregated into their Frederickson type and assayed for their uric acid content. The sera from a control group of individuals with normal cholesterol and triglyceride content following a 10 hour fasting period were also investigated for their uric acid content.

(6)Distribution of uric acid.

a) Erythrocytes

Ion exchange resin: Dowex 2XS (100-200 mesh) in the acetate form using a 2cm x 0·4 cm diameter column.

Eluant 500 ml M HCl and 500 ml M NaCl

10 ml fresh venepuncture blood collected in lithium heparin was centrifuged at 3000 rpm and the plasma and buffy coat removed. The cells were washed twice with physiological saline. 1 ml of the packed cells were diluted 1:10 with distilled water and haemolysed by freeze thawing twice. 0·5 ml of the haemolysate was passed through the ion exchange column. The column was washed twice with 10 ml distilled water and the washings discarded prior to elution with 5 ml of the HCl-NaCl eluant.

The eluant was monitored at 293 nm in a Perkin Elmer 402 scanning spectrophotometer. Many haemolysates were examined by this technique which is a modification of that used by Carr and Pressman (1962).

b) Leucocytes

Stock phosphate buffer pH 7·0

Na_2HPO_4 8·52g

KH_2PO_4 5·44g

made up to 1 litre with H_2O

Glucose oxidase/peroxidase solution: Fermcozyme 952DM, supplied by Hughes and Hughes Ltd.

Working colour reagent

Stock phosphate buffer, pH7·0, 295ml; Fermcozyme 952 DM, 5ml; sodium azide, 300 mg; phenol, 300 mg; 4-aminophenazone, 100 mg;

Uric acid standard ≡ 120 µmol/l

Uricase ≡ 5 u/ml

Preparation of leucocytes

Reagent Lomodex 70 (Leuconostoc mesenteroides N.C.T.C) obtained from Fisons Ltd. (Dextran 70 is a 6% w/v starch solution in 0·9% saline. The average molecular weight of the dextrans is ~ 70,000).

BTL Intermediate centrifuge (1600 rpm ≡ 600g)

Method

2 ml dextran was added to 10 ml heparinised plasma, gently mixed and the sample allowed to separate into two layers at room temperature (approx. 45 minutes). The upper layer was separated from the red cells prior to centrifugation for 10 minutes at 1600 rpm. The supernatant was discarded, the pellet washed with 4 ml

0·9% saline and centrifuged at 1600 r.p.m. The supernant was discarded and 2 ml water added to the pellet followed by 2 ml 1·8% sodium chloride. Following centrifugation at 1600 r.p.m. the supernant was discarded and the pellet was freeze thawed thrice in isopropanol. The leucocyte preparation was diluted with distilled water to contain 12 g/l protein immediately prior to use.

1·0 ml of colour reagent and 0·1 ml uricase were added to 0·1 ml leucocytes in a test tube. A blank and a control tube were also prepared by substituting 0·1 ml water and 0·1 ml uric acid standard for the 0·1 ml leucocyte. The tubes were examined during the course of the day and at 48 hours. The experiment was repeated but with liquid paraffin covering the solution in the three tubes to exclude atmospheric oxidation. Several leucocyte preparations were examined by this technique.

7) Enzymology

a) PRPP synthetase

The assay involves the measurement of AMP production and is essentially a modification of that reported by Valentine and Kurschner (1972) which uses the following linked indicator system:-

$$\text{R-5-P} + \text{ATP} \xrightarrow{\text{PRPP synthetase}} \text{PRPP} + \text{AMP}$$

$$\text{AMP} + \text{ATP} \xrightarrow{\text{adenylic kinase (AK)}} 2\text{ADP}$$

$$\text{PEP} + \text{ADP} \xrightarrow{\text{pyruvic kinase (PK)}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + \overset{+}{\text{H}} \xrightarrow{\text{lactic dehydrogenase (LDH)}} \text{lactate} + \text{NAD}^{+}$$

Buffer Stock solutions $Na_2 HPO_4 2H_2O$ 16·02 g/l Solution A

$K H_2PO_4$ 12·25 g/l Solution B

Working solution 276 ml Solution A and 24 ml Solution B yield a buffer 90 mM phosphate which is routinely checked for pH 7·88 Solution C.

Stock reagent : $MgCl_2$ 81·3 mg; KF 38·6 mg; PEP (mono-cyclohexyl ammonium salts) 106·9 mg; ATP (disodium salt) 161 mg contained in 150 ml phosphate buffer C. The pH, 7·4, is checked before use. Solution is stable for 1 week at 4°.

Working reagent : NADH 13·2 mg, AK 200 µl, PK 80 µl, LDH 60 µl are added to 60 ml of stock reagent and the volume is made up to 80 ml with water. This solution is stable for 4 hours at room temperature.

R-5-P (disodium salt) stock solution. 19 mg R-5-P per ml water. Stable for 1 week at 4°.

(i) Assay of enzymic activity.

2 ml working reagent and 200 µl haemolysate are added to a test and blank cuvette and incubated at 37° for 30 minutes. The reaction is initiated by addition of 20 µl of stock R-5-P to the test cuvette and 20 µl water to the blank. The change in absorbance at 340 nm at 37° is measured at 5 minute intervals for 30 minutes on a MSE Vitatron or monitored continuously on a Gilford 3500 rate analyser linked to a recorder. The final concentration in m mol/l of reagents in the assay cuvette are $MgCl_2$ 1·8; ATP 1·2, PEP 1·8, KF 3·0, NADH 0·21, R-5-P 0·60 in 61 mM phosphate buffer pH 7·4.

Calculations for PRPP synthetase.

Molar extinction NADH = 6220

Enzymic activity as μ mol/mg/ml protein/hr

$$= \frac{\Delta \cdot A \times 2 \cdot 22 \times 10^6}{2 \times 6220 \times 0 \cdot 2}$$

$$= 892 \cdot 3 \ \Delta \ A$$

where Δ A = change in absorbance/hour

The effect of variation in concentration of the substrates, magnesium and phosphate was studied by preparing the reagents at the above concentrations but omitting the substance being investigated. Varying amounts of the later were then diluted and the effect on enzymic activity at each concentration measured.

(ii) Variation of R-5-P

Stock solution of R-5-P: 274 mg R-5-P was dissolved in 5 ml aqueous solution (200 mmol/l)

A range of concentrations of R-5-P from 0·002 to 2·0 mmol/l was prepared in water and 20 μl aliquots added to a series of reaction cuvettes prepared as indicated above. The assay was monitored on a MSE Vitatron and were at least duplicated.

(iii) Effect of pH

The pH of the buffer solution was varied by altering the relative amounts of phosphate solutions A and B. A pH greater than 8 could not be obtained since complex magnesium phosphates are precipitated on addition of the standard reagents. A range of pH values from 6·0 to 8·0 was studied using the procedure for the usual assay.

(iv) Magnesium concentration curve.

A Gilford 3500 enzyme rate analyser was programmed for a preincubation time of zero, an incubation time of 3 minutes and a read time of 20 seconds to study the variation of magnesium concentration at constant R-5-P (0·60 mmol/l) and ATP (1·2 mmol/l) concentrations. This instrument was used for these investigations to enable a result to be obtained before the reagents complex to form an ammonium magnesium phosphate precipitate.

Double strength reagents, containing no magnesium, and a suitable dilution of magnesium were placed on the two dispenser towers of the instrument. This procedure was repeated for various concentrations of magnesium and the ratio Mg^{2+}: ATP in the final reaction mixture were 1·00, 1·33, 3·33 and 13·33.

(v) ATP concentration curve.

Stock solution of disodium ATP

110 mmol/l solution was prepared by dissolving 121·04 mg disodium ATP in 20 ml aqueous solution.

Other reagents, except for R-5-P and ATP were prepared in 4/3 times the concentration used for assay of PRPP synthetase. 0·5 ml aliquots of the different concentrations of ATP were added to 1·5 ml of the working reagents in a series of cuvettes. A blank cuvette contained 0·5 ml water and 1·5 ml of the reagents.

The reaction was initiated after incubation for ½ hr. at 37° by addition of 20 µl R-5-P to all cuvettes. An additional cuvette was set up using the standard assay reagents except for R-5-P to which was added 20 µl water. The reaction was monitored at 340 nm at 37° using a MSE Vitatron.

(vi) Variation in phosphate concentration

This experiment was designed to vary the phosphate concentration but maintaining the buffer capacity of the reaction. This was achieved by preparing stock Tris/HCl and phosphate buffers at90 mM, pH 7·4

Tris buffer

Stock solution : 43.61g Tris made up to 1 litre with water (360 mM)

Working buffer : 50 ml stock Tris buffer and 144 ml 0·1 M HCl diluted to 200 ml with water (90 mM, pH 7·4)

Phosphate buffer prepared as for the assay of total enzymic activity. The reagents were prepared in both Tris/HCl (solution X) and phosphate buffer (solution Y), the pH of both solutions were adjusted to 7·4. Four working solutions of the reagents containing varying amounts of phosphate, were prepared from appropriate mixtures of solutions X and Y as shown in the following Table 9.

Table. 9 Phosphate content of reaction mixture.

Phosphate content	Solution X(ml)	Solution Y(ml)
None	30	0
1 mM	29·5	0·5
20mM	20·1	9·9
60mM	0	30

Haemolysates were prepared in water from freshly collected heparinised blood samples.

Conditions and procedure of assay for enzymic activity were identical with those used for total PRPP synthetase activity.

(vii) Inhibition studies

The working reagent solution is prepared at 4/3 times the concentration for all reagents cited for the assay of PRPP synthetase. The following procedure is used for all inhibition studies, only the nature and concentration of the inhibitor are varied.

200 µl of haemolysate is added to 2 test and 1 blank cuvettes containing 1·5 ml of working reagent. 500 µl of inhibitor is added to one of the cuvettes and 500 µl of water is added to the other two cuvettes. The solutions are incubated at 37°, 20 µl R-5-P is added to the 2 test cuvettes and 20 µl water is added to the blank cuvette. The progress of the 2 reactions, one inhibited and the other uninhibited is monitored for 30 minutes at 340 nm at 37° against the blank reaction.

Calculation

$$\% \text{ Inhibition} = \frac{(A_{\text{uninhibited}} - A_{\text{inhibited}})}{A_{\text{uninhibited}}} \times 100$$

Where $A_{\text{uninhibited}}$ is absorbance of uninhibited reaction after blank correction $A_{\text{inhibited}}$, is absorbance of inhibited reaction after blank correction during a fixed time interval (say 20 minutes) provided that all reactions are linear.

The inhibitors studied and the range of concentration investigated are given in Table 10 . or using fixed concentration Table 11 .

b) Glutathione Reductase.

In the presence of NADH or NADPH, the flavin enzyme glutathione reductase catalyses the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH)

$$GSSG + NADH + H^+ \xrightarrow{\text{Glutathione reductase}} 2GSH + NAD^+$$

Buffer Stock solution A) 0·2M Na_2HPO_4 $2H_2O$ 35·61 g/l

B) 0·2M Na H_2PO_4 $2H_2O$ 31·21 g/l

Working buffer solution

C) 32 ml A, 68 ml B and 2·92 mg EDTA.

Reagent: To 40 ml buffer C) add 6·6 mg NADH

Stock GSSG 139·7 mg GSSG in 2 ml water (114 mmol/l)

Method: 200 µl haemolysate or plasma is added to each of two cuvettes containing 1·8 ml working reagent and incubated for 10 minutes at 37°, 20 µl substrate (stock GSSG) is added to one cuvette and 20 µl water added to blank. The change in absorbance of the substrate against the blank is monitored at 340 nm for 10 minutes at 2 minute intervals using an MSE Vitatron.

Reaction strength of reagents GSSH = 1·13 mmol/l

NADH = 0·23 mmol/l

Calculations for Glutathione reductase.

Molar extinction coefficient for NADH = 6220

Enzymic activity as µ mol/mg/ml protein/hr

$$= \frac{\Delta A \times 2{\cdot}02 \times 10^6}{0{\cdot}2 \times 6220}$$

$$= 1623{\cdot}8 \ \Delta A$$

where ΔA = change in absorbance/hr

For Plasma

Enzymic activity = $\Delta A \times 1623{\cdot}8$ µ mol/ml/hr

(c) Adenosine Deaminase

Serum adenosine deaminase is assayed by using an indophenol reaction to monitor the ammonia liberated. (Martinek 1963).

$$\text{Adenosine} \xrightarrow{ADA} \text{Inosine} + \text{Ammonia}$$

Buffered substrate: 1·79 g Na_2HPO_4 and 0·78 g KH_2PO_4 are dissolved in 50 ml preboiled distilled water. 18 mg adenosine are then added and the volume made up to 100 ml using the same water. The pH, 7·05, is checked. Reagent is stable for 3 months at 4°.

Phenol colour reagent: 5·4 ml liquified phenol and 25 mg sodium nitroferricyanide were dissolved in 500 ml distilled water. This solution is stable for 3 months at 4°.

Alkaline hypochlorite reagent: 62·5 ml M NaOH and 4·2 ml sodium hypochlorite (5%) were made up to 500 ml with distilled water. Reagent stable for 1 year at 4°.

Nitrogen standard

Stock solution: 353·9 mg anhydrous ammonium sulphate made up to 250 ml in distilled water. 1 ml of this solution contains 0·3 mg of nitrogen.

Working solution: The stock solution was diluted 1 in 5 so that 1 ml of this solution contains 60 µg nitrogen.

Method.

A blank and test cuvette containing 0·5 ml buffered substrate are incubated at 37° for 5 minutes prior to the addition of 50 µl of unhaemolysed serum or plasma to the test cuvette. The cuvettes are stoppered and mixed prior to incubation at 37° for 1 hour. 2·5 ml phenol colour reagent is added to both cuvettes. This reagent stops the reaction. 50 µl of the serum is added to the blank cuvette and 2·5 ml of the alkaline hypochlorite reagent added to both tubes. Each cuvette is mixed and incubated for 15 minutes at 37° for colour development. The colour is stable for 20 minutes. The absorbance of the test is read at 640 nm on a Gilford Stasar III spectrophotometer against the reagent blank adjusted to 100% transmittance.

A calibration curve is prepared by substituting 50 µl of standard solutions of differing nitrogen content for 50 µl serum in the usual assay procedure. A graph, which is checked at regular intervals, relates the units of ADA activity to absorbance. One unit of ADA activity is the amount that will liberate 1 µg of ammonium nitrogen per ml of serum or plasma per hour at 37°.

d) Hypoxanthine-guanine phosphoribosyl transferase.

This analysis measures GTP production and is essentially the continuous spectrophotometric assay reported by Giacomello and Salerno (1977) which uses the following linked reactions

$$\text{Guanine} + \text{PRPP} \xrightarrow{\text{HGPRT}} \text{GMP} + \text{PP}_i$$

$$\text{GMP} + \text{ATP} \xrightarrow{\text{GK}} \text{ADP} + \text{GDP}$$

$$\text{ADP} + \text{GDP} + 2\text{PEP} \xrightarrow[Mg^{++}, K^{+}]{\text{PK}} \text{ATP} + \text{GTP} + 2 \text{ pyruvate}$$

$$2 \text{ pyruvate} + 2\text{NADH} + 2H^{+} \xrightarrow{\text{LDH}} 2 \text{ lactate} + 2NAD^{+}$$

Buffer Tris/HCl, 0·05 M, pH 7·5 consisting of 25 ml 0·2 M Tris and 40 ml 0·1 M HCl made up to 100 ml with water.

Stock reagent.

ATP (disodium salt) 36·3 mg, PEP (monocyclohexyl-ammonium salt) 46·5 mg, $MgSO_4\ 7H_2O$ 985·9 mg and KCl 969·2 mg made up to 100 ml with Tris HCl buffer.

Stock guanine solution.

17·8 mg guanine made up to 50 ml with distilled water containing 1 ml 0·1 M NaOH.

Working reagent.

Disodium NADH 2.28 mg, stock guanine solution 1·7 ml PK 32·0 µl LDH 16·0 µl GK 200 µl made up to 18 ml with stock reagent.

Stock PRPP solution. Tetra sodium PRPP 16·73 mg dissolved in 250 ml water. Prepared immediately before use.

Method.

1·8 ml working reagent and 200 µl of haemolysate preparation contained in 2 cuvettes are incubated for 10 minutes at 37°. 20 µl stock PRPP solution is added to the test cuvette and 20 µl water added to the blank cuvette. The reaction is monitored at 2 minute intervals for 30 minutes at 340 nm on a MSE Vitatron.

The reaction mixture contains a final concentration of reagents of Tris/HCl 0·05M, pH 7·5; 0·6 ATP; 1·0 PEP; 40 $MgSO_4$; 130 KCl; 0·16 NADH; 0·2 guanine; 1·0 PRPP (mmol/l) and 0·21 u/ml GK; 3·2 u/ml PK and 3·2 u/ml LDH.

8) Electrophoretic methods.

(1) Agarose electrophoresis.

Reagents. Litex agarose HSIF with low electroendosmosis and Gel Bond electrophoresis film from Marine Colloids Ltd. Other reagents and enzymes obtained from BDH Ltd or Boehringer Corporation Ltd.

Shandon Vokam power pack.

Hoeoscht electrophoresis tank with cooling plates.

Stock bridge and gel buffer.

Sodium barbitone 20·6g, barbitone 3·7g, Calcium lactate 0·5g made up to 1 litre with water.

Working bridge and gel buffer.

The stock buffer is diluted 1 in 2. This gives a working buffer of ionic strength 0·05, pH 8·6

Preparation of gel.

1% (w/v) agarose was prepared in the buffer. The solution was magnetically stirred and heated to the boil to clarify it. 30 ml aliquots can be stored at 4° for 4 days.

The gel was cast by placing a sheet of Gel Bond film (114x225 mm) with the hydrophilic side uppermost onto a wetted glass plate of the same dimension. Excess water was squeezed from under the film and a gel casting frame placed over the film and clamped into position with spring clips.

The film and casting frame were placed on a levelling table and prewarmed with a hair dryer before the agarose was poured. When the gel had set a scalpel was run around the edge of the casting frame prior to its removal. The agarose plates were then kept at 4° for 1 hour before use.

Sample application.

5 µl of sample was applied to precut wells.

Running conditions.

All gels were run at constant current of 80 mA for 1½ hours and cooled to 10°.

(II) Agarose isoelectrofocusing.

Agarose IEF and ampholines obtained from Pharmacia Fine Chemicals.

Other reagents from BDH.

LKB 2103 High voltage power supply.

LKB 2117 Multiphor electrophoresis tank.

Gel composition.

Agarose (IEF) 0·3g; Sorbitol 3·6g, distilled water 27·0 ml and ampholine (range 3·5-9·5) 1·9 ml.

Gel preparation.

The agarose and sorbitol were dissolved in water, heated with magnetic stirring until a clear solution was obtained. The solution was cooled to 60°, the ampholine added and, after mixing, the solution was poured onto a Gel Bond film prewarmed and prepared as outlined for agarose electrophoresis.

Electrode solutions.

Cathode 1M NaOH

Anode 0·05M H_2SO_4

Sample application.

Samples were obtained from the deep frozen stock of haemolysates. After thawing, 2 mercaptoethanol was added to give a final concentration of 20 mmol/l. 20 µl samples applied to gels on filter paper applicators which are removed after 45 minutes.

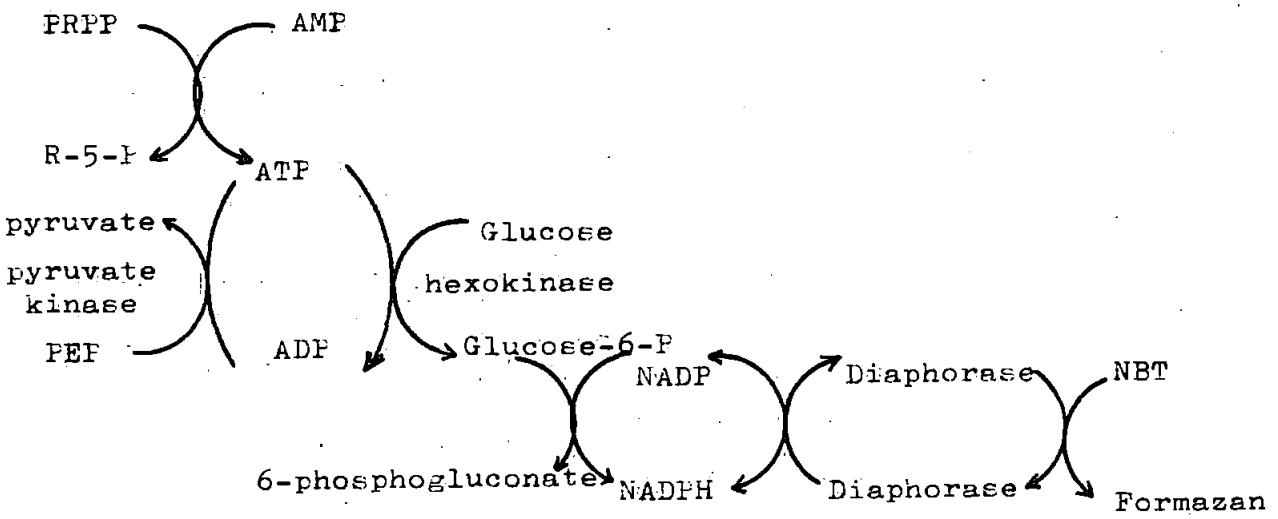
Running conditions.

The constant power supply was set to deliver a maximum of 15W at 1500 V. The gels were run for 1½ hours.

(iii) Staining methods.

a) PRPP synthetase isoenzymes.

This method is based on the Switzer (1968) assay for enzymic activity. It is essentially the reverse of the reaction used for assay in the current study. Switzer's method has been modified to include an ATP regenerating stage in an attempt to intensify the staining reaction. (Lebo and Martin 1977).



Stain preparation.

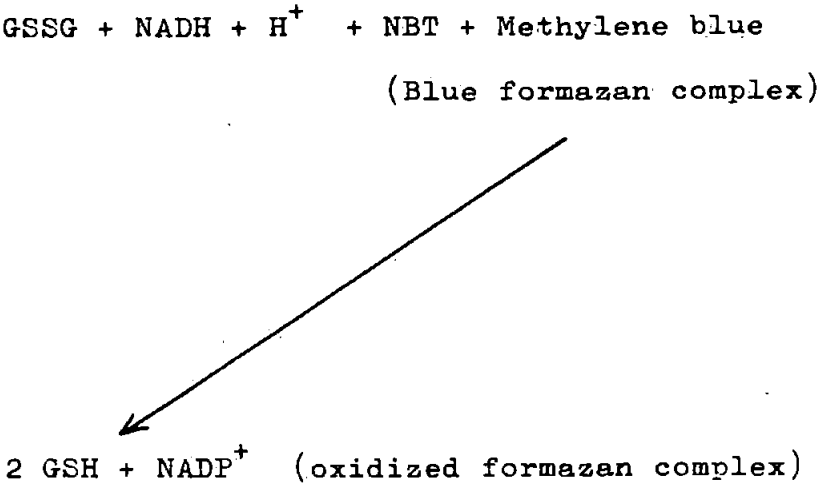
Sorensen's phosphate buffer pH 7·4, 60 mM containing 2 mmol/l magnesium chloride and 2 mmol/l EDTA.
D glucose 1·8 mg, disodium PRPP 4·7 mg. AMP 7·0 mg, $NADP^+$ 3·3 mg, G-6-PD/HK in glycerol 100 µl (180 u/ml/100 u/ml) PK 10 µl, diaphorase 20 µl (1050 u/ml), NBT 3 mg made up to 10 ml with buffer.

Stain application.

10 ml stain reagent was added to 10 ml of 2% Oxoid ion agar in water at 60°. The agar was poured quickly and evenly as an overlay to the film following electrophoresis. The film was incubated for 2 hours in the dark at 37° for the colour reaction development.

b) Glutathione reductase isoenzymes.

A modified method of Long's (1967) two stage method employing a reverse form agar staining technique has been developed.



Stain buffer

Phosphate	100 mM pH 7·4	
Stock buffer solutions	200 mM	
	$Na_2HPO_4 2H_2O$	35·61g/l
	$NaH_2PO_4 2H_2O$	31·21g/l

Working buffer solution

Stock	$Na_2HPO_4 2H_2O$	81·0 ml
Stock	$NaH_2PO_4 2H_2O$	19·0 ml
	EDTA	175 mg
	KCl	11 mg

made up to 200 ml with distilled water.

Staining solution.

NADPH 6mg, GSSG 15mg, NBT 9mg, 0·5% methylene blue 200 µl made up to 10 ml with stain buffer.

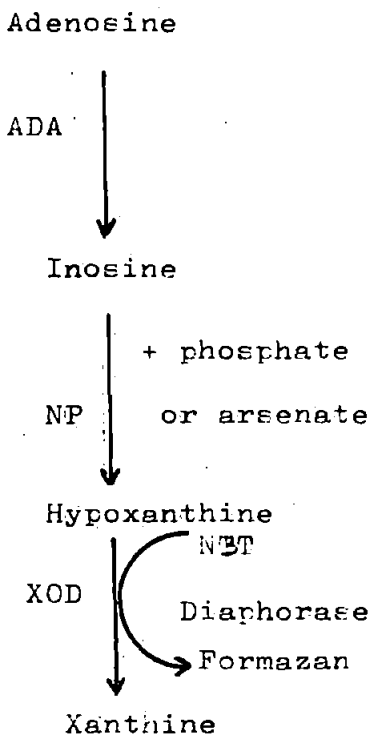
Stain application

The stain was applied as an overlay to the electrophoresed film after mixing 10 ml of 2% Oxoid ion agar in water at 60° with 10 ml staining solution. The overlaid gel film was incubated in the dark at 37° for reaction development.

No mercaptoethanol was added to the haemolysates. No plasma was investigated.

c) Adenosine deaminase isoenzymes

The staining technique was a modification of that used by Spencer et al (1968). Diaphorase was used as hydrogen carrier instead of phenazine methasulphate.



Standard haemolysate preparations were used with the addition of 2-mercaptoethanol at a final concentration of 20 mmol/l.

Stain buffer

Stock solution Solution A 0·2M, $Na_2HPO_4 2H_2O$, 35·61g/l

Solution B 0·2M, $NaH_2PO_4 2H_2O$, 31·21g/l

Working solution 84 ml solution A and 16 ml solution B made up to 400 ml with distilled water. (50 mM phosphate pH 7·5.)

Reaction stain

Adenosine 8 mg, NBT 2 mg, Xanthine oxidase 10 µl (4u/ml) nucleoside phosph. orylase 20 µl (20 u/ml), diaphorase 20 µl (1050 u/ml) made up to 10 ml with phosphate buffer 50 mM, pH 7·5.

Stain application

By the overlay technique using a mixture of 10 ml stain solution and 10 ml 2% ion agar in water at 60°. The overlaid gel was incubated at 37° in the dark for the colour reaction to develope.

(d) HGPRT isoenzymes

The staining method developed was based on the assay used by Giacomello and Salerno (1977) using a reversed formazan staining technique as follows:-

Buffer

Stock solutions Solution A, 0·2M Tris, 24·23 g/l

Solution B, 0·1M HCl

Working solution 25 ml solution A and 40 ml of solution B made up to 100 ml with H_2O, 0·05 M Tris/HCl, pH 7·5

Reagents.

Stock guanine 17·8 mg guanine were dissolved in 50 ml water containing 1 ml of 10M NaOH,

Stock stain reagent. Disodium ATP 38·3 mg, PEP (monocyclohexyl ammonium salt) 46·5 mg, $MgSO_4$ 985·9 mg, KCl 969·2mg made up to 100 ml with buffer.

Working stain reagent. Monosodium PRPP 6·7 mg, NADH 1·14 mg, stock guanine 850 µl, PK 16µl, LDH 8 µl, 0·5% methylene blue 200 µl, diaphorase 20 µl (1050 u/ml) made up to 10 ml with stock reagent and prepared immediately before use.

Stain application.

10 ml working stain mixed with 10 ml of 2% Oxoid ion agar in water at 60° and poured as an overlay onto the gel following electrophoresis. The overlaid strip was incubated at 37° in the dark for two hours for colour development.

9) Column Chromatography.

Khym (1974) has achieved group separation of ribonucleotides, ribonucleosides, purines and pyrimidine bases on Bio-Gel P-2 mesh 200-400, using borate buffer pH 8·9 as eluant. In the current study, this eluant did not give consistant elution profiles and physiological saline gave better resolution of the absorbance peaks monitored at 290 nm.

Bio Gel P-2, mesh 200-400 (Bio Rad Laboratories Ltd) was washed with 0·9% saline and applied as a slurry to a column (30 cm x 1 cm diameter). The packed gel was washed with 0·9% saline until zero absorbance was recorded on a LKB Uvicord at 290 nm.

Serum and urine samples were filtered through Amicon Centriflo cones at 2000 r p m for fifteen minutes. 0·5 ml of the filtered serum or urine was applied to the column and eluted with 0·9% saline at a flow rate of 0·6 ml/min delivered from a Technicon proportioning pump. The eluant was monitored continuously on a LKB Uvicord at 290 nm. The column was washed with 0·9% saline after each application to zero absorbance at 290 nm.

RESULTS

Urate binding to plasma proteins.

1) Occlusion at diastolic pressure.

The analyses of the blood samples obtained from these experiments are summarised in Table 12. The percentage changes in several serum components together with those of Christiansen et al 1975, are given in Table 13.

2) Ultrafiltration

The uric acid content of serum and the filtrate following filtration with Centriflo membrane cones are presented in Table 14.

3) Electrophoresis

One blue staining band was observed in all sera and this band had the same mobility as the laboratory standard containing uric acid. Counterstaining of the cellulose acetate strips with Ponceau S for the location of serum protein bands indicated that the uric acid migrated towards the anode faster than the pre-albumin bands.

4) Gel Filtration

The elution profile for protein content, as monitored at 280 nm and uric acid content of each fraction collected after gel filtration with Sephadex G 200 is shown in Figure 6 .

Hyperlipidaemia

Figure 7 shows the distribution curves for plasma uric acid values in a population of healthy individuals and in individuals found to have Type IV hyperlipidaemia.

Figure 8 shows the distribution curves for plasma uric acid content in the same healthy controls and in

individuals found to have Type IIb hyperlipidaemia. The mean plasma uric acid content of the healthy group is 317 μ moles/l whilst that for the Fredrickson type IV group is 433 μ moles/l and 400 μ moles/l for the Type IIb group.

Distribution of urates.

1) Erythrocytes

No absorbance peak at 293 nm was observed following the elution of the haemolysate from the Dowex ion exchange resin. Similar treatment of a serum resulted in an absorbance peak at 293 nm. The addition of uricase to the fractions showing absorbance at 293 nm caused a marked decrease in absorbance at this wavelength.

2) Leucocytes

No colour development is seen in the test or blank tubes whereas a red colour develops in the standard tube after 2 hours at room temperature. Re-examination of the tubes after 24 hours shows that a red colour has developed in the reaction tube whereas only a faint colour is apparent in the blank tube. The intensity of colour increases appreciably in the tube after 48 hours at room temperature but no apparent increase occurs in the blank reaction.

The exclusion of air from the surface of each tube by a covering of liquid paraffin produces identical observations to those recorded above.

Serum uric acid content

The serum uric acid content (mean and SD) for 141 individuals divided into their three clinical groups is given in Table 15 . The data has been further analysed by segregation into male and female populations and the corresponding results are included in the same Table.

Enzymology

PRPP synthetase in erythrocytes

1) Enzymic activity

Histograms and confidence intervals of enzymic activities for normal, gouty and hyperuricaemic individuals are shown in Fig. 9 . The enzymic activities (mean and SD) for 141 individuals divided into the three clinical groups studied is given in Table 16 . The data has been further analysed by segregation into male and female and the mean enzymic activity and SD for the various groups is also included in Table 16 .

2) Kinetics

a) Effect of varying concentration of R-5-P

R-5-P profiles were obtained for erythrocyte PRPP synthetase in six individuals (2 controls, 3 gout patients and 1 patient with hyperuricaemia). All curves were very similar and Fig. 10 shows the profiles obtained for i) a control subject and ii) a patient with tophaceous gout. Lineweaver Burke plots for the same two individuals is shown in Fig. 11 . All the data was examined statistically and regression equations were derived from the Lineweaver Burke plots for each individual. This

data is given in Table 17 . Michaelis constants and maximum velocities have been calculated from all such plots and this data is shown in Table 18 .

b) Effect of varying the concentration of magnesium

Fig.12a shows the effect on the enzymic activity of varying magnesium ion concentration, at a fixed concentration of ATP (1·33mmol/l). The Lineweaver Burke plot for these investigations is given in Fig. 12b. This data indicates a Michaelis constant of $7{\cdot}0 \times 10^{-4}$ with a maximum velocity of 1092 umol/ml/hr (Table 18).

c) Effect of varying the concentration of ATP

The effect of variation of ATP concentration on the enzymic activity, at a fixed concentration of magnesium, is shown in Fig. 13 for the haemolysates of four individuals investigated. The corresponding Michaelis constants and maximum velocity's, calculated from Lineweaver Burke plots are given in Table 18 .

d) Effect of varying the concentration of Phosphate

The change in enzymic activity with change in phosphate concentration for three haemolysates is shown in Table 19 .

e) Effect of pH

The effect of varying the pH of the medium on erythrocyte PRPP synthetase activity is shown in Fig. 14 .

3) Inhibitors

a) Alcohols

i) Ethyl alcohol

The mean percentage inhibition of PRPP synthetase using varying concentrations of ethyl alcohol in 25 individuals with their distribution amongst

the three clinical groups is shown in Table 20 . The average inhibition profile of the enzyme with ethyl alcohol is given in Fig. 15 . Two types of profile were observed amongst the 25 individuals and these differing curves are shown in Fig. 16 a and b. The population of 25 were divided according to their ethyl alcohol profiles and the mean inhibition of the enzyme for each alcohol concentration is shown in Table 21 .

ii) Methyl alcohol

The inhibition profile for varying concentration of methyl alcohol is presented in Fig. 17 . No variation in profile was obtained from individuals from the three clinical groups.

iii) Propanol

The range of propanol used for inhibition studies was restricted by the fact that the blank reaction was more sensitive to increasing concentrations of this alcohol than PRPP synthetase. At a concentration of 4·5% propanol only 6% inhibition was observed. Some activation occured at lower concentrations of alcohol but these were of insufficient magnitude to establish the confidence to extend the studies beyond an exploratory 2 or 3 samples from haemolysates of each of the three clinical groups.

iv) Butanol

The mean percentage inhibition of PRPP synthetase from 3 normal, 2 gouty and 1 hyperuricaemia haemolysates is shown in Table 22 .

b) 2,3 Diphosphoglycerate

The inhibition profiles of a) a control and b) a tophaceous gout haemolysate for PRPP synthetase using varying concentrations of DPG are shown in Fig. 18 . The percentage inhibition of the enzyme by 5·4 mmol/l DPG in the haemolysates from 11 individuals is given in Table 23 .

c) Nicotinamide

The inhibition profile of the mean percentage inhibition obtained for PRPP synthetase in the haemolysates from 14 individuals (5 controls, 7 gouts and 2 hyperuricaemics) using a range of concentration of nicotinamide is shown in Fig.19 and Table.24

d) Nicotinic Acid

Similar shaped inhibition profiles were obtained for nicotinic acid and nicotinamide.

e) 2-Mercaptoethanol

At a fixed concentration of 25 mmol/l this inhibitor produced a large range of effect from 58% activation to 29% inhibition for PRPP synthetase in 30 different haemolysates. This distribution of activation or inhibition is shown in Fig. 20a . If the data is divided into three subgroups so that the first group is characterised by an inhibition $>$ 10, the third group is characterised by an activation $>$ 10 then the second group will consist of individuals showing an activation $<$ 10 or an inhibition $<$ 10. The distribution of the three clinical classes amongst these subgroups is shown in Fig. 20b . Averaging the figures

algebraically gives an overall activation for normals = 4·7, for gouty patients , 2·8 and for hyperuricaemic individuals 5·1 but the differences are not significant.

f) Dithiothreitol

A similar effect to 2-mercaptoethanol was obtained but fewer individuals were investigated. Again a large spectrum of results were observed with values from 37% inhibition of PRPP synthetase to 52% activation.

g) Histidine

The inhibition profile for PRPP synthetase using a range of concentrations of histidine is shown in Fig. 21 . The effect of using a fixed concentration of 22·5 mmol/l histidine in many samples showed only experimental variation in percentage inhibition obtained. No difference was observed between the inhibitive characteristics of the enzyme from gouty and normal individuals.

h) Uric acid

No inhibition of PRPP synthetase was observed in many haemolysates using 1·13 mmol/l uric acid. This concentration was the limit of solubility of uric acid . It is possible to prepare metastable solutions of higher uric acid content but these were unsuitable for our use.

j) Formaldehyde

Inhibition profiles for PRPP synthetase from a control individual and a patient with tophaceous gout with a range of concentrations of formaldehyde are shown in Fig. 22 . The percentage inhibitions

using two fixed concentrations of formaldehyde for a number of individuals are shown in Table 25 .

k) Other inhibitors

No inhibition of PRPP synthetase was observed using 1·8 mmol/l lithium carbonate. Neither tryptophan nor caffeine were found to inhibit the enzyme at the concentrations used. Solubility was the determinant.

4) Electrophoretic studies

A photograph of a typical agarose gel stained for PRPP synthetase following electrophoresis is shown in Fig. 23 . A single band which migrates toward the anode, slower than haemoglobin, has been observed for all samples.

Glutathione reductase in erythrocytes

1) Enzymic activity

The enzymic activities (mean and SD) for 141 individuals divided into the three clinical groups is shown in Table 26 . The distribution of the activities and confidence intervals in the three groups is shown in Fig. 24 . The data has been further analysed by segregation into male and female and the mean enzymic activity and SD for the various groups is also included in Table 26 .

2) Electrophoretic studies

Fig. 25 shows a typical electrophoresis after staining for glutathione reductase in several different haemolysates. Residial staining was observed on the origin. All samples had one band which migrated towards the anode just faster than haemoglobin. A few samples had an additional slower band which was located between the origin and haemoglobin. This variant did not segregate

into a clinical type.

Glutathione reductase in plasma

1) Enzymic activity

The distribution of enzymic activities and confidence interval of samples in each of the clinical groups studied is shown in Fig. 26 . The mean activity and SD for 141 individuals, divided into 3 clinical groups and subdivided according to sex, is given in Table 27 .

Adenosine deaminase in erythrocytes

1) Electrophoretic studies

In the absence of mercaptoethanol a single isoenzyme was found running anodically to haemoglobin. Multiple staining bands were obtained after the addition of mercaptoethanol (20 mmol/l) and these are shown in Fig. 27 . In some of the samples, 3 anodic bands migrating faster than haemoglobin were visualised in addition to the comparativily slow anodic band migrating behind the haemoglobin. Several possible polymorphic variants may be present but none of the variants were confined to one clinical group.

Adenosine deaminase in serum

1) Enzymic activity

Random samples of sera received by the routine laboratory were divided into the three clinical groups defined in the current study. The uric acid content of all specimens were checked and the mean serum ADA activities found in each group are presented in Table 28 .

Hypoxanthine-guanine phosphoribosyl transferase in erythrocytes

1) Enzymic activity

The haemolysates from most of the gout samples and several control samples were assayed for enzymic activity. The results obtained are presented in Table 29 .

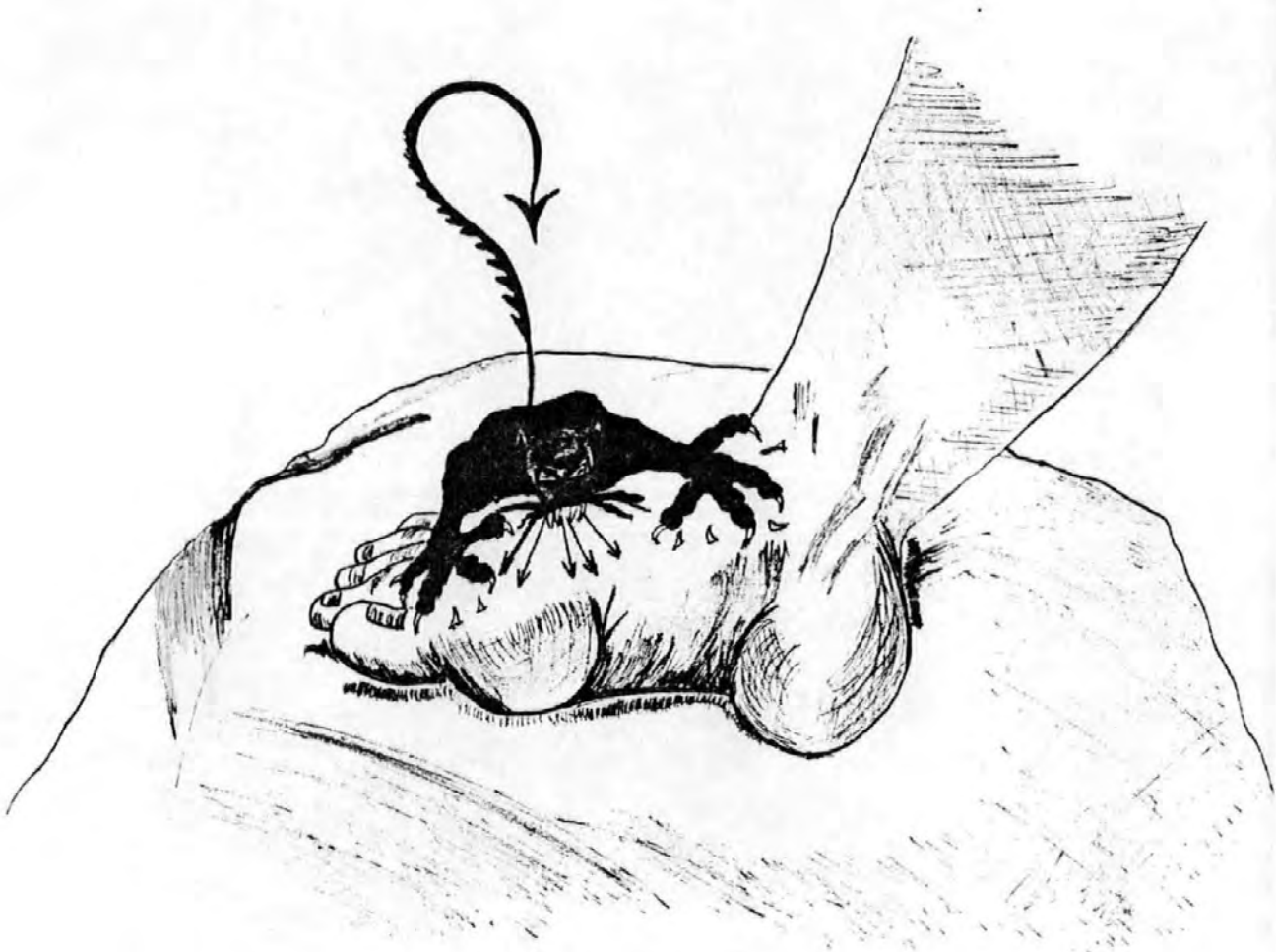
2) Electrophoresis

One or two isoenzymes were visualized in thesamples after staining following electrophoresis (Fig 28). Both bands migrated anodically but at a slower rate than haemoglobin. The two band pattern, although less frequently observed than the single band, was not confined to one clinical group.

Column Chromatography

The elution pattern of serum obtained after elution with 0·9% saline and monitored at 290nm is shown in Fig. 29 . The composite results obtained after the elution of markers added singly to a sample of pooled sera are presented in Fig. 30 . Typical elution patterns for a hyperuricaemic serum and two sera from an individual with gout and another with familial primary gout are given in Fig. 31 .

The Gout



Discussion

The binding of urate to plasma proteins remains a provocative topic and we have investigated this not only by modifications of recognised procedures but also by an occlusion study, which is a first attempt under physiological conditions. It is known that venous stasis induces a dissociation of the protein-bound constituents in blood (Dent 1962; Berry et al 1973, Husdan et al 1974). The phenomenon has been attributed to a change in the protein-bound fractions, parallel to the change in serum proteins while the free fractions are assumed to be constant (Berry et al 1973). It can be seen from our results in Table 12 that we have no evidence from our occlusion experiments that uric acid binding is present at the time of doing our investigations. Moreover this observation is independent of temperature since experiments 3 and 4 are effectivily the same. Each blood sample was divided - one half was processed at 4° and the other at 37°. One must query whether the method of analysis would differentiate between free and protein-bound uric acid. An enzymic colorimetric assay has been used with uricase, catalase and acetylacetone. It would have been desirable to supplement the analysis with a purely chemical method for the assay of uric acid. Preliminary experiments using both chemical and enzymic methods of assay had indicated no statistical differences but overall the enzymic method gave consistently more reliable results with smaller sample aliquots and was selected as our standard assay. The results in Table 13 do however give some indication that the ten minute occlusion has resulted in the release of bound calcium into the serum

in all cases and in amounts comparable to those reported by Christiansen et al (1975). There is an increase in γ-globulin which is probably too small to account for the increase in plasma protein following occlusion. Christiansen et al (1975) report a mean increase in globulin of 7% after venous stasis in nineteen epileptic patients. They also report increases in α_1, α_2 and γ -globulins which we have not investigated. It may be that the apparent increase in all plasma protein fractions may be due to the large oligomeric molecules dissociating into smaller units which would indicate a higher protein content using the modified Biuret analytical procedure outlined in Appendix 1.

We can see from Table 14 that ultrafiltration using Amicon cones gives less clear cut results and in one case there appears to be evidence for appreciable binding ($\sim$14%). This apparent urate binding was found in the serum from a patient with gout and other investigations (e.g. Klinenberg and Kippen, 1970 and Alvaaker 1968)report that urate binding is less in hyperuricaemic individuals than in normal controls. Such a report should not be regarded as definitive since an inspection of their results shows that few individuals have been investigated and moreover a wide range of values are found in controls and the hyperuricaemic group. It is felt that the experimental error in our ultrafiltrations is considerable in so far as the retained high molecular weight complexes are very gelatinous and it is impossible to spin down to a dry mass. Furthermore the physical appearance of the deposit clearly varies from one sample to another. Prior to uric acid assay

the volume of the filtrate was adjusted by addition of water, which varied from sample to sample, to 5ml. In view of the limitations of the method it was felt to be unprofitable to examine more samples. It is noteworthy that Farrell et al (1975) found a range of 0-3% urate binding in heparinised plasma from seven uraemic patients compared with 7% binding in heparinised plasma from one healthy control and 6% binding in citrated normal plasma. Further investigations should compare the effect of anticoagulants on binding. Sera was used in the current study.

Electrophoretic studies have failed to reveal any binding to protein although a fast moving uric acid fraction was observed. The mobility was greater than any of the plasma proteins. The staining technique involved uricase, peroxidase and 4-aminophenazone. Our results can be interpreted in two ways: either the staining technique is specific for only free uric acid or we have no experimental evidence of urate bound to plasma protein.

None of our experiments to study urate binding to plasma protein are devoid of criticism but the integrated picture is indicative of the absence of appreciable binding of urate to plasma protein.

Supportive evidence for this conclusion arises from Figure 6 which depicts the results of molecular sieving of serum through Sephadex G 200. Fractions containing uric acid were located, together with low molecular weight components of serum after the albumin and pre-albumin fractions of the eluant.

The results given in Table 30 give the uric acid

content of sera from fasting individuals segregated into groups following lipid analysis and classification according to Fredrickson. It is apparent that the mean uric acid level of each group of hyperlipidaemics is in the order Type 1V > Type 11b > Type 11a ≫ normal control. In other words it is probable that individuals with hyperuricaemia will be classified as Fredrickson Type 1V hyperlipidaemia. We can see from Appendix I that the serum cholesterol level of this Type 1V group is either normal or slightly increased whereas the triglycerides are moderately increased. Type 11b is classified by greatly increased serum cholesterol and triglycerides. These results suggest that an increased serum triglyceride level may be an ancillary parameter in hyperuricaemia whereas the serum cholesterol level is not. Such an hypothesis is in accord with the work of Nishida et al (1975) who reported elevated plasma triglycerides and free fatty acid levels in 107 gouty patients but no significant differences in plasma cholesterol and phospholipid levels as compared with control subjects.

It was suggested by Gibson and Grahame (1974) with support from Nishida et al (1975) that an excessive intake of alcohol may play an important role in inducing hyperlipidaemia in gout. However Naito and Mackenzie (1980) found that neither alcohol intake nor hyperuricaemia per se appeared to be the cause of the lipid and lipoprotein disorder. These workers selected 30 patients with primary gout, rendered asymptomatic by therapy, to examine the frequency and type of hyperlipidaemia and compare the findings with an age group of controls. It was found that Type IV and IIb lipoprotein electrophoretic patterns

were most prevalent in the gout group and the study suggested that there was no evidence of liver or kidney dysfunction but diet and possibly defective clearance of triglycerides may be etiological factors associated with hyperlipidaemia in gout. Obesity did appear to be a major underlying factor associated with the lipidaemia.

Blood samples collected from the three groups of individuals investigated in the present study did not fulfil the requirements of a 10 hour fasting period so that lipoprotein electrophoresis was not practical in these samples. However it is apparent from our hyperlipidaemia typing together with the observations of Naito and Mackenzie (1980) that future work should embrace not only patients with uncontrolled gout but others having hyperuricaemia without gout.

There is at present no evidence for the occurence of uric acid in red blood cells, but the current study has indicated the presence of uric acid or its precursors in leucocytes. It seems improbable that free uric acid occurs in the cytoplasm of the leucocyte since a colour reaction was not visible immediately after addition of the chromogenic reagents. Twenty-four hours were required for the colour to be observed and one must query whether the colour production could be attributed to atmospheric oxygen. Such an hypothesis seemed unlikely since the 'blank' reaction showed only a faint coloration which, in contrast to the leucocyte and standard uric acid tubes, did not intensify appreciably after forty eight hours. The possibility of atmospheric oxidation was eliminated when identical observations were obtained if the surfaces of the

solutions in each tube were covered with a layer of liquid paraffin. The colour observed may be formed by a reagent other than uric acid but such a concept is very improbable since one of the reactions preceding the formation of the colour complex is dependent on uricase which is specific for uric acid and moreover the same type of colour developed, albeit more quickly, in the tube containing only uric acid following the addition of the chromogen reagents. Our observations do, however, indicate that uric acid is either bound in a sack like structure such as a lysosome or is adsorped to the cell wall or to proteinous components of the leucocytes. If this were true then uric acid could be released slowly by degeneration of the cell wall or by desorption. It would appear worthwhile to extend these investigations by pretreating the leucocytes with lysozyme or a protease to find out whether a colour reaction developes quickly after the addition of the chromogenic reagents. If, after such treatment, the slow colour development persists it would seem likely that the precursors to uric acid production are present in the leucocyte suspension and are still functioning.

The selection of the individuals in our gout controls was made from the diagnosis of a G.P. or consultant who were requested to send heparinised blood samples from such patients. It is significant that 47/54 patients were male. This uneven distribution of the sexes in our sample is indicative that more males than females are diagnosed clinically as gout in the Torbay area.

The selection of the control group and hyperuricaemic

individuals was essentially identical and therefore the distribution of the sexes should be the same in these two groups. We see from Table 15 however that there is a higher incidence of hyperuricaemia in males than in females. Appendix II represents a crude analysis of the serum uric acid levels reported by a routine clinical laboratory of all samples assayed during the period 1970-1979. Any sample with a uric acid content greater than 420μ mol/l is classified as abnormal or hyperuricaemic. The results show no significant variation in the proportion of hyperuricaemic sample from month to month over a six year period. (Fig. 32) but during this period there has been an apparent 10% increase in the percentage of hyperuricaemics reported (Table 31). This may be due to our survey containing repeat samples from the same individual during the period that the hyperuricaemia was being controlled. It would appear rather more significant that from 1975 onwards the percentage of abnormal or hyperuricaemic blood samples remains remarkably constant (Table 32) in spite of the ever increasing requests for serum uric acid assays each year (Fig. 33). The samples from January 1976-April 1978 inclusive have been separated by sex and the percentage with hyperuricaemia determined. These results are presented in Table 33 and show that the incidence of hyperuricaemia in males is almost twice that found in females. The selection of samples sent to the clinical laboratory for serum uric acid assay is certainly not sex biased, but these results do provide convincing ancillary evidence to the deduction from our current study that hyperuricaemia has a higher frequency in males

than females.

It was necessary to select an assay for PRPP synthetase which was not only accurate and reproducible but adaptible to semi-automation. This was desirable because it was planned to screen a statistically valid number of samples of our three groups of individuals for PRPP synthetase activity and ultimately extend the work to the use of enzyme inhibitors with a view to examining their usefulness as diagnostic aids for the condition of clinical gout. Such a tool would be invaluable for determining any genetic factor that might be present in our sample of gout patients. Many of the currently used assays for PRPP synthetase involve a procedure in which the PRPP formed by the enzyme is reacted with a radioactively labelled base such as (8 ^{14}C) adenine (Becker et al 1973) or (8 ^{14}C) hypoxanthine (Hershko et al 1969) in the presence of the appropriate partially purified purine phosphoribosyl transferase. The corresponding isotopically labelled nucleotide produced is separated by high voltage electrophoresis (Fox and Kelley 1971) or paper or thin layer chromatography (Hershko et al 1969, Becker et al 1973). These methods are expensive and time consuming. The same objection can be raised when (^{14}C)ATP is used as substrate (Fox and Kelley 1971) or ATP-γ-^{32}P is used for the assay of PRPP synthetase based on enzymic transfer of radioactivity from ATP-γ-^{32}P to PRPP (Johnson et al 1974). There was no β counter available in the department. The assay of AMP production described by Valentine and Kurschner (1972) appeared to be most suitable for our requirements. Using apparently identical conditions to those described by these authors many difficulties

were encountered and reproducibility was abysmal. It was subsequently shown that whereas the assay was reported to be performed at pH 8, the buffer capacity was quite inadequate to accomodate the reagents. (pH lowered from 8 to 6·8). Moreover varying amounts of NaOH were required to restore the pH of the medium to 8 depending on which aspect of kinetics was being investigated. Increasing the buffer strength from 20 mM to 40 mM still did not maintain the pH at 8 on addition of the reagents (pH lowered to 7·4). It was ultimately decided to use a phosphate buffer of 60 mM and to maintain the pH of the assay at 7·4 in spite of the sub-optimal pH of the reaction (Fig. 14).

There was a reasonable doubt about the validity of the method since the linked enzyme system could be measuring the endogenous AMP present in the haemolysate and not the product of the PRPP synthetase. This objection was surmounted by estimating blanks containing no R-5-P for each haemolysate studied.

Other methods were tried for the assay of PRPP synthetase to check and substantiate our confidence in our modified "Valentine" procedure. The same linked system of assay had been used by Ferrari et al (1978) who dialysed all erythrocytes against 8 mM sodium phosphate buffer pH 7·4 containing 1 mM EDTA and 2mM mercaptoethanol for two hours prior to lysis. PRPP synthetase was assayed by a two step procedure. First the enzymic reaction was terminated by addition of perchloric acid, second the reaction producing AMP was monitored at 340 nm by using the following NADH linked method.

$$AMP + ATP \xrightarrow{AK} 2\ ADP$$

$$2\ ADP + 2PEP \xrightarrow[Mg^{++}K^{+}]{PK} 2\ ATP + 2\ pyruvate$$

$$2\ pyruvate + 2NADH + 2H^{+} \xrightarrow{LDH} 2\ lactate + 2\ NAD^{+}$$

Dialysis of the haemolysate was not found to be necessary but the method was not suitable for routine assay of a large number of samples. Similar enzymic activities were found in samples assayed by Ferrari and by our modified "Valentine" procedures.

The enzyme was also assayed by the reverse reaction according to Switzer (1969) by coupling the action of hexokinase and glucose-6-phosphate dehydrogenase.

$$PRPP + AMP \longrightarrow R\text{-}5\text{-}P + ATP$$

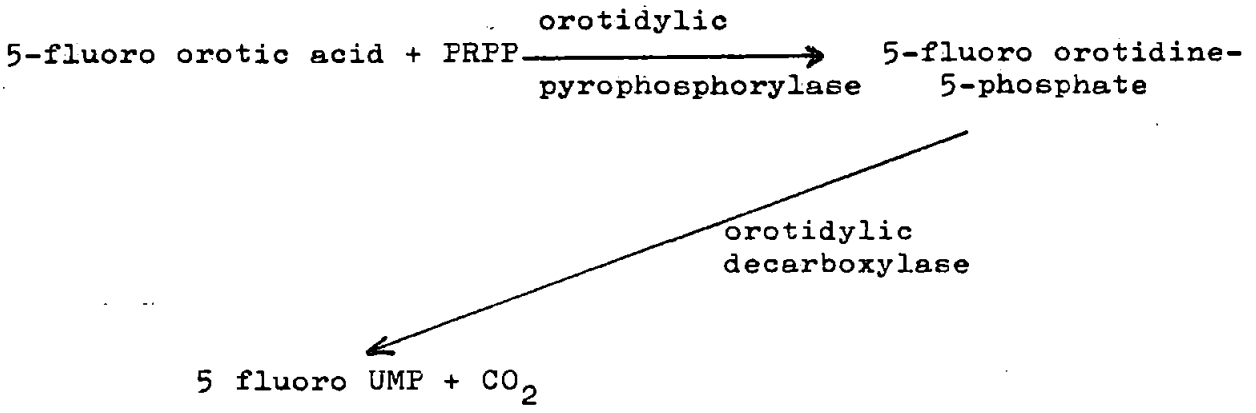
$$Glucose + ATP \xrightarrow{HK} G\text{-}6\text{-}P + ADP$$

$$G\text{-}6\text{-}P + NADP^{+} \xrightarrow{G\text{-}6\text{-}PD} 6\text{-phosphogluconate} + NADPH + H^{+}$$

This method was rejected since in all cases less activity was observed ($\sim$ 50%) of that found using our "Valentine" modification. There was additionally a problem of reagent precipitation due to the fortifying enzymes being present in saturated ammonium sulphate. Precipitation of magnesium ammonium phosphate was avoided either by dialysing the fortifying enzymes or by using these enzymes contained in glycerol . Our kinetic studies using this assay were not only non linear but a wide variance of duplicates was observed. The commercial

substrate PRPP, although expensive and difficult to obtain, was often contaminated with inorganic phosphate, R-5-P and pyrophosphate and such impurities would explain our unsatisfactory results using this assay.

Valentine and Kurschner (1972) also assayed PRPP synthetase by measurement of PRPP using the following linked reactions:-



The reaction is followed spectrophotometrically at 295nm during the conversion of fluoro orotate to fluoro-UMP. This is a sensitive assay but the cost and slow delivery of the reagents for the coupled reactions eliminated its usefulness for our purposes. It was moreover an unsuitable method for even semi-automation. All PRPP synthetase studies in the current work used our modified "Valentine" procedure. Good reproduction was obtained using this assay and for nine repeats of the same haemolysate the average activity was 45·08 μ mols/mg protein/ml (SD=1·19).

The apparent instability of PRPP synthetase has been the cause of many established workers in the field of purine metabolism neglecting to study possible changes in properties in the enzyme caused by pathological conditions (Simmonds, private communication). Fox and Kelley (1971) report that the human erythrocyte enzyme

can be protected at -70° by 1 mM dithiothreitol but the enzyme is inactivated by 1 mM mercaptoethanol whilst any purification of the enzyme is apparently accompanied by inactivation with dithiothreitol. Reports by Kornberg et al (1955) and Flaks (1963) that pigeon liver PRPP synthetase requires high levels of reduced glutathione for maximal activity prompted the examination of a number of thiols for their effect on the erythrocyte enzyme. Reduced glutathione and 2 mercaptoethanol at 1 mmol/l concentration resulted in marked denaturation of our enzyme preparations during storage at -20°. Fox and Kelley (1971) also report an absolute requirement for phosphate which they demonstrated by desalting the enzyme with a Sephadex G 100 column equilibrated with 50 mM Tris chloride pH 7·4. No assayable activity was observed under such conditions but it is possible that the enzyme is inhibited by 50 mM Tris chloride at pH 7·4. Our observations in the current study show that the enzyme is inactive in Tris buffer in the absence of phosphate but the effect of decreasing the amount of Tris and increasing the amount of phosphate on the enzymic activity is sigmoidal (Fig. 34). The presence of inorganic phosphate is now accepted as an essential requirement for the expression of PRPP synthetase activity since replacement of inorganic phosphate by dialysis of the enzyme against buffers containing either Tris-HCl or HEPES results in no major loss of enzymic activity provided that enzyme assay occurs in the presence of inorganic phosphate (Roth et al 1974). Washed, packed erythrocytes stored at -20° without addition of protective agents retain their enzymic activity for about two weeks

whilst crude haemolysates (1:3 haemolysis) have been found to have lost half their enzymic activity in 48 hrs. at 4°(Yip et al 1978). No loss of PRPP synthetase activity has been found in the crude haemolysates stored with 0·3 mM ATP after 48 hours at 4° (Yip et al 1978). No data from these authors is available for longer storage of haemolysates. But the changes in PRPP synthetase from normal human erythrocytes upon storage have been investigated by gel filtration on Sephadex G 200 (Yip et al 1978). PRPP synthetase from fresh normal erythrocytes had the majority of its enzymic activity eluted in the void volume. Storage for 17 days at -70°, although producing only a slight decrease in enzymic activity, showed a drastic shift of the PRPP synthetase molecular size with the elution of two active components (one high and one low molecular weight). After three months storage at -70° only trace amounts of the large molecule remained and 40% of the activity was lost. When haemolysates were stored at 4° a rapid decrease in the PRPP synthetase activity and molecular size occured. The addition of 0·3 mM ATP to the haemolysate during storage preserved the enzymic activity for 48 hours at 4° but did not prevent the dissociation of the enzyme molecule into subunit fractions. In all instances the decrease of the larger molecule results in a concomittant appearance of the smaller enzyme molecule with decrease in activity

An overview of the reported storage experiments does suggest that the same preparation has been used for the assay of enzymic activity of PRPP synthetase by many workers. If this is true then it is not surprising that a decrease in both enzymic activity and

molecular weight is observed on storage. Repeated freezing and thawing of the same sample produces denaturation associated with loss in activity and hybridization of enzyme molecules, such as lactate dehydrogenase, presumably by initial dissociation into sub-units of the large enzyme molecule. In this study it was decided to store haemolysates of all blood samples in small aliquots, any one of which could be used for the day's investigation. All haemolysates contained 45mM phosphate and 2 mmol/l EDTA. Under these conditions the PRPP synthetase was maintained following storage at -20° for six months. We did not investigate any changes in molecular character as determined by gel filtration. Electrophoretic investigations were performed after the addition to haemolysates of 2 mercaptoethanol (20 mmol/l) which is known to eliminate the storage bands which sometimes appear on staining following electrophoresis of old samples. No mercaptoethanol was used for the glutathione reductase electrophoresis.

Van Maris et al (1980) have measured PRPP and some enzymes of purine metabolism in erythrocytes from 43 young hyperuricaemic males. They report that the mean erythrocyte PRPP synthetase activity in their hyperuricaemic individuals was slightly but significantly ($P < 0.01$) higher than the means of their control group. The latter consisted of only 10 males which is insufficient. Meyskens and Williams (1971) have assayed the concentration and synthesis of PRPP in erythrocytes from normal, hyperuricaemic and gouty subjects and found no difference in PRPP synthetase activity amongst the 3 groups of individuals. Table 16 summarises the PRPP synthetase

activities found in our three groups of individuals. In the case of the gout patients, the increase in SD found overall is mainly due to the SD found in female patients. In the small number of female gouts investigated, the figures are biased by this one individual and if we eliminate her from the sample the mean PRPP synthetase activity found in gout compared to normals increases by the same amount for males or females. Thus it is possible to ignore the sex and look at the combined figures. These show a significant increase in enzymic activity for the gout and hyperuricaemic groups compared with the healthy controls.($0.001 < P < 0.01$), Table 16. Such an increase is inadequate for the use of PRPP synthetase activity in the diagnosis of gout. The histograms and confidence limits, Fig. 9, clearly illustrates these differences in the mean PRPP synthetase activities of the three groups . We have as yet no evidence for a genetic variant of PRPP synthetase characterized by high enzymic activity as reported by Becker et al (1973). It is however proposed to investigate in depth repeat samples from the female gout patient already mentioned and investigate her family if any unusual properties of the enzyme emerge.

Very similar substrate profiles were obtained for PRPP synthetase activity when the concentration of R-5-P was varied. In all, six profiles were obtained and the data used to calculate regression equations from the double reciprocal plot of initial velocity against variable concentration of R-5-P and fixed ATP (Table 17).

All substrate profiles appeared to be hyperbolic and this was substantiated by the linear Lineweaver Burke plots. Michaelis constants were calculated and compared with values obtained by other workers using PRPP synthetase obtained from different sources (Table 34). Our mean value for Km_{R-5-P} is very similar to the one reported by Fox and Kelley (1972) and Sperling et al (1973) for the erythrocyte enzyme. There is no apparent difference in R-5-P profile between individuals classified as normal, gouty or hyperuricaemic. The mutant PRPP synthetase found in the erythrocytes of a gouty patient with excessive purine production and the normal enzyme did not differ in respect to their K_m's reported for ribose-5-phosphate (Sperling 1973 and Table 34).

The ATP profile shown in Fig. 13 appears to follow a hyperbolic curve with increasing amounts of ATP. But at high ATP concentration the curve ceases to be asymptotic and starts to dip (Fig. 13). It was initially believed that this "dip" may be due to the fall in pH (7·4-7·2) which occurs when high ATP concentrations are used; in other words the buffer capacity of medium is insufficient to maintain the pH under these conditions. Another explanation would appear to be more apt since the fall in pH mentioned above is $\sim$ 0·2. An inspection of Fig. 14 shows that a drop in pH of this magnitude does have an appreciable effect on PRPP synthetase activity at pH 7·4. But the true substrate for the enzyme in addition to R-5-P is ATP Mg^{2-} not ATP and at the high values of ATP used, we have insufficient Mg^{2+} present in the medium to form the complex salt. It is therefore essential that the design of future

experiments must have due regard to the amountof Mg^{2+} required to convert ATP into the complex ATP Mg^{2-}. Additionally ATP induces aggregation of the small molecular weight erythrocyte PRPP synthetase. Therefore the possibility that the dissociation produces an inactive enzyme molecule, which appears to be active due to the presence of ATP in the assay mixture, cannot be completely ruled out (Yip et al 1978).

The activation profile shown in Fig. 12 shows a hyperbolic increase in enzymic activity of PRPP synthetase with increasing concentrations of magnesium ions. It can be calculated that at least 99·8% of the ATP added was present in the current assay conditions as Mg-ATP^{2-} (Martell et al 1964, Keech and Barritt 1967). Our data shown in Fig. 12 clearly indicates that free Mg^{2+} stimulates PRPP synthetase. Such an effect has been reported by Murray and Wong (1967) in their studies of the enzyme isolated from Ehrlich ascites tumour cells. In the presence of an excess of Mg^{2+} the initial velocity of PRPP synthetase shows hyperbolic responses to increasing concentrations of both $MgATP^{2-}$ and R-5-P from sources of enzyme as diverse as Ehrlich ascites tumour cells (Wong and Murray 1969), human erythrocytes (Fox and Kelley 1972, Becker et al 1973) rat liver (Roth et al 1974) and Samonella typhimurium (Switzer 1971). Although the role of magnesium in our investigations can be classified as "bound" or "ionic" it is highly probable that $MgATP^{2-}$ cannot account for all the bound magnesium. In fact the fortifying enzymes are usually supplied in saturated solutions of ammonium sulphate and unless the ammonium salt is removed by dialysis against a suitable

buffer, precipitation of magnesium ammonium phosphate occurs during the assay. Additionally magnesium phosphate may be precipitated at high phosphate or high magnesium concentrations. Magnesium also has an affinity for R-5-P and some workers query whether the magnesium R-5-P complex is one of the true substrates for PRPP synthetase. (Switzer 1971 c). There is however no doubt that Mg^{2+} is an activator for the enzyme and it is believed to function by direct action on the enzyme by binding at an active site remote from the R-5-P active site. The dependence of initial velocity on $MgCl_2$ and ATP concentration may be analysed by comparison to various steady state kinetic models for metal ion activation (Dixon and Webb 1964). The treatment of such data is very complex when the total concentrations of substrate and activators are used but also the concentration of substrate $MgATP^{2-}$ and activator cannot be varied independently. It is therefore desirable to perform two sets of experiments, one in which the total $MgCl_2$ is held constant and the total ATP concentration varied and the other in which the roles are reversed. A set of conditions can then be found in which one reactant is essentially constant and a profile can be deduced for a range of concentrations of the second reactant. This has not been done in the present study. Due regard should be paid to the pH of the reaction. It seems probable that the complex kinetics encountered in the present study could be attributed to the distribution of the ATP among two or more ionic forms (e.g. $MgHATP^-$ and $MgATP^{2-}$) at pH 7·4 whereas at pH=8 nearly all the ATP is present as $MgATP^{2-}$ (O'Sullivan and Perrin 1964).

At the time the current study was started, most workers were using buffers at 7.4 and this determined our working conditions.

Gibson and Switzer (1980) have investigated the stereochemistry of the complex Mg ATP^{2-} substrate for PRPP synthetase from S.typhimurium. They report that the enzyme shows absolute specificity for the A(S) enantiomer of adenosine 5-O-(1-thiot riphosphate). The enzyme has similar affinity for ATP and the thio derivative which enabled Gibson and Switzer to propose the structure and stereochemical configuration for the Mg-ATP^{2-} complex at the active site of PRPP synthetase (Fig. 35).

Inorganic phosphate is neither a substrate nor a product of PRPP synthetase but Switzer (1969) reports that although PRPP synthetase from S. typhimurium has an absolute and specific requirement for inorganic phosphate the effects of phosphate are complex and appear to involve two modes of action : activation and inhibition. His kinetic data indicates activation of PRPP synthetase at high phosphate concentration but inhibition of the enzyme at low phosphate concentration Hershko et al (1969) have found that the formation of PRPP in human red cells is characterized by a sigmoid inorganic phosphate curve whereas in haemolysates the curve becomes hyperbolic. The hyperbolic inorganic profile of PRPP synthetase becomes increasingly sigmoidal when increasing amounts of ADP are added to the reaction (Hershko et al 1968). Atkinson and Fall (1967) suggested that the inhibition of the enzyme by ADP acts as a regulatory device of the erythrocyte to

obviate excessive expenditure of energy involved in the formation of PRPP and its subsequent utilisation in the biosynthesis of nucleotides. Flaks (1963) has suggested that the essential requirement of PRPP synthetase for inorganic phosphate may be ascribed to its stabilising effect on PRPP by prevention of the metal catalysed decomposition of PRPP by sequestration of the divalent cations in the reaction medium. But although EDTA, a potent metal chelator, is more efficient than inorganic phosphate in preventing the decomposition of PRPP during the course of incubation, it neither activates PRPP synthetase nor overcomes inhibition of the enzyme by ADP. Our apparent sigmoidal dose-response curve with inorganic phosphate, which is very different to that described by Fox and Kelley (1972), indicates that the role of this metabolite is likely to be described in terms of an allosteric mechanism, although the action of inorganic phosphate is unlikely to potentiate subunit aggregation of the molecule. We have found that the stimulation of the enzyme in human erythrocyte with increasing phosphate concentration continues to at least 60 mM, which is well above the accepted physiological level in erythrocytes (0·5-1·5 mM). The phosphate profile (Fig. 34) lacks precise definition and further studies at other phosphate concentrations are clearly desirable. Sperling et al have reported a hyperbolic response of a mutant PRPP synthetase to increasing inorganic phosphate concentration in a gout patient's haemolysate but our limited investigations have not shown such a response .

The pH profile shown in Fig. 14 is very similar in shape to the data produced by Switzer (1969) for PRPP synthetase from S. typhimurium and by Fox and Kelley (1972) for the human erythrocyte enzyme following gel filtration on Sepharose 4B. In all cases there is a pronounced shoulder from pH 7 to 8 and the activity is low below 6.

After indulgence of alcoholic beverages there is an increase in serum uric acid with an increase in gouty attacks in some susceptible individuals. This was believed to be caused by deposition of urates in consequence of a diminished renal uric acid excretion induced by alcohol hyperlactaemia(Lieber et al 1962). Such an explanation is not really plausible because the increased serum lactate observed after alcohol indulgence is much lower than that required for decreased uric acid output (Grunst et al 1977). The relationship between ethanol metabolism and increased hepatic uric acid production is not clear. It could be that ethanol may induce the de novo synthesis of purines or enhance nucleotide catabolism in the liver. Oxidation of the alcohol would cause a shift of the redox system to a more reduced state with a consequential decrease in ATP content in the liver. The changes in cellular ATP, which are known to regulate many enzymes of purine metabolism could account for the increased serum uric acid after alcohol. In the current work the effect of ethyl alcohol on PRPP synthetase shows that an in vitro inhibition of the enzyme occurs (Fig. 16). However the concentrations of ethyl

alcohol used in these experiments was very much in excess of those found in vivo after a heavy drinking session. At low concentration of alcohol some activation of the enzyme is found. These effects of activation of an enzyme at low concentration of alcohol and inhibition at high concentration is not unusual (e.g.plasma cholinesterase Whittaker 1968). The inhibition effect is probably caused by denaturation of the protein. Bull and Breese (1978) have shown that the denaturation of protein by alcohol is first order with respect to the protein but between eleventh and eighteenth order with respect to alcohol. They report neither dependence on pH nor difference in susceptibility to alcohol denaturation between the various proteins.

The two types of inhibition curve found for ethyl alcohol (Fig. 16) is however more difficult to explain. Neither curve is a characteristic of any one of our clinical groups. One can however speculate that some people, notably the Japanese are very susceptible to alcohol whilst others are able to imbibe freely. The two groups have been characterised by differences in their liver alcohol dehydrogenase. Are we now meeting such a polymorphism? This hypothesis seems improbable but it is worthy of some speculation.

The effect on PRPP synthetase activity of altering the chain length of the alkyl radical has been studied using methyl, ethyl, propyl and n-butyl alcohols. No difference in concentration profile for a single alkyl alcohol has been observed in any of the three clinical groups. The concentration of alcohol for maximum enzymic

activity of PRPP synthetase was observed to decrease with increasing chain length of the alkyl radical. High concentrations of the higher alcohols were not feasible owing to low miscibility with the aqueous medium. A technical difficulty was the ready appearance of air bubbles after the addition of n-butanol to the enzyme assay system. This was overcome by preincubation of butanol for a fixed period prior to initiation of reaction by the addition of R-5-P. Unlike ethyl alcohol, the other alcohols did not yield two types of inhibition profile for PRPP synthetase . Hershko et al (1969) reported that 2,3-DPG, at concentrations within the range of the high physiological amounts found in human erythrocytes (Barlett 1959), produced a marked inhibition of PRPP synthetase in human erythrocytes. This observation was not only confirmed by Fox and Kelley (1972) but the inhibition was shown to be competitive with R-5-P and PRPP. These workers also proposed, by analogy to the control of the state of haemoglobin oxygenation by 2,3-DPG, Mg^{2+} and MgATP (Bunn et al 1971), that the rate of erythrocyte PRPP synthesis may be indirectly affected by haemoglobin oxygenation. This aspect of 2,3-DPG has not been investigated in the current study but identical inhibition profiles have been found for a normal control and a patient with tophaceous gout (Fig. 18). No statistical difference in the percentage inhibition of PRPP synthetase using a fixed concentration of 2,3-DPG (5·4 mmol/l) has been found in our three clinical groups. Such an observation is in agreement with the findings of Meyskens and Williams (1971) who showed that the inhibition of PRPP synthetase by 2,3-DPG

was similar in erythrocyte lysates from normal, hyperuricaemic and gouty patients. It has been reported that purified rat liver PRPP synthetase responds to a comparativily wide range of inhibitory compounds in the absence of albumin, EDTA or dithiothreitol but "stabilisation" of the enzyme by addition of these compounds results in a markedly restricted range of inhibitors (Roth and Deuel 1974). The "stabilised" enzyme was found to be unaffected by 2,3-DPG. In spite of the presence of EDTA in all haemolysates, appreciable inhibition of PRPP synthetase has been found with 2,3-DPG during the current work (Fig.18).

Previous work on the inhibition of PRPP synthetase from sources as diverse as human erythrocytes (Hershko et al 1969, Fox and Kelley 1972) Ehrlich ascites tumour cells (Wong and Murray 1969) rat liver (Roth and Deuel 1974) and S. typhimurium (Switzer 1971) has been confined to two main classes of inhibitor: purine and pyrimidine derivatives such as nucleosides, nucleotide and phosphonucleotides and carbohydrate derivatives such as phosphomonosaccharides and glycolytic substrates.

The charicature of an old man with gout sitting in an armchair, heavily bandaged foot on a stool, a bottle of port at his side and smoking a pipe provided the stimulus to study nicotine as a potential inhibitor of PRPP synthetase. It was impossible to maintain the pH of the reaction medium using the standard buffer capacity in the presence of nicotine and it seemed probable that the observed activation of PRPP synthetase activity could be attributed to a pH effect. Nicotinamide and nicotinic acid were investigated for their inhibitory

effect on the enzyme. Similar inhibition profiles were obtained for the two inhibitors and no difference was apparent in several samples taken from the three clinical groups.

The effect of uric acid on the activity of PRPP synthetase was studied and for the purpose of a preliminary screening, the laboratory standard uric acid was used. Appreciable inhibition of the enzyme was observed which could not be reconciled with the lack of inhibition observed with slightly lower concentrations of uric acid made up either in phosphate buffer or water. The laboratory standard was made up in lithium carbonate, formaldehyde and sulphuric acid. Any component in this mixture could be expected to affect the enzymic activity but no inhibition was found with lithium carbonate. Although the inhibition profiles of PRPP synthetase, using varying amounts of formaldehyde for a control individual and a patient with tophaceous gout, appear to diverge at 0·08% formaldehyde, no difference in percentage inhibition of the enzymic activity was observed with several haemolysates using 0·08% formaldehyde. Some differences were found with 0·02% formaldehyde but more screening at this concentration is required to substantiate our preliminary results.

Inhibition studies of PRPP synthetase with a fixed concentration of 2 mercaptoethanol produced a wide range of results (Fig. 20). In spite of this vast spectrum, some remarkable results were obtained- albeit not confined to one particular clinical group. One gout patient had been bled twice; the samples differed in age by three months and yet haemolysates gave activation with

mercaptoethanol and showed good agreement (26·5% and 32% respectively). A second gout patient had two samples taken at an interval of one week. Both haemolysates showed inhibition of 4% and 7·5% respectively. Although it is not possible to predict the characteristic inhibition pattern of PRPP synthetase of any of the three clinical groups using 2-mercaptoethanol, repeat samples from the same individual do show the same inhibition characteristics. Similar effects were found using dithiothreitol as differential inhibitor. The two inhibitors, used singly, did not give the same inhibition for PRPP synthetase and some haemolysates gave enzymic activation with one thiol derivative and inhibition with the other. Neither negative nor positive correlation was obtained. There would appear to be a total population polymorphism which is worthy of further investigation and family studies.

Histidine is a metabolite of an alternative metabolic pathway to de novo purine biosynthesis for PRPP. The use of this compound as inhibitor did not differentiate the samples into their three clinical groups. An atypical inhibition profile for histidine, compared with other inhibitors was obtained for PRPP synthetase . It is however very similar in shape to the inhibition profile of PRPP synthetase for ADP reported by Sperling et al (1978). Further studies are clearly necessary. The effects of other pharmacologic and chemical agents, such as tryptophan, caffeine and allopurinol, on the activity of PRPP synthetase were investigated. These experiments were limited either by the low solubility of the chemical or by the lack of ready access to the pure chemical

present in commercial samples of the drug. No useful data was produced from these studies which need drastic revision prior to future experimentation. None of the inhibitors used in the current work were able to differentiate between the three clinical groups investigated but some extension of these studies may reveal a population polymorphism.

Erythrocyte glutathione reductase activity is increased in patients with neoplastic disease (Kerpola et al 1959) hepatitis and obstructive jaundice (loc. cit) and in most patients with anaemia (Ramachandran and Iyer, 1974). The enzymic activity is reported to be reduced during protein-calorie malnutrition (Verjee and Behal 1976). A decrease in erythrocyte glutathione reductase, compared to normals and hyperuricaemics, was found in gouty patients (Table 26). Overall this decrease in enzymic activity is significant (just less than 1 in 100) compared with controls but 1 in 1000 compared with hyperuricaemics. There is no significance between the glutathione reductase activities of the normal and hyperuricaemic groups,(Table 38). But the difference between the control group and the gouty patients may be masked by an age effect. Long (1962) has reported an elevated activity of red cell glutathione reductase in a group of Caucasians with untreated primary gout, and in 23/28 American negroes with gout (Long 1967). Although Long (1967) has found a correlation between the increased glutathione reductase activity and a fast moving electrophoretic variant of the same enzyme, some caution is counselled in the interpretation of his results. The blood samples used in his study were collected for the investigation of

G-6-PD variants occuring with sickle cell anaemia. It is true that many of this negroid group did have gout but West et al (1961) has found increased values of glutathione reductase activity in most patients with sickle cell anaemia. Such findings have prompted Beutler (1969) to question the reliability of previous measurements of glutathione reductase activity which is dependent not only on certain disease states but on vitamin compounds such as riboflavin and nicotinamide.

Plasma glutathione reductase levels (Table 38) show no difference between normals and hyperuricaemias but significant difference between gouts and normals ($p < 0{\cdot}01$) and between gouts and hyperuricaemics ($p < 0{\cdot}001$). There is remarkable constancy in SD calculated from group to group.

Nishizawa et al (1975) found a significant increase of ADA activities in erythrocytes of gouty patients whereas Van Maris et al (1980) has found no elevation of ADA activity in erythrocytes from the same number of hyperuricaemic males. ADA activities in the haemolysates collected in the current study have not been measured, but a random of sera have been segregated according to their uric acid content and the serum ADA activities measured in normals and hyperuricaemic individuals (including gout). The results presented in Table 28 show a significant increase in serum ADA activity of hyperuricaemics compared with normal controls.

The contribution of reduced purine salvage to the hyperuricaemia associated with HGPRT deficiency has been studied by measurement of enzymic activities in the haemolysates from our clinical groups. The results

shown in Table 29 indicate no significant difference between the enzymics activities of the haemolysates from the gout patients compared with normal controls. This is in accord with the findings of Van Maris et al (1980). No HGPRT deficiency was detected in the current work.

It seemed worth while to find out whether any correlations existed amongst the parameters that had accumulated during the current study. Age was the first consideration. The two pathological groups were older than the control group and so any changes found in gouty patients or hyperuricaemic individuals could be an age effect and not the result of the malady. A positive correlation (0·5) with age has been found for both erythrocyte and plasma glutathione reductase (Table 36). Both enzymic activities increase with age in normals but there is only slight evidence for this correlation with plasma glutathione reductase in the hyperuricaemic group (0·27) and none for the gouty group. It can be assumed that there is something swamping the age correlation in gout but it is pointless to eliminate the age factor in this group because the effect is real and would be magnified by taking age into account. There is no correlation of PRPP synthetase activity with age in any of the three clinical groups (Table 36). It can therefore be concluded that there is no need to consider age any further. There is a positive correlation between erythrocyte glutathione reductase and plasma glutathione reductase (0·51) but only low correlation for the two enzymes in the hyperuricaemic group (0·25) and none in the gout group (0·2). On the other hand we find good correlation between erythrocyte PRPP synthetase activity

and erythrocyte glutathione reductase in both the hyperuricaemic and gout group 0·45 and 0·43 but low correlation of these two enzymes in the control group (0·25)(Table 37). The mechanism causing the reduction of erythrocyte glutathione reductase activity is not responsible for the increase in PRPP synthetase activity in the gout or hyperuricaemic patients since a negative correlation between the two is observed, whereas in the normal controls the two enzymes are linked with a positive correlation. Plasma uric acid showed negligible correlation with the enzymes studied in the current work in any of the clinical groups apart from a positive correlation (0·32) with plasma glutathione reductase in the hyperuricaemic group.

None of the electrophoretic studies produced any variants which could be confined to one of our clinical groups. In all systems there was some indication of probable polymorphisms which were undoubtedly a population effect. Repeat samples and family follow up studies will be necessary to confirm the polymorphism and at this stage it is impossible to decide whether there may be an uneven distribution of variants amongst the three clinical groups.

The electrophoretic characterization of human erythrocyte PRPP synthetase was first described by Boer et al (1974) using a radiochemical assay following electrophoresis on cellulose acetate. An anodic single band was observed which migrated faster than haemoglobin at pH 8·5. A slower second band was observed in stored haemolysate Johnson et al (1974) also used cellulose acetate strips for electrophoresis but used the reverse reaction of PRPP synthetase coupled to the hexokinase, G6PD and $NADP^+$ reactions according to Switzer (1968).

They also included creatine phosphate and creatine phosphokinase in their staining fluid as an ATP generating system. A single anodic band, migrating at a slower rate than the "nothing dehydrogenase" was found in all samples except for their mutant enzyme (Becker et al 1973) which also was a single band coincident with the "nothing dehydrogenase". Current investigations have been done on agarose and in agreement with these workers a single band has been observed in all cases but it migrated at a slower rate than haemoglobin. The change in supporting medium could account for the differing relative migrations between the current and previous work. A large variation in intensity of stain has been observed in the current work and this variation cannot be attributed to the relative size of the applied sample since the intensity of the haemoglobin fraction is not proportional to PRPP synthetase intensity. All investigations have been done at pH 8·6 and it seems desirable not only to change the pH using the present supporting medium but to use acrylamide or starch gel for better resolution. Isoelectric focusing on ultra thin acrylamide gels or two dimensional electrophoresis should resolve any multiple bands that are not unmasked by the present technique.

Long (1967) has reported that glutathione reductase migrates as a single band in starch gels at a rate slower than haemoglobin. He also found a variant electrophoretic band which is associated with gout in negroes. This variant has a greater mobility than the usual band and family studies indicate that it has a recessive inheritance with a broad band indicative of

the heterozygous individuals. Anselsletter and Weidemann (1979) have separated glutathione reductase in human serum by gradient polyacrylamide gel electrophoresis at pH 8·2. Two glutathione reductase bands were seen. The faster one could be resolved into a triplet by addition of 2-mercaptoethanol at a final concentration of 12·8 mmol/l. These authors had earlier used agarose electrophoresis and homogenous polyacrylamide gel and found only one slowly moving glutathione reductase band which remained after incubation of all sera with 2-mercaptoethanol (Weidemann and Anselsletter 1977). The present observations are in agreement with the polymorphism described by Long (1967). Some haemolysates have one band just anodically faster than haemoglobin whilst others have an additional band behind haemoglobin. The samples with two bands are presumably the heterozygotes described by Long (1967). No single slow band has yet been observed but one must remember that Long's patients and healthy controls were negroid and it is not unusual for the gene frequencies of enzyme polymorphisms to be altered by ethnic factors (Harris 1975). Isoelectric focusing of erythrocyte glutathione reductase indicated two or three bands on the alkaline side of haemoglobin. More complex patterns may be revealed as the number of samples screened is increased. The serum isoenzymes have not been investigated during the current study.

Electrophoresis of several haemolysates followed by staining for ADA revealed the well established polymorphism (Harris 1975). No new variants were observed and our data is incomplete for the analysis of the distribution of the various genes controlling this

polymorphism amongst our three clinical groups.

It is noteworthy to recall that a mutant HGPRT with significantly reduced activity in erythrocytes has been found in a few patients with gout (Kelley et al 1969, Yu et al 1972). A number of studies have demonstrated the presence of electrophoretic variants of normal human HGPRT, a deficiency of which would cause impaired purine salvage. Arnold and Kelley (1971) have questioned whether the electrophoretic heterogeneity could be due to a nongenetic alteration of the protein which may occur during the preparation of the sample. The haemolysates used in the current study received identical treatment and so any variation found ought not to be attributed to preparation of sample. It is not possible however to eliminate the role, if any, assumed by drug therapy in some individuals. However Fox and Lacroise (1977) found a different pattern of isoenzymes in haemolysates from four patients with partial HGPRT deficiency as compared with normal. There was variation in isoenzymes amongst these patients and their observations suggest that electrophoretic variations is a common occurance in partial HGPRT deficiency and support the existence of structural gene mutations with genetic heterogeneity in this disorder.Gulumian and Wakid (1975) have isolated four components from purified human erythrocyte HGPRT by isoelectric focusing. Variation in electrophoretic migration of HGPRT has certainly been observed in the present study but such variation is not confined to one clinical group. Until repeat specimens and family studies have been persued it is not prudent to claim a polymorphism.

Column chromatography of serum on Bio Rad with subsequent elution using physiological saline shows real potential as the initial step in the identification of any unusual metabolite, apart from uric acid, having absorbance at 290 nm which may occur in sera from gouty patients or individuals with hyperuricaemia. The preliminary investigations reported in this work have shown that it is possible to align synthetic markers to a definate position in the elution profile using saline as eluant. Although the work has been concentrated on purine and pyrimidine bases, nucleosides and nucleotides, we can also identify the elution of tryptophan and tyrosine. The bases such as adenine and guanine are not resolved by this technique but it is then relativily easy to resolve them by HPLC or by other means. It is also highly probable that ATP or GTP are not resolved using this non specific elution method but we are confident that complete resolution can be achieved with HPLC following the initial purification step on Bio Rad which aimed at a broad crude separation of purine pyrimidine derivators. The elution profiles shown in Figs 29&31 do indicate that there are some different components in the sera of random controls and sera from gouty individuals or individuals with hyperuricaemia. This crude chromatography could not as yet be used as an ancillary aid to the diagnosis of gout.

Nature of future work.

The enigma of urate binding to plasma proteins persists but as more sensitive assays for the detection of uric acid are developed, a clear cut resolution will emerge. The effect of different anticoagulants on ultrafiltration should be investigated. It seems worth while either to denature plasma proteins using agents such as sodium dodecyl sulphate which will disrupt the globular proteins into subunit structure or to degrade the proteins with enzymes such as neuraminidase, phospholipases or proteases which will change the structure of the plasma protein molecules. By such techniques it seems probable that the uric acid bound to the proteins could be released. This free uric acid would then be easy to detect .

The study of the association of lipidaemia types and hyperuricaemic should be extended to patients with uncontrolled gout and to other individuals having hyperuricaemia without gout.

The presence of uric acid in leucocytes demands confirmation. Disruption of the cells with enzymes such as lysozyme or proteases would be worthwhile. A rapid detection of uric acid following such treatment would be indicative of the presence of uric acid within the leucocyte whereas a slow appearance of uric acid would favour the presence of precursors, with subsequent synthesis, of uric acid.

The present study has indicated anomalous results for a few individuals and for example one female gout patient had an exceptionally high activity of PRPP synthetase. It is essential not only to confirm such a result with a repeat sample but to check her reported

chemotherapy and to extend our investigations to other close relatives in her family.

The preceding discussion has indicated that the kinetic studies for PRPP synthetase should be repeated at pH 8 preferably in Tris buffer, of adequate buffer capacity to maintain the pH, with the addition of a fixed amount of inorganic phosphate which is essential for enzymic activity. Whenever a linked enzyme assay system is used, the linked enzyme must be dialysed in order to remove ammonium sulphate which curtails the usefulness of the method by precipitation of complex magnesium salts.

Any linked enzyme system has limited application when studying inhibitors. The current work on PRPP synthetase has been frustrated by interference of an inhibitor with the coupled reactions. It is therefore desirable to use an alternative assay and the most promising appears to be the measurement of the rate of disappearance of the substrate ATP using the principle of chemiluminescence. It would be useful to study the effect of some inhibitors such as alcohol and nicotine using this new assay for PRPP synthetase.

We should also investigate whether the high activity of PRPP synthetase found in gout patients is due to the lack of substrate for the purine salvage enzyme HGPRT.

The current electrophoretic survey was essentially a preliminary scan. New staining techniques for the detection of the three enzymes studied have been developed during the current work and these techniques should be used for other gel support systems as well as for the techniques of isoelectric focusing. The apparent

polymorphisms should be consolidated by family studies. It appears from unreported current work that the isoenzyme pattern for serum ADA is coincidental with serum lipoprotein staining. Such an observation demands further investigation and it is proposed to selectively degrade the lipoproteins in serum with enzymes such as cholesterol oxidase and phospholipases prior to electrophoresis and subsequent staining for ADA and phospholipids.

The potential of column chromatography of serum on Bio Rad with subsequent elution using physiological saline should be fully explored with wider application not only to gout patients but to other hyperuricaemic individuals.

TABLE 1 ASSAY OF URIC ACID

Oxidation of Uric Acid to Allantoin

Discipline	Method	Chromaphore	Wave length	*pH	Reference
a) Chemical					
Colorimetric	Reduction of ferric chloride	1,10 phenanthroline	500	5.5	Morin and Prox (1973)
Spectrophotometric	Reduction of potassium ferricyanide	-	293	11.3	Morin (1974)
Colorimetric	Reduction of ferric chloride	2,4,6 pyridyl-s-triazine	593	4.95	Morin (1974)
b) Automated					
Spectrophotometric	Uricase/Catalase/Aldehyde dehydrogenase	$NADP^+$	334	8.5	Barti et al (1979)
Colorimetric	Reduction of phospho-tungstate		660	9.6	Lolekha et al (1978)
Colorimetric	Reduction of ferric chloride	1,10 phenanthroline	505	3.6	Nelson and Batra (1975
Colorimetric	Uricase/Catalase	4-amino phenazone	505 or 520	9.0	Klose et al (1978)
Colorimetric	Uricase/peroxidase	3-methyl-2-benzothiazolinone hydrazone and N-N-dimethylaniline	600	9.2	Kamoun and Douay (1978
Fluorimetric	Immobilised uricase/ peroxidase	p-hydroxyphenylacetic acid and 5,5' dicarboxymethyl-2,2' dihydroxybiphenyl			
Colorimetric	Immobilised uricase/ peroxidase	Aminophenazone and dichlorophenol	508	7.0	Sundaram et al (1978)
Colorimetric	Immobilised uricase/ peroxidase	3-methyl-2-benzolinone	590	9.2	Endo et al (1979)
c) Enzymic (Manual)					
Spectrophotometric	Uricase	-	293	8.6	Praetorius (1949)
Spectrophotometric	Uricase	-	286	9.4	Remp (1970)
Fluorimetric	Uricase/peroxidase	Dichlorofluoresein and cycloheptа amylose	-	7.5	Kato et al (1979)
Colorimetric	Uricase	3,5 dichloro-2-hydroxybenzene sulphonic acid and 4-amino phenazone	520	7.0	Fossati et al (1980)
Colorimetric	Uricase/peroxidase	o-dianisidine	N.G.	N.G.	Lorentz and Berndt (1967)
Spectrophotometric	Uricase/Catalase/aldehyde dehydrogenase	NAD^+ or $NADP^+$	340	8.5	Haeckel (1976)
Colorimetric	Uricase/Catalase	2-acetylacetone	410	N.G.	Cunningham and Heavey (1978)
Colorimetric	Uricase/Catalase	3,5 diacetyl-1,4-dihydrolutidine	410	7.0	Kageyama (1971)
Colorimetric	Uricase/peroxidase	crystal violet	590	9.2	Meites et al (1974)
Colorimetric	Uricase	p-hydroxybenzoate and 4-amino antipyrine	500	7.0	Trivedi et al (1978a)
Spectrophotometric	Uricase/catalase/alcohol dehydrogenase	NAD^+	293	7.0	Trivedi et al (1978b)
Chromatographic					
HPLC	Electrochemical detection	-	-	5.25	Slaunwhite et al (1975)
HPLC	Spectrophotometric detection	-	280	4.0	Kiser et al (1980)
HPLC	Electrochemical detection	-	-		Pachla and Kissinger (1975)
HPLC	Mass spectroscopy detection	-	-		Lim et al (1978)
HPLC	Chromatographic detection	-	-		Brown et al (1980
Others					
Anion exchange resin	Uricase	-	292-5	8.0	Simmonds (1967)
Polargraphic	Oxygen consumption with uricase	-	-	9.4 or 8.5	Meites et al (1974)
Mass fragmentographic	Gas chromatography-mass spectrometry	-	-	-	Ohman (1979)

* Values quoted for pH refer either to reaction or elution conditions according to the method.

Table 2 Conditions associated with hyperuricaemia

Decreased renal excretion	Increased nucleic acid turnover	Other causes
Nephritis	Chronic leukaemia	Eclampsia
Urinary obstruction	Polycythemia	Chronic lead poisoning
Urinary suppression	ACTH or cortisone therapy	Hypertension
Destructive renal lesions	Pernicious anaemia	Obesity
Renal hypogenesis	Sickle cell anaemia	Degenerative vascular disease
Congestive heart failure	Thalassemia	Sarcoidosis
Adrenocorticol insufficiency	Lymphoblastoma	Chronic beryllium disease
Hepatic disease	Macroglobinaemia	Psoriasis
Drug therapy (nicotinic acid)	Starvation	Down's syndrome
Oral thiazide diuretics	Gout	Neoplastic disease
Myocardial infarction	Pneumonia	Multiple myeloma

Table 3 Drug induced hyperuricaemia

Drug	Mechanism
Diuretic agents (except spironolactone)	Not fully understood Increased proximal tubular resorption of uric acid and/or impaired tubular secretion.
Alcohol	Lactic acid inhibits uric acid secretion
Aspirin	Low doses inhibit uric acid secretion
Cytotoxic drugs	Increases nucleic acid turnover and excretion
Ethambutol	Decreases uric acid clearance
Levodopa	Unknown
Nicotinic acid	Increases purine production, decreases uric acid excretion
Pyrazinamide	Inhibits uric acid secretion

Table 4 Genetic Aspects of Hyperuricaemia and Gout

Molecular defects	Type	Metabolic disturbance	Inheritance
Primary hyperuricaemia			
Specific enzyme defects	PRPP synthetase variants	Increased PRPP and Uric Acid production	Unknown
"	HGPRT partial deficiency	Increased purine biosynthesis denous by surplus PRPP. Increased uric acid production	X linked
Undefined	Normal excretion of uric acid	Over production or retention of uric acid	Polygenic
"	Over excretion of uric acid	Increased uric acid formation	Polygenic
Secondary hyperuricaemia			
Increased purine biosynthesis denovo	Glucose-6-phosphatase deficiency or absence	Increased production and decreased excretion of uric acid	Autosomal recessive
"	HGPRT deficiency	Increased uric acid production Lesch-Nyhan syndrome	X linked

Table 5 Reactions of PP-Ribose-P

Phosphorobosyl Acceptor	Product	Enzyme Commission Number
Glutamine, NH_3	Phosphoribosylamine	2.4.2.14
Adenine	Adenylate	2.4.2.7
Guanine, hypoxanthine	Guanylate, enosinate	2.4.2.8
Xanthine	Xanthylate	
Orotate	Orotidylate	2.4.2.10
Uracil	Uridylate	2.4.2.9
2,4-Diketopyrimidines, 2,6-diketopurines	Uridylate, oroticylate, 3-phospho-ribosyl-uric acid, 3-phosphoribosyl-xanthine	
Nicotinate	Nicotinate ribonucleotide	2.4.2.11
Nicotinamide	Nicotinamide ribonucleotide	2.4.2.12
Quinolinate	Quinolinate ribonucleotide + CO_2	
Imidazoleacetate (+ATP)	Phosphoribosyl-imidazoleacetate (+ADP + Pi)	
Histamine (+ ATP)	Phosphoribosyl-histamine (+ADP + Pi)	
Anthranilate ATP	Phosphoribosyl-anthranilate 1-(5-Phosphoribosyl)ATP	

Table 6 Subunit Analysis of PRPP synthetase

Electrophoresis S D S - Polyacrylamide 10M Urea-Polyacrylamide:	 Single Protein Band, MW 33,500 Single Protein Band,
Amino Acid Analysis:	18 Arginine + 20 Lysine Residues per 33,500 MW Subunit
Tryptic Peptide Mapping	37 Peptides Identified (Fluorescamine Reagent)
Amino-Terminal Analysis	Threonine Only (Dansyl Chloride Method)
Analytical Ultracentrifugation In 0.01 N HCl:	Molecular Weight 33,200 (Sedimentation Equilibrium)

Table 7 Aggregated Forms of PRPP synthetase

Relative S20, w	Stokes Radius	Molecular Weight	No. of Subunits	Enzyme Activity
	Å			
4.8	31	65,000	2.0	<3%
7.1	43	133,000	4.0	<3%
9.7	61	258,000	7.7	<3%
15.9	74	513,000	15.4	100%
22.1	108	1,042,000	31.1	100%

TABLE 8 Enzyme mutations found by Electrophoretic studies in patients with Gout or Hyperuricaemia

Enzyme	Number of Variants	Technique	Number of cases	Reference
PRPP Synthetase	1	Cellulose acetate electrophoresis	3	Becker et al (1973)
		" " "	1	Lebo and Martin (1976)
HGPR transferase	1	Polyacrylamide gelelectrophoresis	4	Bakay et al (1972)
	1	Isoelectric focusing	1	Fox et al (1975)
	1	Isoelectric focusing	1	Gutensohn and John (1979)
APR transferase	1	Polyacrylamide gel electrophoresis	1	Nyhan and Bakay (1975)

Table 10 Inhibitors studied and range of concentrations investigated.

Inhibitor	Concentration range
Ethyl alcohol	0- 22·5%
Methyl alcohol	0- 22·5%
Propanol	0- 4·5%
Butanol	0- 2·7%
2.3.Diphosphoglycerate	1·8- 7·2 mmol/l
Nicotinamide	22·5-225·0 mmol/l
Nicotinic acid	0- 18·0 mmol/l
Histidine	0- 90·0 mmol/l
Formaldehyde	0- 0·08mmol/l

Table 11 Inhibitors studied at a fixed concentration

Inhibitor	Concentration
2.3.Diphosphoglycerate	5·4 mmol/l
2 Mercaptoethanol	25·0 mmol/l
Dithiothreitol	25·0 mmol/l
Histidine	22·5 mmol/l
Uric acid	112·6 mmol/l
Formaldehyde	0·08%
Formaldehyde	0·016%
Lithium carbonate	1·8 mmol/l
Tryptophan	11 ·3mmol/l
Caffeine	18· Ommol/l

Table 2 Urate binding to plasma proteins by occlusion at diastolic pressure

Expt. Number	Time (mins)	Total Protein g/l	Albumin g/l	Total bilirubin µmol/l	Conjugated bilirubin µmol/l	Alkaline phosphatase u/l	Ca^{2+} mmol/l	PO_4^{111} mmol/l	IgG g/l	IgA g/l	IgM g/l	T_4 nmol/l	Temp °C	Uric Acid µmol/l
1	0	75	47.5	11.5	0.2	64.5	2.58	0.975					37	359.5
	4	75.5	46.5	11.5	0.2	62.0	2.58	0.970					37	359.5
	10	84.5	52.0	12.0	0.2	67.5	2.75	0.980					37	358.9
2	0	77.0	46.0	11.0	4.0	74.0	2.57	1.01	14.13	1.77	0.60	123	37	345.2
	10	85.0	49.0	12.0	4.0	75.0	2.69	1.05	14.57	1.91	0.71	138	37	342.3
3	0	72.0	44.5			85.0	2.41	1.16					37	292
	10	83.5	55.5			96.0	2.66	1.055					37	289
4	0	72.0	45.0			80.0	2.385	1.050					4	304
	10	82.0	54.0			92.5	2.595	0.960					4	307

Table 13 Changes in mean serum levels of total protein, albumin, calcium and uric acid induced by venous stasis in different individuals

Experiment	Total Protein g/l	Albumin g/l	Calcium m mol/l	Uric Acid μ mol/l
1	12.7	9.4	6.2	-1.7
2	10.4	6.5	4.7	-0.8
3	16.0	19.8	10.4	-1.0
4	13.9	20.0	8.8	+1.0
5*	14.0	11.0	5.4	N.G.

* Data of Christiansen et al (1975) - mean values in epileptic patients

Table 14 Urate binding to plasma proteins as estimated by ultrafiltration using Amicon cones

Sample	Total in serum μ mol/l	Ultrafiltrate μ mol/l	Bound μ mol/l	% Bound
B	309.5	309.5	0	0
C	708.3	607.1	101.2	14.3
R	169.6	163.7	5.9	3.5
G	261.9	258.9	3.9	1.1

Table 15 Uric acid content (mean and S.D.) in 3 clinical groups of individuals

Clinical Group	Male			Female			Total		
	Number of individuals	Uric acid µ mol/l (mean)	S.D.	Number of individuals	Uric acid µ mol/l (mean)	S.D.	Number of individuals	Uric acid µ mol/l (mean)	S.D.
Normal	21	336	67	30	301	80	51	315	76
Gout	47	492	137	7	404	135	54	481	138
Hyperuricaemic	24	509	143	12	564	166	36	527	151

Table 16 PRPP synthetase activities (mean and S.D.) in 3 clinical groups of individuals

Clinical Group	Male			Female			Total		
	Number of individuals	Enzymic acitivity (mean)	S.D.	Number of individuals	Enzymic activity (mean)	S.D.	Number of individuals	Enzymic activity (mean) †	S.D.
Normal	21	106.4	29.6	30	88.3	30.6	51	96	31.2
Gout	47	120.4	37.6	7	133.7	79.1	54	122	44.2
Hyperuricaemic	24	121.9	35.3	12	98.8	29.2	36	114	34.8

Activity Units: μ moles substrate/mg./ml. protein/hr

† Average standard error of the difference between means = 7·74

Table 17 Statistical analysis of the regression equation for the Lineweaver-Burke plots $\frac{1}{v}$ v $\frac{1}{[S]}$, where v is the velocity of reaction and [S] is the substrate concentration, of the effect of varying concentrations of R-S-P on the red cell PRPP synthetase activity in six individuals.

Individual	Clinical condition	Regression equation†	S.D. of Y about regression line	R^2	Analysis of Variance
H	Gout	y = 0.0014 + 0.0001x	0.0004954	99.4	0.000442
S	Control	y = 0.0004 + 0.0003x	0.001870	99.2	0.004751
B	Tophaceous gout	y = 0.0014 + 0.0001x	0.0007220	98.4	0.000348
Du	Hyperuricaemia	y = 0.0010 + 0.0001x	0.0003019	99.5	0.000190
Da	Gout	y = 0.0005 + 0.0001x	0.0009962	98.0	0.000545
F	Control	y = 0.0012 + 0.0001x	0.0003387	99.6	0.0003198

† $y = \frac{1}{v}$, $x = \frac{1}{[S]}$

Table. 18

Mean Michaelis constants and maximum velocities for R-5-P, $MgCl_2$ and ATP determined by Lineweaver Burke plots for PRPP synthetase activity.

Substrate	Mean Km	Mean Vmax	Activity units
R-5-P	$5{\cdot}6 \times 10^{-5}$	860	μ mol/ml/hr
$MgCl_2$	$7{\cdot}0 \times 10^{-4}$	1092	μ mol/ml/hr
ATP	$2{\cdot}9 \times 10^{-5}$	907	μ mol/ml/hr

Table 19 Effect of varying the concentration of phosphate on PRPP synthetase activity.

Sample number	Phosphate concentration m mol/l			
	0	1	20	60
1	0	1·3	13·2	105·8
2	0	6·2	23·8	59·7
3	0	0	10·8	97·0
Mean	0	2·5	15·9	87·5

Table 20 Mean percentage inhibition of PRPP synthetase with varying concentrations of Ethyl alcohol in three groups of individuals.

% Concentration of ethyl alcohol	Mean % Inhibition			
	Total all groups	Normal	Gout	Hyperuricaemic
2.25	-0.7	-1.7	-0.4	0.3
4.5	-0.1	0.8	-0.5	-0.4
9.0	3.7	7.0	2.1	2.2
13.5	16.0	22.6	12.5	16.0
18.0	31.2	27.2	29.2	49.2

Table21 Mean percentage inhibition of PRPP synthetase with varying concentrations of Ethyl alcohol showing the two different shape curves observed.

% Concentration of Ethyl alcohol	Mean % Inhibition		
	Combined curves	Curve shape 1	Curve shape 2
2.25	-0.7	-1.5	1.2
4.5	-0.1	-1.1	2.3
9.0	3.7	-0.7	11.5
13.5	16.0	9.9	31.7
18.0	31.2	29.6	56.3

Table22 Mean percentage inhibition of PRPP synthetase with varying n-butanol in three groups of individuals.

% Concentration n-butanol	Mean % Inhibition			
	All groups	Normal	Gout	Hyperuricaemic
0.23	-2.4	-3.8	-1.8	-2.4
0.45	-1.8	-3.3	1.3	-1.8
0.90	2.2	-0.2	3.8	2.2
2.30	7.3	6.8	6.0	12.9

Table. 23

Percentage inhibition of PRPP synthetase activity using 5·4 m mol/l 2:3 diphosphoglycerate.

Group of individuals	Mean % inhibition	S.D.	Number of individuals
Normal	40·5	2·6	3
Gout	41·0	4·3	6
Hyperuricaemic	41·2	0·2	2
All groups	41·0	3·1	11

Table 24 Mean percentage inhibition of PRPP synthetase with varying concentrations of nicotinamide in three groups of individuals.

Concentration of nicotinamide mmol/ℓ	Clinical condition					
	Normal		Gout		Hyperuricaemia	
	% Inhibition	S.D.	% Inhibition	S.D.	% Inhibition	S.D.
22.5	3.7	0.7	2.5	1.2	3.95	0.1
90.0	15.2	1.8	12.9	2.4	13.70	3.2
180.0	48.5	6.4	48.3	2.7	47.10	6.8
225.0	64.1	4.6	64.8	2.7	61.25	0.1

Table 25 Mean % Inhibition of PRPP synthetase using two fixed concentrations of Formaldehyde.

Group of individuals	Concentration of Formaldehyde.			
	0·08%		0·016%	
	mean % inhibition	S.D.	mean % inhibition	S.D.
Normal	94·6	2·9	48·2	2·9
Gout	93·1	4·2	43·0	4·2
Hyperuricaemics	93·3	2·2	56·6	3·2

Table 26 Erythrocyte Glutathione reductase activities (mean and S.D. in 3 clinical groups of individuals

Clinical Group	Male			Female			Total		
	Number of individuals	Enzymic activity (mean)	S.D.	Number of individuals	Enzymic activity (mean)	S.D.	Number of individuals	Enzymic activity (mean)†	S.D.
Normal	21	101.55	69.9	30	110.98	56.70	51	107.1	62.0
Gout	47	81.93	43.1	7	87.26	42.60	54	82.6	42.7
Hyperuricaemic	24	128.03	74.8	12	106.58	72.9	36	120.9	73.8

Activity units: μ moles substrate/mg. protein/ml/hr

† Average standard error of the difference between means = 12·15

Table 27 Plasma Glutathione reductase activities (mean and S.D.) in 3 clinical groups of individuals

Clinical Group	Male			Female			Total		
	Number of individuals	Enzymic activity (mean)	S.D.	Number of individuals	Enzymic activity (mean)	S.D.	Number of individuals	Enzymic activity (mean)†	S.D.
Normal	21	571	260	30	665	198	51	626	228
Gout	47	520	238	7	445	203	54	511	233
Hyperuricaemic	24	648	200	12	648	200	36	683	218

Activity units: μ moles substrate/ml/hr

† Average standard error of the difference between means = 46·83

Table. 28 Mean activity and standard deviation of Adenosine deaminase in normal and hyperuricaemics (including gout).

Group of Individuals	Mean ADA Activity u/l	Standard Deviation	Number of Individuals
Normal	2·1	0·6	40
Hyperuricaemic	3·4	1·2	43

Table 29 Mean and S.D. for HGPRT in two clinical groups of individuals

Mean and S.D.	Clinical group	
	Normal	Gout
Mean	143	156
S.D.	85	74
Number of individuals	10	35

Table. 30 Uric acid content of normal control samples and hyperlipidaemia samples classified according to Fredrickson.

Fredricksons classification	Number of individuals	Uric acid µmols/l		
		Mean	S.D.	Range
Normal	93	317	95	90-568
Type IV	103	433	108	120-700
Type IIb	53	400	91	84-658
Type IIa	26	331	80	120-485

Table 31 Total number of sera analysed for uric acid content each year showing the number of hyperuricaemic samples and their percentage of the total

Year	Number	Number of hyperuricaemics	% hyperuricaemics
1970	1270	301	23.7
1971	1270	353	27.8
1972	1711	538	31.4
1973	1996	699	35.0
1974	2207	833	40.0
1975	2736	977	35.7
1976	3154	1169	37.1
1977	3273	1123	34.3
1978	3451	1276	37.0
1979	3314	1199	36.2

Table32 Number of hyperuricaemic samples found in a routine clinical laboratory. Distribution of abnormal values according to sex during a $2\frac{1}{3}$ year period.

Year	Number of Individuals investigated	% hyperuricaemic samples		
		Male	Female	Total
1976	1876 male 1278 female	46.5	23.2	37.1
1977	1946 male 1327 female	42.8	21.9	34.3
1978 (Jan-April)	709 male 479 female	42.5	24.0	35.0
$2\frac{1}{3}$ Year	4531 male 3084 female	44.2	22.8	35.6

Table 33 Number of hyperuricaemic samples found in a routine clinical laboratory. Month by month analysis with sex segregation over a 2 year period.

		% hyperuricaemic samples											
	Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Total Population	1976	35.5	35.7	37.8	37.1	37.8	41.5	38.0	40.2	38.0	33.3	33.6	34.9
	1977	31.0	31.6	31.8	35.6	35.0	35.6	38.7	34.6	37.0	36.5	32.0	33.2
Male Population	1976	43.0	48.6	43.8	42.3	50.7	52.4	45.9	53.5	51.6	45.0	37.2	47.0
	1977	42.5	35.9	38.5	46.7	40.4	46.5	46.1	43.7	44.5	46.8	39.4	44.0
Female Population	1976	22.0	11.1	26.0	29.6	20.4	28.4	26.0	21.2	24.5	18.3	27.7	17.8
	1977	14.8	25.5	22.0	22.0	27.1	19.6	27.9	20.5	23.4	18.9	21.5	17.4

Table 34 Kinetic properties of PRPP synthetase.

ENZYME SOURCE	MICHAELIS CONSTANTS				REFERENCE
	R-5-P	ATP	Mg^{2+}	Pi	
Human erythrocytes	$1{\cdot}5\times10^{-5}$M	$5{\cdot}0\times10^{-5}$M		$1{\cdot}0\times10^{-3}$M	Hersko et al (1969)
Human erythrocytes	$3{\cdot}3\times10^{-5}$M	$1{\cdot}4\times10^{-5}$M	$2{\cdot}0\times10^{-4}$M		Fox and Kelley (1972)
Rat liver	$2{\cdot}9\times10^{-4}$M	$2{\cdot}2\times10^{-4}$M			Roth et al (1974)
Lymphocytes	$5{\cdot}1\times10^{-5}$M	$1{\cdot}8\times10^{-5}$M			Danks and Scholus (1974)
S. typhimurium	$1{\cdot}6\times10^{-4}$M	$4{\cdot}6\times10^{-5}$M		$2{\cdot}0\times10^{-3}$M	Switzer (1971)
				$4{\cdot}0\times10^{-3}$M	
Human erythrocytes	$1{\cdot}2\times10^{-5}$M	$7{\cdot}5\times10^{-5}$M		$5{\cdot}2\times10^{-4}$	Sperling et al (1973)
Mutant erythrocytes	$1{\cdot}2\times10^{-5}$M	$6{\cdot}0\times10^{-5}$M		$4{\cdot}8\times10^{-4}$	Sperling et al (1973)
Human erythrocytes	$1{\cdot}4\times10^{-5}$M	$6{\cdot}0\times10^{-6}$M			Becker et al (1973)
Human mutant erythrocytes	$2{\cdot}1\times10^{-5}$M	$1{\cdot}1\times10^{-5}$M			Becker et al (1973)
Ehrlich Ascites tumour cells	$2{\cdot}0\times10^{-4}$M			$3{\cdot}3\times10^{-3}$M	Murray and Wong (1967)
Human erythrocytes	$5{\cdot}6\times10^{-5}$M	$2{\cdot}9\times10^{-5}$M	$7{\cdot}0\times10^{-4}$M		Present study

Table. 3 5 Correlation of red cell Glutathione reductase and plasma glutathione reductase activities in three clinical groups.

Enzyme	Clinical group											
	Normal				Gout				Hyperuricaemic			
	Mean	SD	corr. coef.	significance†	Mean	SD	corr. coef.	significance†	Mean	SD	corr. coef.	significance†
Glut. reductase (Erythrocyte)	107·1	62·0			82·6	42·7			120·9	73·8		
v			0·512	* * *			0·199	N S			0·247	N S
Glut. reductase (Plasma)	626·0	228			511·0	233			683·0	218		

† significant at 1 in 1000 * * *
significant at 1 in 100 * *
significant at 1 in 20 *
Not significant N S.

Table 36 Correlation of Age with Enzymic activities in 3 clinical groups of individuals

Enzyme	Clinical type	Number of individuals	Correlation coefficients	Significance†
PRPP Synthetase	Normal	51	0.10	NS
	Gout	54	-0.21	NS
	Hyperuricaemia	36	-0.19	NS
Glutathione reductase (Erythrocyte)	Normal	51	0.53	***
	Gout	54	0.04	NS
	Hyperuricaemia	36	0.002	NS
Glutathione reductase (Plasma)	Normal	51	0.49	***
	Gout	54	0.05	NS
	Hyperuricaemia	36	0.27	NS

† significant at 1 in 1000; ***
significant at 1 in 100 ; **
significant at 1 in 20 ; *
Not significant ; NS

Table. 37 Correlation of red cell PRPP synthetase and erythrocyte Glutathione reductase activities in three clinical groups of individuals.

Enzyme	Clinical group											
	Normal				Gout				Hyperuricaemic			
	Mean	SD	corr. coef.	significance	Mean	SD	corr. coef.	significance	Mean	SD	corr. coef.	significance
PRPP synthetase	96·0	31·2			122·0	44·2			114·0	34·8		
v			0·245	*			0.445	* *			0.430	*
Glut. reductase (Erythrocytes)	107·1	62·0			82·6	42·7			120·9	73·8		

significant at 1 in 1000 * * *
significant at 1 in 100 * *
significant at 1 in 20 *
Not significant N S

Table. 38 Standard error of difference between mean enzymic activities (students t test) of red cell PRPP synthetase, red cell Glutathione reductase and plasma Glutathione reductase in three clinical groups:- normal, gout and hyperuricaemic.

Clinical group	PRPP synthetase			Glutathione reductase (Erythrocytes)			Glutathione reductase (Plasma)		
	t	Degrees of freedom	significance †	t	Degrees of freedom	significance †	t	Degrees of freedom	significance †
Normal v Gout	-3·399	103	* * *	2·634	84	* *	2·380	104	*
Normal v hyperuricaemic	-2·377	75	*	-0·848	71	N S	-1·324	78	N S
Gout v hyperuricaemic	1·012	91	N S	-3·065	93	* * *	-3·550	93	* * *

† significant at 1 in 1000 * * *
significant at 1 in 100 * *
significant at 1 in 20 *
Not significant N S

Fig. 1 Formation of Uric acid by deamination of Adenine and Guanine.

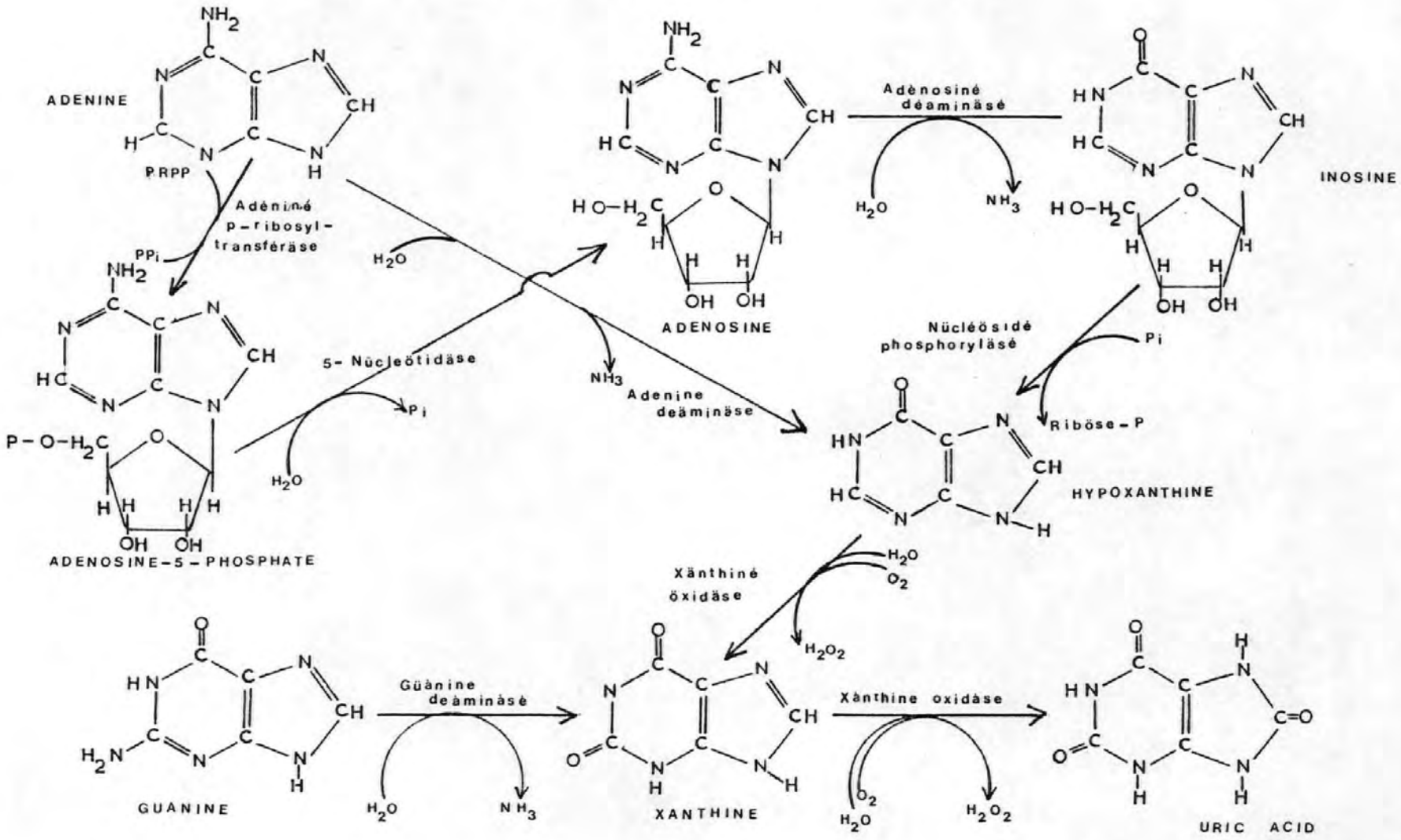
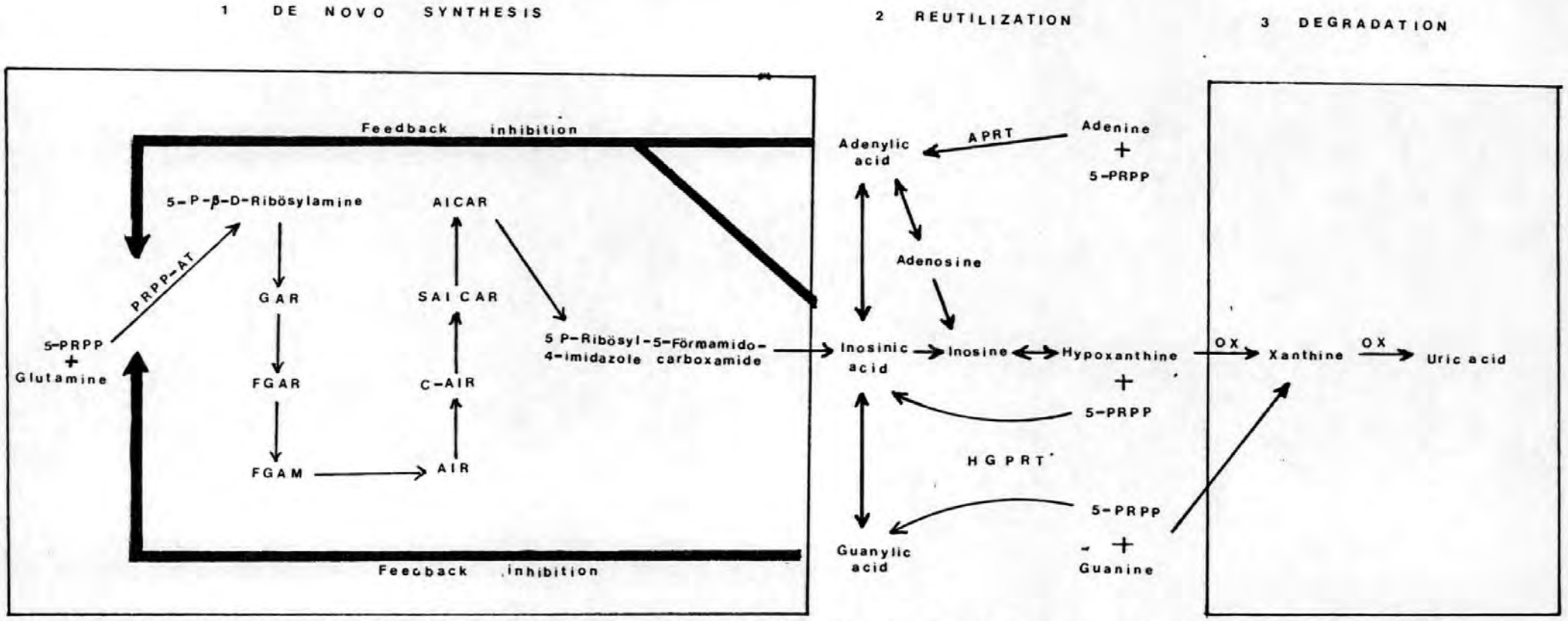


Fig. 2 Purine metabolism and Uric acid production, showing 1. De novo synthesis, 2 reutilization and 3 degradation.



Key:- 5-PRPP, 5-phosphoribosylpyrophosphate; PRPP-AT, PRPP aminotransferase; GAR, glycinamide ribonucleotide; FGAR, ∝-N-formyl GAR; FGAM, ∝-N-formylglycinamidine ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; C-AIR, 5-aminoimidazole-4-carboxylic acid ribonucleotide; SAICAR, 5-aminoimidazole-4-N-succinocarboxamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; APRT, adenine phosphoribosyltransferase; HGPRT, Hypoxanthine guanine phosphoribosyl transferase; XO, Xanthine oxidase.

Fig. 3

Correlation between A/A phosphotungstate method (y) and Uricaquant method (x) for the determination of uric acid concentration in serum. The straight line indicates a 1:1 correlation

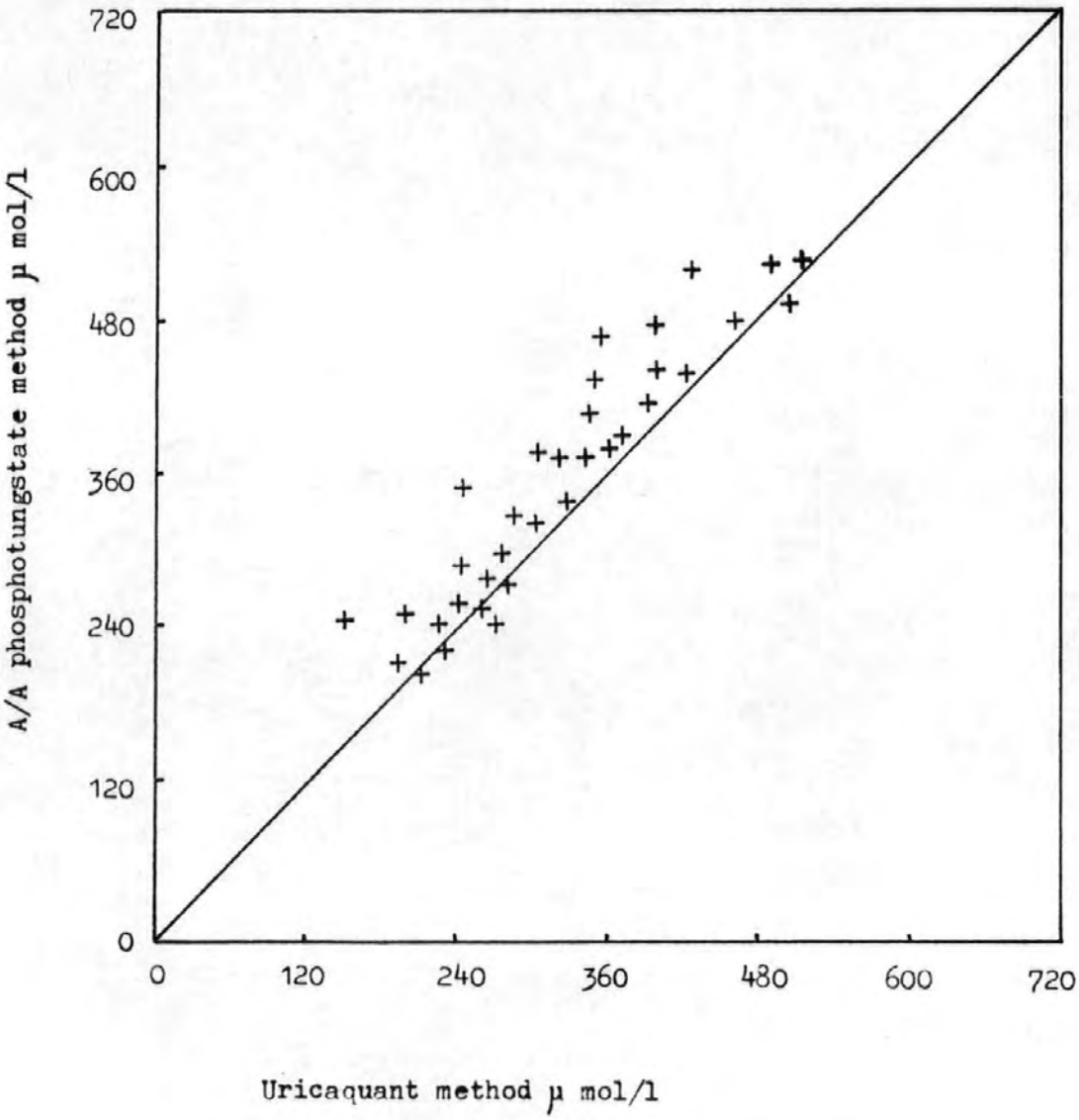


Fig. 4

Correlation between TPTZ method (y)
and Uricase method (x) for the determination
of uric acid concentration in serum
The straight line indicates a 1:1 correlation.

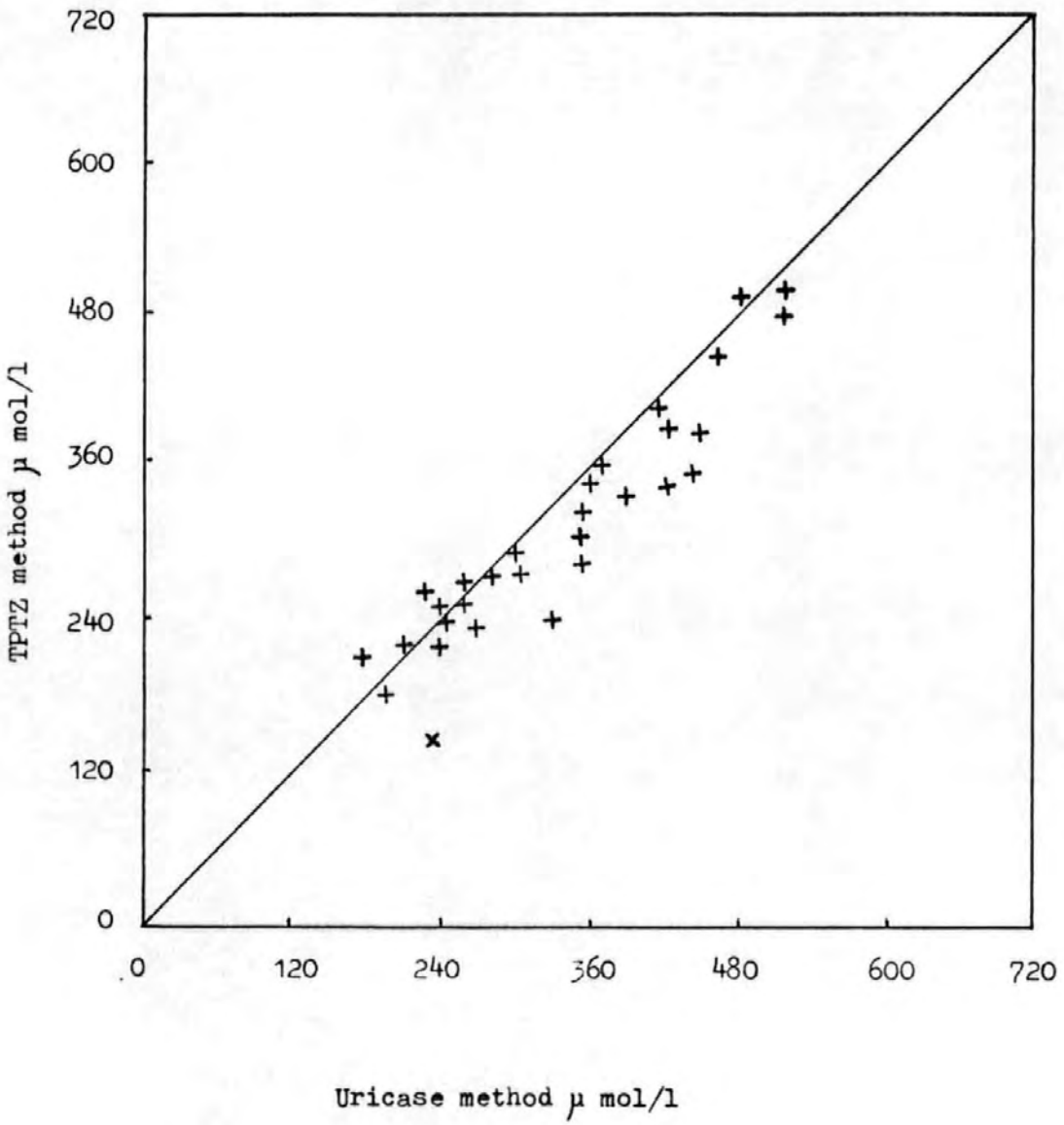


Fig. 5

Correlation between Uricase method (y)
and Uricaquant method (x) for the determination
of uric acid concentration in serum.
The straight line indicates a 1:1 correlation.

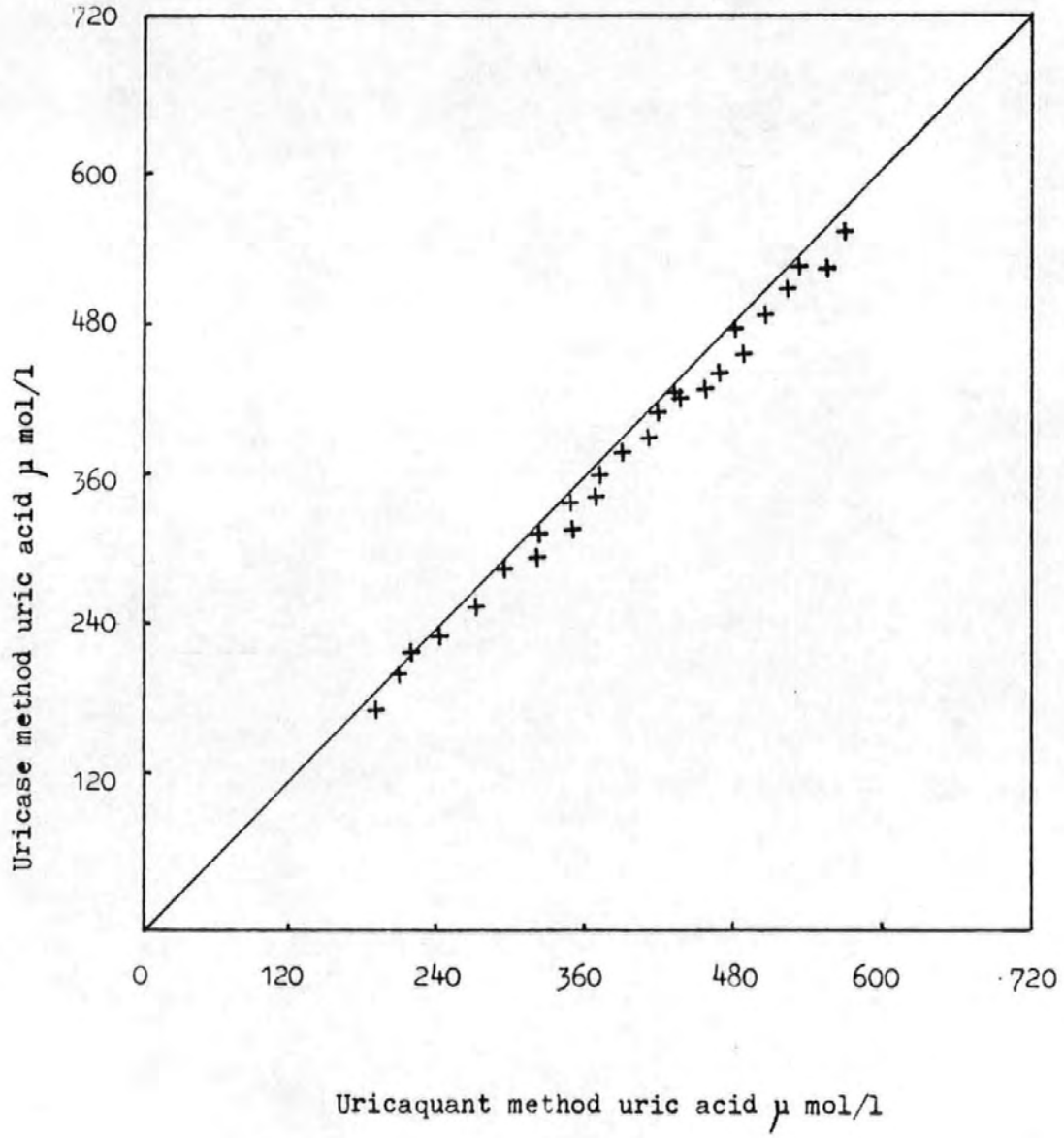
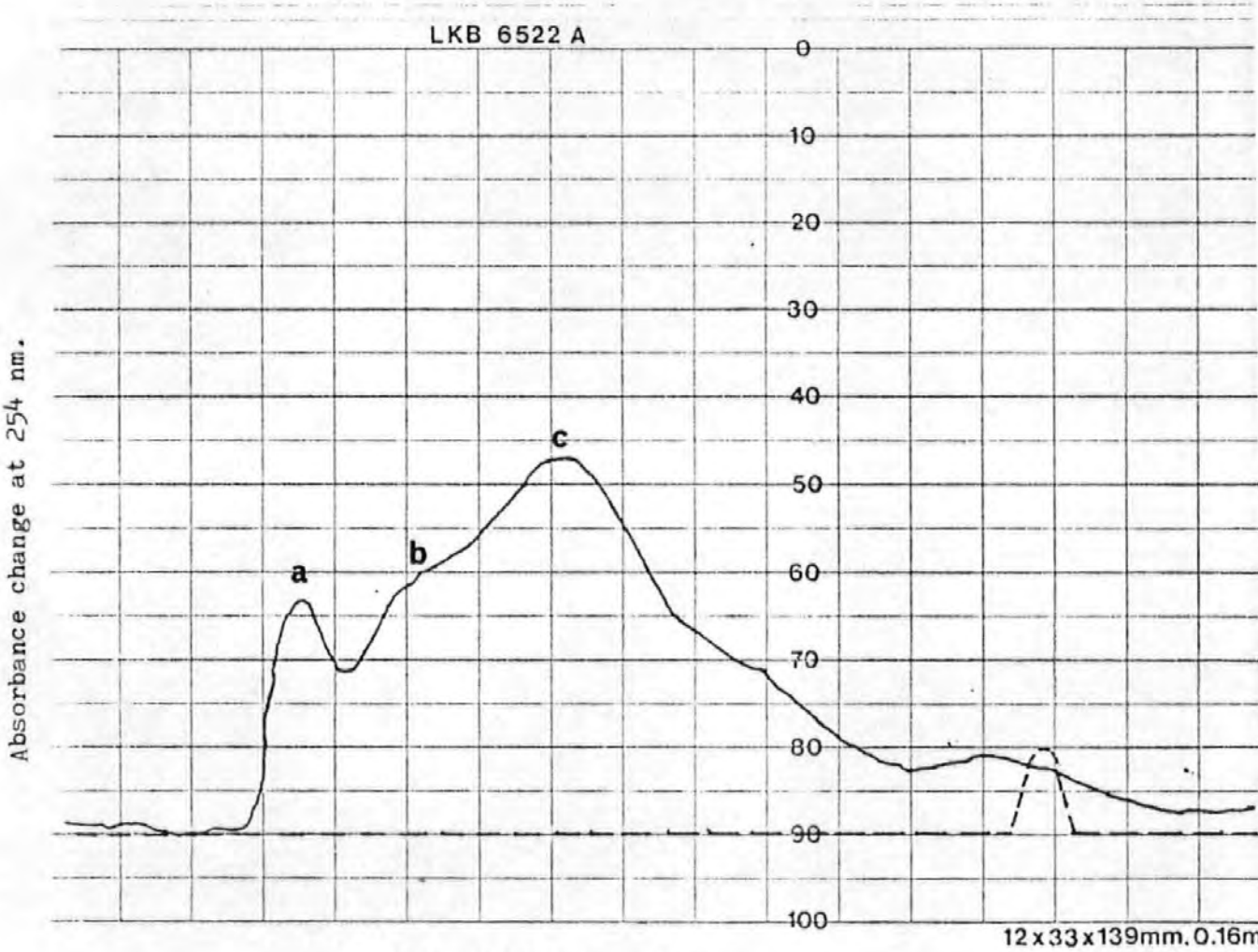


Fig. 6 Scan of eluant at 254nm following elution of 0·2 ml of serum by 0·9 % saline after Sephadex G200 gel filtration.



——— Scan of eluant at 254 nm.

------ Fractions of eluant collected indicating detected uric acid position.

Main componants under peaks a), b) and c).

a) α_2 macro; IgM and β globulins.

b) IgG, α_2 and β globulin.

c) Albumin, α_1 antitrypsin, transferin, α_1 and α_2 globulin.

Fig. 7 Distribution of serum uric acid concentration in individuals with Type IV hyperlipidaemia and in individuals with normal lipid profiles.

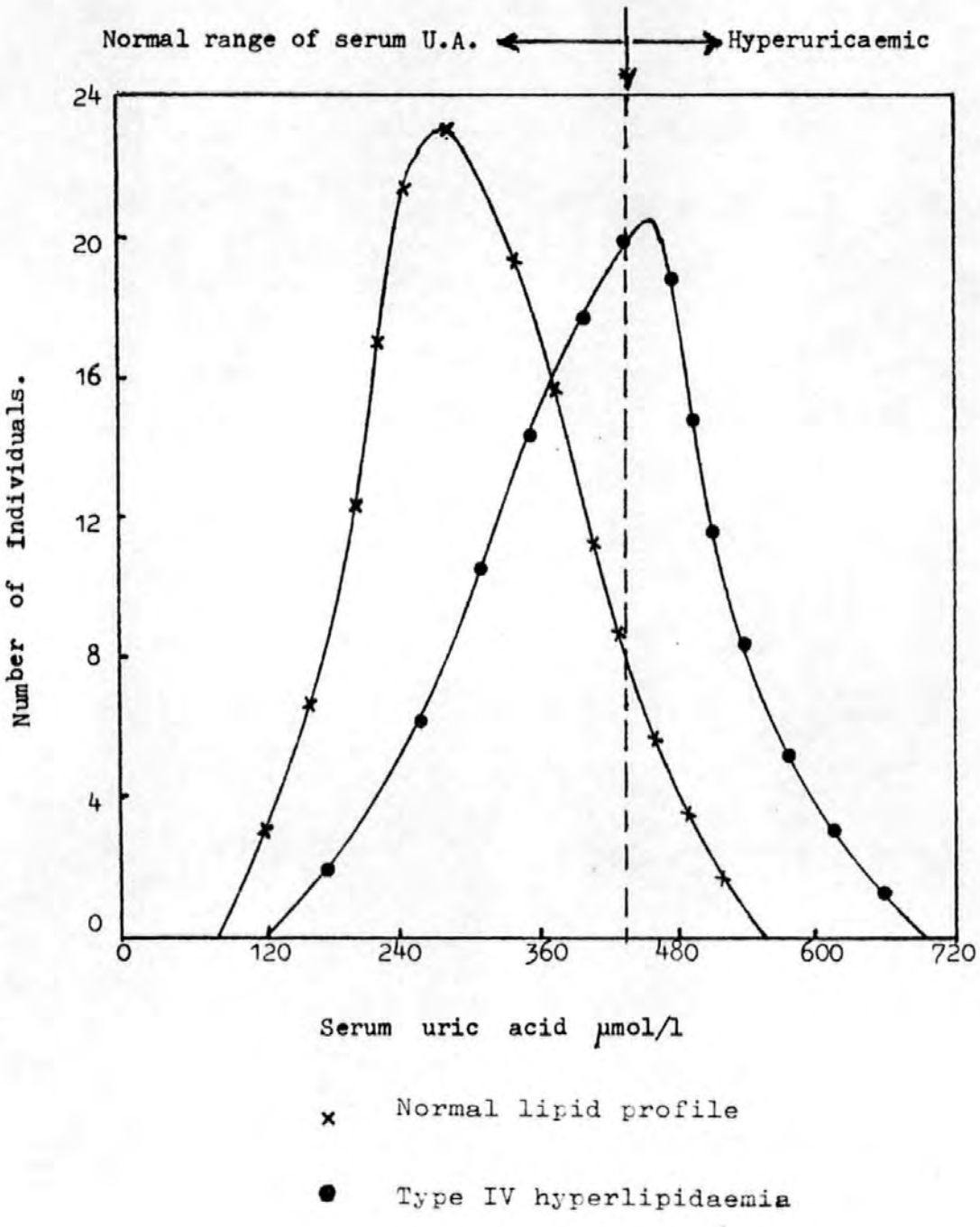


Fig. 8 Distribution of serum uric acid concentration in individuals with Type IIb hyperlipidaemia and in individuals with normal lipid profiles.

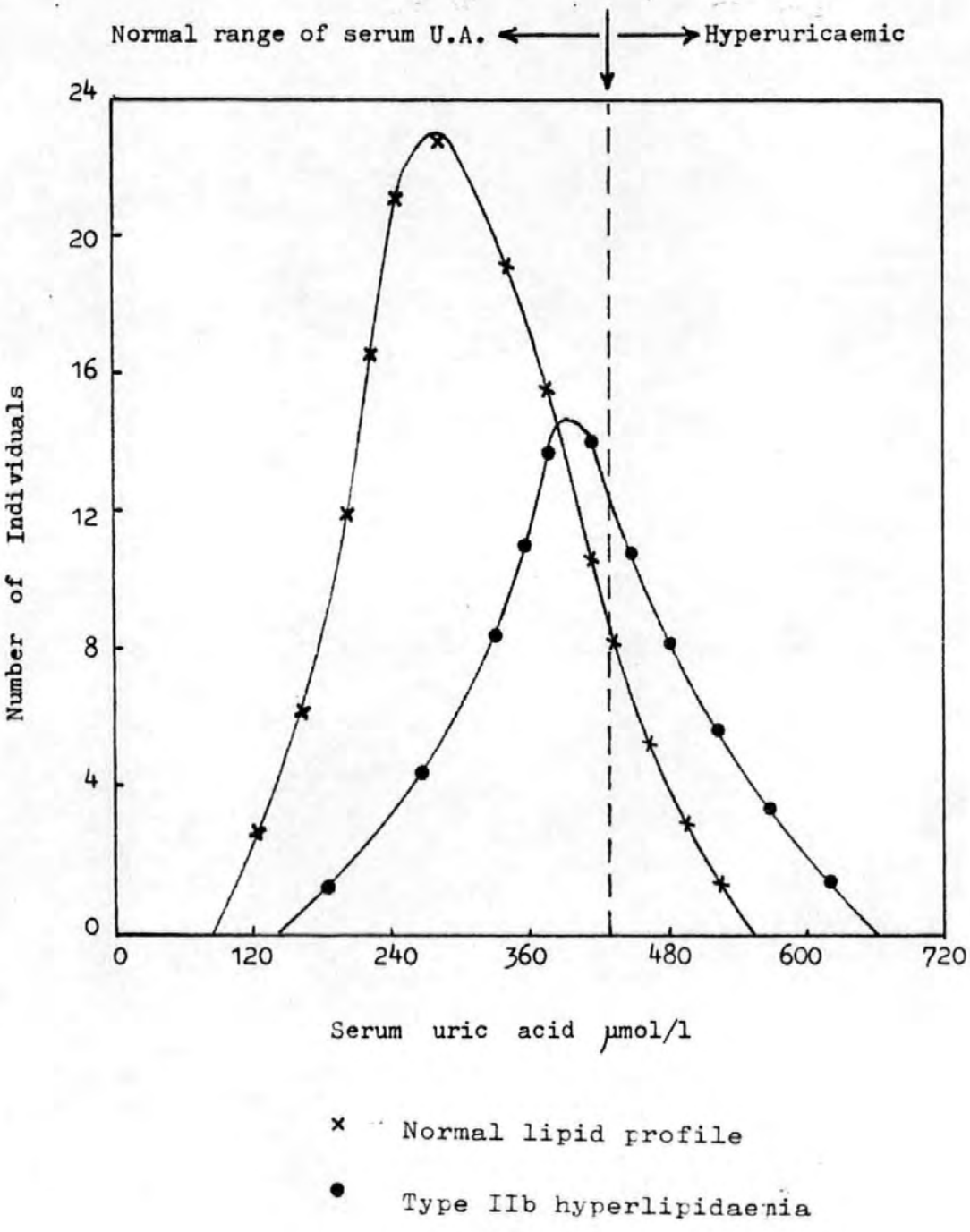


Fig. 9 Distribution and confidence intervals of red cell PRPP synthetase in three clinical groups.

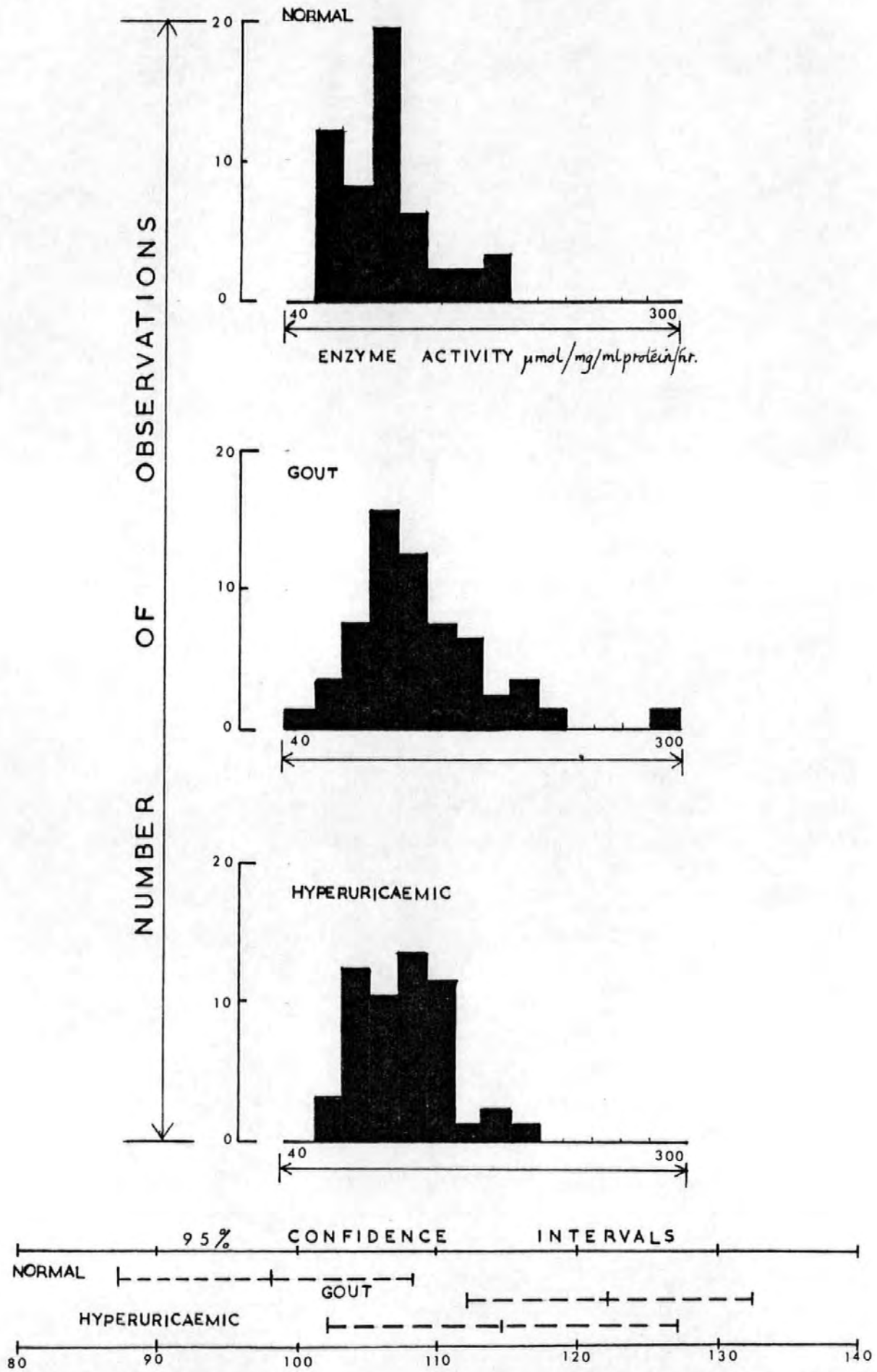


Fig. 10. The effect of varying R-5-P concentration on PRPP synthetase activity in

(i) control sample (ii) tophaceous gout

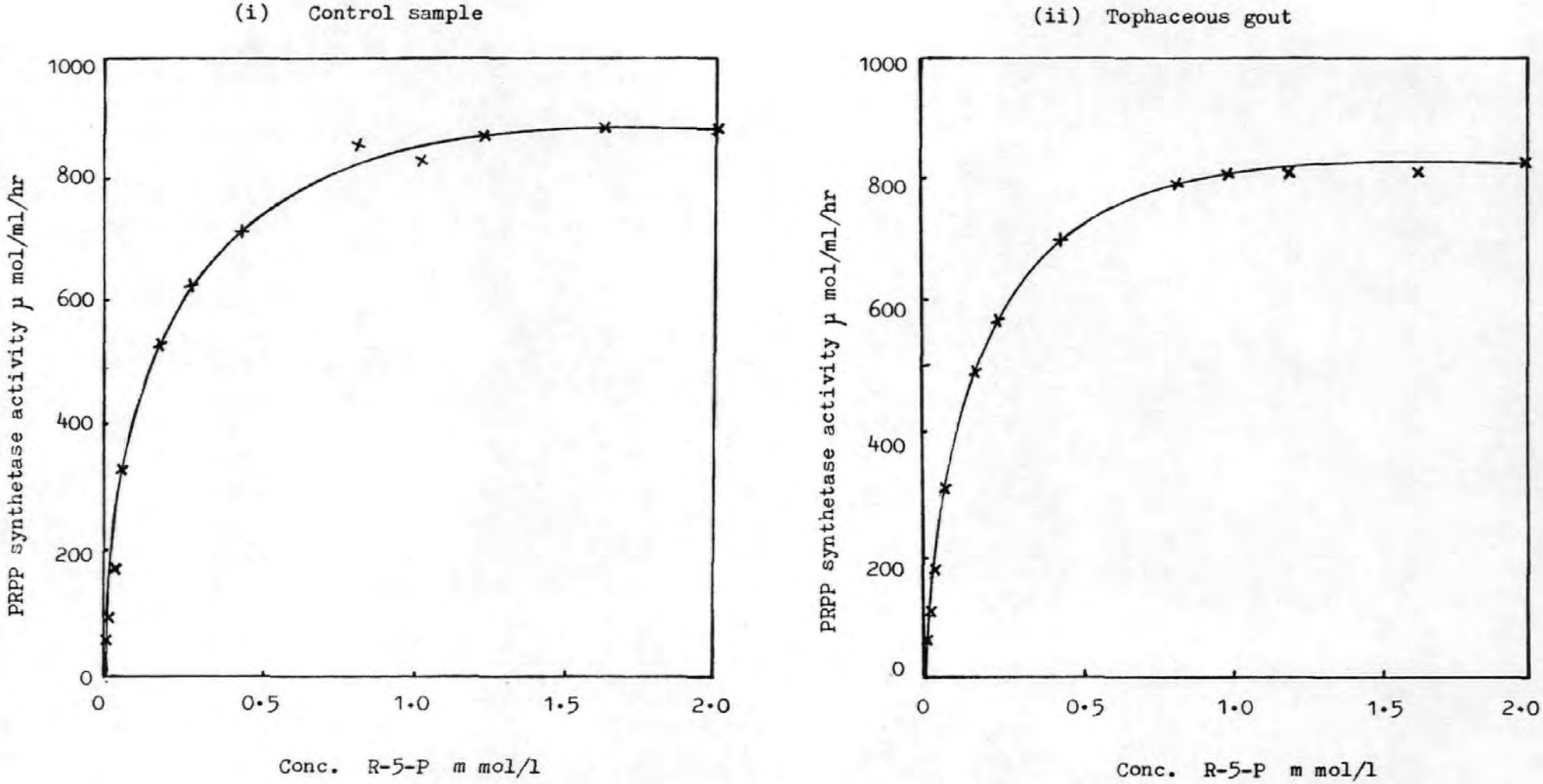
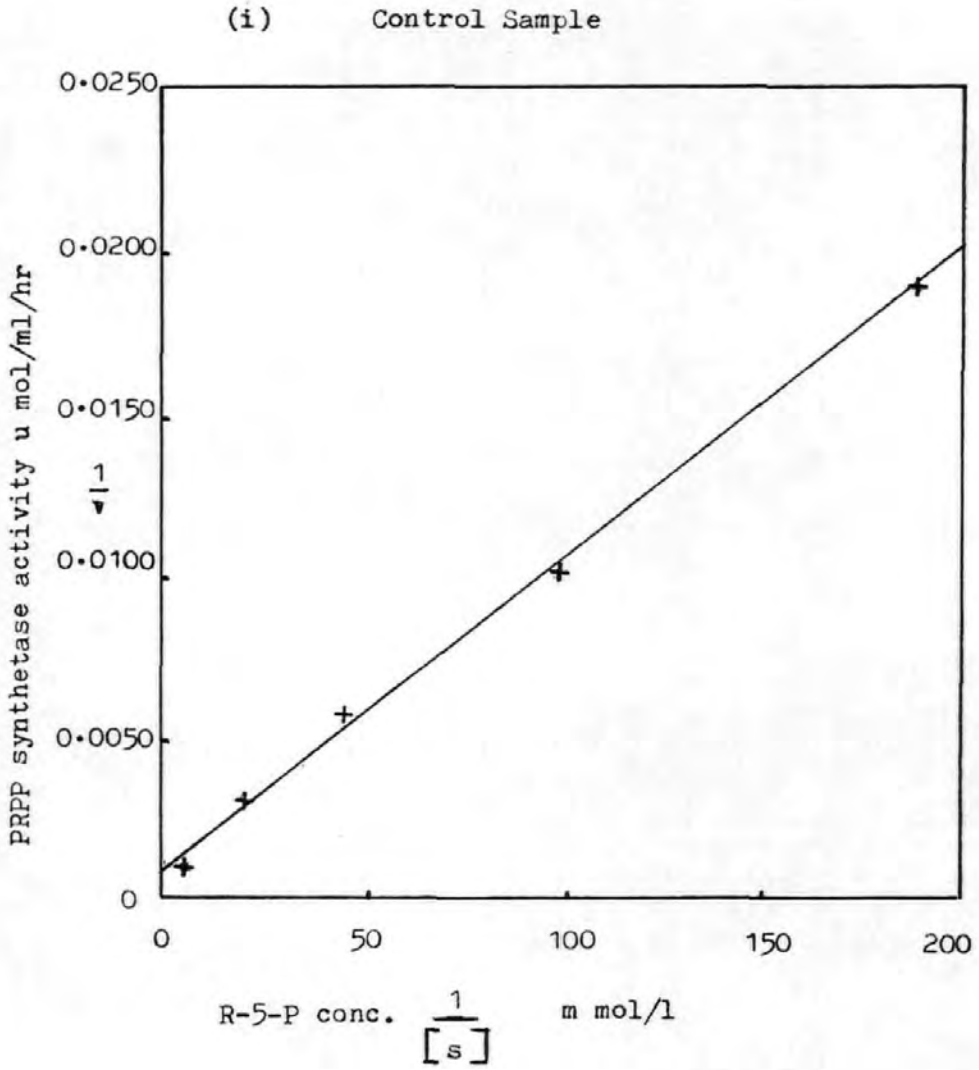


Fig. 11 The effect of varying concentrations of R-5-P on PRPP synthetase activity in (i) control sample (ii) tophaceous gout sample shown by Lineweaver Burke plots $\frac{1}{v}$ v $\frac{1}{[R\text{-}5\text{-}P]}$



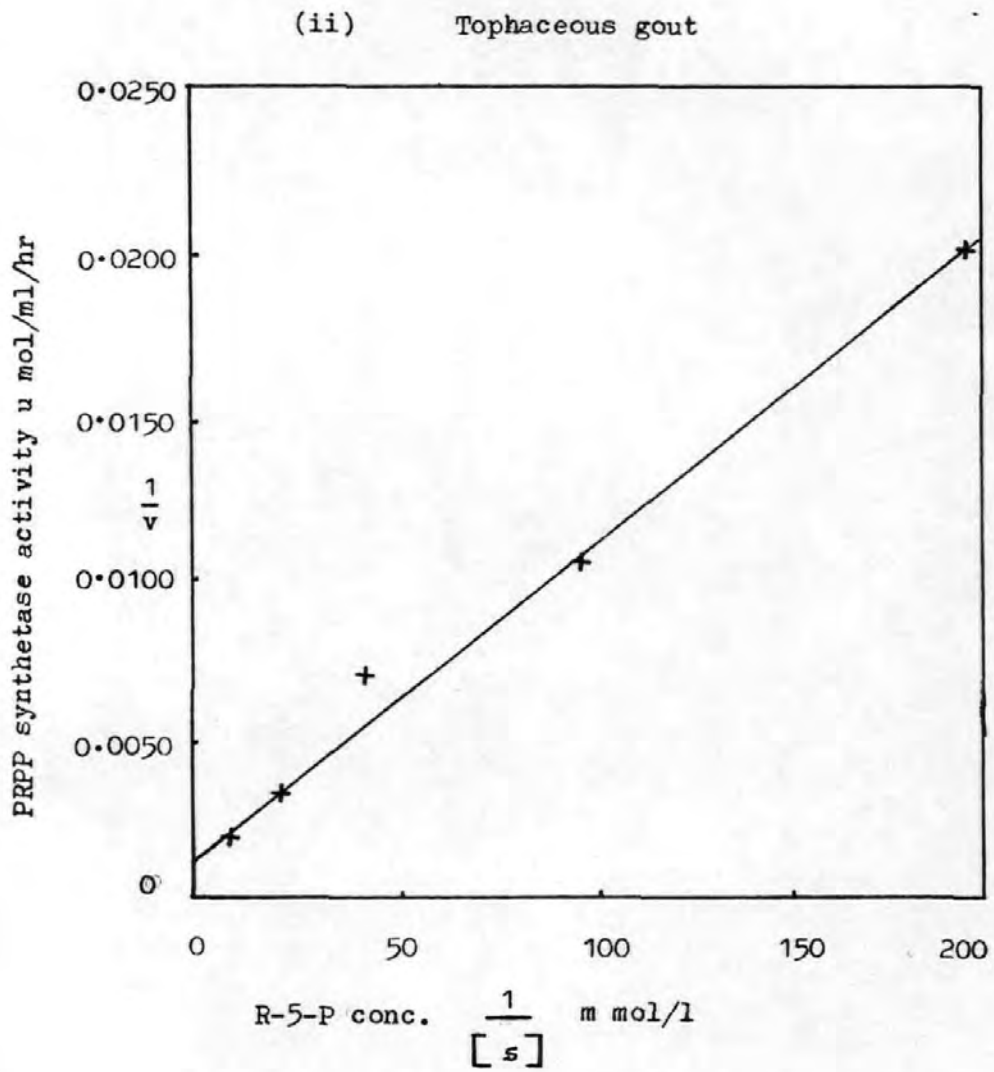
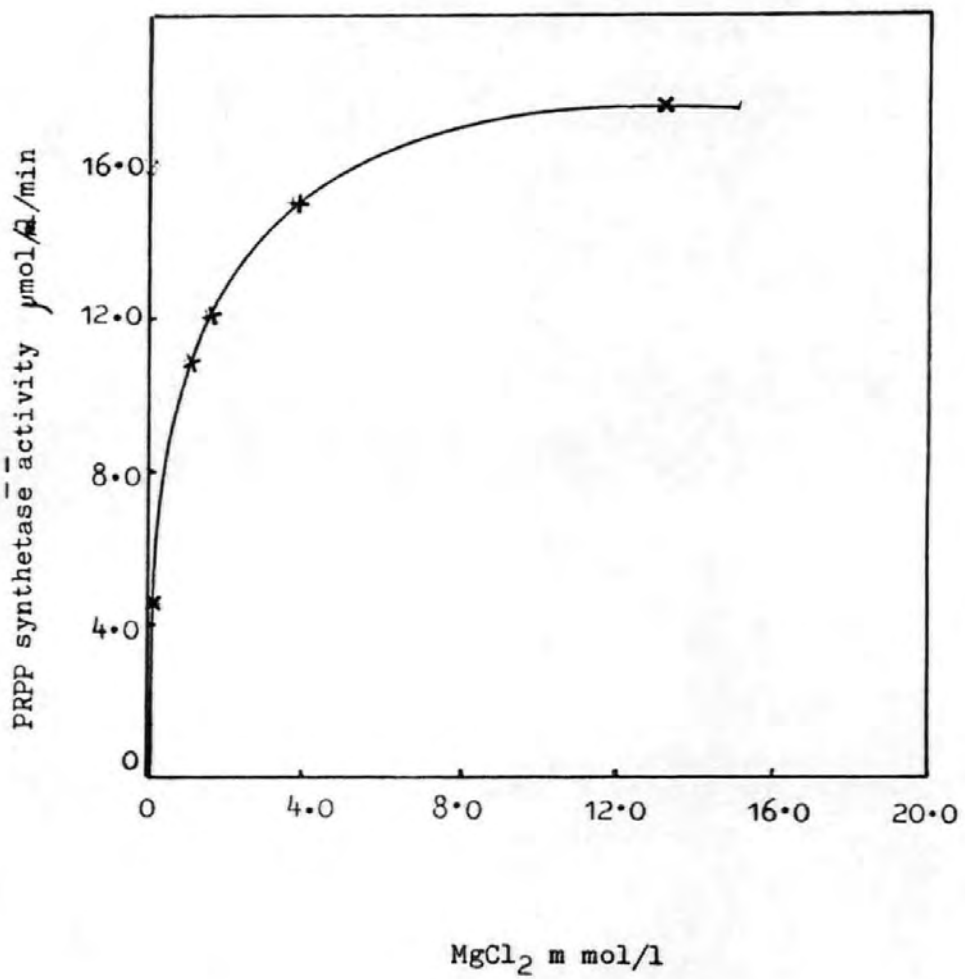
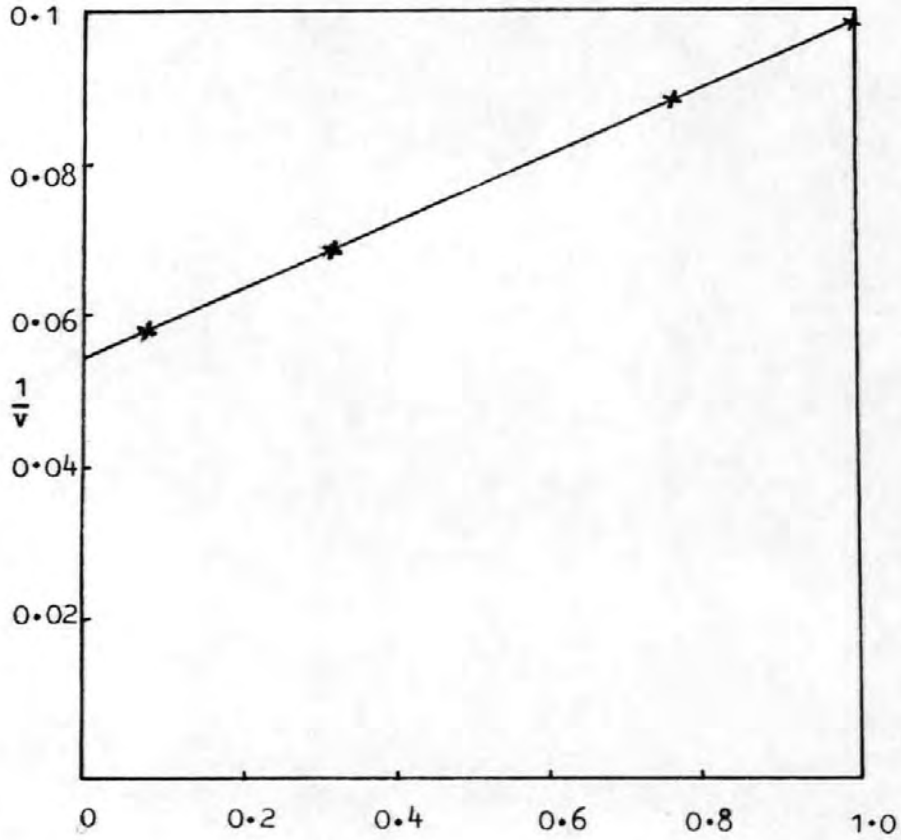


Fig. 12 The effect of varying concentrations of $MgCl_2$ ion on PRPP synthetase activity in the presence of 1·33 m mol/l ATP. a) concentration profile b) Lineweaver Burke plots $\frac{1}{v}$ v $\frac{1}{MgCl_2}$



a)



b)

Fig. 13

The effect of variation of ATP concentration on PRPP synthetase activity at a fixed concentration of magnesium. (1·8 m mol/l)

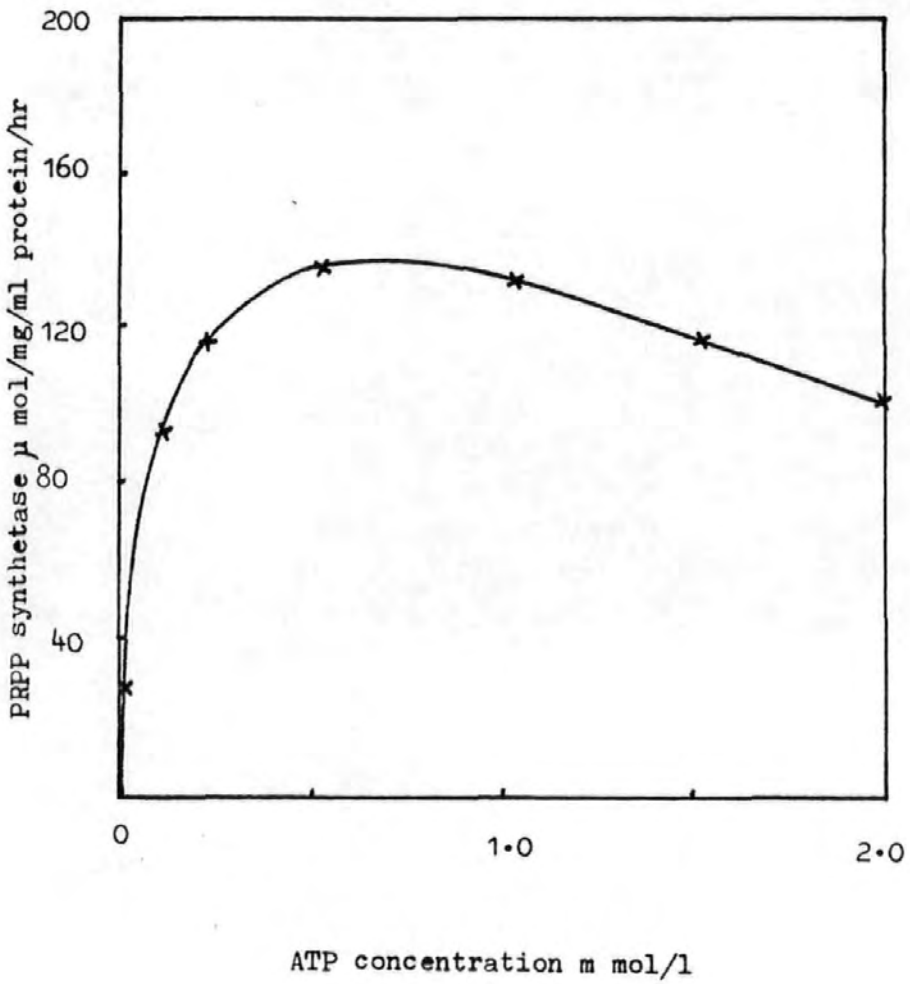


Fig. 14 The effect of varying pH on the enzymic activity of PRPP synthetase.

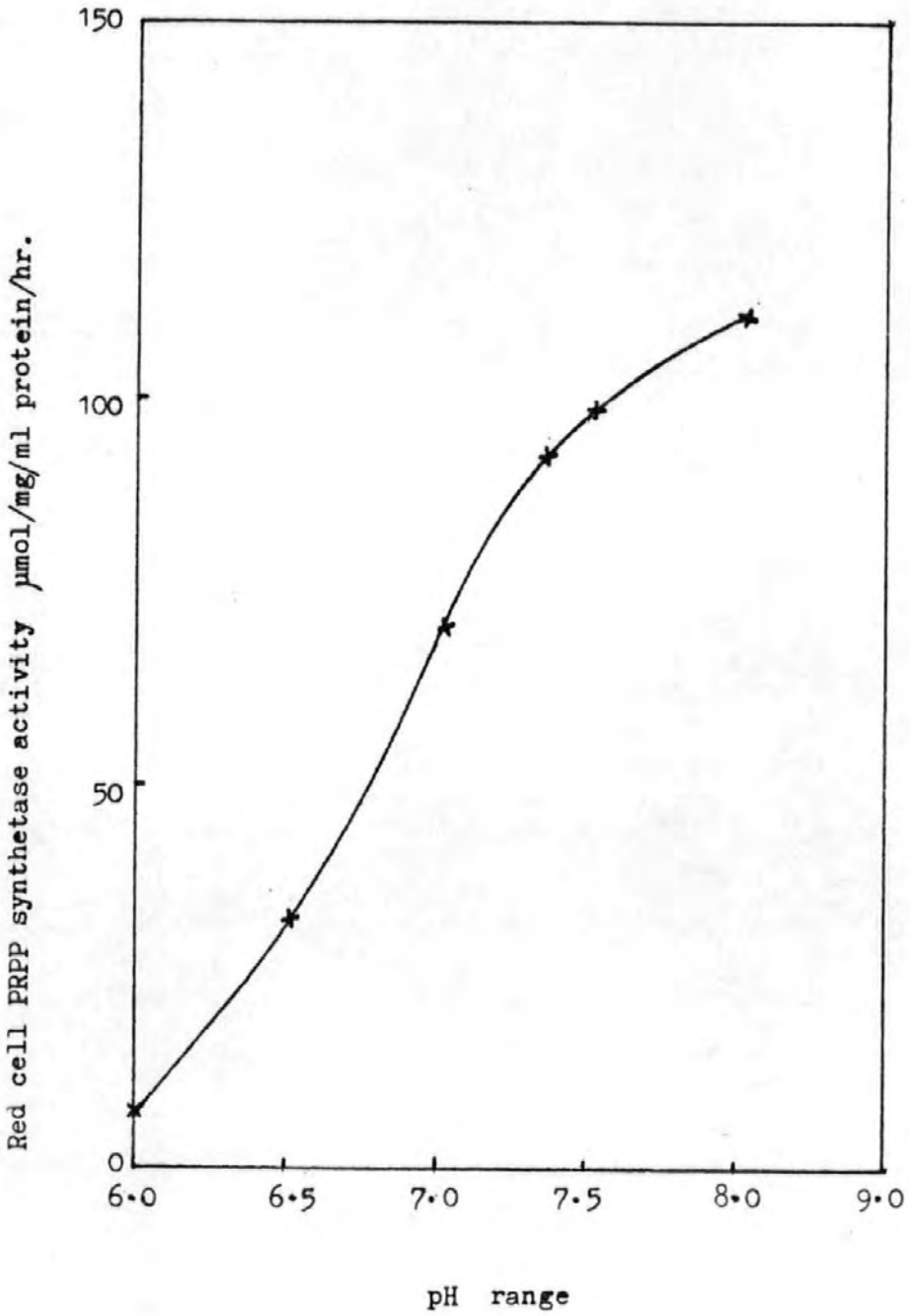


Fig. 15. The mean % inhibition profile of PRPP synthetase activity with ethyl alcohol.

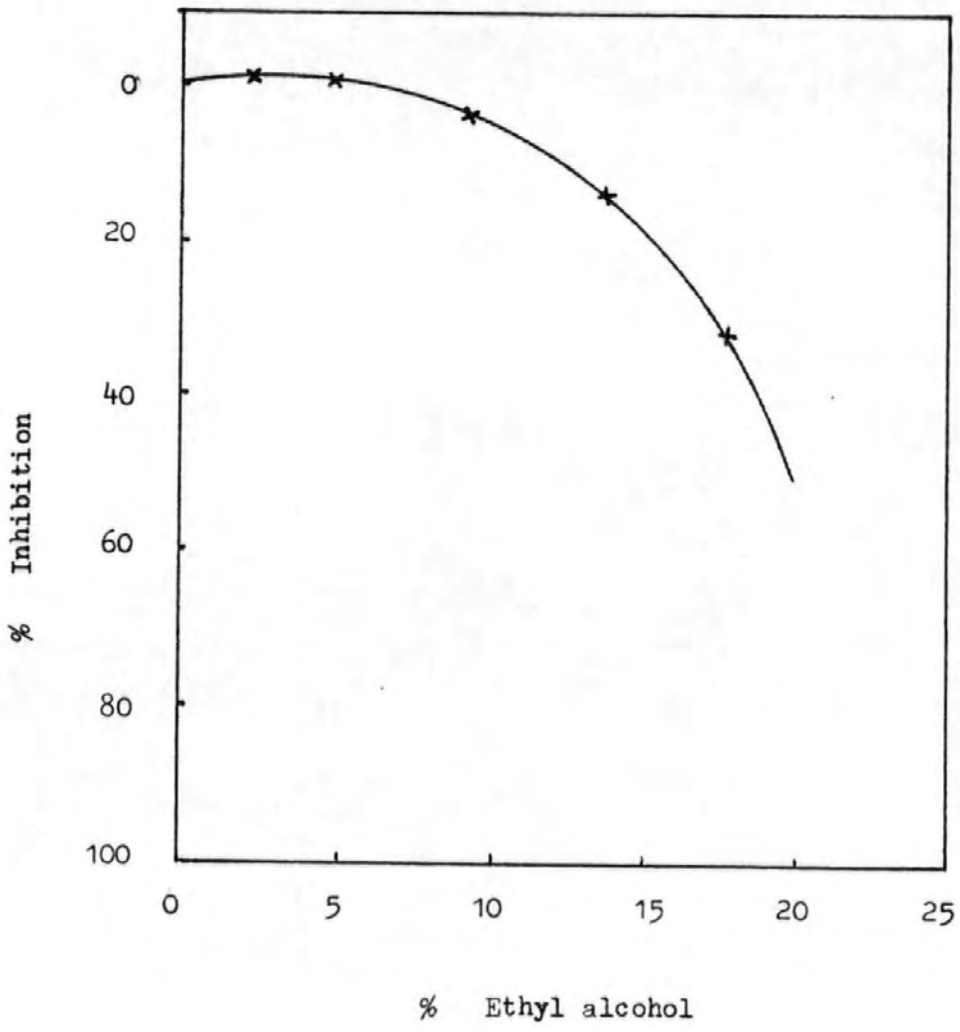


Fig. 16 The mean % inhibition of PRPP synthetase activity with ethyl alcohol in two groups of individuals showing two different profiles a) and b).

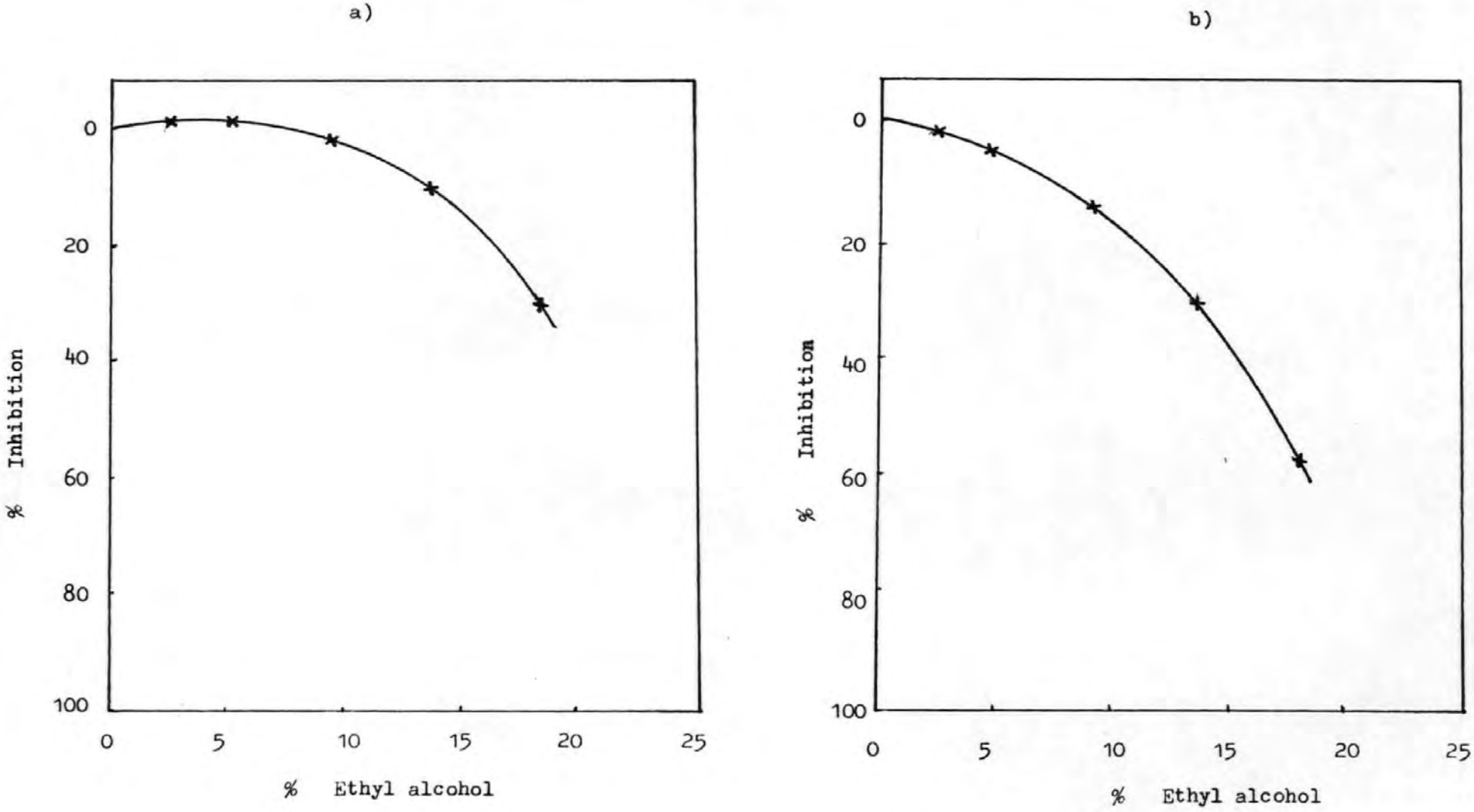


Fig. 17 Inhibition profile of PRPP synthetase activity with methyl alcohol

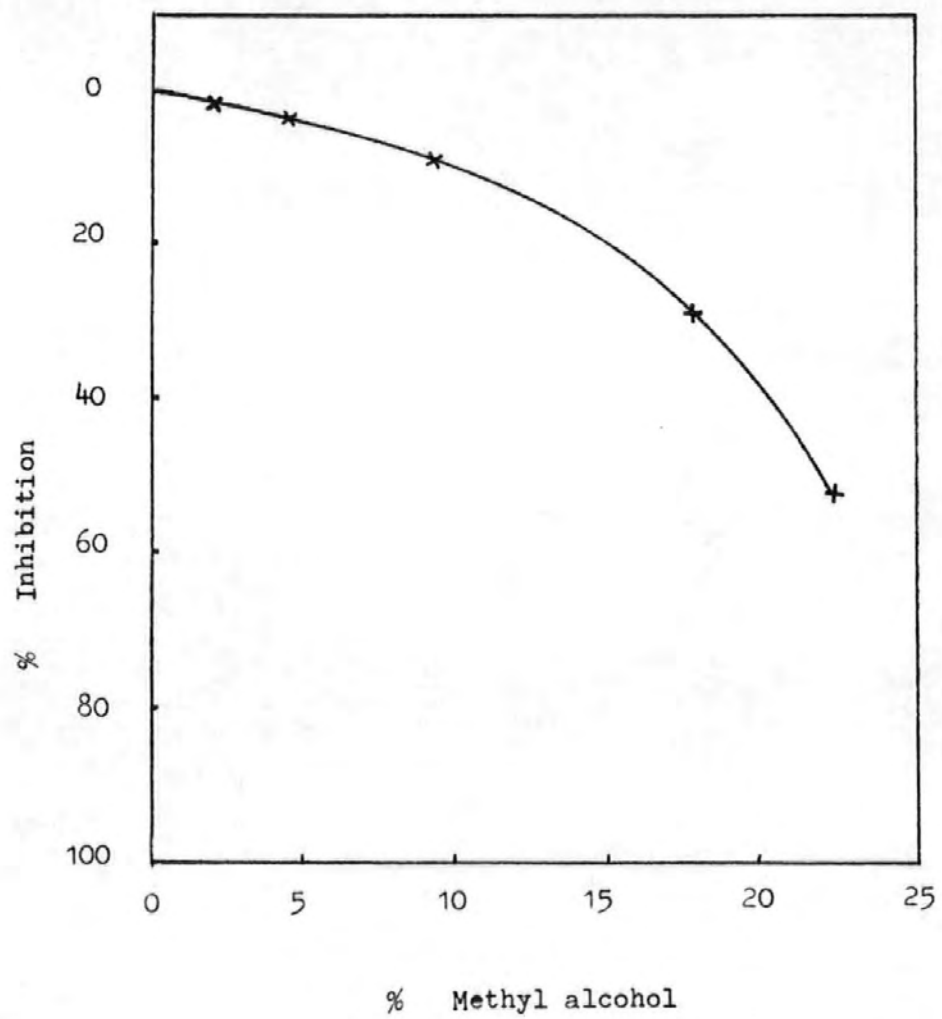
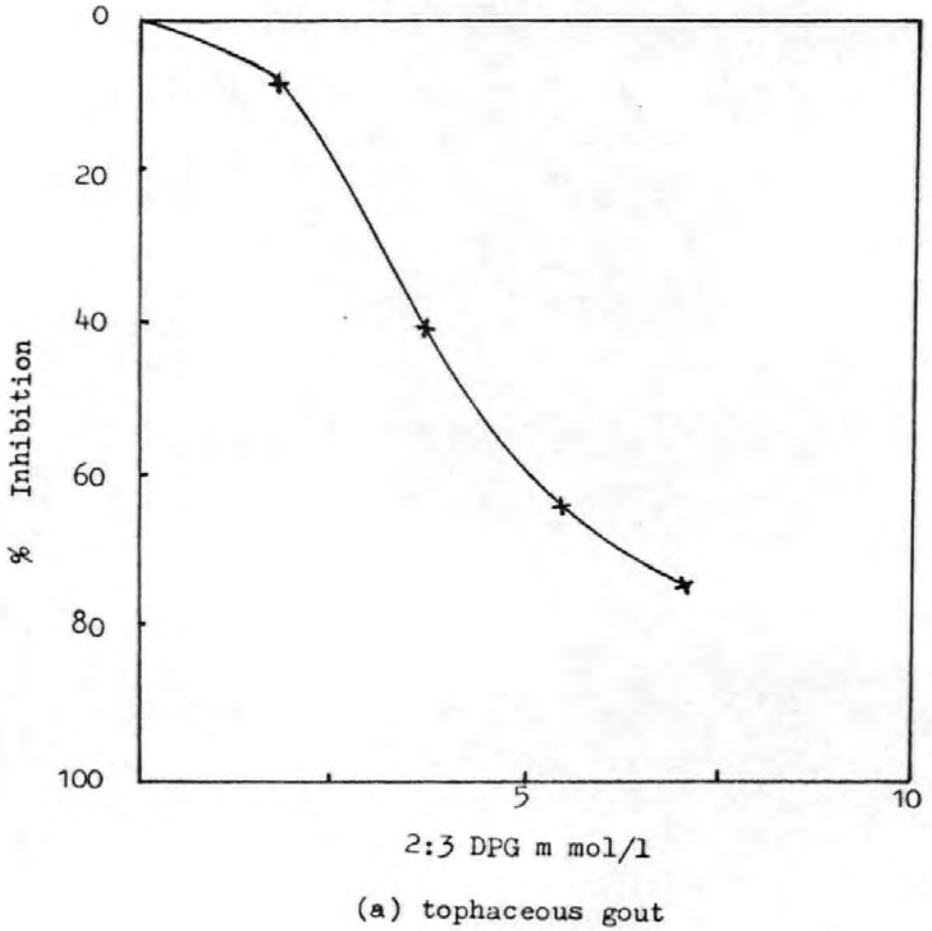
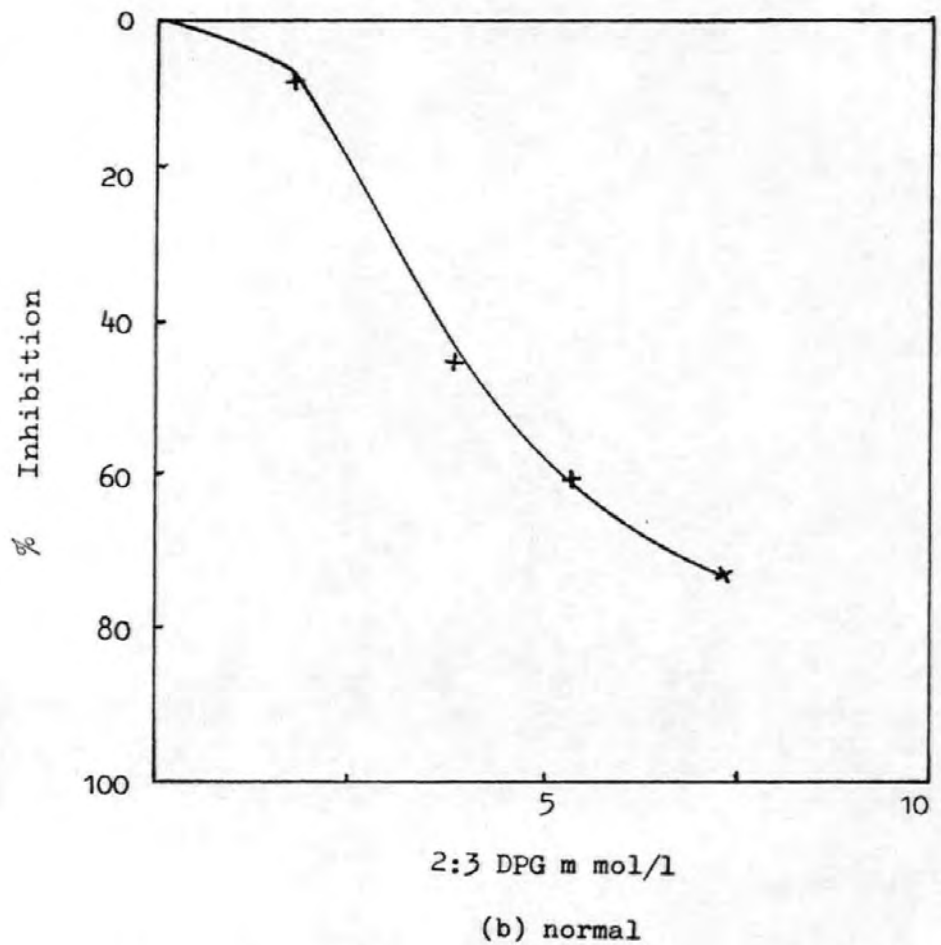


Fig. 18 Inhibition profile of (a) tophaceous gout (b) normal, for PRPP synthetase activity with 2:3 diphosphoglycerate



(a) tophaceous gout



(b) normal

Fig. 19 Inhibition profile for PRPP synthetase activity with nicotinamide

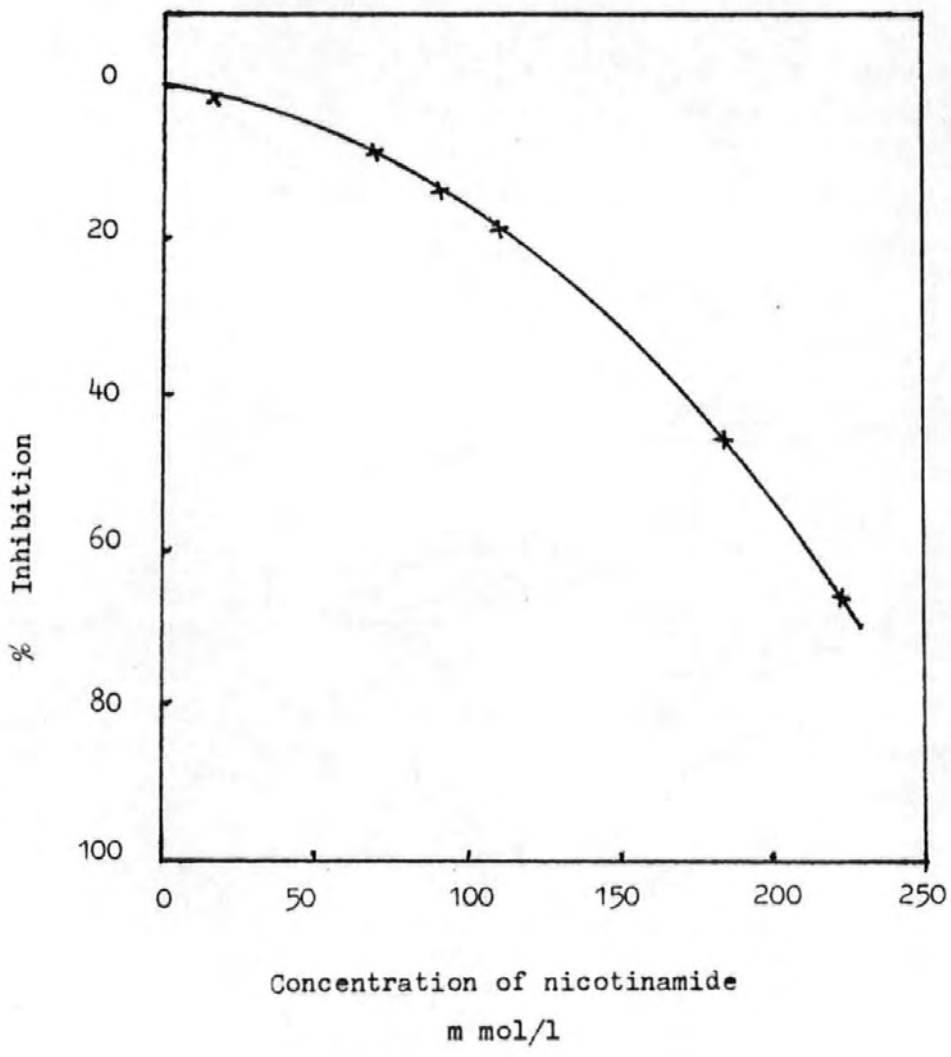


Fig.20 Effect of 25 mmol/l 2-mercaptoethanol on PRPP synthetase activity a)distribution of activation or inhibition b) data divided into three clinical subgroups.

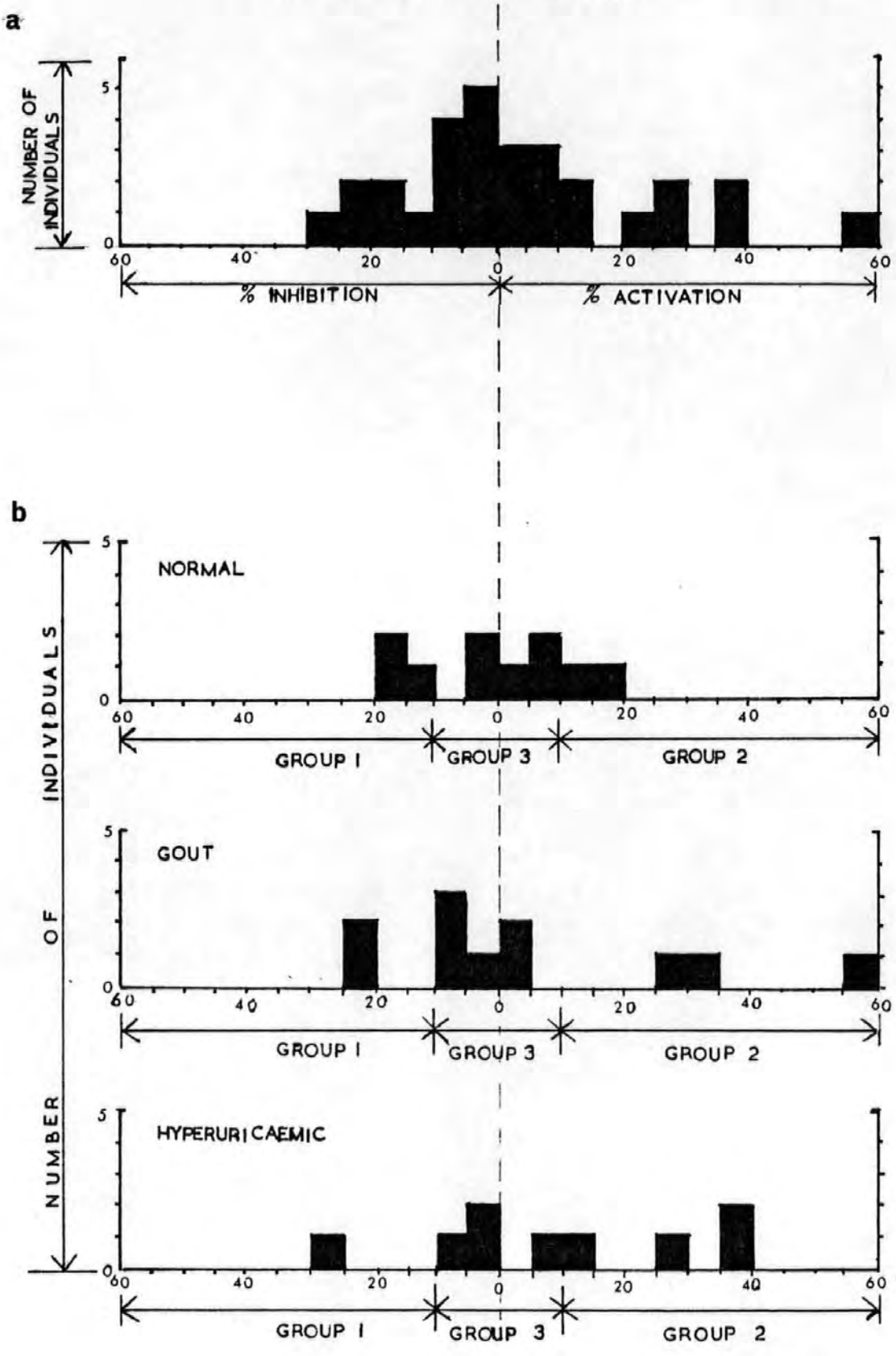


Fig. 21 Inhibition profile of

PRPP synthetase with Histidine.

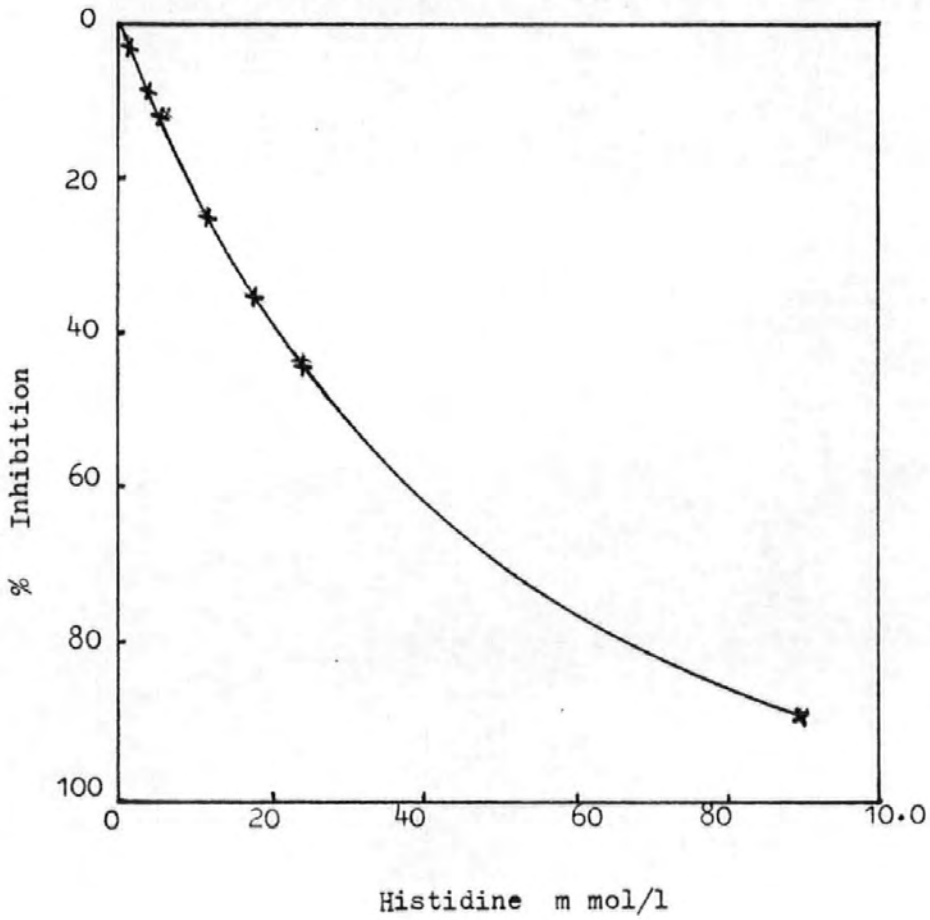


Fig. 22 Inhibition profile of PRPP synthetase activity with varying concentrations of formaldehyde in a normal sample and a patient with tophaceous gout.

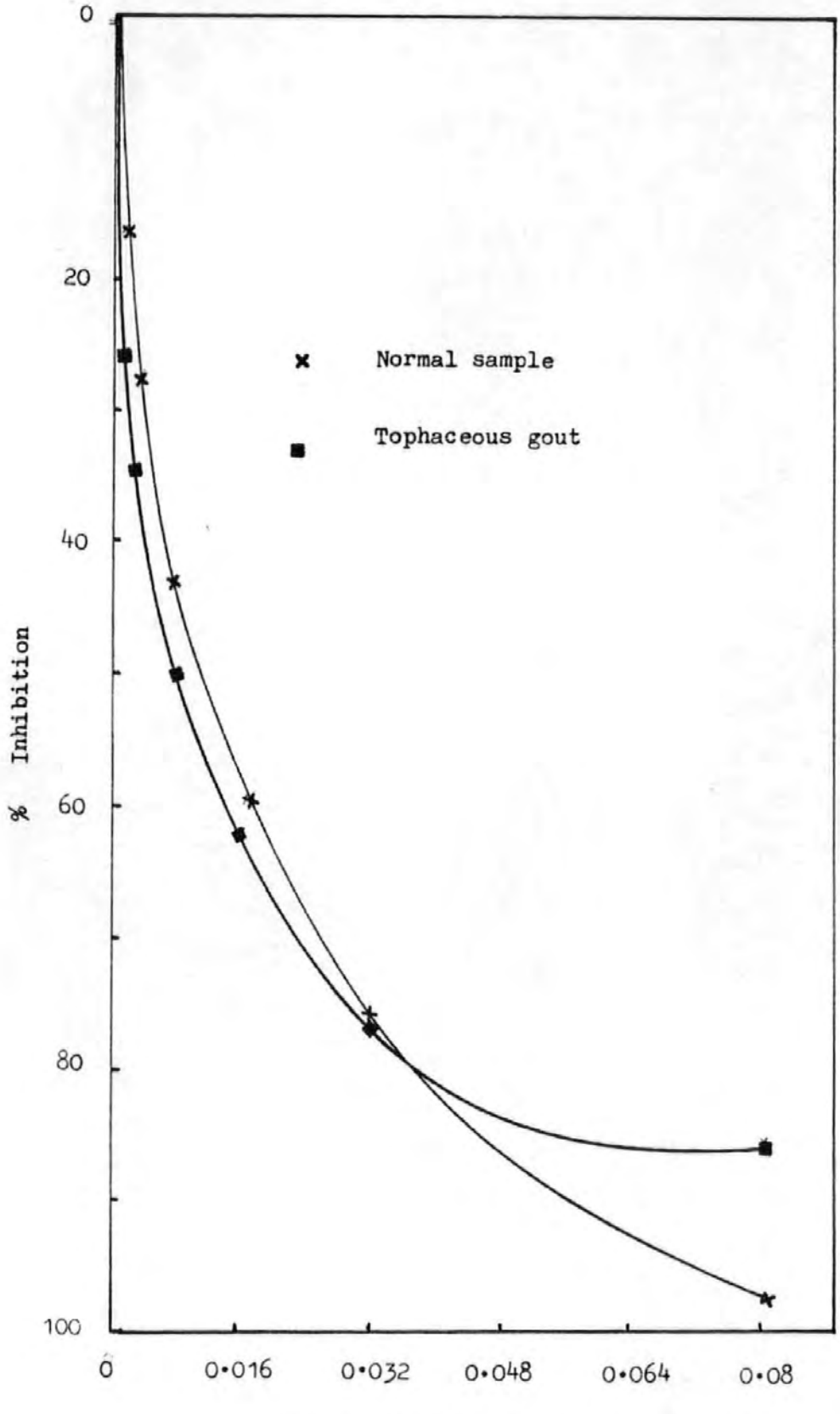
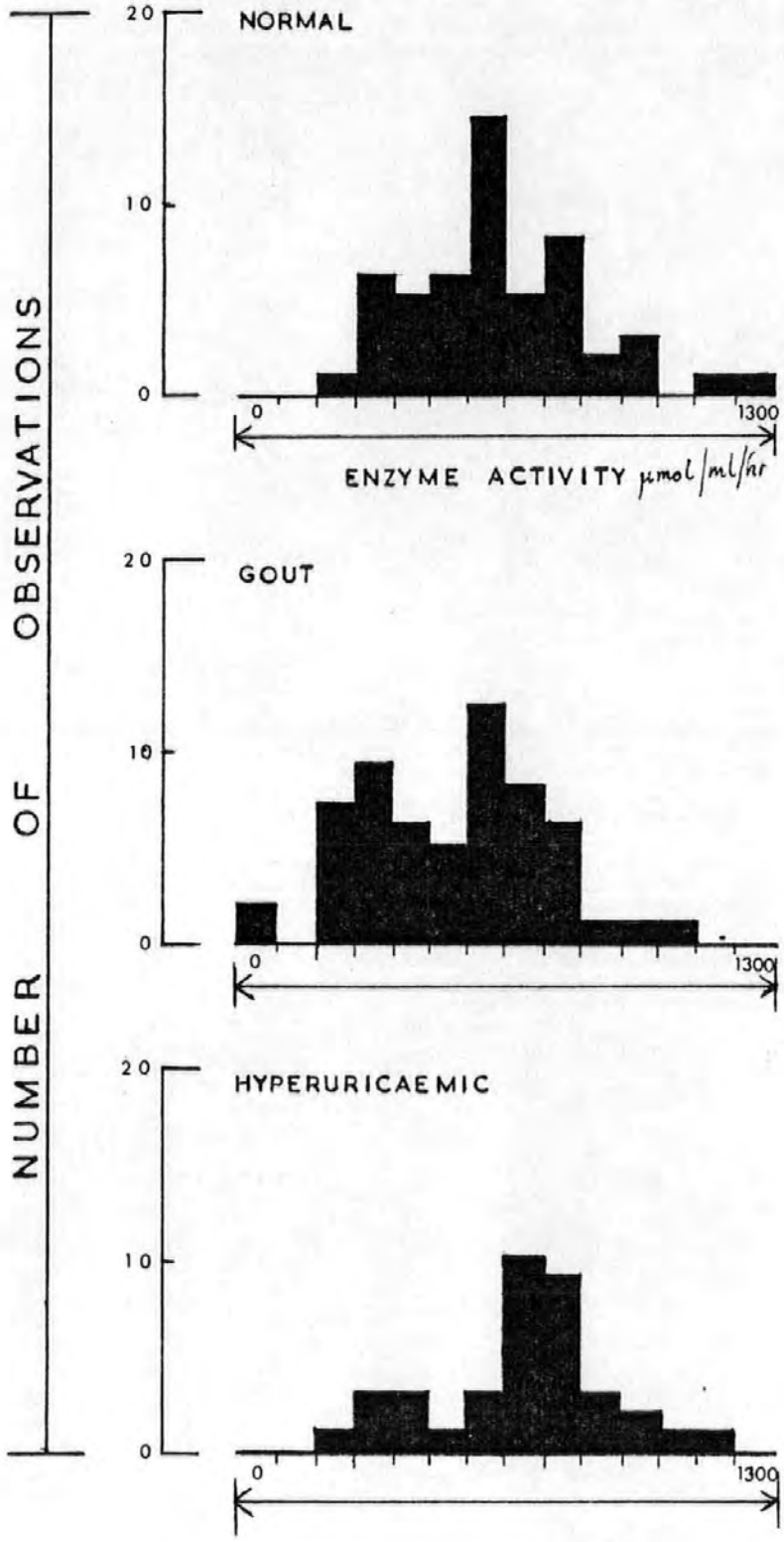


Fig.26 Distribution and confidence intervals of plasma Glutathione reductase in three clinical groups.



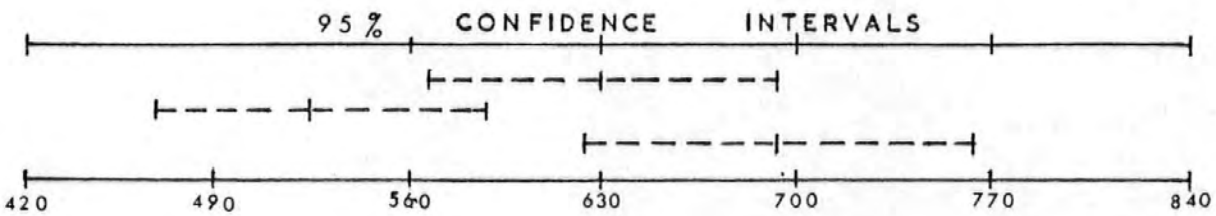


Fig. 27 Agarose electrophoresis of haemolysates for Adenosine deaminase isoenzyme activity: Block diagram indicating detected activity positions in the three clinical groups; normals, gouts and hyperuricaemics.

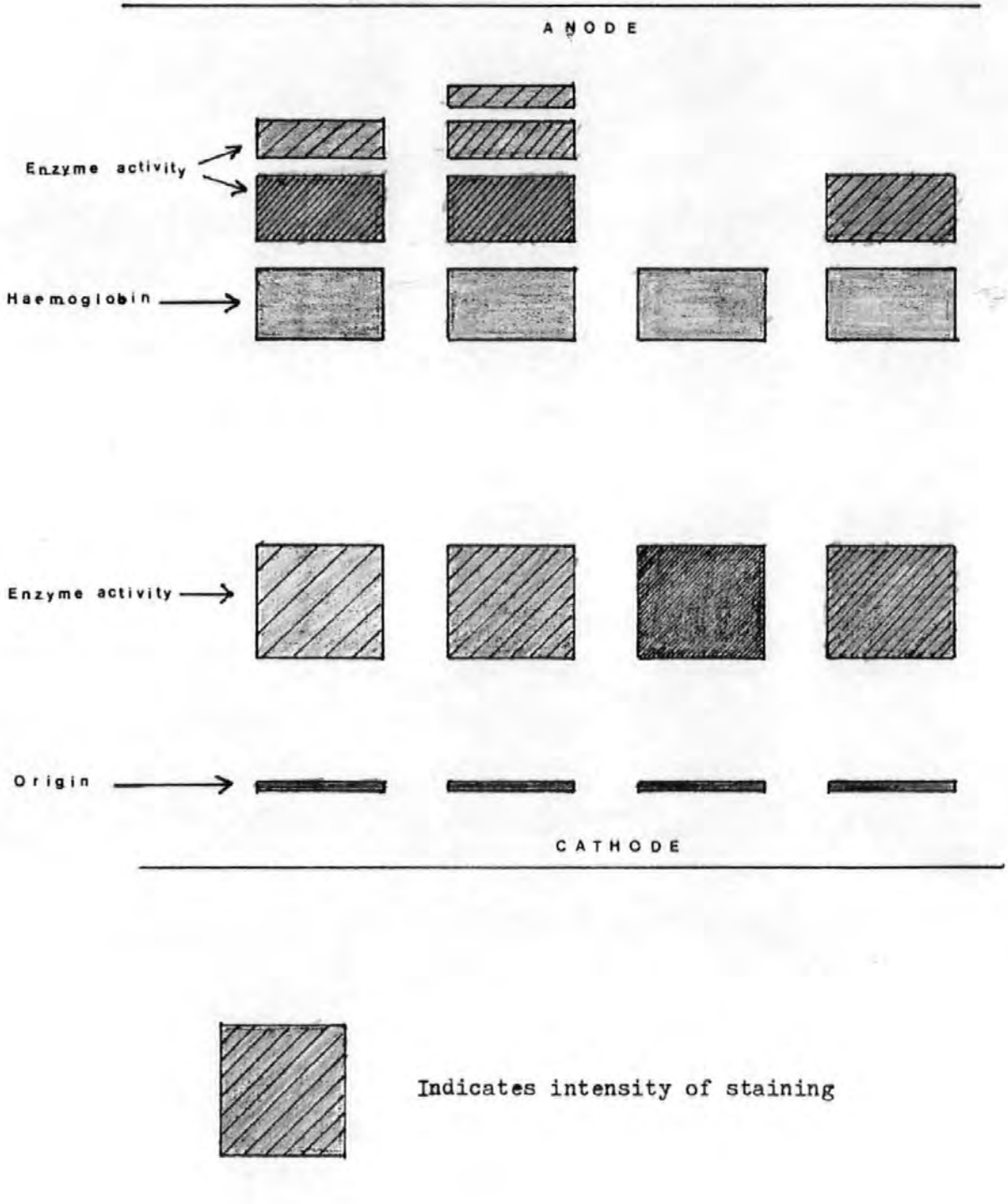


Fig. 2 8 Agarose electrophoresis of haemolysates from normal, gout and hyperuricaemic samples for HGPRT isoenzyme activity.

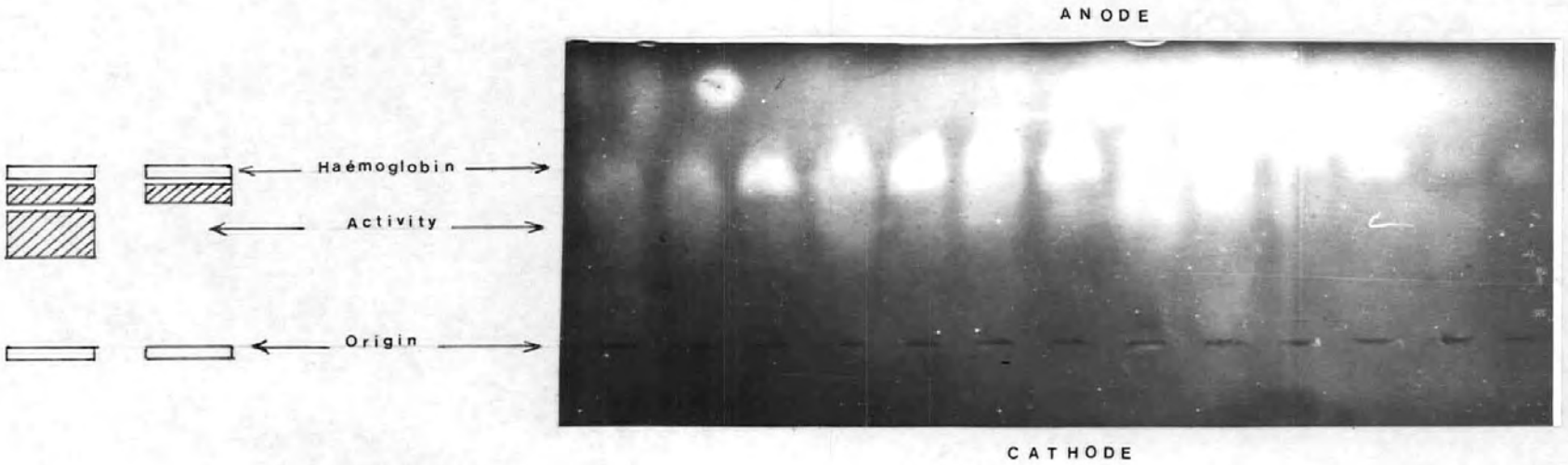


Fig. 29

A typical elution pattern of a normal serum on Biol-Gel P-2, mesh 200-400 using 0·9% saline as eluant monitored at 290 nm.

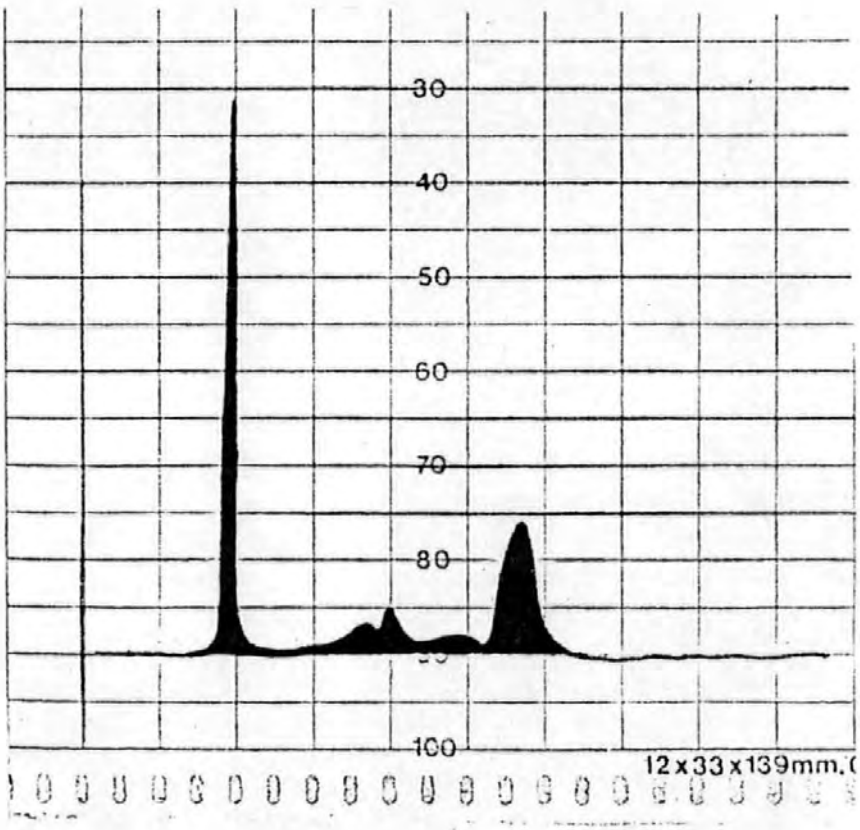
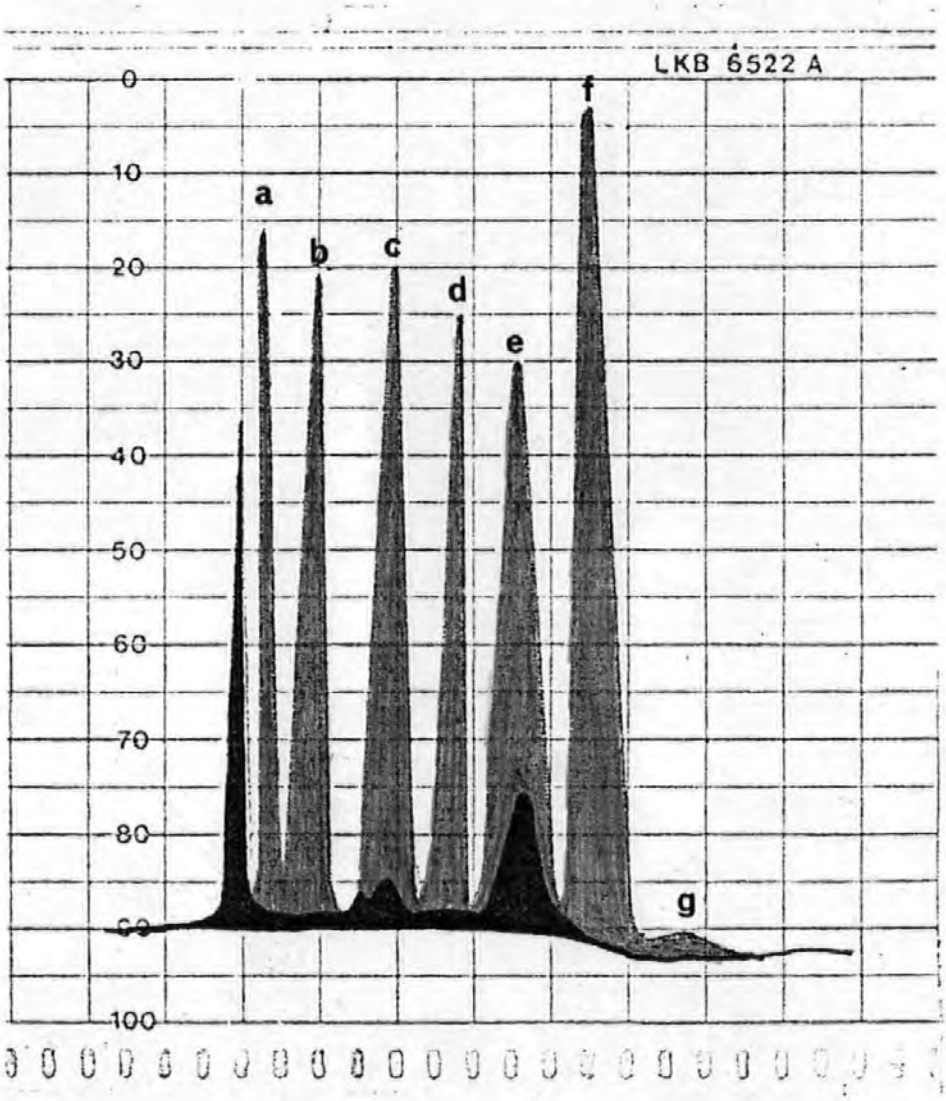


Fig. 30

The composite elution pattern of pooled sera and markers, added singly, on Bio-Gel P-2 mesh 200-400 using 0·9% saline as eluant monitored at 290 nm.



a) A.T.P.

b) A.M.P.

c) Creatinine.

d) Cytosine.

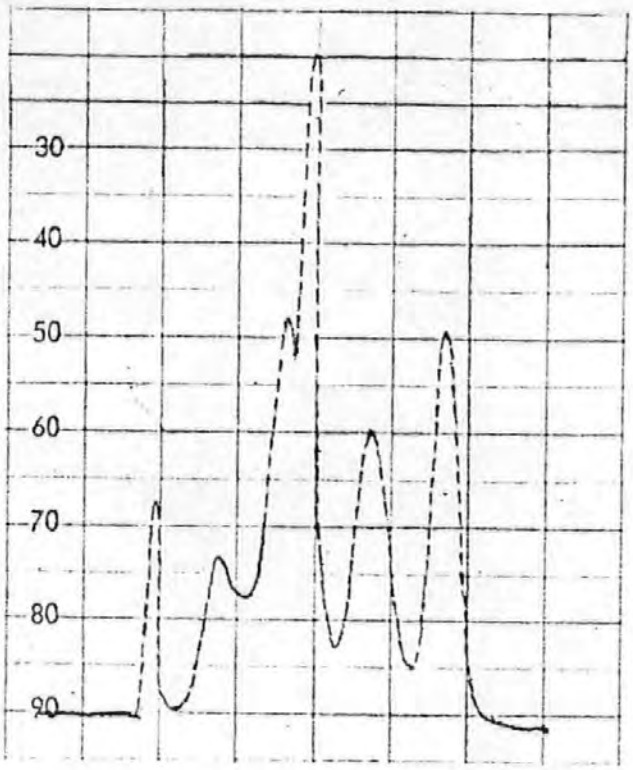
e) Uric Acid, Uracil, Hypoxanthine, Adenosine.

f) Adenine, Xanthine, Guanosine.

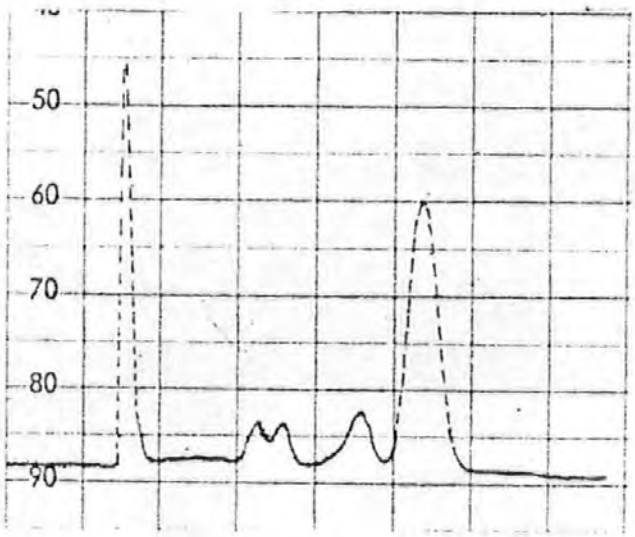
g) Guanine.

Fig.3 1 Typical elution patterns for a) hyperuricaemic b) gout c) familial primary gout serum on Bio-Gel P-2, mesh 200-400 using 0·9% saline as eluant monitored at 290 nm.

a



b



c

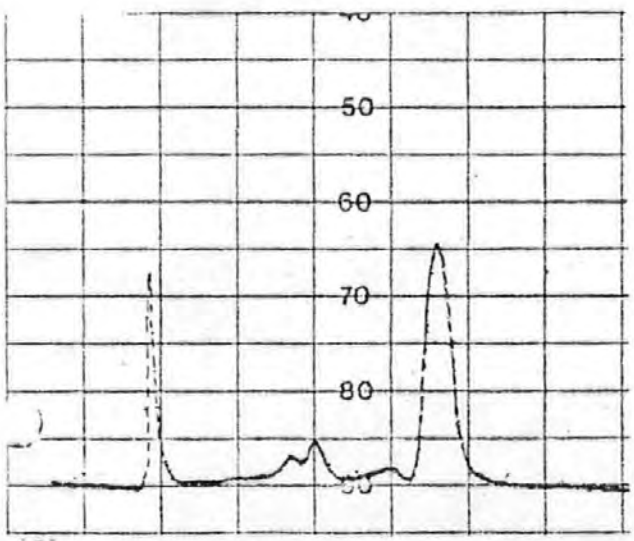


Fig. 32 Percentage hyperuricaemic samples from 1970-1975 per month

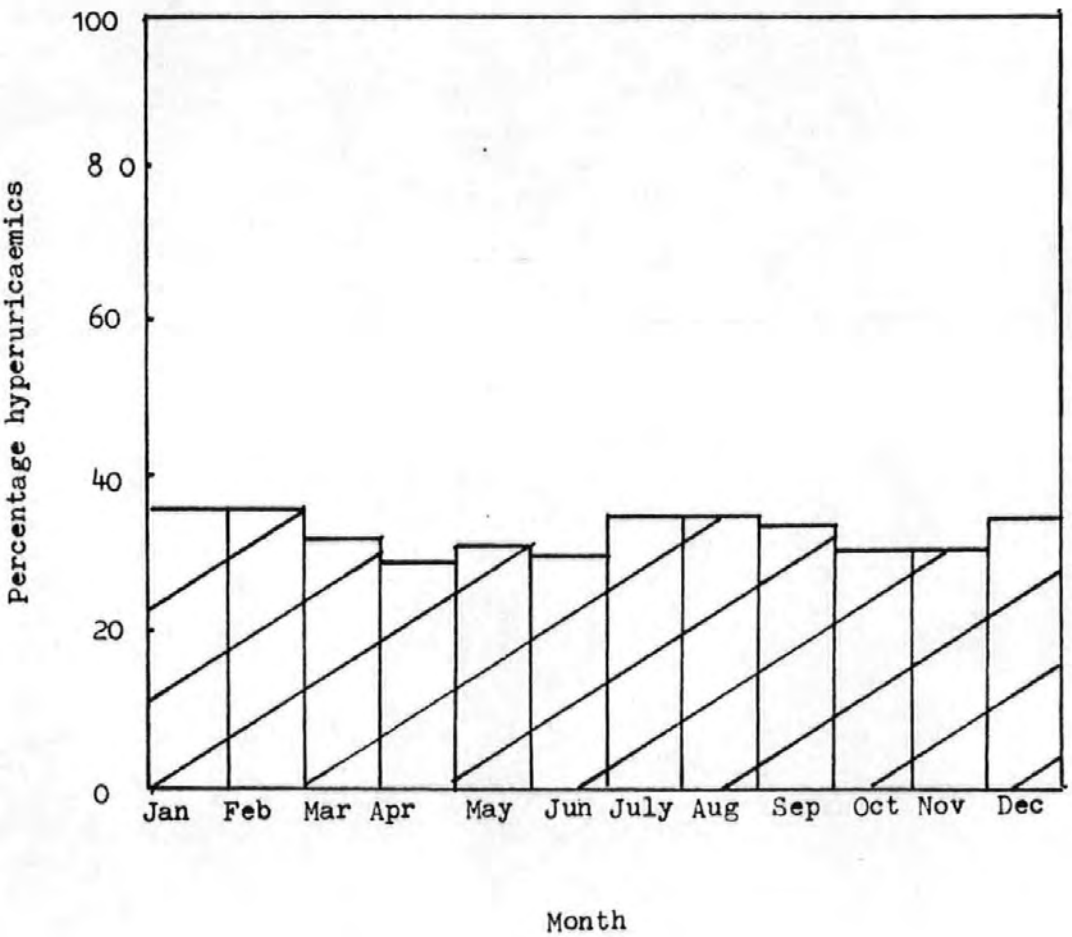


Fig. 3 3 Number of uric acid samples analysed per year

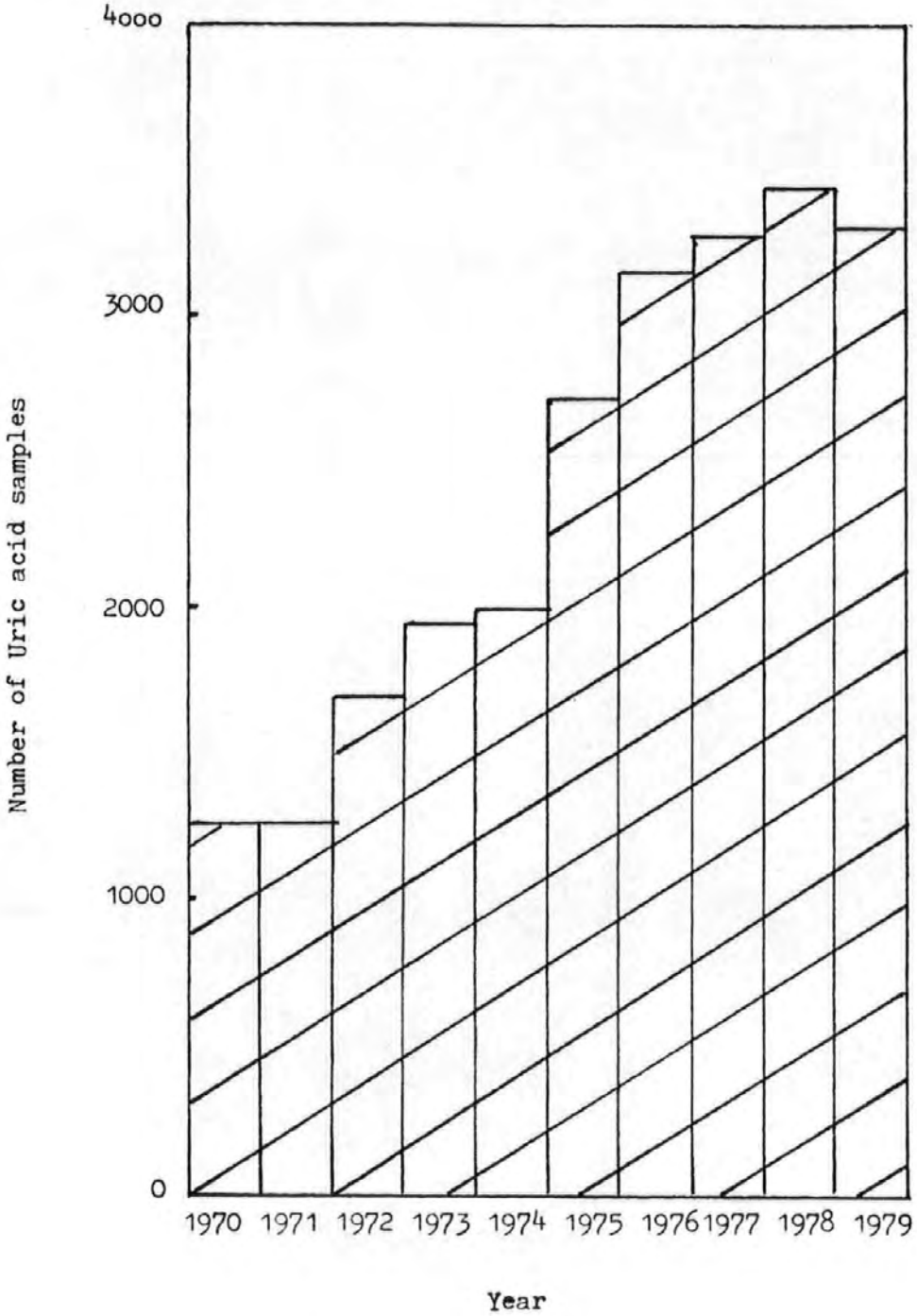


Fig. 34 The effect of varying phosphate concentration on PRPP synthetase activity.

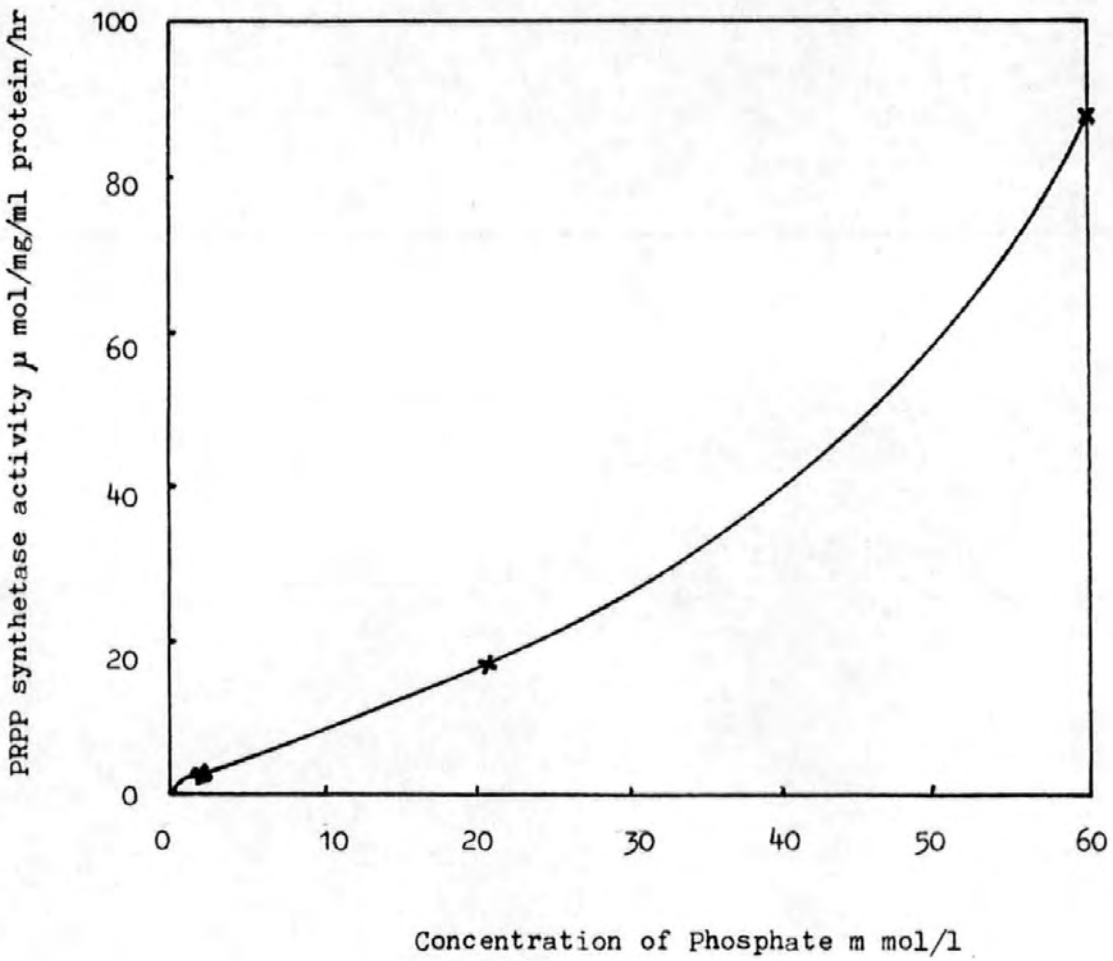
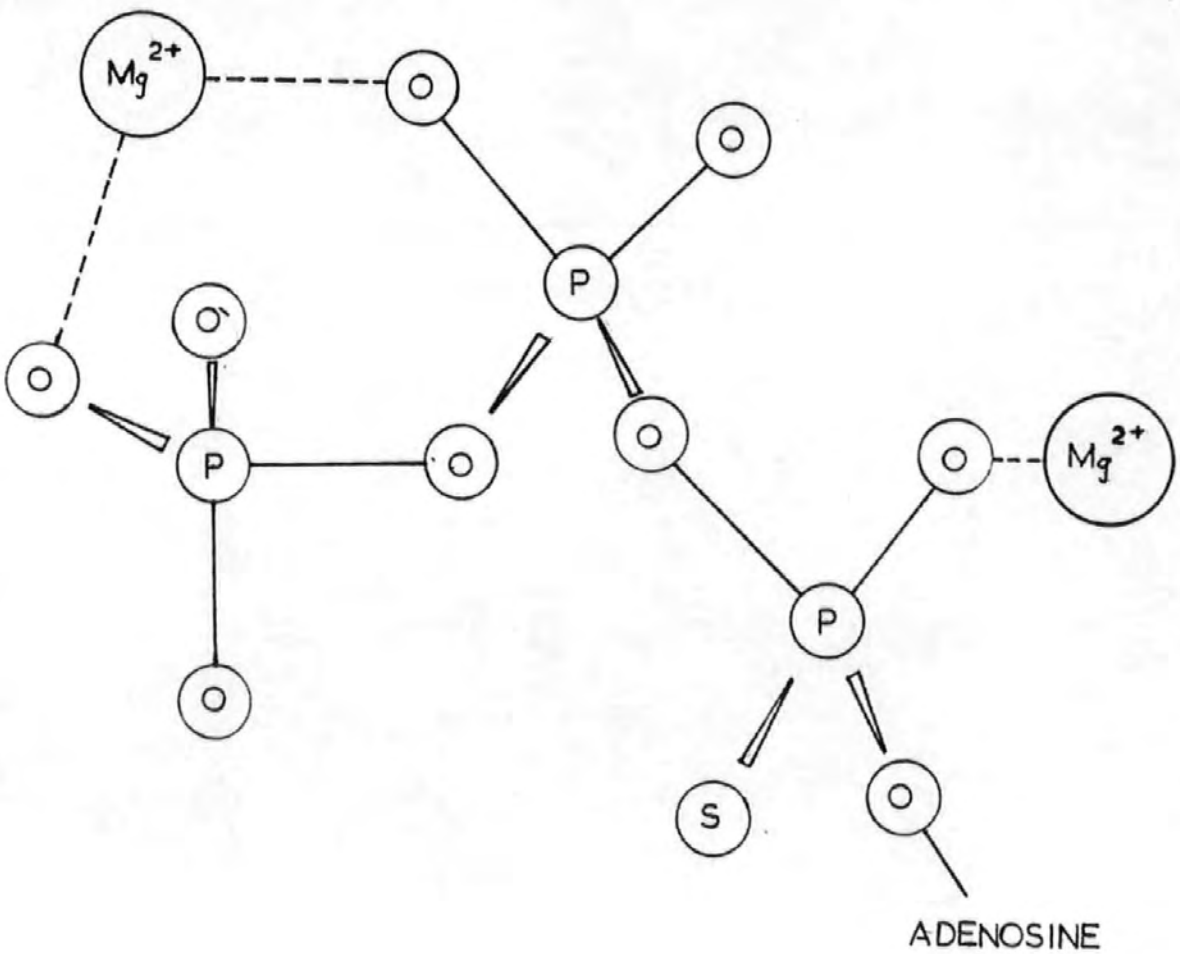


Fig. 3 5 Proposed configuration of the Mg^{2+} ATP∝5 complex required at the active site of PRPP synthetase.



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APPENDIX 1

Automated methods of analysis for occlusion experiments.

Assays were performed with the following equipment.

Technicon SMA multi channel biochemical analyser.

Nuclear Enterprise NE 1600 gamma counter.

Hyland laser nephelometer PDQ.

Reagents were obtained from Technicon Instruments and the Radiochemical Centre, Amersham.

a) Total Protein. (Technicon method "SDA-0014 PK 7"). Estimated by absorbance at 550 nm following the reaction of protein with a biuret reagent. A blank reagent is necessary to compensate for endogenous absorbance of sample at 550 nm.

b) Albumin. (Technicon method "SD4-0030 PK 7"). Albumin reacts with bromcresol green at pH 4·20 to form a coloured complex, the absorbance of which is measured at 630 nm. No blank is necessary.

c) Bilirubin. (Technicon method "SD4-0018 PK 7"). The method uses a strong alkaline buffer and measures alkaline azobilirubin as the end product at 600 nm. A blank reaction is necessary.

d) Alkaline phosphatase. (Technicon method "SD4-0006 PK 7") The method is based on the enzymic hydroysis of p-nitrophenyl phosphate in 2-amino-1-propanol at pH 10·25 in the presence of magnesium ions. The absorbance of the analytical stream is measured at 410 nm following dialysis which separates the p-nitrophenol from bilirubin which absorbs at the same wavelength.

e) Calcium. (Technicon method "SD4-0003 PK 7"). Calcium is estimated colorimetrically at 570 nm by complexing the metal with cresol phthalein complexone

in the presence of 8-hydroxy quinoline to eliminate magnesium interference and potassium cyanide to eliminate heavy metal interference and to stabilise the base reagent. Calcium ion is dialysed before complexing with the dye.

f) Inorganic phosphate. (Technicon method "SD4-0004 PK 7") Phosphate is estimated after dialysis by conversion of acidified ammonium molybdate solution to phospho-molybdate followed by reduction of the phosphomolybdate by stannous chloride to molybdenum blue. The absorbance of the latter is measured at 660 nm.

The flow diagrams for these systems are shown in Diagrams

Thyroxine (T_4) by radio immuno assay (RIA). The radio immuno assay depends on competition for a limited number of binding sites on a T_4 specific antibody between T_4 in serum and ^{125}I thyroxine. The proportion of ^{125}I thyroxine bound to antibody is inversely proportional to the concentration of unlabelled T_4 present in serum. This proportion is measured by separating free T_4 by adsorption on a solid matix and estimating the radioactivity of the bound fraction remaining in solution. By measuring the proportion of ^{125} I thyroxine bound in the presence of reference standard serum containing varying amounts of T_4, that present in other sera can be interpolated.

Immunoglobulins (IgG, IgA and IgM) by laser nephelometry. The nephelometer is designed to quantitate specific protein concentrations by utilizing the principle of molecular light scatter. Monospecific antiserum reacts with a specific antigen to form antigen-antibody complexes in solution which scatter an incident light beam. The amount of light scattered is proportional to the concentration

of the antigen. The processing circuits in the instrument and the selected angle of scattered light are designed to optimise the measurement of forward light scatter for these reactions and to selectivily minimise the forward light scatter produced by potentially interfering substances. The samples after dilution and reaction with the appropriate antiserum are read in a laser nephelometer and the results calculated from a prepared standard curve.

Fredrickson's classification of abnormal Lipoproteins.

Fredrickson (1969) distinguished five different types of hyperlipoproteinaemia by staining the lipoprotein separation following electrophoresis of sera from patients with lipoprotein abnormalities. This classification was modified by the WHO (1970) by subdividing the predominant Type II group into Type IIa and Type IIb. Stone and Thorp (1966) had separated the lipoproteins by size analyses into small (S), medium (M) and large particles(L) and expressed the resulting pattern as a S M L profile. This classification has been related to the Fredrickson types (Stone and Thorp 1971) and the characteristics of both are summarised in Diagram 13 .

The relationship of serum cholesterol and triglycerides occuring in the different Fredrickson types is shown in Diagram 14 .

Triglyceride Assay.

The method (Bucolo and David, 1973) is based on the enzymic hydrolysis of triglyceride to glycerol and the corresponding fatty acid. The liberated glycerol is then assayed at 340 nm by the following linked reactions.

$$\text{Glycerol} + \text{ATP} \xrightarrow[\text{kinase}]{\text{glycerol}} \text{Phosphoglycerol} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \text{lactate} + \text{NAD}^{+}$$

The change in absorbance at 340 nm is proportional to the concentration of glycerol from the triglycerides and free glycerol in the serum. The latter is estimated by the link reactions prior to addition of glycerol kinase. A blank reaction without serum is run in parallel. All measurements were done with Calbiochem Triglyceride kits on an MSE Vitatron.

Serum Cholesterol

A Technicon AAIwas used to estimate total cholesterol by the method of Annan and Isherwood (1969). Prior extraction of serum is not required and a single colour reagent (sulphosalicylic acid in acetic and sulphuric acids) is used. The method is calibrated using standard solutions of cholesterol in propan-2-ol and the absorbance measured at 630 nm.

Lipid typing by nephelometry.

Thorp (1969) developed a classification of hyperlipidaemia, based on the size of the lipoprotein particles in sera, by measurement of light scattered by the lipoproteins using a Thorp micronephelometer. The amount of light scattered depends on the difference between the refractive index of the sera and that of the particles. Three types of low-density lipoprotein particles are designated.

S = small (low density or B-lipoproteins S = 0-20)

M = medium (very low density or pre-β lipoprotein S=20-400

L = large (chylomicra, S over 400)

A sample of fasting serum is analysed for total cholesterol content (C). An aliquot of the serum is diluted by one tenth and the light scattered by the sample measured on a nephelometer(A). The solution is filtered through a membrane of pore size 0·1 μ, which filters off the chylomicrons and the light-scattering measured againsgt (B).

Equations relating A,B and C to the quantity of S,M and L particles in the serum have been devised by Thorp (1969).

L = constant x (A-B)

M = 11 (B-5) or if B is greater than 50, M = 5·7 (B+37)

S = 2·1, C-0·19, M-10·5

Lipoprotein electrophoresis.

Buffer Sodium barbital, pH 8·6, 0·05M with 0·035% EDTA was used as bridge buffer and for the preparation of 1% agarose electrophoretic plates containing 0·04% EDTA.

Apparatus Corning Black Box electrophoresis equipment.

Procedure

1·0 μl aliquots of fasting sera were applied to sample wells on agarose plates. Excess moisture was drained from the plates following electrophoresis for 35 minutes at 90 volts. The agarose was dried at 60° for approximately 20 minutes prior to staining in freshly prepared 0·02% Fast Red 7B in 84% aqueous methanol for 2-3 minutes. The film is washed with gentle agitation in 50% aqueous methanol until the background is pale pink.

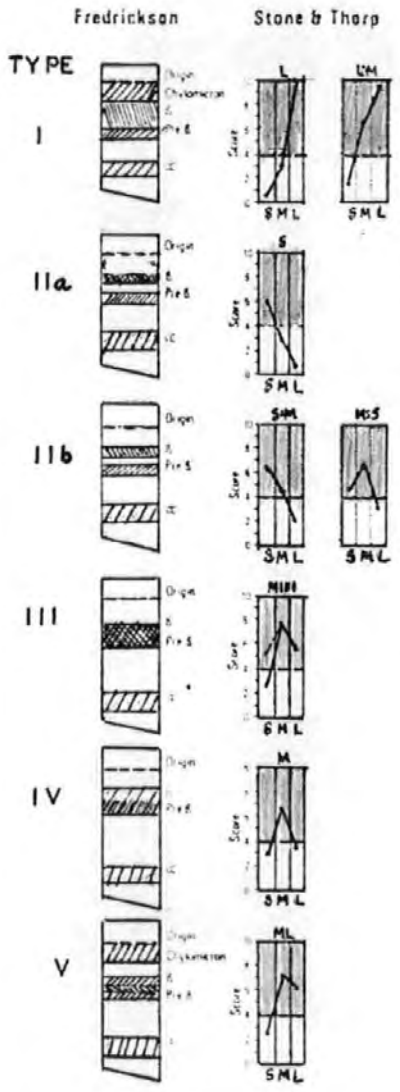
The resulting stained bands are examined for lipoprotein fractions and the order of bands from cathode to anode are chylomicrons, β-, pre β - and α -lipoprotein.

Measurement of Creatinine

The assays were performed on a suitably programmed Gilford 3500 enzyme rate analyser.

The reaction is based on the Jaffa reaction whereby creatinine reacts with a saturated solution of picric acid in sodium hydroxide producing a red colour which is monitored at 510 nm and read against a standard creatinine sloution, equivalent to 100 μ mol/l.

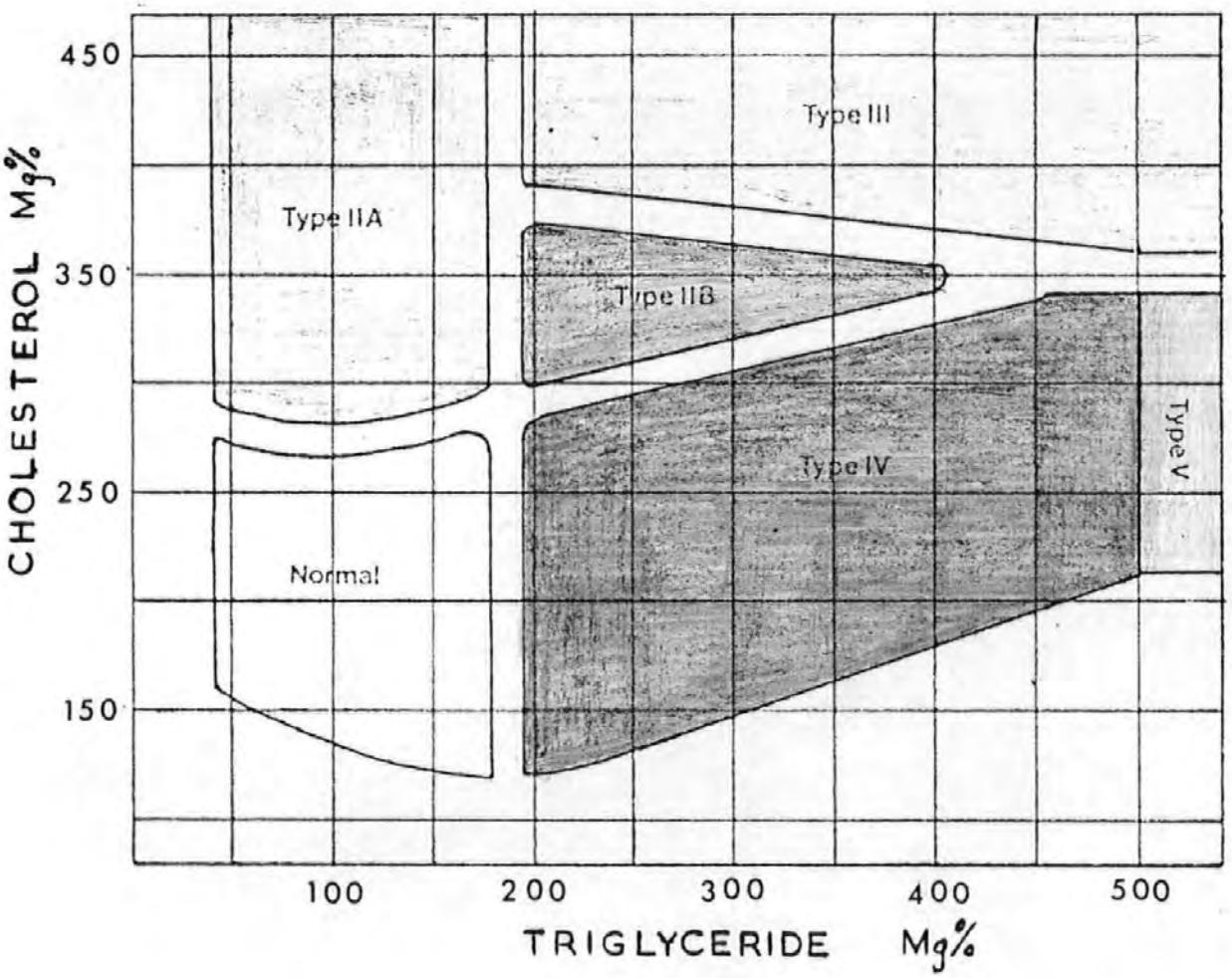
Diagram. 13 Fredrickson's classification of abnormal lipoprotein compared with Stone and Thorp's SML profile.

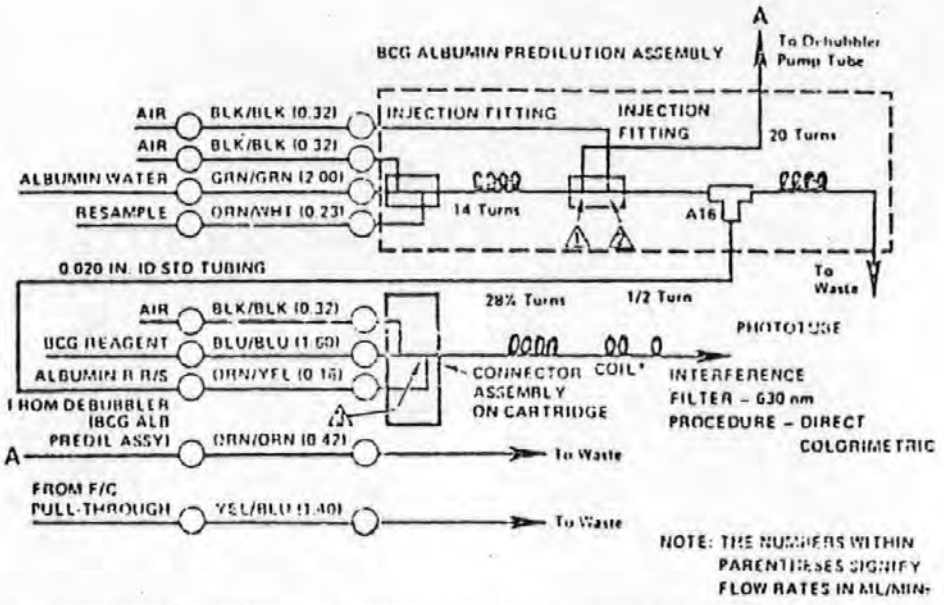


Lipoprotein	Plasma	Cholesterol	Triglyceride	Cholesterol/ Triglyceride ratio
chylomicra sometimes + pre-β	creamy	+	+++	< 0·1
β	clear	+++	−+	> 1·5
β & pre-β	clear faintly turbid	+++	+++	0·70-2
"floating" β	turbid	++	++	may vary from 0·3 to > 2·0 usually close to 1·0
pre-β	slightly turbid	−+	++	0·4-1
pre-β and chylomicra	turbid	+	+++	0·15-0·75

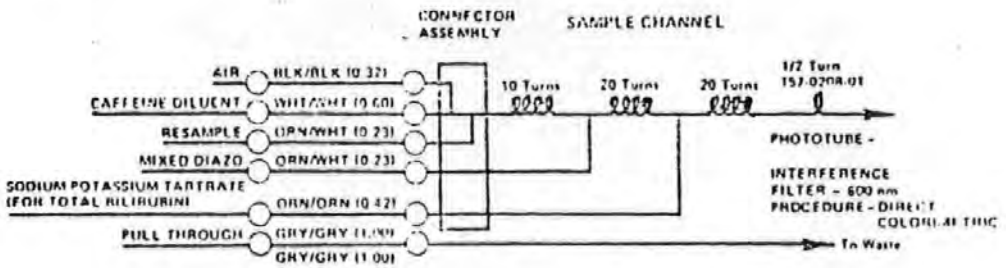
KEY:
−+ normal to slightly increased
\+ slightly increased
++ moderately increased
+++ greatly increased

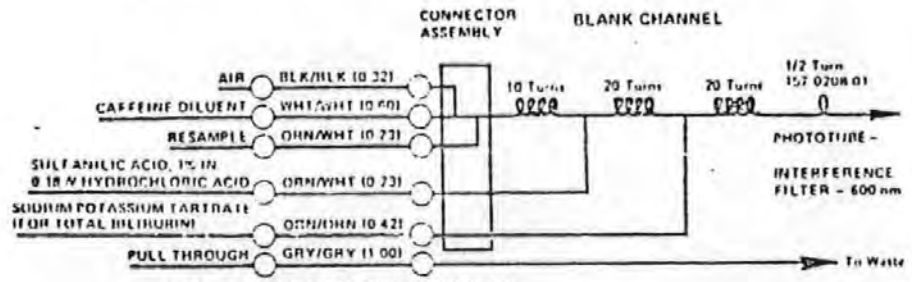
Diagram. 14 The relationship of serum cholesterol and triglycerides occuring in the different Fredrickson types.





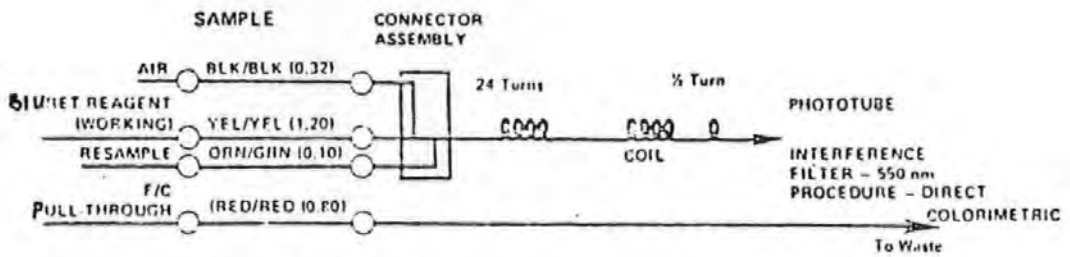
SIMPLIFIED FLOW DIAGRAM OF THE ALBUMIN (BCG) CHANNEL FOR THE TECHNICON SMA SYSTEM

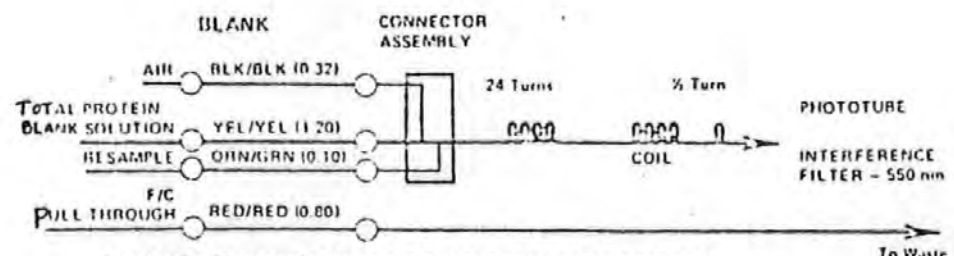




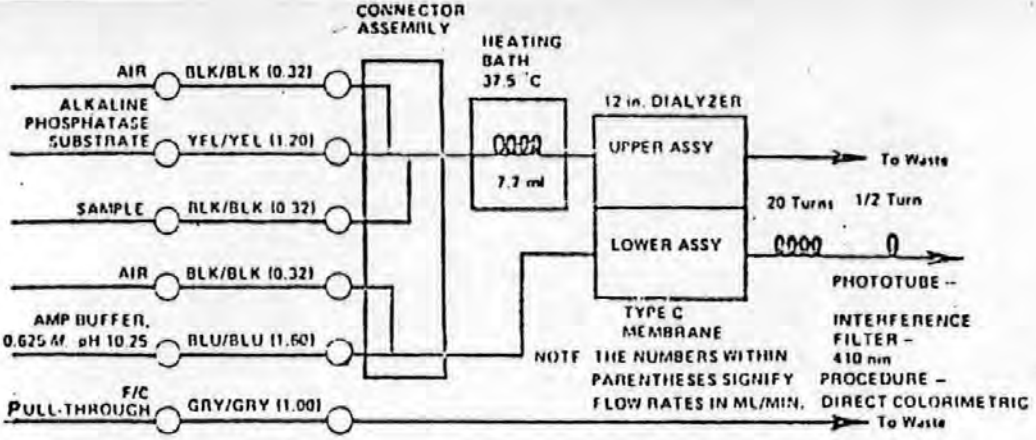
NOTE: THE NUMBERS WITHIN PARENTHESES SIGNIFY FLOW RATES IN ML/MIN

SIMPLIFIED FLOW DIAGRAM OF THE TOTAL BILIRUBIN CHANNEL FOR THE TECHNICON SMA SYSTEM

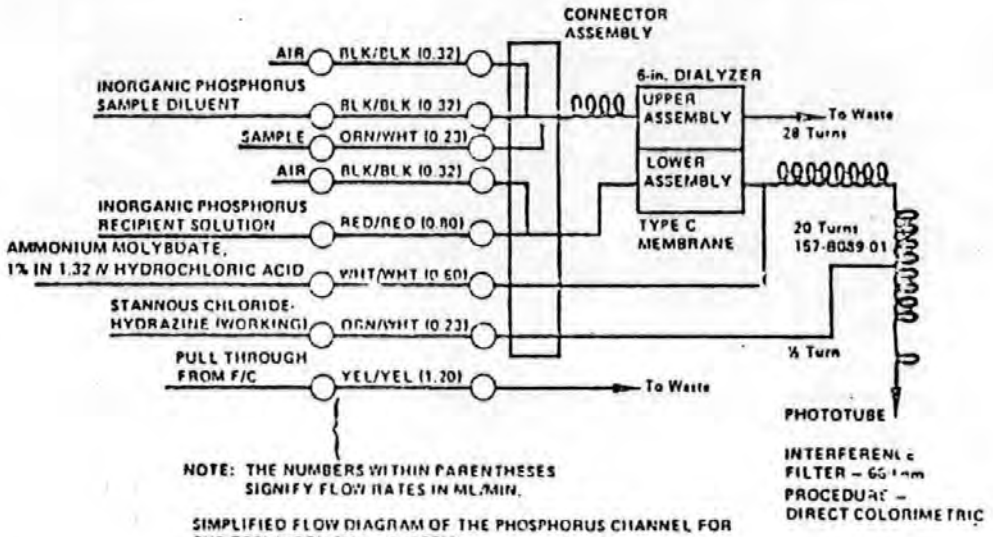




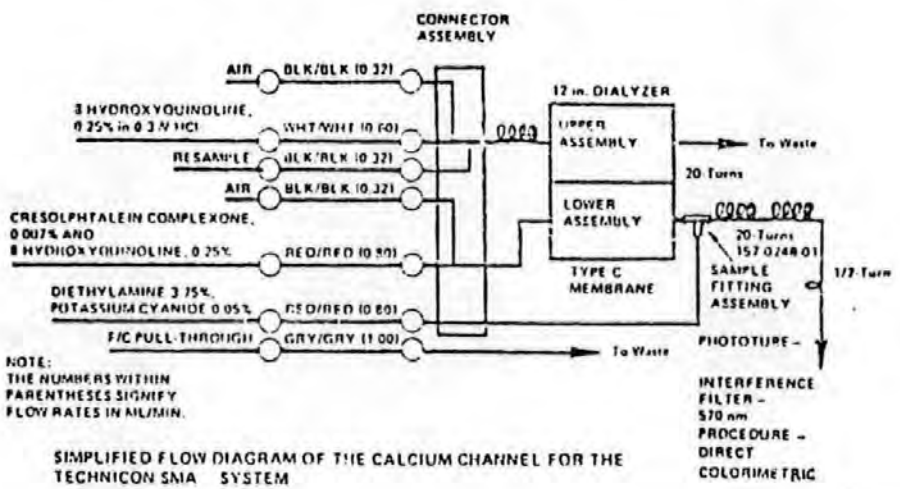
SIMPLIFIED FLOW DIAGRAM OF THE TOTAL PROTEIN CHANNELS FOR THE TECHNICON SMA SYSTEM



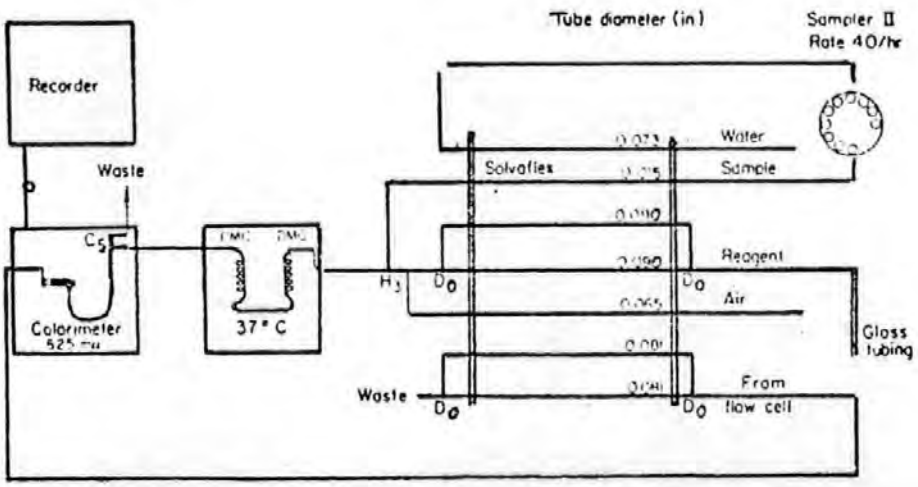
SIMPLIFIED FLOW DIAGRAM OF THE ALKALINE PHOSPHATASE CHANNEL FOR THE TECHNICON SMA SYSTEM



SIMPLIFIED FLOW DIAGRAM OF THE PHOSPHORUS CHANNEL FOR THE TECHNICON SMA SYSTEM



SIMPLIFIED FLOW DIAGRAM OF THE CALCIUM CHANNEL FOR THE TECHNICON SMA SYSTEM



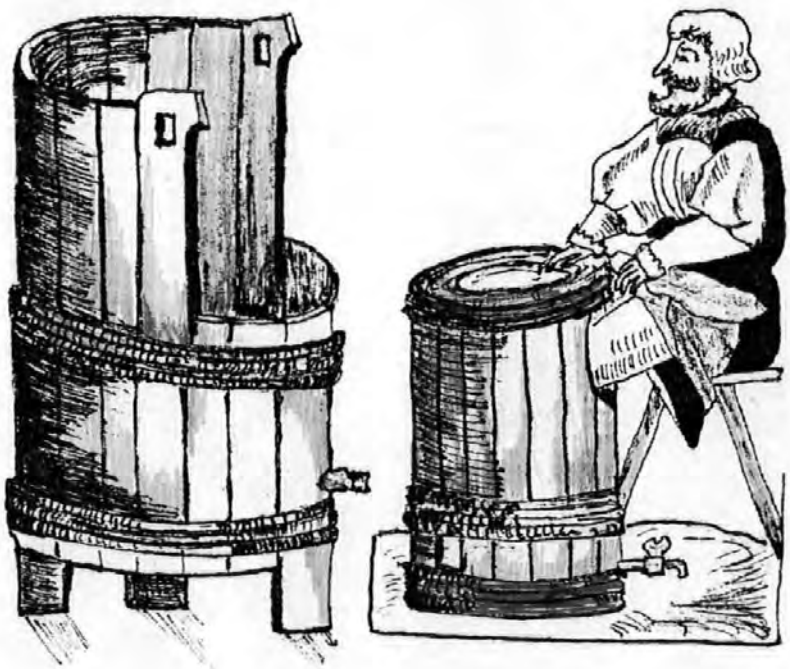
Flow diagram, CHOLESTEROL 10

Appendix II

The results from a routine clinical laboratory assay of serum uric acid using an autoanalyser over a ten year period have been analysed. The samples have been crudely separated into abnormal and normals. A serum uric acid content was classified as "abnormal" for all values greater than 420 μ mols/l. These abnormal sera are of course hyperuricaemic specimens. The distribution of the abnormal serum uric acid year by year during this 10 year period is shown in Fig. 33 and the percentage of hyperuricaemic sera month by month during the period 1970-1975 is shown in Fig. 32 . The total number of sera analysed for their uric acid content each year is shown in Table 32 together with the number of hyperuricaemic blood samples per year.

The data for the period from January 1976 to April 1978 inclusive has been further analysed for the distribution of the hyperuricaemic samples amongst men and women. This data is summarised in Table 31 . The month by month distribution of hyperuricaemia sera for men and for women is compared with that found for the total population in Table 31 .

Steam-bath Treatment for Gout
16th Century



Appendix III

The Development of Understanding of Gout.

Gout in the Earliest times.

Gout at the Time of the Renaissance.

Gout in the Seventeenth Century.

Gout in the Eighteenth Century.

Gout in the Nineteenth Century.

The history of gout is an interesting one, and it has taken many years as well as men of renown to interpret the nature of the malady. The word 'gout' was first recorded in Roman times and was derived from the Latin word gutta, meaning a drop, which referred to the four humours, which when in equilibrium were said to maintain the health of the body, any excess of one of these humours would drop or flow into a joint causing pain. This classification of a clinical condition would not be restricted to the flow of humours only to joints as the flow could occur to other parts of the body and examples would have been 'gouty' migraine, diarrhoea and haemorrhoids. Catarrh and 'rheumatism' were words also used to express the concept of flowing humour.

It was a Dominican monk, Randolfus of Bocking who used the word 'gout' as we would understand it today as a painful periodical swelling of the big toe.

In the fifth century B.C. Hippocrates wrote the oldest known medical textbook, known as the works of Hippocrates, based upon clinical observations. He also believed that gout was a result of an excessive accumulation of one of the body's humours, probably phlegm, which distended the affected joint painfully. He attributed the cause to sexual excess or too rich a diet with a

sedentary life. Later Galen added an hereditary factor to the Aphorisms, and noted that those who inherited gout suffered the consequences more severely.

The three most well known Aphorisms of Hippocrates which refer to gout are basically as follows:-

(i) 'Eunuchs do not take the gout nor become bald'

(ii) 'A woman does not take the gout unless her menses be stopped'

(iii) 'A young man does not take the gout until he indulges in coitus.' It is thought that Hippocrates meant before the age of puberty.

Hippocrates also noted that attacks of gout were related to the seasons and that 'gouty' afflictions were more prominant in the Spring and the Autumn as were arthritic diseases in the Spring. Celsus explained tha Autumn afflictions as nature's way of expelling bad humours to the extremities after their build up in the body during the heat of the summer.

It would appear that Hippocrates was able to distinguish between acute gout and acute rheumatism which he termed arthritis. In his 'Afflictions of the Parts' he compared the two diseases. He referred to gout as 'Podagra' which he described as the most violent of all joint afflictions. "It lasts long and becomes chronic. The pain may remain fixed in the great toes- it is not fatal." He also stated that 'In arthritis, fever comes on, acute pain affects the joints of the body, and the pains which vary between mild and severe flit from joint to joint;

it is of short duration, and often very acute, but not mortal. It attacks the young more frequently than the old.'

The treatment advocated for gout at this time by Hippocrates and his Greek followers were diet and included types of food and its preparation, the nature of wines and the taking of a barley water. When chronic gout was noted, a stronger form of treatment was recommended in the form of a purgative, white hellebore, because Hippocrates thought that 'an attack of dysentery was natural relief for the affliction.'

The Greeks introduced the art of medicine to Rome and the Roman, Celsus (25B.C.-A.D.50), although not a physician, classified all known human and vetinary maladies. He noted one type of joint affliction which he termed arthritis but applied more specific terms to local sites e.g.- podagra, chiagra etc. Many literary Romans e.g. Virgil, Martial and Avid were not impressed by the results of treatments of the day which included bleeding from a vein at the onset of an attack and external local treatment to affected joints. These writers also held a fascination for gout and the 'Demon Goddess Podagra' was the star of Lucian's comic tragedies, Trapopodagra. The remedies that the Goddess refers to in the play included the use of herbs and mineral medications as well as raw toads and human excretment. All were used against gout in the second century A.D.

The second century physicians Galen and Arataeus subscribed to most of Hippocrates' teachings. Arataeus suggested that there was a specific 'toxic substance'

in the blood of gouty patients: this hypothesis was the first indication of the existence of uric acid but was not proven until the 19th century. He also left an accurate description of an initial attack of gouty arthritis as follows:- 'The joints begin to be affected in this way:- pain seizes the great toe, then the forepart of the heal on which we rest; next it comes into the arch of the foot, but the ankle joint swells last of all.' All sufferers at first wish to blame the wrong cause:- some friction of a new shoe, others a long walk, others an accident, or being trodden upon... but the true cause is seldom believed by the patient when he hears it from a physician.

> 'The first affected (in gout)are the ligaments of the joints, such as have their origin in the bones. Now there is a great wonder in regard to these; there is not the slightest pain in them should you cut or squeeze them; but if they become spontaneously painful, as in gout, no other pain is more severe than this, not iron screws, nor cords, not the wound of a dagger, nor burning fire, for all these may be had recourse to as cures for the still greater pains. And if one cut them whilst they are painful the smaller pain of the incision is obscured by the greater; and if it prevail, they experience pleasure in forgetting their former sufferings.... in a word, any part which is very compact

is insensible to touch or to a wound. For pain consists of an irritation, but what is compact cannot be inflamed, and hence is not sensible to pain; but a spongy tissue is very sensitive.'

At the end of the fourth century after the fall of the Roman empire many new and exotic cures for gout were introduced from the east, but were found to be of no use although many persisted in the middle ages. Two Bygantine physicians contributed to the knowledge of gout: Alexander of Tralles introduced the use of hermodactyl (colchicum) as the specific treatment for gout and Paul of Aegmar thought that gout must be due to some congenital weakness. He speculated that sorrow, anxiety, or other passions of the mind might precipitate attack, and also introduced the colchicum plant (hermodactyl) as the specific treatment for gout.

Even in the eighth century the teachings of Galen still persisted in the Moslem Empire and the use of colchicum was advocated for the treatment of gout.

The great physicians of the Caliphate-Mesue (d.857), Rhages (d.923-924), and Avicenna (980-1037), 'Prince of Physicians'- applied the new botanical knowledge which was creeping in from India, Persia, and possibly even China, to the materia medica, but for gout, colchicum remained the standard remedy.

Physicians of the Middle Ages merely carried on applying many of the strange cures that were in vogue in the Byzantine era. One such remedy for gout sufferers being:-

'Roast a fat old goose and stuff with chopped kittens, lard incense, wax and flour of rye. This must all be eaten, and the drippings applied to the painful joints.'

Even during the period of the European Renaissance of learning no progress was made in man's understanding of gout. Two centuries later the humoral hypothesis, although not in the pure form of humoralism as defined by Galen, was questioned. The concept that gout might only be attributed to an alteration in the proportion or nature of humours was doubted and it was suggested that gout might be caused by some chemical change in blood, or even the production in the body of some toxic substance. At this stage interest was shown in the possible nature of such a substance and the site of its formation and storage in the body.

Gout had been well recognised in England since the thirteenth century as a clinical entity. The reason for this could well have been due to the almost exclusively meat eating habits of the upper classes and nobility. The size of the meals were enormous and washed down with large quantities of heavy sweet wines; vegetables and fresh fruit were never eaten by this 'class' of person.

John of Gaddesden, physician to King Henry V of England, was responsible for writing the first English textbook of medicine, the 'Rosa Anglica' 1280 in which some progress in the understanding of gout was stated. This progress was his personal observation that heredity in gout seemed to come more importantly through the mother's side of the family and might often skip a generation.

The Tudor dynasty from Henry VII were affected by gout and King Henry VIII licensed John Gilbert, the first specialist rheumatologist on record, 'to cure the goute, the crampe and all sore eyes.'

Ambrose Pare, the French surgeon (1510-1590) suffered personally from gout which he described in Book XVIII of his Workes: "The paines of the goute are rightly accounted amongst the most grievous and acute; so that through the vehemency of the agony many are almost mad, and wish themselves dead." He noted that the natural duration of an attack was forty days, although those who had developed tophi were scarcely ever free. He also observed a stricter periodicity in attacks of those who suffered with the inherited type of disease; and in some others a purely individual rhythm. His views on the origin of gout were in the Galenic tradition i.e. the humours, he pointed out that it was the toxic virulence of the peccant matter distilled from the humours and not the quantity that was important, 'as it causeth extreme paines not by abundance, because it happens to many who have no signs of swelling in the jointes.' He was convinced that the disease was caused by dietary and sexual overindulgence. He recommended frequent copious bloodletting, vomiting, sweating, diuresis, and purging, particularly with hermodactyl.

Thomas Sydenham (1624-1689) was named by his countrymen the 'English Hippocrates'. He studied and wrote extensively about being a sufferer, which was probably why he wrote a classical description of the disease. His description, differentiating finally

between gout and rheumatism, has never been surpassed. In fact he wrote more about gout than any other physician in the 17th century. He did not advocate the use of violent purges which was rather unfortunate for fellow sufferers since seammony and coloajrth, together with a small dose of colchicum, formed the basis for most purgative prescriptions and this resulted in the use of colchicum being banished throughout Europe for the next hundred and fifty years. Sydenham suffered his first gouty attack at the age of thirty, seven years later he developed haematuria from a 'renal calculus' and was rarely out of pain. His Treaise on the Gout (1683) is prefaced with an account of his own sufferings: 'my health prevents me from troubling the world much more with medical treatises.... I am at a loss to know whether the stone or the gout be most severe.' After the writing of this treatise he retired permanently to the country. His treatment comprised dietetic restriction, simple cooking, few drugs, and an ample, non-alcoholic fluid intake, a tranquil life devoid of excesses with regular horse-back exercise. Opium was his favourite medicament during the acute attack, and he introduced a liquid tincture of laudanum which brought him early fame throughout the continent of Europe. Jesuits' bark (quinine) was used by him as a prophylactic between attacks although he did not believe in the use of analgesics judging their effect to be non-significant.

The contents of Sydenham's treatise describe the symptoms of gout and some of the complexities which still exist today.

'There is no doubt but that men will conclude either that the nature of the disease, which is my present subject, is incomprehensible, or that I who have been afflicted with it for these thirty-four years past am a person of very slender abilities -insomuch as my observations concerning this distember and the cure thereof fall short of their expectations. But not withstanding this I will faithfully deliver my remarks concerning the difficulties and intricacies occuring in the history of the disease and the method of cure, leaving the illustration thereof to time, that discoverer of the truth.'

He continues that the disease, is common among elderly people living lives of luxury, particularly those that have been athletic in their youth but have given up exercise completely. 'The gout, however, does not only seize the gross and corpulent, but sometimes, though less frequently, attacks lean and slender persons.... sometimes in the prime of life, when they have received the seeds of it from gouty parents, or have otherwise occasioned it by an over-early use of venery.'

'The regular gout generally seizes in the following manner.: it comes on a sudden towards the close of January or the beginning of February, giving scare any sign of its approach except that the patient has been afflicted for some weeks before with a bad digestion.... and the day preceeding the fit the appetite is sharp but preternatural. The patient goes to bed and sleeps quietly till about two in the morning, when he is awakened

by a pain which usually seizes the great toe, but sometimes the heel, the calf of the leg or the ankle. The pain resembles that of a dislocated bone.... and this is immediately succeeded by a chillness, shivering and a slight fever. The chillness and shivering abate in proportion as the pain increases, which is mild in the beginning but grows gradually more violent every hour, and comes to its height towards evening, adapting itself to the numerous bones of the Tarsus and Metatarsus, the ligaments where it affects; sometimes resembling a tension or laceration of those ligaments, sometimes the gnawing of a dog, and sometimes a weight and constriction of the parts affected, which become so exquisitely painful as not to endure the weight of the clothes nor the shaking of the room from a person's walking briskly therein. And hence the night is not only passed in pain, but likewise with numberless endeavours to ease the pain by continually changing the situation of the body and part affected, which notwithstanding abates not till two or three in the morning: that is till after twenty-four hours from the first approach of the fit, when the patient is suddenly relieved.... and being now in a breathing sweat he falls asleep, and upon waking finds the pain much abated, and the part affected to be swollen; whereas before only a remarkable swelling of the veins thereof appeared, as is usual in all gouty fits.

The next day, and perhaps two or three days afterwards, if the gouty matter be copious, the part affected will be somewhat paines, and the pain increases towards evening and remits about the break of day. In a few days it seizes the other foot in the same manner, and if the pain be violent in this, and that which was first seized be quite easy, the weakness of this soon vanishes and it becomes as strong and healthy as if it had never been indisposed: nevertheless the gout affects the foot just seized as it did the former both in respect of the vehemence and duration of the pain; and sometimes.... it affects both at the same time with equal violence.'

He points out that an attack of gout is always comprised of a number of these recurrences or exacerbations, and will often last two months, although those who possess "strong constitutions, and such as seldom have the gout" may recover in fourteen days. In patients who suffer frequent attacks or who are debilitated from age or disease, the attack may last until the end of the following summer, when it usually disappears.

'During the first fourteen days the urine is high coloured, and after separation lets fall a kind of red gravelly sediment and not above a third part of the liquids taken in is voided by urine, and the body is generally costive during this time. The fit is accompanied throughout with loss of appetite, chillness of the whole body towards eventide, and a heaviness and uneasiness even of those parts that are not affected by the

disease. When the fit is going off a violent itching seizes the foot, especially between the toes, whence the skin peels off, as if the patient had taken poison. The disease being over, the appetite and strength return sooner or later, according as the immediately preceding fit hath been more or less severe, and in consequence of this the following fit comes on in a shorter or longer space of time; for if the last fit proved very violent the next will not attack the patient till the same season of the year returns again.

In this manner does the regulas gout, accompanied with its genuine and proper symptoms appear; but when it is exacerbated either by wrong management or long continuance..... and Nature is unable to expel it according to her usual way, the symptoms vary considerably from those just described above. For whereas the pain hitherto only affected the feet..... it now seizes the hands, wrists, elbows, knees and other parts, no less, severely that it did the feet before.... and at length forms stony concretions in the ligaments of the joints, which destroying the skin of the joints, stones not unlike chalk or crab's eyes, come in sight and may be picked out with a needle.'

These tophi he considered to be due to "undigested gouty matter thrown out around joints in liquid form, and changed to their peculiar hardness by the heat and pain of the joint."

Later he describes the chronic phase:

'In the last place, before the disease came to such a height, the patient not only enjoyed long intervals between the fits, but likewise had no pain in the limbs and the other parts of the body, all the bodily functions being duly performed. Whereas now his limbs, during the intermission of the disease, are so contracted and disabled that though he can stand, and perhaps walk a little, yet it is very slowly and with great and lameness, so that he scarse seems to move at all..... the feet in this state of the disease are never quite free from pain.

But besides the above mentioned symptoms, viz. pain, lameness, inability to motion of the parts affected..... the gout breeds the stone in the kidneys in many subjects, either

(1)because the patient is obliged to lie long on his back, or

(2)because the secretary organs have ceased performing their proper functions, or else

(3)because the stone is formed from a part of the same morbific matter; which, however, I do not pretend to determine. And sometimes a suppression of urine, caused by the stones sticking in the urinary passages, destroys him.

He then states his much quoted and famous passage:

'But what is a consolation to me, and may be so to other gouty persons of small fortunes and slender abilities, is that kings, great princes, generals, admirals, philosophers and several other great men have thus lived and died.

Inshort it may, in a more especial manner, be affirmed of this disease that it destroys more rich than poor persons, and more wise men than fools, which seems to demonstrate to the justice and strict impartiality of Providence, who abundantly supplied those that want some of the convenience of life with other advantages, and tempers its profusion to others with equal mixture of evil. So it appears to me universally and absolutely decreed that no man shall enjoy unmixed happiness or misery, but experience both. Since those whom she favours in one way she afflicts in another- a mixture of good and evil pre-eminently adapted to our frail mortality - Nihil est ab omni parte beatum.'

At the end of his Treatise he states:-

'The gout seldom attacks women, and then only the aged, or such as are of a masculine habit of body..... nor have I hitherto found children or very young persons affected with the true gout. Yet I have known some who have felt some slight touches of it before they came to that age, but they were such as begot by gouty parents. And let this suffice for the history of this disease.'

Robert Boyle (1627-1691), the discoverer of Boyles' law, hypothesizes that gout could not be cured psychologically by frightening the victim. He told of a peasant who, while his hands and feet were wrapped in poultices, was fightened by a sow entering his house through the open door. The sow was attracted by the smell of the poultice and proceeded to devour it,....."whereupon

the man was put into such a fright that his pains decreased that very day..... and never returned."

Another remedy for gout was that advocated by Dr. Thomas Dover (1660-1742), a follower of Sydenham, who prescribed 'Diaphoretic Compound Ipecacuanha Powder.' The patients receiving this treatment were advised by the apothecaries to make a will, the doses being so large.

In the 18th century the age of the Spa Therapy began and saw the end of the drastic treatments mainly due to the work of Drs. William Cadogan (1711-1797) and George Cheyne (1671-1743). George Cheyne was known as the Gout Doctor and himself was a sufferer of the gout. In his own words he was born of healthy parents in the prime of their days. He became a tutor in a gentleman's family until persuaded by Professor Archibald Pitcairne to study medicine under him. Not only was Pitcairne professor of medicine at Edinburgh he was involved in the mathematical school of medical science, and Cheyne being a good mathematician soon adopted the teachings of his master. After the publication of one of his books he obtained his Aberdeen M.D. and moved to London and in 1701-1702 was elected F.R.S.

Until Cheyne came to London he had always been 'of sedentary and temperate habits,' but when he started in practice in London he changed his whole manner of living. He became a frequenter of Taverns and made friends among the younger gentry and free-livers, not only was this way of life enjoyable to him but was useful in bringing him professional business.

As this pace of life told on his health he lost

his friends and his prosperous career. He was persuaded by his friends to 'take the Waters of Bath.' After a few years he established a fashionable practice in Bath and wrote his book 'An Essay on the Nature and Due Method of Treating the Gout (1720). Dr. George Cheyne was the first to advocate completely milk and vegetarian regime for certain cases of gout and thus became the advent of a more rational and civilised attitude towards the treatment of gout.

Horace Walpole and many others during the 18th century supported the hypothesis that gout was a remedy and not a disease and that by having the gout they would be free from more serious diseases.

Sir Alfred Garrod (1819-1907) used the interest in chemistry of the day to rationalise scientific inquiries into the nature of gout.