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# HUMAN ERYTHROCYTE PYRIMIDINE 5'-NUCLEOTIDASE

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A Dissertation Submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, for the Degree of Master of Science (Medicine)

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HUMAN ERYTHROCYTE PYRIMIDINE 5'-NUCLEOTIDASE

WHITTAKER, Deborah Anne, for M.Sc., University of the Witwatersrand, 1984.

This dissertation is divided into three parts; the first presents the purification and properties of human erythrocyte pyrimidine 5'-nucleotidase, the second the enzyme activity in normal and enzyme deficient subjects, and the third the distribution of erythrocyte nucleotides in normal and enzyme deficient subjects.

Two methods were used to assay the enzyme activity. These were a chemical method which depended upon the measurement of inorganic phosphate liberated from CMP during a 2 hour incubation and a radiometric method in which the [2-$^{14}$C]cytidine formed from [2-$^{14}$C]CMP during a 30 minute incubation was measured.

The enzyme was purified 250,000-fold using a combination of DEAE-cellulose chromatography, ammonium sulphate fractionation, gel filtration, and isoelectric focusing. The enzyme was found to have a pI of 5.0 and a molecular weight of 28,000 by gel filtration. The enzyme had a pH optimum at 7.5 when assayed radiometrically and was most stable between pH 6 and 7.5. When the chemical assay was used with UMP as substrate, the purified enzyme

showed two pH optima at pH 6.25 and 7.2. In haemolysates, pyrimidine 5'-nucleotidase had a single optimum at pH 7.2 with CMP and at 6.75 with UMP as substrate. The $K_M$ of the purified enzyme was 10 μM, compared to 40 μM when measured in haemolysate. The enzyme was inhibited by both its products - inorganic phosphate and pyrimidine nucleoside. Inorganic phosphate appeared to be a non-competitive inhibitor of pyrimidine 5'-nucleotidase with $K_i$ = 1.5 mM. Contrary to previous reports, adenosine and inosine did not inhibit the enzyme.

The mean erythrocyte pyrimidine 5'-nucleotidase activity in 158 normal fresh blood samples was 130±SD 29.8 mU/gHb. There was no significant difference between males and females. The enzyme level in the index case of pyrimidine 5'-nucleotidase deficiency was 6 mU/gHb. Five of 31 relatives had enzyme levels below 50 mU/gHb and were clearly heterozygous for the enzyme defect. The father, an obligatory heterozygote, had an enzyme level of 87 mU/gHb which fell within the low values of the normal range. It was not possible to identify carriers by enzyme kinetic studies, electrophoresis, chromatographic examination of acid extractable nucleotides or measurement of enzyme levels in young and old erythrocyte populations. The last-mentioned technique showed that erythrocyte 5'-nucleotidase activity in reticulocytes could be as high as 1785

mU/gHb and declined rapidly as the cell aged, reaching about 50 mU/gHb in the oldest cells.

The composition of the erythrocyte nucleotide pool was examined in both heterozygous and homozygous enzyme deficiency by ion exchange chromatography on Dowex formate columns using a linear ammonium formate elution gradient and compared with the results obtained with normal and reticulocyte rich blood. In normal erythrocytes adenine nucleotides accounted for more than 96% of the nucleotides, whereas in the enzyme deficient cells adenine nucleotides accounted for only 32% of the nucleotide pool. The remainder consisted of 50% cytidine and 16% uridine nucleotides. The remaining 2% was not identified. The most abundant compound appeared to be UDP glucose whilst high levels of CTP, CMP and an unidentified cytidine compound less polar than CMP accounted for most of the cytidine nucleotide pool. The possibility that the abnormal nucleotides were due to an elevated reticulocyte count was excluded and it was also shown that erythrocytes from subjects heterozygous for pyrimidine 5'-nucleotidase deficiency did not have detectable levels of the abnormal nucleotides.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree Master of Science (Medicine) in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any other University.

Deborah Ann Whitelaw

24th day of February, 1984.

For my husband and our children.

CONTENTS


| | | Page |
|---|---|---|
| ABSTRACT | | ii |
| PREFACE | | xvii |
| Chapter | | |
| 1. | INTRODUCTION | 1 |
| | The Erythrocyte | 1 |
| | Human Erythrocyte Pyrimidine 5'-Nucleotidase Deficiency | 2 |
| | Early Studies | 3 |
| 2. | METHODS USED FOR ASSAY AND PURIFICATION OF HUMAN ERYTHROCYTE PYRIMIDINE 5'-NUCLEOTIDASE AND FOR STUDYING ERYTHROCYTE NUCLEOTIDES | 9 |
| | Paglia and Valentine (1975a) Assay for Pyrimidine 5'-Nucleotidase | 9 |
| | Preparation of sample haemolysates | 9 |
| | Incubation | 10 |
| | Fiske and SubbaRow (1925) Phosphate Assay | 11 |
| | Reagents | 11 |
| | Procedure | 12 |
| | Bartlett (1959b) Phosphorus Assay in Column Chromatography | 13 |
| | Reagents | 13 |
| | Procedure | 13 |
| | Torrance, West and Beutler (1977b) Assay for Pyrimidine 5'-Nucleotidase | 14 |

Page

Preparation of radioactive substrate ..... 15

Preparation of $Ba(OH)_2$ and $Zn(SO)_4$ solutions ..... 16

Preparation of sample haemolysates ..... 17

Determination of enzyme activity ..... 18

Radiometric Method with UMP as Substrate ..... 19

Storage of Frozen Erythrocytes Intact for Later assay ..... 20

Preservation of erythrocytes ... 20

Recovery of erythrocytes ..... 20

Erythocyte Cell Density Separation and Haemolysate Preparation ..... 21

Procedure ..... 21

Enzyme Purification Method ..... 22

Preparation of haemolysate ..... 22

First DEAE-cellulose extraction ..... 23

Ammonium sulphate concentration procedure ..... 23

Second DEAE-cellulose extraction ..... 24

Sephacryl S-200 column gel filtration ..... 24

Isoelectric focusing ..... 25

Modified Lowry Protein Determination (Lowry, Rosebrough, Farr and Randall 1951) ..... 27

Reagents ..... 27

Procedure ..... 27

Page

Starch Gel Electrophoresis ..... 28

Extraction of Cell Nucleotides using 6% Perchloric Acid ..... 29

Ion exchange Chromatography of Perchloric Acid Extracts ..... 30

Preparation of resin ..... 30

Procedure ..... 31

Identification of elution peaks. 31

3. PURIFICATION AND PROPERTIES OF HUMAN ERYTHROCYTE 5'-NUCLEOTIDASE ..... 33

Purification of the Enzyme ..... 33

Some Properties of the Enzyme ..... 37

Molecular weight ..... 39

Effect of pH on purified enzyme activity ..... 39

Enzyme activity in haemolysate with four substrates and over a range of pH ..... 40

Extending the pH range ..... 43

Purified enzyme activity with four substrates and over a range of pH ..... 45

Effect of pH on purified enzyme stability ..... 48

Optimum $Mg^{++}$ concentration ..... 50

Kinetic Studies of the Enzyme ..... 50

$K_M$ of purified enzyme and haemolysate ..... 51

Inhibition Studies ..... 53

Page

Inorganic phosphate ..... 53

Purine and pyrimidine nucleosides ..... 56

4. STUDIES OF NORMAL AND DEFICIENT LEVELS OF HUMAN ERYTHROCYTE PYRIMIDINE 5'-NUCEOTIDASE ..... 59

Study of the First Case of Human Erythrocyte Pyrimidine 5'Nucleotidase Deficiency Reported in South Africa . 59

Enzyme Levels of Family of Enzyme Deficient Child ..... 61

Linkage Studies ..... 63

Enzyme Levels Found in Normal Population Studies ..... 65

Pyrimidine 5'-Nucleotidase Level in Frozen Blood ..... 67

Variation in Pyrimidine 5'-Nucleotidase Levels in a Single Subject ..... 71

Changes in Pyrimidine 5'-Nucleotidase with Cell Density ... 71

Electrophoretic Studies ..... 75

Enzyme Kinetics in Heterozygotes and Normals ..... 76

5. DISTRIBUTION OF ERYTHROCYTE NUCLEOTIDES IN PYRIMIDINE 5'-NUCEOTIDASE DEFICIENCY .. 79

Preparation of Perchloric Acid Extracts ..... 80

Study of Whole Extracts Before Ion Exchange Chromatography ..... 81

Page

Ion Exchange Chromatography ..... 83

Elution patterns ..... 85

6. DISCUSSION ..... 92

Background to the Purification and Studies of the Properties of Pyrimidine 5'-Nucleotidase ..... 92

Purification of the Enzyme ..... 93

Inhibitors and properties of Enzyme in Haemolysates and Purified Form ... 94

Effect of pH on enzyme activity ..... 94

Kinetic studies ..... 95

Inhibition studies ..... 96

Distribution of Erythrocyte Pyrimidine 5'-Nucleotidase Levels in Normal Subjects ..... 100

Distribution of Erythrocyte Pyrimidine 5'-Nucleotidase in the Deficient Family ..... 103

Use of Frozen Glycerolized Blood for Measurement of Pyrimidine 5'-Nucleotidase ..... 105

Density Gradient Separation of Erythrocytes ..... 108

Erythrocyte Nucleotides in Enzyme Deficiency and Normals ..... 109

Spectroscopic examination of PCA extracts of erythrocytes ... 109

Ion exchange chromatography of PCA extracts of erythrocytes ... 110

Page

Study of abnormal nucleotide pool and recent identification of cytidine compound ..... 111

Heterozygote study ..... 113

High reticulocyte blood study .. 114

Source of abnormal nucleotides . 115

Possible causes of haemolytic anemia ..... 116

LIST OF REFERENCES ..... 117

TABLES


| Table | | Page |
|---|---|---|
| 1. | Purification of human erythrocyte pyrimidine 5'-nucleotidase ..... | 38 |
| 2. | The effect of various purine and pyrimidine compounds on the pyrimidine 5'-nucleotidase activity of a crude haemolysate and a 250,000-fold purified preparation ..... | 57 |
| 3. | Haematological data on the patient and his parents ..... | 62 |
| 4. | Kinetic studies on the pyrimidine 5'-nucleotidase in haemolysates from two heterozygotes and two normals one of whom was from the deficient family .... | 78 |
| 5. | Concentration of acid-soluble phosphate compounds in erythrocytes from a pyrimidine 5'-nucleotidase deficient subject, a subject heterozygous for this condition, a normal subject and a subject having 16% reticulocytes ..... | 88 |

FIGURES


| Figure | | Page |
|---|---|---|
| 1. | Isoelectric focusing of Sephacryl S-200 fractions that had pyrimidine 5'-nucleotidase activity ..... | 34 |
| 2. | Polyacrylamide disc electrophoresis of 250,000-fold purified pyrimidine 5'-nucleotidase from human erythrocytes .. | 36 |
| 3. | The effect of pH on erythrocyte pyrimidine 5'-nucleotidase activity using the radiometric method with 0.3 mM [2-$^{14}$C]CMP as substrate ..... | 41 |
| 4. | The effect of pH on the 5'-nucleotidase activity of dialyzed haemolysate using the chemical method with 2 mM substrates . | 44 |
| 5. | The effect of pH on 5'-nucleotidase activity of the purified enzyme using the chemical method ..... | 46 |
| 6. | The stability of erythrocyte pyrimidine 5'-nucleotidase at 37°C as a function of pH using the radiometric method with [2-$^{14}$C]CMP as substrate ..... | 49 |
| 7. | The double reciprocal plots of human erythrocyte pyrimidine 5'-nucleotidase using a crude haemolysate and the purified enzyme ..... | 52 |
| 8. | The effect of inorganic phosphate on the pyrimidine 5'-nucleotidase activity of a 1:20 haemolysate ..... | 54 |

Figure Page

9. a The double reciprocal plots of purified enzyme pyrimidine 5'-nucleotidase activity in the presence of increasing concentrations of inorganic phosphate.
b Graphical determination of $K_i$ from the plot of reciprocal estimated initial reaction rate against the phosphate concentration ..... 55

10. Pedigree of the family of the patient with pyrimidine 5'-nucleotidase deficiency ..... 64

11. Histogram showing the distribution of erythrocyte pyrimidine 5'nucleotidase activity in 160 normal subjects and in erythrocytes from the deficient patient and members of his family ..... 66

12. Histogram showing the distribution of erythrocyte pyrimidine activity in 151 samples of normal blood stored in glycerol at -20°C for longer than one year ..... 70

13. Changes in erythrocyte pyrimidine 5'-nucleotidase activity in a single subject during an 18 month period ..... 72

14. Pyrimidine 5'-nucleotidase levels in erythrocytes separated according to their density ..... 74

15. Comparison of the starch-gel pyrimidine 5'-nucleotidase pattern of the deficient family with a normal specimen and a specimen which had been partially purified on DEAE cellulose ..... 77

16. Spectral scan of a perchloric acid extract from a subject with pyrimidine 5'-nucleotidase deficiency, from normal blood, and from both parents of the deficient subject ..... 82

Figure Page

17. Phosphate compounds in normal erythrocytes ..... 86

18. Phosphate compounds in pyrimidine 5'-nucleotidase deficient erythrocytes ... 87

PREFACE

This work was undertaken in the Joint University of the Witwatersrand/ South African Medical Research Council Iron and Red Cell Metabolism Unit, Department of Medicine, University of the Witwatersrand, Johannesburg. The work was supported by a grant from the South African Atomic Energy Board.

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Torrance, J.D., Whittaker, D. and Jenkins, T. (1980) Erythrocyte Pyimidine 5'-Nucleotidase. British Journal of Haematology. 45: 585-597.

Torrance, J.D. and Whittaker, D.A. (1981) Some Properties of Human Erythrocyte Pyrimidine 5'-Nucleotidase. Enzyme. 26: 64-73.

# 1. INTRODUCTION

## The Erythrocyte

The erythrocyte is a specialized cell, common to all vertebrates, evolved to transport oxygen throughout the body. The human erythrocyte has an extremely flexible cell membrane with a biconcave shape at rest that can assume many other shapes in order for it to pass through very confined spaces in the circulatory system. It has a far larger surface area than needed to enclose the normal cell contents and this facilitates its ability to distort its shape. However, the membrane is unable to stretch appreciably and expansion of cell contents easily causes it to rupture. Loss of membrane flexibility may also be a factor in some types of haemolytic anemia (Bessis and Mohandas 1975; La Celle and Kirkpatrick 1975).

The interior of the cell is structureless and contains a very concentrated aqueous solution of the respiratory pigment, haemoglobin. It also contains a host of other substances necessary to facilitate the survival of the cell and the oxygen transport function of the haemoglobin. The mature human erythrocyte has

sacrificed many of the metabolic functions of other body cells, however it is still metabolically active and utilizes glucose as its principal source of energy. Deprived of glucose it is unable to survive. It becomes echinocytic, then spherocytic and eventually suffers osmotic lysis.

There are several conditions in which nonspherocytic haemolysis occurs, leading to nonspherocytic haemolytic anaemia. Many of these conditions result from an hereditary deficiency of one or more enzymes necessary for the normal function of the red cell. One of these enzymes facilitates the dephosphorylation of pyrimidine nucleotides within the cell (Valentine et al. 1974). This enzyme is pyrimidine 5'-nucleotidase.

## Human Erythrocyte Pyrimidine 5'-Nucleotidase Deficiency

A deficiency of pyrimidine 5'-nucleotidase affects nucleotide metabolism within the red cell, resulting in haemolytic anaemia. In all cases of this deficiency there is marked basophilic stippling of erythrocytes. Similar basophilic stippling has also been noted in cases of lead poisoning and studies have shown that the enzyme is highly sensitive to lead inhibition (Paglia et al. 1975b, 1977; Buc & Kaplan 1978). This stippling effect, discernable in heparinized blood, is assumed to be due to aggregation of RNA within the cell (Valentine et al. 1974).

Clinical manifestations (Valentine et al. 1972, 1973, 1974) of this enzyme deficiency are characterized by a mild anaemia. When tested, most patients exhibit an abnormal autohaemolysis that is not corrected by glucose, a Selwyn and Dacie (1954) Type II pattern. In most cases splenomegaly is present, however splenectomy appears to have little beneficial effect in the treatment of the anaemia. Red cells show normal or only slightly increased osmotic fragility. Increased activity of some other enzymes have been reported and raised levels of glutathione have been noted. There also appears to be a decreased level of ribosephosphate pyrophosphokinase activity. In all cases, basophilic stippling is present.

## Early Studies

In 1972 Valentine, Anderson, Paglia, Jaffe, Konrad and Harris reported the discovery of a new type of haemolytic anaemia in a 29 year old black woman. A drop in red cell ribosephosphate pyrophosphokinase activity, basophilic stippling and raised glutathione levels were reported. The patient's haemolytic disorder was of long standing and an hereditary basis was suspected. Her only known relative, a son of six years, was located and his erythrocytes studied. His cells did not appear to exhibit the abnormalities found in his mother.

A further paper presented by Valentine, Bennet, Krivit, Konrad, Lowman, Paglia and Wakem was published in 1973. This disclosed the discovery of three more cases of an unusual haemolytic anaemia exhibiting the same characteristics as reported in the previous paper. These three patients consisted of a caucasian woman of 37 whose symptoms had only become manifest during adolescence and an unrelated family , two sisters, one of 23 years and the other of 12 years, white, both of whom had displayed abnormalities from birth.

In both papers, mention was made of an increase in levels of ATP and other adenine nucleotides in the patient's red cell. This later proved to be a mistaken diagnosis, but at the time it appeared valid. Adenine nucleotides make up 97% or more of the nucleotide pool in normal erythrocytes, with guanosine nucleotides levels from less than 1% to up to 3% of the normal pool and pyrimidine nucleotides present in negligable quantities (Minakami et al. 1965; Bartlett 1959; Bishop 1961; Bishop et al. 1959; De Luca et al. 1962). Appreciable amounts of pyrimidine nucleotides were never suspected to be present in the enzyme deficient cell, hence it had not been considered necessary to devise a completely specific adenine assay. In these studies, AMP, ADP and ATP had been measured using a conventional enzymatic method (Minakami et al. 1965). In this assay nonadenosine nucleotides would also react, but at a slower rate. In both papers a disturbingly slow

reaction rate in the assay of the deficient erythrocytes had been noted. This, coupled with the discovery of a shift in maximal ultraviolet absorbance from the normal 256-257 nm to the patient's approximately 266-270 nm when deproteinized red cell extracts were examined, led to clarification of the exact nature of this nucleotide pool (Valentine et al. 1974). Pyrimidines were found to be present and a pyrimidine specific enzyme was discovered.

In 1974 Valentine, Fink, Paglia, Harris and Adams published a paper concerning the discovery of the true nature of the anaemia previously reported. The prime cause of the problem appeared to be a deficiency of the enzyme pyrimidine 5'-nucleotidase necessary to facilitate the dephosphorylation of pyrimidine nucleotides within the erythrocyte. Previous patients as well as two additional patients were studied in the light of this discovery. One of the new patients was described as a 24 year old woman of Jewish ancestry with haemolytic anaemia diagnosed in childhood. This patient's parents were first cousins. Both parents and a sister were reported to be clinically well and haematologically normal. A detailed history of the second new patient was not given. This paper described an assay to measure pyrimidine 5'-nucleotidase activity. A second paper was published in the following year, presenting the assay and further studies of the enzyme (Paglia and Valentine, 1975a). This assay measured $P_i$

released from pyrimidine phosphate substrates in the presence of haemolysates containing the enzyme. Magnesium cation was necessary to facilitate the reaction for optimal results. Findings were expressed in μmol of inorganic phosphate liberated per hour per gram of haemoglobin. Paglia and Valentine (1975a) reported a normal average liberation of 7.3 μmol $P_i$ with UMP and 6.2 μmol $P_i$ with CMP as substrate. When TMP was used, about half these amounts of $P_i$ were liberated per hour per gram of haemoglobin. Michaelis constants were Ø.33mM UMP, Ø.15mM CMP and 1.ØmM TMP. Optimum pH was obtained between 7.Ø and 7.5. Moderate decrease in activity was noted with acidification with rapid decrease when the pH was raised. They also reported that the enzyme was heat sensitive. All these measurements were made using haemolysate without any purification. Although the method was accurate it was cumbersome, and therefore inappropriate for use in large population studies.

A second method of assaying the enzyme using radioactive substrates was devised by Torrance, West and Beutler (1977b). In this assay the radioactive substrate used was [2-$^{14}$C]CMP. When CMP was incubated with haemolysates in the presence of $Mg^{++}$, both cytidine and $P_i$ were products of the reaction. When [2-$^{14}$C]CMP was the substrate it produced [2-$^{14}$C]cytidine. This was followed by a single $BaSO_4$ precipitation that stopped the reaction and at the same time precipitated all

protein and substrate remaining, leaving radioactive cytidine in solution. Aliquots of this supernatant were counted by a beta-ray emission counter. The activity of the enzyme in this assay was expressed in mU/gHb where one unit (U) was equal to one μmole of CMP hydrolyzed per minute per gram haemoglobin. Paglia and Valentine's (1975a) results expressed in μmol of $P_i$ liberated per hour per gram haemoglobin could be expressed in mU/gHb by multiplying their results by a factor of 1,000/60. For this dissertation, the radiometric method has been the method of choice. The chemical method was only used to verify Valentine's studies, compare methods or when suitable radioactive substrates were unobtainable. Torrance et al. (1977b) reported that a comparison between the radiometric and chemical assay showed excellent agreement. These findings have been confirmed.

From 1974 onwards more patients were discovered world wide and by 1977, at least 16 cases from all over the world had been reported (Valentine et al. 1972, 1973, 1974; Rochant et al. 1975; Ben-Basset et al. 1976; Oda and Tanaka 1976; Miwa et al. 1977; Vives-Corrons et al. 1976) and it appeared that this enzyme deficiency would prove to be a common cause of nonspherocytic haemolytic anaemia.

In 1977 the first case of this enzyme deficiency was discovered in South Africa and a report published. This

constituted the beginning of the author's involvement in the case studies and in further studies of the enzyme.

## 2. METHODS USED FOR ASSAY AND PURIFICATION OF HUMAN ERYTHROCYTE PYRIMIDINE 5'-NUCLEOTIDASE AND FOR STUDYING ERYTHROCYTE NUCLEOTIDES

### Paglia and Valentine (1975a) Assay for Pyrimidine 5'-Nucleotidase

With the discovery of pyrimidine 5'-nucleotidase in the erythrocyte, Paglia and Valentine (1975a) presented a method of assay of enzyme activity levels by incubating dialyzed haemolysates with UMP or CMP as substrates and measuring $P_i$ liberated during incubation. Results were expressed in units of nucleotidase activity, one unit being defined as "the amount of enzyme catalyzing the release of 1μmole of $P_i$ per hour per gram of haemoglobin".

Preparation of sample haemolysates:
Venous blood samples were anticoagulated with heparin. Leukocytes and platelets removed by sedimentation with 'Plasmagel' or by filtration through cotton wool. The erythrocytes were washed three times with 40 volumes of isotonic saline, packing lightly between washes by centrifugation. After removal of the last wash, the cells were resuspended in saline to approximately 3 x $10^6$ per μl. Aliquots were removed to determine haemoglobin content. At the same time erythrocytes,

reticulocytes and contaminating leukocytes were counted.

Cell suspensions were rapidly frozen in dry ice-alcohol mixture and then thawed. This was repeated a total of 3 times. The haemolysates were then dialysed 16 to 20 hours against Tris-HCl buffered isotonic saline, containing 0.01 M Tris-HCl pH 8.0, 10 mM $MgCl_2$ and 0.02 mM EDTA. Haemoglobin was re-determined on this haemolysate after dialysis.

Incubation:

For incubation, 0.5 ml of dialyzed haemolysate was added to a reaction mixture consisting of 0.85 ml of 0.05 M Tris-HCl buffer pH 8.0, 0.15 ml of 0.1 M $MgCl_2$, 0.05 ml of 1:1000 2-mercaptoethanol, and 0.2 ml of 0.02 M UMP or CMP. The $Mg^{++}$ was needed for optimum results and 2-mercapto-ethanol was added to protect the enzyme. Final pH of the total mixture was 7.7 to 7.8 varying only by 0.1 throughout the incubation.

Incubation was usually 2 hours in duration carried out at a temperature of $37^{\circ}C$. The reaction was stopped and the mixture deproteinized by the addition of 1 ml of 20% trichloroacetic acid. Reagent blanks were run identically save for the absence of substrate, which was added after the TCA. The acid extracts were then individually filtered and assayed for $P_i$ using the Fiske and SubbaRow (1925) method.

Fiske and SubbaRow (1925) Phosphate Assay

Purification of 1-amino-2-napthol-4-sulphonic acid: The 1-amino-2-napthol-4-sulphonic acid used in this assay was purfied by recrystallization. This was accomplished by heating 1 litre of distilled water to 90°C and dissolving in it 150 g sodium bisulphite and 10 g crystalline sodium sulphite. To the hot mixture was added 15 g of the crude sulphonic acid and mixed again until dissolved. This mixture was filtered hot and allowed to cool. 10 ml concentrated HCl was added and the mixture immediately placed in the dark to crystallize. The crystals were filtered off with suction and washed with 300 ml $H_2O$ and finally with ethanol after which the crystals were allowed to air dry. This was all done in a dark room. When dry the crystals were stored in a brown bottle.

Reagents:

Two solutions were used in this method of $P_i$ assay. Solution A consisted of 15 g sodium bisulphite, 0,25 g recrystallized 1-amino-2-naphthol-4-sulphonic acid, and 0,5 g sodium sulphite in distilled water to a total volume of 100 ml. This solution was made by dissolving the sodium bisulphite in about 90 ml of heated distilled water. A heated magnetic stirrer was useful for dissolving the solid rapidly. The recrystallized 1-amino-2-naphthol-4-sulphonic acid was then added and well mixed and then the sodium sulphite was added and

the solution mixed again. The solution was cooled by swirling the flask under running tap water and when at room temperature the volume was checked and distilled water added as necessary to make up to 100 ml. This solution was then filtered into a dark bottle and kept at room temperature. It remains stable for about three weeks. Care was taken not to inhale fumes from this reagent. Solution B was 5% ammonium molybdate.

Procedure:

Acid extracts from the Paglia and Valentine (1975a) assay were filtered or centrifuged and the supernatant used. Centrifugation was the most sucessful method. It was essential that all test tubes were acid washed and that no traces of detergent were present. 0.5 ml of sample supernatant was put into a phosphate free glass test tube, 0.1 ml of solution A was then added followed by 0.1 ml of solution B. Finally 2.2 ml of distilled water was added and then the mixture was vortexed and inverted for thorough mixing. The tubes were left to stand and incubate at room temperature for 25 minutes and then read in a glass cell in a spectrophotometer at 830 nm. A blank was run at the same time as the samples by substituting 0.25 ml of 20% trichloroacetic acid plus 0.25 ml distilled water for the sample. A standard curve was prepared using aliquots of $KH_2PO_4$ containing 10 µg/ml instead of distilled water used in the blanks.

Bartlett (1959b) Phosphorus Assay in Column Chromatography

Reagents:

1Ø N sulphuric acid

5% ammonium molybdate

30% hydrogen peroxide

Fiske-SubbaRow reagent (prepared weekly)

Preparation of Fiske-SubbaRow reagent:

The 1-amino-2-napthol-4-sulphonic acid was purified as described in the previous method. Ø.5 g purified 1-amino-2-naphthol-4-sulfonic acid was added to 2ØØ ml of freshly prepared 15% anhydrous sodium bisulphite followed by the addition of 1 g sodium sulphite. This was filtered and stored in a brown bottle.

Procedure:

2 ml aliquots of eluate were placed in phosphate free test tubes. Ø.5 ml of 1Ø N $H_2SO_4$ was added to each sample and the test tubes heated at 15Ø-16Ø$^{o}$C for at least 3 hours. Two drops of 30% hydrogen peroxide were then added to each test tube and the samples returned to the oven for at least 1.5 hours to complete combustion and to decompose the peroxide. The samples were allowed to cool, after which 4.8 ml of a freshly prepared

solution was added. The solution consisted of:

Ø.2 ml 5% ammonium molybdate

Ø.2 ml Fiske-SubbaRow reagent

4.4 ml water

The tubes were mixed well and heated in a boiling water bath for seven minutes, then cooled and read on a spectrophotometer at 83Ø mn.

A blank was prepared using 2 ml water in place of eluate and treated in the same manner as the samples. A standard curve was prepared using aliquots of $KH_2PO_4$ containing 1Ø µg/ml.

Torrance, West and Beutler (1977b) Assay for Pyrimidine 5'-Nucleotidase

A second method of assaying the enzyme using radioactive substrate was devised by Torrance, West and Beutler (1977b). In this assay the radioactive substrate was [2-$^{14}$C]CMP. When CMP was incubated with haemolysates in the presence of $Mg^{++}$, both cytidine and $P_i$ were products of the reaction. When [2-$^{14}$C]CMP was the substrate it produced [2-$^{14}$C]cytidine. The procedure used to measure the radioactive cytidine released in the reaction, which was a measure of the activity of the enzyme present, consisted of a single $BaSO_4$ precipitation. This stopped the reaction and at the same time precipitated all protein and substrate leaving the radioactive cytidine in solution. The activity of the enzyme in this assay

was expressed as mU/gHb where one unit was one μmole of CMP hydrolyzed per minute per gram of haemoglobin.

Preparation of radioactive substrate:

Low blank counts were only obtained with very pure radioactive substrate. It was necessary to purify commercially available [2-$^{14}$C]CMP by ion-exchange chromatography on a Dowex column. A Pasteur pipette with a little glass wool in the bottom served as a column. This was filled to a height of approximately 5 cm with a slurry of Dowex 1x8 $Cl^-$ (200-400 mesh) and washed with 10ml of distilled water. This gave a packed volume of approximately 0.4 ml (2 cm). When purifying substrate for assay of erythrocyte enzyme levels, 0.3 ml of 40 mM cytidine-monophosphate was added to 10 μC of [2-$^{14}$C]CMP and passed through the column. When substrate was prepared for kinetic studies only the radioactive cytidine was put through the column, without added unlabelled cytidine.

The column was first washed with 10 ml distilled water to remove unbound substances. The CMP was then eluted with 0.01 N HCl. Tubes containing 0.2 ml of 1 M Tris-HCl pH 7.6 in 50% ethanol were placed in an ice-water bath. Ten 0.8 ml fractions were collected into these tubes. The CMP fractions were located by counting 5 μl samples in 5 ml Instagel (Packard, Downers Grove, Ill., USA) containing 0.5 ml distilled water. The peak fractions were pooled and CMP concentration was

determined by measuring the optical density of a 1:50 dilution of the pool at a wave length of 271 nm. 100 mM pH 7 phosphate buffer was used as the diluent. The concentration was calculated according to the formula:

$$(CMP) = \frac{OD \times 50}{9} \text{ mM}$$

A final concentration of 3 mM was achieved by diluting with 0.4 mM pH 7.6 Tris-HCl in 10% ethanol. This solution was then divided into approximately 0.5 ml aliquots and frozen, to be thawed as needed. When kept frozen, the substrate remained stable for at least two weeks. The concentration was not measured or adjusted when purifying labelled CMP without the addition of non-radioactive CMP for kinetic studies.

Preparation of $Ba(OH)_2$ and $Zn(SO)_4$ solutions:
It was essential that barium hydroxide and zinc sulphate solutions be prepared of identical molarity for use in the assay. The lowest possible ionic concentration had to be achieved on mixing equal volumes of these two reagents in order to precipitate all unreacted substrate at the end of the assay. The freshly distilled water used to prepared 0.15 M $Ba(OH)_2$ was de-gassed and then saturated with $N_2$ gas. The bottle used to store the solution was first filled with $N_2$ gas. The 0.15 M $Ba(OH)_2$ solution was quickly filtered into the bottle and the solution protected from $CO_2$ contamination by an

air filtering $CaCl_2$ system. Ø.155 M $Zn(SO)_4$ was prepared with freshly distilled water.

The molarity of the $Zn(SO)_4$ was then adjusted to exactly the molarity of the $Ba(OH)_2$ solution by titration with the aid of a conductivity meter. The end point was at minimum conductivity. The $Zn(SO)_4$ solution was then diluted to obtain minimum conductivity with equal volumes of the two solutions.

Preparation of sample haemolysates:
Venous blood was collected and anticoagulated with heparin or ACD and stored at $4^{o}C$ until assayed. Platelets and leukocytes were removed by passing through a short column of a mixture of equal weights of microcrystalline cellulose and alpha cellulose (Sigma). This column was freshly poured to a height of 2 cm in a 2 ml syringe with a cotton wool plug. It was thoroughly washed with ice-cold isotonic saline before the red cells were passed through (Beutler et al. 1976). These cells were washed through the column with very small quantities of cold saline. The erythrocytes were washed and spun and checked for the presence of any contaminating cells by microscopy. The cellulose column was very effective and it was never found necessary to repeat the above procedure. An haemolysate was prepared by adding 19 volumes of an aqueous solution containing 2.7 mM neutralized EDTA and Ø.7 mM 2-mercaptoethanol. Alternate freezing and thawing ensured that all cells

were lyzed. Phosphate levels were not measured in this assay so overnight dialysis was not necessary. The 1:20 haemolysate was assayed for haemoglobin level using the cyanmethaemoglobin method.

Determination of enzyme activity:

To determine the activity of pyrimidine 5'-nucleotidase in the sample, a reaction mixture was prepared containing:

5 µl 0.1 M $MgCl_2$

10 µl 3 mM [2-$^{14}$C]CMP

25 µl distilled $H_2O$.

This mixture was placed in a 5 ml test tube and quickly brought to 37°C in a water bath. Samples were normally assayed in triplicate. To start the reaction, 60 µl of prepared haemolysate was added to each tube at regular time intervals and left to react in the 37°C water bath for exactly 30 minutes. After this time the reaction was stopped by adding 0.2 ml of 0.15 M $Ba(OH)_2$ and mixing, followed by 0.2 ml of 0.15 M $ZnSO_4$. This was mixed and then 0.5 ml of distilled $H_2O$ added. A 0 time blank was prepared by adding chilled haemolysate to chilled reaction mix immediately followed by the precipitating solutions. The tubes were centrifuged and then 0.5 ml of clear supernatant was added to 5 ml of Instagel (Packard Instrument Company, Inc., Downers Grove, Ill., U.S.A.) and counted in a beta-ray emission counter. A total available count was obtained by adding 40 µl of assay reaction mix to 960 µl distilled $H_{2O}$,

mixing and adding Ø.5 ml of this mixture to 5 ml Instagel and counting. A ten minute counting period was usually adequate. Counts per minute were used in calculations.

To obtain the activity of the enzyme (E) the calculations were as follows:

$$E = \frac{(\text{Counts T} - \text{Counts O Time}) \times Ø.Ø3 \times 1{,}ØØØ}{(\text{Counts of Total} - \text{background}) \times T \times Ø.ØlHb \times Ø.Ø6}$$

T was the incubation time in minutes and Counts T the counts per minute of the sample incubated at 37°C for T minutes. The activity (E) was expressed in mU/gHb with one unit (U) equal to one μmole of CMP hydrolyzed per minute per gram of haemoglobin.

Radiometric Method with UMP as Substrate

The radiometric method with radioactive UMP as substrate remained the same as with CMP substrate until the precipitation procedure, however UMP was strongly bound to the resin column during the substrate ion-exchange chromatography purification procedure. Double the number of fractions were collected as UMP was eluted at an acid concentration greater than that needed to release CMP. The reaction mix was prepared with radioactive UMP and incubated with haemolysate as in the the assay with CMP as substrate. At the precipitation stage the reaction was stopped by adding Ø.2 ml of Ø.15

M $Ba(OH)_2$ followed by 0.5 ml of 0.1 M HCl, this was well mixed and then 0.2 ml of 0.15 M $ZnSO_4$ added. The tube was thoroughly mixed and centrifuged immediately. 0.5 ml of supernatant was then mixed with 5 ml Instagel and counted as in the assay with CMP as substrate. 0.5 ml $H_2O$ was added to 0 time blanks and to totals at the beginning of the assay in order to keep volumes identical.

Storage of Frozen Erythrocytes Intact for Later Assay

Erythrocytes were frozen intact for use in a variety of studies. In normal population studies, it was useful to be able to draw on samples previously stored.

Preservation of erythrocytes:
Venous blood samples were collected into ACD and within 24 hours the plasma and buffy layer was removed and packed cells were mixed with an equal volume of glycerol preservative (5.47 M glycerol, 20 mM $NaH_2PO_4$, 20 mM $Na_2HPO_4$, 60 mM tripotassium citrate). The preservative solution was added dropwise to the packed cells with constant mixing and the cells were stored at $-20^{\circ}C$.

Recovery of erythrocytes:
Before these samples were assayed by the method of Torrance et al. (1977b), they were thawed and dialyzed overnight at $4^{\circ}C$ against physiological saline containing 0.7 mM 2-mercaptoethanol and 2.7 mM neutralized EDTA.

This removed the glycerol from the mixture. The thawed erythrocytes were then handled in the same way as blood samples that had not been frozen.

## Erythrocyte Cell Density Separation and Haemolysate Preparation

Enzyme levels were measured in haemolysates prepared from erythrocytes separated according to their density by the method of Danon and Marikovsky (1964).

Procedure:

Venous blood was collected and the red cells filtered and washed (Beutler *et al.* 1976, Torrance *et al.* 1977b). Packed erythrocytes were suspended in an equal volume of physiological saline and layered above phthalate ester in microhaematocrit tubes. A set of tubes having phthalate ester densities ranging from 1.122 to 1.070 $g/cm^3$ was prepared by mixing methyl phthalate and di-n-butyl phthalate in appropriate proportions. The tubes were centrifuged at $4^{\circ}C$ for 15 min at 12,000 g using a Hawksley microhaematocrit centrifuge. The heavy and light packed cells were haemolysed by the addition of 19 volumes of a 2-mercaptoethanol (0.7 mM) EDTA (2.7 mM) solution.

Enzyme Purification Method

Pyrimidine 5'-nucleotidase was purified for use in studying the enzyme.

Preparation of haemolysate:

Large-scale preparation of haemolysate was achieved by passing erythrocytes through a 5 cm column of mixed equal weights of alpha cellulose and microcrystalline cellulose (Beutler et al. 1976). The column was poured in a sintered-glass funnel of approximately 10 cm diameter and washed with chilled saline. Erythrocytes were extracted from 350 ml of normal blood, collected in ACD as an anticoagulant. The whole blood was centrifuged and the plasma and buffy layer aspirated. The remaining cells were resuspended in chilled saline and washed through the column with more saline. The cells collected were centrifuged and washed three times with cold saline. The final wash was aspirated after checking that all white cells had been removed. The packed cells were haemolysed by the addition of four volumes of 0.7 mM 2-mercaptoethanol 2.7 mM EDTA 10 mM Tris-Bistris-HCl pH 6.5 buffer, this stabilizing buffer was used throughout the purification procedure. After haemolysis was completed, the pH of the solution was readjusted to pH 6.5.

First DEAE-cellulose extraction:

A 5 cm column of DEAE-cellulose was poured into a 10 cm sintered glass funnel and the column washed with stabilizing buffer until the cellulose was equilibrated. The haemolysate was passed through the column and washed with stabilizing buffer until all traces of haemoglobin were removed. Gentle suction from a water-vacuum pump was employed when using the sintered glass funnel columns. The absorbed pyrimidine 5'-nucleotidase was washed with 500 ml of stabilizing buffer containing 50 mM NaCl before being eluted with the same buffer containing 150 mM NaCl.

Ammonium sulphate concentration procedure:

A 50% concentration of ammonium sulphate was achieved in the above eluted enzyme solution through the addition of solid ammonium sulphate, this was added slowly with constant stirring. When 50% concentration was reached, the pH was immediately readjusted to pH 6.5, and the solution allowed to precipitate at $0^{o}C$ for 20 minutes. The precipitate was then removed by centrifugation at 14,000 x g for 30 minutes. The ammonium sulphate concentration in the supernatant was gradually increased to 80% by addition of more solid ammonium sulphate and the pH again immediately adjusted to pH 6.5. The solution was left to precipitate overnight at $0^{o}C$ and then centrifuged for 60 minutes at 14,000 x g. The supernatant was discarded and the precipitate dissolved in a minimal amount of stabilizing buffer. The

remaining ammonium sulphate was removed by buffer exchange into the same buffer using a Sephadex G-25 column.

Second DEAE-cellulose extraction:

A 1.6 by 20 cm column of DEAE-cellulose was poured and equilibrated with stabilizing buffer. The enzyme-containing solution was pumped through the column at a constant speed of 1.0 ml per minute and the absorbed enzyme washed with 30 ml of stabilizing buffer and then eluted with 500 ml of a linear NaCl gradient, 0 to 250 mM in stabilizing buffer. Fractions of 5 ml were collected and assayed for the presence of the enzyme using the radiometric method previously described and the fractions containing the enzyme pooled. This pool was concentrated by precipitation with an 80% concentration of ammonium sulphate and centrifugation as described above. The precipitate was again dissolved in a minimal volume of stabilizing buffer.

Sephacryl S-200 column gel filtration:

A 1.6 by 90 cm column of Sephacryl S-200 was poured and washed well with stabilizing buffer. The concentrated enzyme solution was applied and chromatographed. NaCl and $(NH_4)_2SO_4$ were removed as well during this step due to the buffer exchange taking place in the column. The column was eluted with stabilizing buffer at a constant speed of 0.5 ml per minute and 2.5 ml fractions collected. These fractions were assayed for enzyme

activity by the radiometric method and the fractions containing the enzyme were pooled.

Isoelectric focusing:

The enzyme was purified further by isoelectric focusing on a 110 ml LKB electrofocusing column. A 0 to 45% sucrose gradient containing 1% Ampholine (LKB) of pH range 4 to 6 and 1.4 mM 2-mercaptoethanol was slowly pumped into the column according to the manufacturer's instructions. The following solutions were used:

Anode electrolyte solution (bottom electrode):

12 ml $H_2PO_3$

14 ml $H_2O$

12 g sucrose

Cathode electrolyte solution (top electrode):

0.5 ml 5N NaOH

9.5 ml $H_2O$

Dense gradient solution:

2 ml 40% pH 4-6 Ampholine (LKB)

36 ml $H_2O$

25 g sucrose

0.005 ml 2-mercaptoethanol

Light gradient solution:

0.44 ml 40% pH 4-6 Ampholine (LKB)

32.2 ml $H_2O$

0.005 ml 2-mercaptoethanol

Enzyme sample light gradient solution (pooled fractions from Sephacryl S-200 column):

23 ml pooled enzyme fractions

0.31 ml 40% pH 4-6 Ampholine (LKB)

0.005 ml 2-mercaptoethanol

The sucrose gradient was layered between electrolyte solutions with the cathode connection at the top of the column. The enzyme sample was introduced into the middle of the sucrose gradient while the column was being prepared. Focusing was carried out at 6$^{o}$C for 48 hours at a constant 500 V. The column was then collected in 2.5 ml fractions which were assayed for enzyme activity and pH. The pyrimidine 5'-nucleotidase focused sharply at a pH of 5.0. The pooled peak fractions were rechromatographed on the same Sephacryl S-200 column with the same elutant used in the previous step, to remove sugar and Ampholine contamination.

Modified Lowry Protein Determination (Lowry, Rosebrough, Farr and Randall 1951)

Reagents:

Solution A = 2% $Na_2CO_3$ in Ø.1 N NaOH.

Solution B = 1% $CuSO_4$ in 2% tri-sodium citrate.

Solution C = 5Ø% dilution of Folins phenol reagent.

Procedure:

5Ø ml of solution A was mixed with Ø.5 ml of solution B and 2.5 ml of this mixture poured into a glass test tube. Up to 2ØØ µl of protein solution or standard solution was then added to each tube, mixed and allowed to stand at room temperature for 1Ø minutes. Ø.25 ml of solution C was added to each tube in succession while being vortexed. This addition was usually carried out at 3Ø second intervals and timed with a stop watch. The tubes were allowed to stand at room temperature for exactly 3Ø minutes and the optical density of samples, standards and blanks read on a spectrophotometer at 7ØØ nm zeroed with distilled water. A standard curve was plotted on graph paper for each assay, optical density against standard concentration. Sample protein concentrations were calculated from this graph from sample density readings and corrections made for dilutions. The values obtained using this method are all related to the bovine serum albumin standard used and different proteins may react slightly differently, however this method is the most reliable and widely used

method for protein determination.

A sample volume of 100 or 200 µl was chosen arbitrarily depending upon the roughly estimated protein concentration of the sample. If the range was unknown a preliminary determination could be made by using a total volume of 200 µl and running the sample at different dilutions. Dilutions of sample were usually made with distilled water as some buffers, such as Bistris, affected the assay. Interfering buffers were first removed by dialysis against distilled water. This buffer removal method could be adapted for proteins sensitive to low ionic concentrations by dialysis against isotonic saline. A standard curve was prepared for each run using 10 to 100 µl of bovine serum albumin 1mg/ml in 11 dilutions and making up to a total chosen sample volume with distilled water. Two distilled water blanks were also prepared for each run, these contained all reagents but substituted water for the sample.

## Starch Gel Electrophoresis

Starch gel electrophoresis was run using either the purified enzyme or an haemolysate. The haemolysate was prepared by freezing and thawing packed washed erythrocytes. The samples were submitted to electrophoresis at 75 V for 17 hours at $4^{o}C$ in the starch gel using a histidine-citrate buffer pH 7.0. The pyrimidine 5'-nucleotidase bands on the gel were

specifically stained using the method of Anderson et al. (1974). The sliced gel was overlayed with a 1% ionagar gel prepared by mixing 10 ml of 2% ionagar with an equal volume of 0.1 M Tris-HCl pH 7.8 containing 20 mg UMP, 5 mg GSH, and 246 mg $MgSO_4.7H_2O$. After 2 hours this agar overlay was removed and replaced by a 1% ionagar gel prepared by mixing 10 ml of 2% ionagar and 10 ml of $(NH_4)_2SO_4$ containing 0.25 g ammonium molybdate and 1.0 g of ascorbic acid which was added immediately before mixing. The phosphate liberated from the UMP during incubation with the first overlay stained blue indicating the position of the enzyme.

## Extraction of Cell Nucleotides using 6% Perchloric Acid

Blood samples from the antecubital vein were anticoagulated with heparin. The blood was centrifuged immediately at room temperature for 10 minutes at 3,000 x g. The plasma was removed and the precipitated cells were first haemolysed with an equal volume of water and then extracted with 4 volumes of ice-cold 6% perchloric acid. The precipitate removed by centrifugation was then re-extracted with the same volume of perchloric acid and the supernatant solutions were pooled and neutralized with $K_2CO_3$. The extracts were stored at $-20^{\circ}C$ until chromatographed.

## Ion Exchange Chromatography of Perchloric Acid Extracts

Ion Exchange chromatography was carried out on the above PCA extracts. A 1.0 x 30 cm column of Dowex 1 x 8 formate (Bartlett, 1968) was used.

Preparation of resin:

The column resin was prepared by washing 1 kg of Bio-rad Analytic Grade Anion Exchange Resin AG 1-8 200-400 mesh in chloride form with several volumes of distilled water. The resin was then washed with at least 25 volumes of 1 M NaOH to remove the $Cl^-$ group. This was done in a large funnel but not under vacuum. The resin was tested for complete removal of this ion by testing the effluent. A few drops of concentrated $HNO_3$ were added to 2 ml of effluent and mixed and then 0.5 ml of 1% $AgNO_3$ was added. A white precipitate indicated the presence of $Cl^-$. When no further $Cl^-$ could be detected the resin was washed with at least 6 volumes of distilled water. Rinsing was completed when the pH of the effluent was less than pH 9. The resin was then converted to the formate form by washing with at least 4 volumes of 1 N formic acid. The resin was fully converted to the formate form when the pH of the effluent dropped to below pH 2. The resin was then rinsed under vacuum with about eight volumes of distilled water until the effluent pH rose to over 4.5.

Procedure:

A 1.Ø x 3Ø cm column of prepared resin was poured and equilibrated with distilled water using a Gilson peristaltic pump delivering 1 ml effluent per minute to an automatic fraction collector set to collect 5 ml fractions. The neutralized PCA extract sample was applied and the column was eluted with one litre of a linear gradient of Ø to 5 molar ammonium formate pH 3. This was made by mixing one part of 5 M formic acid with four parts of 5 M ammonium formate. After the run was completed, the column was regenerated by washing with 5 M formic acid followed by distilled water until the pH of the effluent again rose to above pH 4.5.

Identification of elution peaks:

To establish the elution pattern of known nucleotides, commercially available cytidine, uridine and adenosine phosphates and nucleotide sugars were mixed together and dissolved in 6% PCA, neutralized with $K_2CO_3$ and run on the same column with identical eluent. If there was any doubt as to identification of these compounds, different concentrations could be used or several columns run with only a few products in each run. Tracer amounts of radioactive cytidine and uridine monophosphate were added to the neutralized cell extract prior to chromatography as an aid in peak identification. Aliquots of the fractions were then examined for the presence of radioactivity and the elution pattern plotted. These radioactive tracers were also used to

verify the elution peaks of the duplicate column run with commercially obtainable compounds. Fractions of 5 ml were collected and the optical density of each fraction was measured spectrophotometrically at 260 and 280 nm. Phosphate content was also determined with aliquots from each fraction using the method of Bartlet (1959b).

It was necessary to correct all spectrophotometer reading for the linear increase in the optical density of the formate gradient. In addition to elution position, various compounds could be identified from their ultraviolet spectra after removal of the formate which interfered with the spectrum. The formate was removed from the sample in a vacuum oven at 80$^{o}$C. The residue was then dissolved in water. This dissolved residue was examined spectrophotometrically three times, first in the distilled water, then with HCl added to make the solution 0.02 N, and then again after the addition of solid Tris to bring the pH up to 10. The pH of the eluted fractions changed with the gradient and the pH of each fraction was determined as soon as collected in order to aid calculation of approximate concentrations. Concentrations could be calculated assuming a constant pH of 3, but in reality, this was only achieved half way through the gradient and underestimated cytidine nucleotide levels. Correct values could also be obtained by spectrophotometry using the fracions with formate removed.

# 3. PURIFICATION AND PROPERTIES OF HUMAN ERYTHROCYTE 5'-NUCLEOTIDASE

## Purification of the Enzyme

A 250,000-fold purification of the enzyme was achieved from human erythrocytes. The methods employed consisted of DEAE-cellulose chromatography, ammonium sulphate fractionation, gel filtration on Sephacryl S-200, and isoelectric focusing. These steps have been described in detail in Chapter 2. Assay of enzyme activity in the fractions collected after electrofocusing showed that the enzyme focused in a sharp band at pH 5.0 (Figure 1). After focusing, this enzyme peak was finally rechromatographed on the Sephacryl S-200 column to remove all sugar and ampholine contamination. All purification was carried out at a temperature between 0 and 6$^{o}$C. All buffers used in the purification process contained 0.7 mM 2-mercaptoethanol and 2.7 mM EDTA to stabilize the enzyme.

During the entire purification procedure, the presence of pyrimidine 5'-nucleotidase and its activity levels were monitored through the use of the radiometric assay of Torrance *et al.* (1977b). This assay was adapted by using samples of extract diluted to suitable strength with stabilizing buffer (10 mM Tris-Bistris-HCl pH 6.5

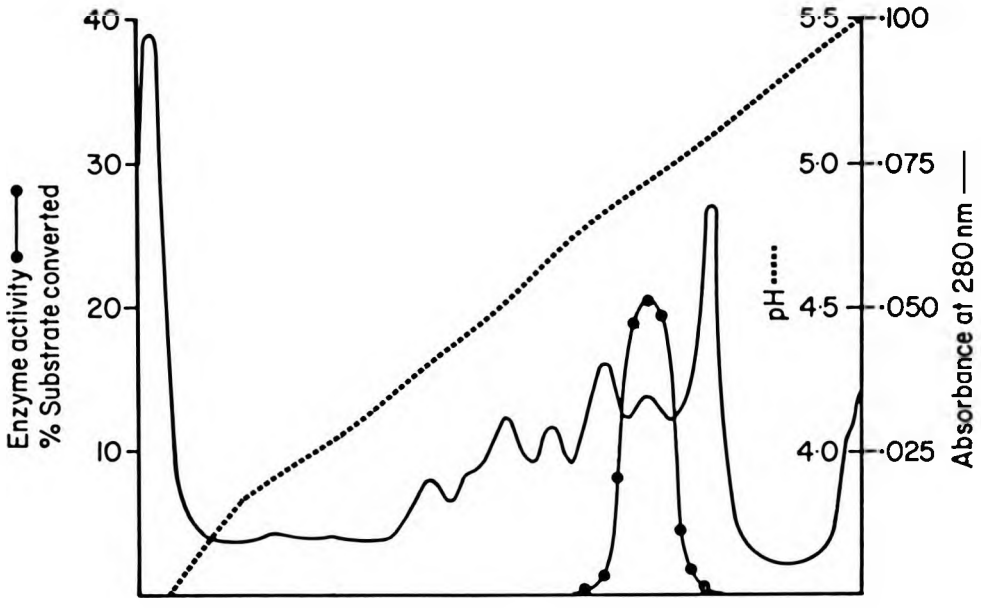



Figure 1. Isoelectric focusing of Sephacryl S-200 fractions that had pyrimidine 5'-nucleotidase activity. Focusing was carried out at 500 V for 48 hours at 6° C with the cathode at the top in a 1% Ampholine column stabilized by a 0-45% sucrose gradient. Protein was estimated by absorbance at 280 nm and enzyme activity is expressed as % substrate converted by a 10 µl sample in 6 minutes. The pH values were determined at 6° C immediately after removal of the sample from the column.

containing 0.7 mM 2-mercaptoethanol and 2.7 mM EDTA) in place of haemolysate. The original haemolysate was diluted and assayed and the haemoglobin content determined by the cyanmethaemoglobin method. By correcting dilution calculations to that of the original haemolysate it was possible to determine the amount of enzyme purified in each step. Protein content was measured by absorption at 260 and 280 nm. This was not entirely satisfactory and a Lowry protein determination (Lowry et al. 1951) was also carried out. The samples had to be dialyzed against distilled water to remove the Tris-Bistris-HCl buffer used in the purification method as this interfered with the Lowry protein assay.

The final purity of the electrofocused enzyme was assessed first by disc electrophoresis using the method of Davis (1964) as modified by the Buchler Co. (1971), and secondly through the use of starch gel electrophoresis. Four protein bands were found when the polyacrylamide electrophoresed discs were stained, two major bands and two very faint bands (Figure 2). The polyacrylamide gels could not be effectively stained for the presence of enzyme activity. As an alternative, the purified enzyme was electrophoresed in histidine-citrate buffer pH 7 on a starch gel. This gel was fixed and stained for the presence of enzyme after

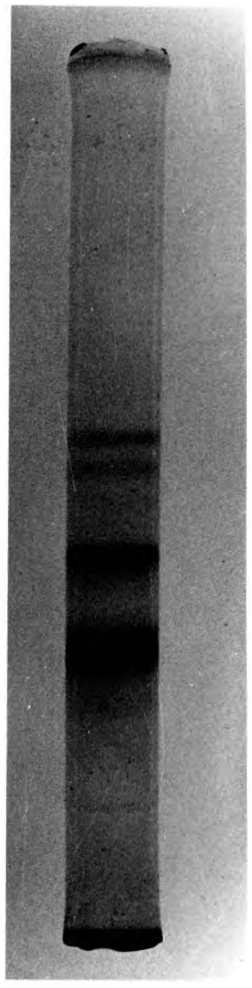


Figure 2. Polyacrylamide disc electrophoresis of 250,000-fold purified pyrimidine 5'-nucleotidase from human erythrocytes. Migration is toward the anode, at the bottom, and protein is stained with Coomassie blue.

electrophoresis (Anderson et al. 1974). Two blue bands appeared showing the presence of pyrimidine 5'-nucleotidase. This suggested that the extra bands stained for protein on the acrylamide gel indicated the presence of remaining impurities. The two distinct enzyme bands on the starch gel seemed to suggest that the enzyme is composed of two proteins carrying different charges, each capable of hydrolysing UMP.

On the basis of both protein determination methods, it was concluded that at least a 250,000-fold purification had been achieved. Table 1 gives the yield and degree of purification in each step taken.

An attempt was made to further purify the enzyme by using cytidine bound to Sepharose 4B in an affinity chromatography column. The enzyme bound exceedingly well but could not be released from the column. The enzyme bound to Sepharose 4B beads was still capable of effecting the hydrolysis of CMP in the radioactive assay. If an effective method of releasing the enzyme could be devised, this could prove to be an excellent purification step.

## Some Properties of the Enzyme

The effect of pH on the activity and the stability of the purified enzyme was studied and the amount of $Mg^{++}$ needed for optimum activity determined. The molecular

Table 1. Purification of human erythrocyte pyrimidine 5'-nucleotidase

| | Enzyme activity | | Spectrophotometric protein assay | | | Lowry protein assay+ | | |
|---|---|---|---|---|---|---|---|---|
| | Milliunits++ | Yield, % | Protein, mg | Specific activity+++ X10$^3$ | Purification, fold | Protein, mg | Specific activity+++ X10$^3$ | Purification, fold |
| Hemolysate | 5,000 | 100 | 45,000 | 0.11 | 1 | 45,000 | 0.11 | 1 |
| Batchwise DEAE-cellulose | 5,559 | 111 | 15.1 | 367 | 3,339 | 34 | 164 | 1,486 |
| Ammonium sulfate | 4,511 | 90 | 9.58 | 471 | 4,280 | 15.5 | 291 | 2,646 |
| DEAE-cellulose | 2,790 | 55.8 | 2.56 | 1,092 | 9,923 | 5.98 | 466 | 4,241 |
| Sephacryl S-200 | 2,233 | 45 | 0.31 | 7,200 | 65,454 | 0.45 | 4,962 | 45,111 |
| Electrofocused | 888 | 18 | 0.023 | 38,947 | 354,067 | 0.032 | 27,750 | 252,272 |

+ Dialyzed samples were used for protein assays by the Lowry method.

++ One unit of pyrimidine 5'-nucleotidase is defined as the amount of enzyme required to hydrolyze 1 μmol of CMP per min under the assay conditions.

+++ The specific activity is defined as units per mg of protein.

weight of the enzyme was estimated. The effect of potential inhibitors of the enzyme, both in purified form and in haemolysates was studied. The radiometric assay of Torrance et al. (1977b) was used in most cases. The method was modified when using [2-$^{14}$C]UMP as substrate. The chemical assay method of Paglia and Valentine (1975a) was modified and used to determine enzyme activity on a large range of substrates at varying pH. Both crude haemolysates and purified enzyme were studied in this respect.

Molecular weight:

The molecular weight of the purified enzyme was estimated by descending chromatography using a 1.6 by 90 cm column of Sephadex G-150. Reference proteins chromatographed in stabilizing buffer containing 0.15 M NaCl were used to standardize the column. These markers were bovine serum albumin, ovalbumin and cytochrome C. The enzyme was then chromatographed in 10 mM Tris-Bistris-HCl pH 6.5 stabilizing buffer used in the purification methods. From the elution position, pyrimidine 5'-nucleotidase appeared to have a molecular weight of 28,000.

Effect of pH on purified enzyme activity:

The effect of pH on the activity of the enzyme during the radiometric assay of the purified enzyme was studied. 50 mM Tris-Bistris-HCl buffers of differing pH were substituted for the distilled water normally used

in the assay. Equimolar amounts of Tris and Bistris were used and the buffers were adjusted to pH rangeing from 8.5 to 5.6 by the addition of HCl. The pH of each sample was determined during incubation of the final assay mixture at 37°C by using a Radiometer thermostated K 497 blood electrode. The purified enzyme had an optimum at pH 7.5 (Figure 3).

Enzyme activity in haemolysate with four substrates and over a range of pH:

The activity of the enzyme in dialysed haemolysate was studied using the chemical method of Paglia and Valentine (1975a) with CMP, UMP, AMP and GMP as substrates. The total phosphate liberated during a two hour incubation at 37°C was measured. The buffer used was stabilized 24 mM Tris-HCl pH 8 and substrates were used in a final reaction concentration of 2.2 mM. This assay confirmed Valentine et al. (1974) findings that more phosphate was liberated under these conditions with UMP as a substrate than was liberated with CMP substrate in the same assay using identical haemolysate. Valentine et al. (1974) had reported an increase of 20 to 30% in the enzyme activity when UMP was used as a substrate compared to enzyme activity with CMP. An even more marked activity increase of 37% was noted with this assay. Valentine et al. (1974) findings were also confirmed with purine substrates. 16.5% $P_i$ was liberated using GMP and 4.9% $P_i$ using AMP as substrates in the same assay in respect to $P_i$ liberated using CMP.

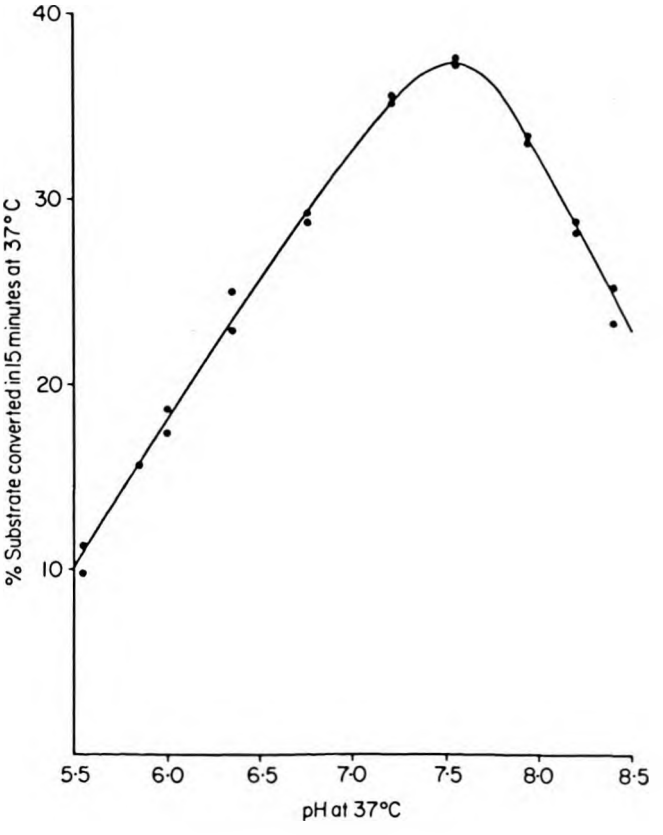



Figure 3. The effect of pH on erythrocyte pyrimidine 5'-nucleotidase activity using the radiometric method with Ø.3 mM [2-$^{14}$C]CMP as substrate. The buffer was an equimolar mixture of Tris and Bistris adjusted to the required pH with HCl. The pH values were measured at 37$^{o}$ C in the final incubation medium.

The chemical method (Paglia et al. 1975a) was slightly altered when studying the effect of different reaction pH on enzyme activity using the same four substrates. Both haemolysate and purified pyrimidine 5'-nucleotidase were tested with this modified method. The substrate concentration was decreased to 2.0 mM and the incubation period shortened to 1 hour. The shortened time was valid because Valentine et al. (1974) had found the phosphate liberation rate to be linear over a 2 hour period. The 0.2 mM reduction in substrate was introduced to simplify the final calculations and make it easier to compare this method with the method of Torrance et al. (1977b). Similar results were achieved in the chemical assay using 2.2 mM substrate so it was felt that this lower concentration of substrate did not affected the validity of the assay. Torrance et al. (1977b) had noted excellent agreement between the two assay methods. This was confirmed with the modified chemical assay where the mean value on 10 haemolysates was 148.2 (SD 18.3) mU/g Hb compared to 152.2 (SD 17.2) mU/g Hb when duplicate assays were made using the radiometric method.

The effect of different pH on activity using the chemical method was determined using Tris-Bistris-HCl in a pH range of 5.6 to 7.7 in the incubation mix at a final buffer concentration of 10 mM. This buffer was made up in the same manner as that used for the preceding radiometric pH assay. When haemolysate was

used, it was only possible to obtain a final pH range of 6.1 to 7.7 because of the strong buffering effect of the dialyzed haemolysate (Figure 4a).

The rate of hydrolysis was consistently higher for UMP at every pH studied when compared with CMP. UMP presented a sharp peak with optimum $P_i$ liberated at pH 6.75. The curve was much flatter for CMP and this substrate had an optimum at pH 7.3. This CMP pH optimum for haemolysate matched the findings of Paglia and Valentine (1975a).

An unexpected finding was that AMP and GMP were hydrolysed at 66 and 23% of the rate for hydrolysis of CMP at pH 6.1. However, as pH increased hydrolysis rates of UMP and CMP increased while rates for AMP and GMP dropped, in the case of AMP the drop was dramatic and at pH 7.2 the hydrolysis rate of both purine substrates was only about 13% of the rate of CMP.

Extending the pH range:

An attempt was made to extend the pH range for studying the haemolysate reaction by using buffers other than Tris-Bistris-HCl. Unfortunately, the buffers themselves affected the activity of the enzyme in the haemolysate. Citrate buffer used for pH 4.6 to 6.8 reduced enzyme activity by about 30%, while glycine buffer increased activity when used with pyrimidine substrates in the pH range of 7.5 to 8.0. In the case of UMP there was a 10%

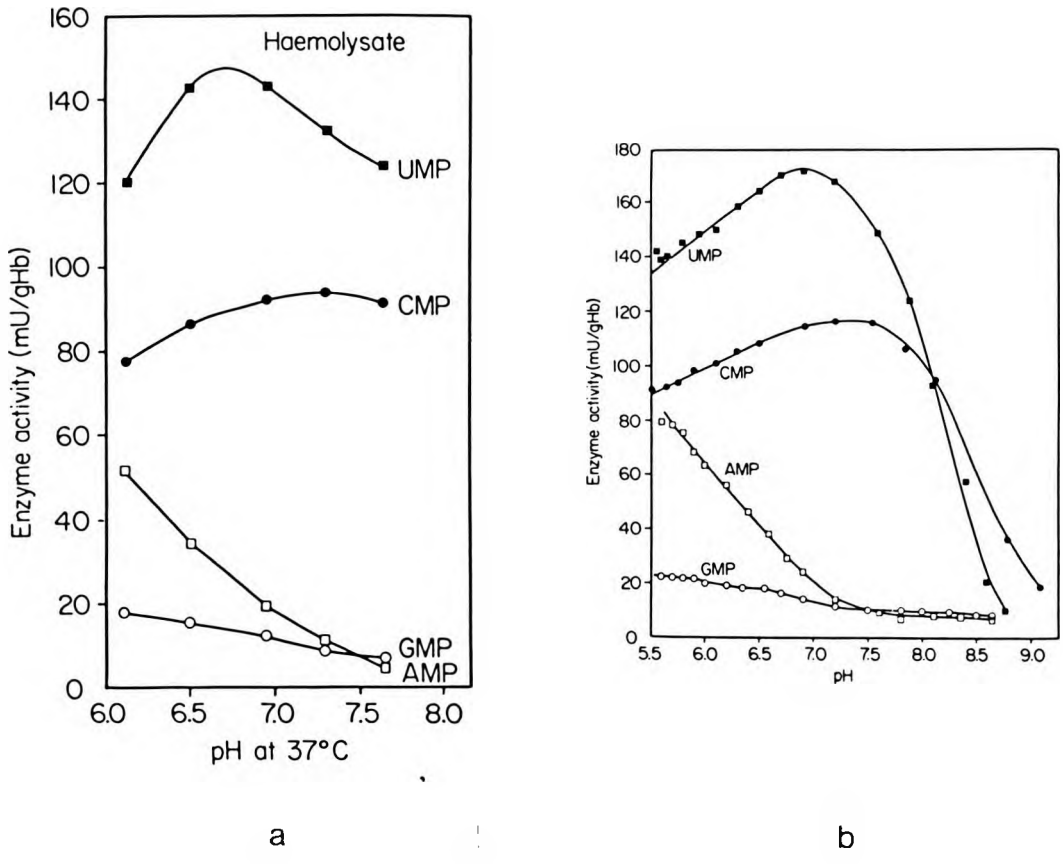



Figure 4. The effect of pH on the 5'-nucleotidase activity of dialyzed haemolysate using the chemical method with 2 mM substrates. Solid squares = UMP, solid circles = CMP, open squares = AMP, open circles = GMP. In figure a the buffer used to obtain the pH range was 10 mM Tris-Bistris-HCl. In figure b this range was widened by using a 50 mM piperazine-Tris-Bistris buffer.

increase and with CMP the activity rose by 30% when compared with results obtained with Tris-Bistris-HCl. An attempt was made to circumvent the problem by expanding the buffering range of Tris-Bistris-HCl with the addition of piperazine (Figure 4b). This buffer gave a continuous range of pH 5.6 to pH 9. However, there was a slight alkaline shift of pH optimum when using this buffer compared to Tris-Bistris-HCl alone. With UMP as substrate the optimum increased to pH 6.9 and with CMP it rose to pH 7.5 using dialyzed haemolysate. It was not felt that this increase of pH 0.2 invalidated the study.

With the piperazine-Tris-Bistris buffer at pH 5.6 there was little difference between hydrolysis rates of AMP and CMP. However, as with Tris-Bistris-HCl buffer, the activity with AMP as substrate dropped dramatically with increase of pH to physiological levels where it was 10% of the activity with CMP as substrate. Increase of pH above physiological levels led to loss in activity with all substrates tested. CMP and UMP dropped to less than 10% of their optimal activity at pH 9.0. GMP was found to be a poor substrate at all pH levels.

Purified enzyme activity with four substrates and over a range of pH:

The purified enzyme was substituted for haemolysate used in the previous study (Figure 5). In the case of the 250,000-fold purified enzyme, the buffering effect of

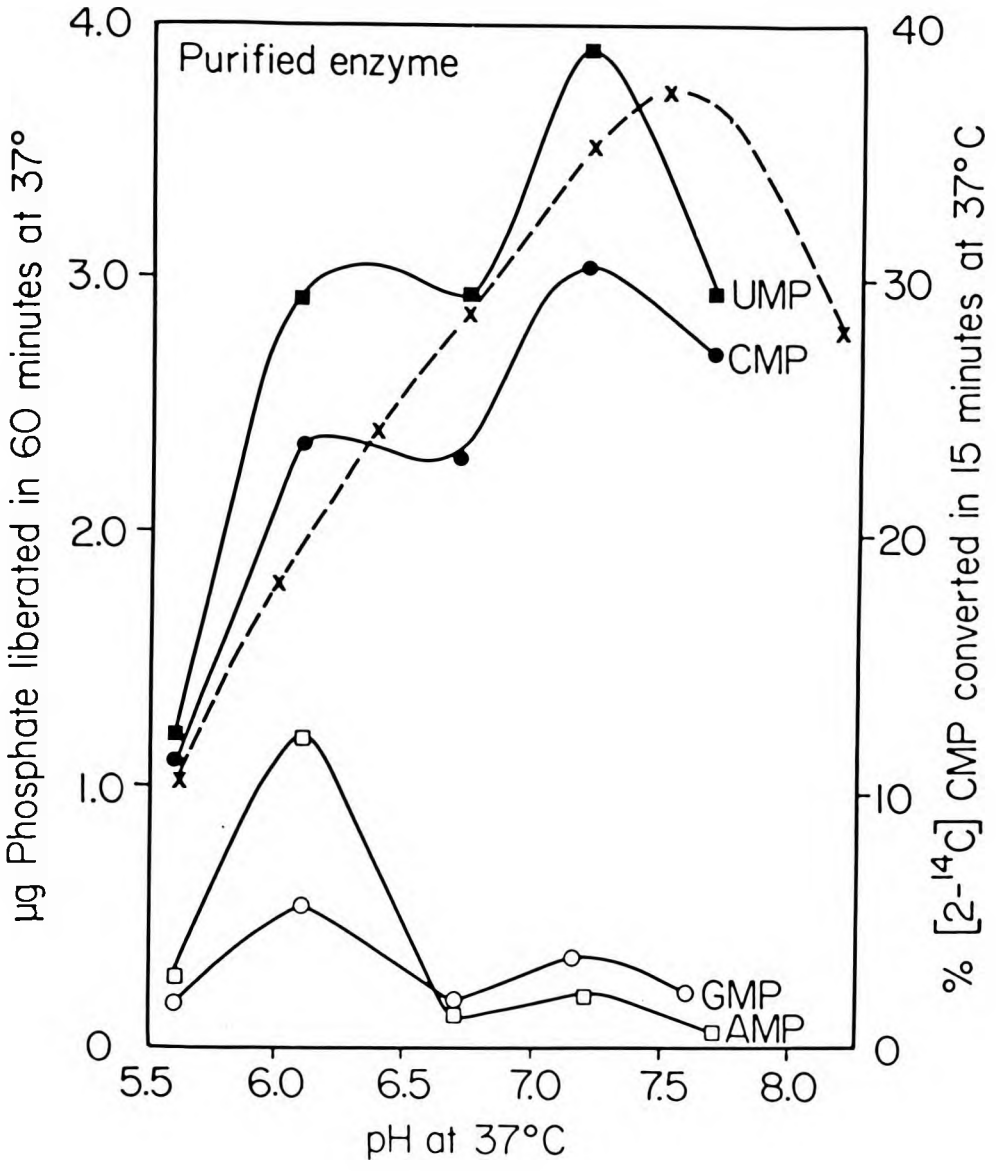



Figure 5. The effect of pH on 5'-nucleotidase activity of the purified enzyme using the chemical method. A 10 mM Tris-Bistris-HCl buffer was used to obtain the pH range. 2 mM substrates were used. Solid squares = UMP, solid circles = CMP, open squares = AMP, open circles = GMP. The broken line shows the result obtained using the radiometric method with 0,3 mM [2-$^{14}$C]CMP as substrate (figure 3 page 41).

10 mM Tris-Bistris-HCl was sufficient to give a fairly broad range of pH levels (pH 5.6 to 7.7). It was not necessary to resort to the addition of piperazine. The same four substrates were studied; UMP, CMP, AMP and GMP. UMP still gave the highest rate of hydrolysis over the whole pH range studied. The optimum for UMP had now increased to pH 7.2 with the purified enzyme and an identical pH optimum was found with CMP. A new development occurred in that a biphasic curve appeared when plotting a range of $P_i$ liberation against pH levels for each substrate, a second peak became evident between pH 6 and 6.5. With the pyrimidine substrates this second optimum was very minor compared to the optimum at pH 7.2 and in some assays of CMP it was only slight or completely absent. In the radiometric assay of the purified enzyme reported previously (Figure 3, page 41) where an optimum of pH 7.5 had been obtained, there was also no evidence of this second minor peak. When the two purine substrates were used, the double peaks became reversed with optimal activity occurring at pH 6.1 followed by a rapid loss of activity as the pH was increased with only a minor rise in activity at pH 7.2. When haemolysate was used the hydrolysis of GMP was slower at pH 7 than that of AMP at this pH while with the purified enzyme the reverse was the case, although still negligible when compared to the hydrolysis rate of CMP. This slight increase might be due to impurities in the GMP substrate. Purifying GMP using a Dowex ion-exchange column had no effect on the subsequent rate

of GMP hydrolysis.

Effect of pH on purified enzyme stability:
The effect of pH on the stability of the purified enzyme (Figure 6) was tested. The radiometric method of analysis with [2-$^{14}$C]CMP substrate was used. Equal quantities of 25 mM Tris and Bistris containing Ø.7 mM 2-mercaptoethanol and 2.7 mM EDTA were mixed and the pH adjusted with HCl to give a range of buffers with pH from 5.6 to 8.1. Samples of purified enzyme in stabilized buffers were incubated at 37$^{o}$C for 3Ø minutes. The pH of the enzyme samples was then re-adjusted towards pH optimum by the addition of Tris buffer pH 7.6, to give a final concentration of 5Ø mM. Substrate reaction mix was added with this final buffer and the reaction was allowed to proceed at 37$^{o}$C for 15 minutes. This time was cut short because of the length of pre-incubation and was sufficient for an accurate assay. The pH values of the first incubation mixtures and the final complete reaction mix were determined at 37$^{o}$C using an Astrup type Radiometer thermostated K 497 blood electrode. The original pH range of 5.6 to 8.1 was changed by the addition of the pH 7.6 buffer to a range of 7.1 to 7.3 at 37$^{o}$C. From the previous study of the effect of differing pH of the reaction mix, it was possible to deduce that activity would be underestimated by 3% at the lowest pH and overestimated by 5% at the highest reaction pH. This would not be

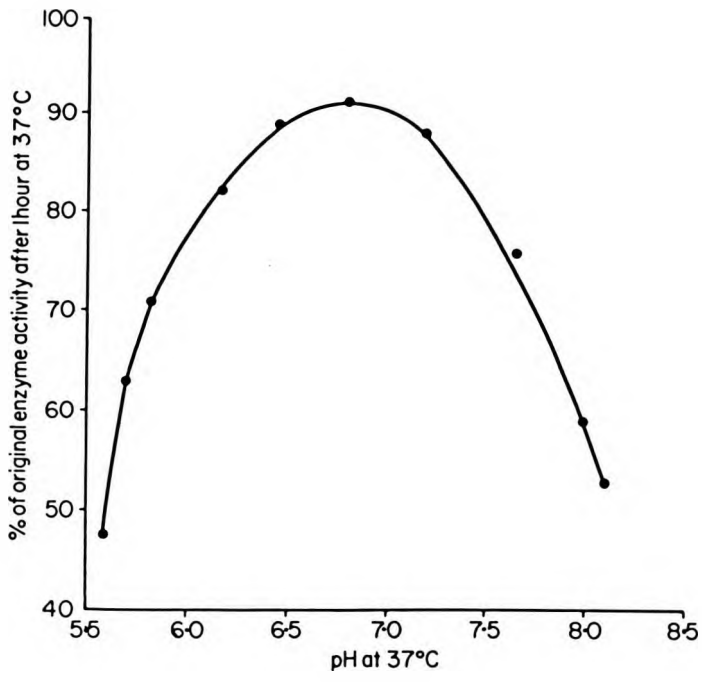



Figure 6. The stability of erythrocyte pyrimidine 5'-nucleotidase at 37° C as a function of pH using the radiometric method with [2-$^{14}$C]CMP as substrate. The buffers were prepared using HCl to adjust the pH of an equimolar mixture of Tris and Bistris. The pH was adjusted back towards the pH optimum with Tris at pH 7.6 before the enzyme activities were measured.

sufficient variation to materially alter the shape of the stability curve. At 37°C the enzyme was most stable at pH 6.5.

Optimum $Mg^{++}$ concentration:

The concentration of $Mg^{++}$ needed to give maximum activity was determined with the purified enzyme using the radiometric method. Valentine et al. (1974) had reported that enzymatic activity in dialyzed haemolysates approached maximal at a concentration of 1.0 mM $Mg^{++}$. Much lower concentrations of $Mg^{++}$ were needed for maximal activity after purification of the enzyme. Only 0.05 mM $Mg^{++}$ was sufficient to give maximum enzymatic activity.

Kinetic Studies of the Enzyme

Kinetic behaviour of pyrimidine 5' nucleotidase was examined using the radiometric method of assay with CMP and UMP as substrates. [2-$^{14}$C]CMP and [2-$^{14}$C]UMP was purified (Chapter 2 page 15) without the addition of non-radioactive CMP or UMP. This was accomplished using a Dowex 1x8 $Cl^-$ (200 to 400 mesh) column. UMP needed 0.023 N HCl for elution as compared with the 0.01 N HCl sufficient to release CMP when bound to the column. The radiometric assay had to be altered slightly when using the more acidic UMP as substrate. Using the standard procedure, after $BaSO_4$ precipitation all cytidine formed remains in the supernatant but only 2% of the CMP is present, however when UMP is used as substrate not only

all the UMP is precipitated, but 30% of the uridine formed is also precipitated resulting in false low activity assays. The addition of HCl after $Ba(OH)_2$ and before $ZnSO_4$ solved the problem and resulted in 98.4% precipitation of UMP with no measurable precipitation of uridine.

$K_M$ of purified enzyme and haemolysate:

The $K_M$ of the purified enzyme and of the haemolysate was determined with both substrates by using purified [2-$^{14}$C]CMP and [2-$^{14}$C]UMP and adding varying amounts of non-radioactive CMP or UMP. The specific activities of the radioactive compounds was 55 mCi/mmol for CMP and 30 mCi/mmol for UMP. The reaction mix was incubated for 30 minutes at 37$^{o}$C at pH 7.6. At the end of this time the reaction was stopped by precipitation and the radioactive cytidine or uridine counted. Double reciprocal plots were drawn of enzyme activity against substrate concentration (Figure 7). The purified enzyme had a mean $K_M$ of 11.0 µM with CMP and 16.6 µM with UMP. When a 1:20 haemolysate was used in the assay a $K_M$ of 30.0 µM with CMP and 33.0 µM with UMP as substrate was found. Using identical samples of enzyme or haemolysate the $V_{max}$ values between substrates were compared. The purified enzyme $V_{max}$ was 50% higher with UMP than with CMP.

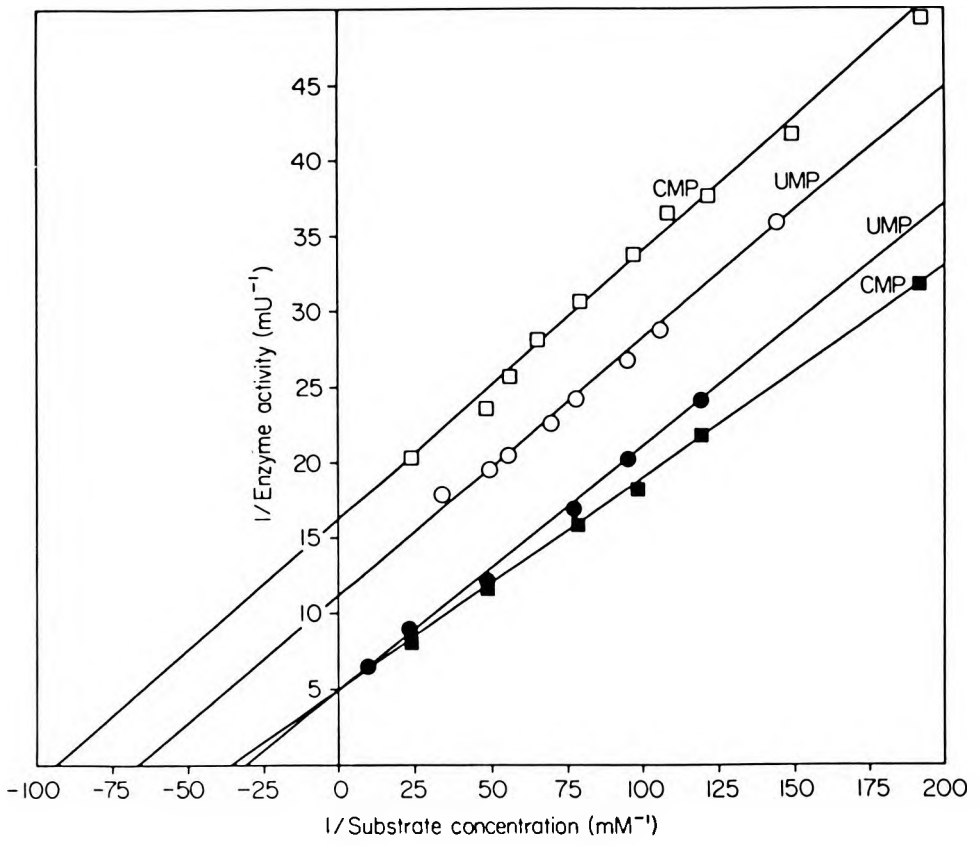



Figure 7. The double reciprocal plots of human erythrocyte pyrimidine 5'-nucleotidase using a crude haemolysate (solid symbols) and the purified enzyme (open symbols). The radiometric method was used. Both [2-$^{14}$]CMP (squares) and [2-$^{14}$C]UMP (circles) were used as substrates.

## Inhibition Studies

### Inorganic phosphate:

The higher value of $K_M$ in haemolysates when compared to purified enzyme suggested the presence of an enzyme inhibitor in the haemolysate. The presence of a dialysable inhibitor was apparent when ten haemolysates were assayed before and after dialysis. Predialysis activities were (139.5 [SD 20.6] mU/g Hb) but after dialysis the activity became significantly higher ($p$ 0.005 in a paired t test) with an activity of (152.2 [SD 17.2] mU/g Hb). It seemed possible that inorganic phosphate could be an inhibitor as it is present at a concentration of 0.21 - 1.0 mM in normal red cells. When inorganic phosphate was added to a haemolysate, enzyme activity decreased as phosphate concentration increased (Figure 8). These assays were carried out with 0.3 mM [2-$^{14}$C]CMP as substrate. This inhibition was then studied using partially purified enzyme with [2-$^{14}$C]CMP as substrate. Previous tests had indicated that reaction rates remain linear for at least 15 minutes, so an approximation of initial reaction velocity was determined by incubating for 10 minutes at 37$^{o}$C. Figure 9 shows that increasing additions of inorganic phosphate resulted in progressive decrease of $V_{max}$ with $K_M$ remaining constant at 0.05 mM at all levels of $P_i$. These results are typical of non-competitive inhibition. In the absence of added inorganic phosphate the $K_M$ appeared to have a value of 0.01 millimole/litre.

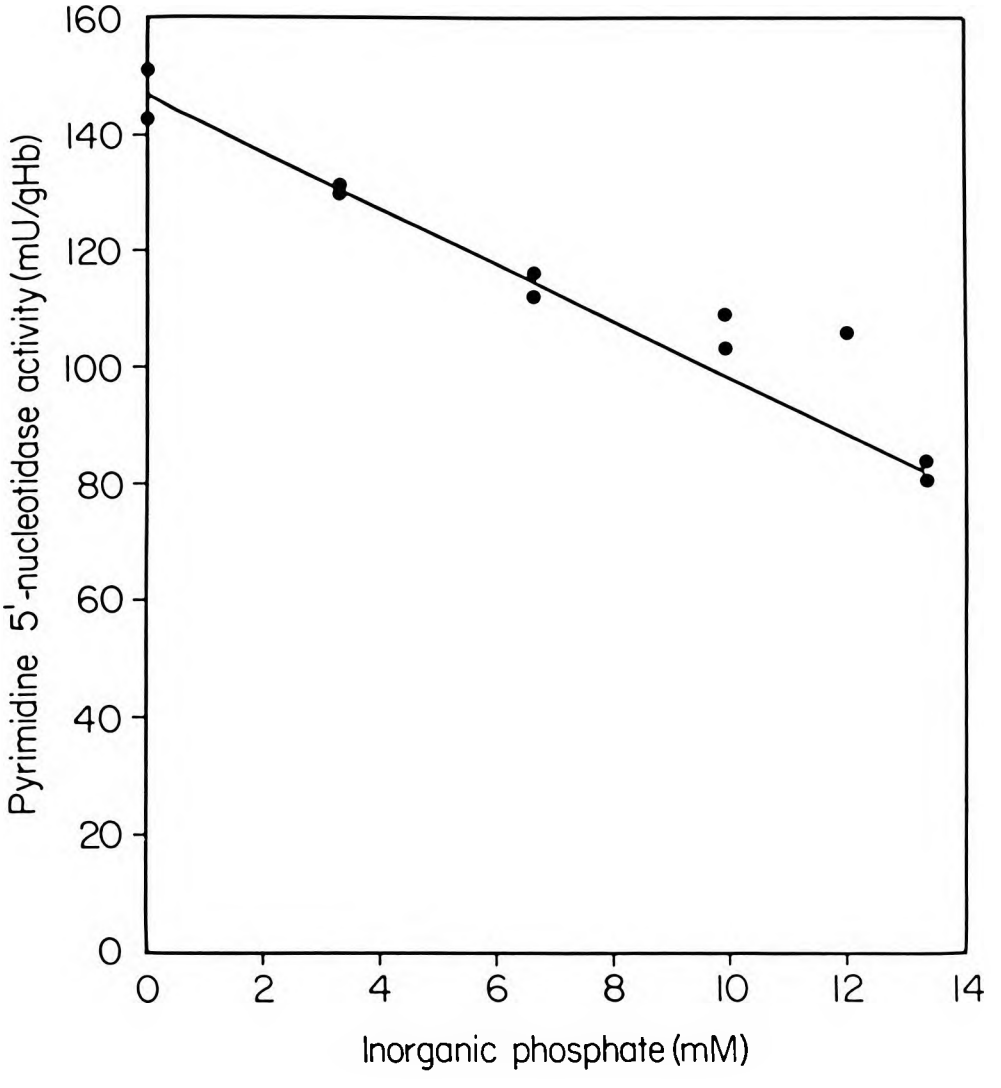



Figure 8. The effect of inorganic phosphate on the pyrimidine 5'-nucleotidase activity of a 1:20 haemolysate. The radiometric method was used with [2-$^{14}$C]CMP as substrate.

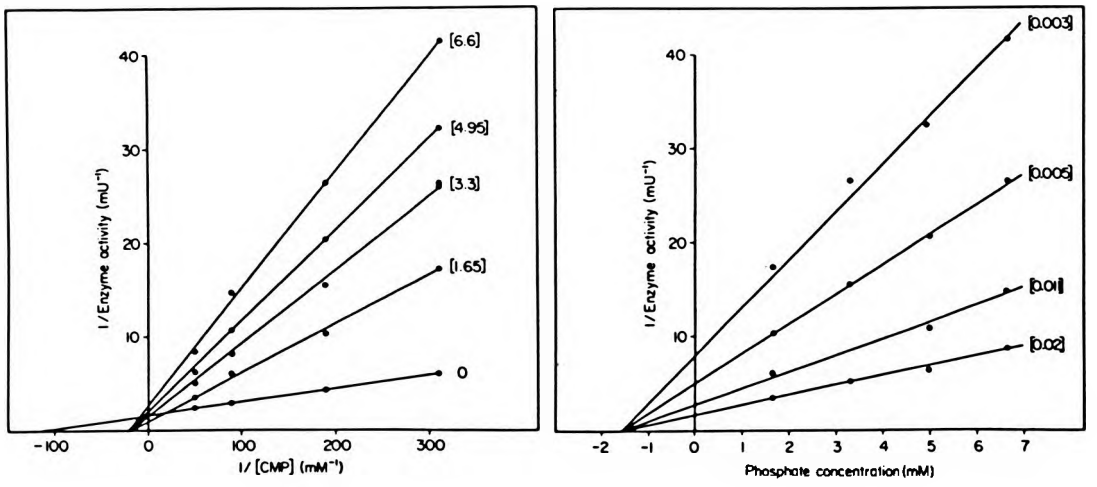



Figure 9. a The double reciprocal plots of purified enzyme pyrimidine 5'-nucleotidase activity in the presence of increasing concentrations of inorganic phosphate. The $P_i$ concentrations are shown in the brackets. b Graphical determination of $K_i$ from the plot of the reciprocal estimated initial reaction rate against the phosphate concentration. The radiometric method was used. The concentration of [2-$^{14}$C]CMP substrate is shown in brackets. The plot shows non-competitive inhibition with $K_i$ = 1.5 millimole/litre.

This finding was inexplicable.

Purine and pyrimidine nucleosides:

The effect of added purine or pyrimidine nucleosides or nucleotides on enzyme activity was tested. Both crude haemolysate and purified enzyme was used for the study with Ø.3 mM substrate ([2-$^{14}$C]CMP or [2-$^{14}$C]UMP). The compounds added were of the same concentration and at a pH of approximately 7.Ø. The final incubation pH was measured at 37$^{o}$C with the Astrup type blood electrode. Using the previously calculated pH optimum curve, the resulting variations in pH could only account for a 1% change in enzyme activity. The results are listed in Table 2 expressed as the percentage of the activity in the absence of added compounds. Of the purine and pyrimidine nucleosides and nucleotides tested, with purified enzyme and with CMP as substrate, only cytidine produced a marked inhibition. With UMP as substrate cytidine remained the strongest inhibitor, but all the nucleotides tested depressed the activity to between 73 and 78% of the activity of the purified enzyme without added compounds. The results of this study suggest that CMP should be the substrate of choice for routine analysis of enzyme activity levels.

When crude haemolysate was used as a source of enzyme, the effect of cytidine and uridine remained very much the same as with the purified enzyme. However, with the haemolysate alone, activity was also inhibited by

Table 2. The effect of various purine and pyrimidine compounds on the pyrimidine 5'-nucleotidase activity of a crude haemolysate and a 250,000-fold purified preparation.

| Substrate | 5'-Nucleotidase activity | | | |
|---|---|---|---|---|
| | purified enzyme | | haemolysate | |
| | [2-$^{14}$C] CMP | [2-$^{14}$C] UMP | [2-$^{14}$C] CMP | [2-$^{14}$C] UMP |
| No addition | 100.0 ± 4.9 (10) | 100.0 ± 2.5 (7) | 100.0 ± 3.6 (21) | 100.0 ± 7.6 (17) |
| AMP | 95.4 ± 2.9 | 73.0 ± 5.3*** | 99.2 ± 2.9 | 87.6 ± 9.6*** |
| ADP | 100.8 ± 1.3 | 76.7 ± 1.2*** | 97.4 ± 1.7 | 73.0 ± 9.7*** |
| ATP | 100.6 ± 2.1 | 73.3 ± 2.5*** | 94.0 ± 6.0* | 63.8 ± 5.5*** |
| GMP | 99.4 ± 4.8 | 75.5 ± 4.0*** | 98.8 ± 2.5 | 96.0 ± 18.0 |
| IMP | 101.0 ± 3.0 | 77.3 ± 1.2*** | 102.8 ± 3.0 | 98.6 ± 17.6 |
| CDP | 97.0 ± 3.0 | 73.3 ± 2.1*** | 66.8 ± 3.6*** | 51.4 ± 10.7*** |
| CTP | 100.4 ± 2.4 | 74.7 ± 4.5*** | 71.0 ± 2.7*** | 58.8 ± 7.0*** |
| UDP | 99.8 ± 1.9 | 76.0 ± 1.7*** | 88.0 ± 3.2 | 66.8 ± 9.4*** |
| UTP | 103.0 ± 2.7 | 77.0 ± 1.0*** | 85.4 ± 3.5*** | 61.4 ± 9.5*** |
| Inosine | 101.0 ± 1.7 | 94.0 ± 2.7* | 102.5 ± 3.9 | 97.3 ± 11.0 |
| Adenosine | 94.5 ± 6.4* | 97.7 ± 2.1 | 103.0 ± 4.7 | 96.9 ± 11.3 |
| Cytidine | 63.3 ± 4.1*** | 72.3 ± 1.5*** | 63.3 ± 4.1 | 74.0 ± 18.0*** |
| Uridine | 92.1 ± 5.3*** | 87.7 ± 7.1* | 92.1 ± 5.3*** | 88.6 ± 7.7*** |

The values are expressed as a percentage ± SD of the value in the absence of any addition. The SD in the uninhibited samples was calculated from the duplicate samples used in each assay. A Student t test was used to calculate the significance of deviations from the uninhibited value (* $p < 0.01$; ** $p < 0.001$; *** $p < 0.0005$). The concentration of [2-$^{14}$C] CMP or [2-$^{14}$C] UMP substrate was 0.3 mmol/l and all inhibitors were present at the same concentration. The mean pH at 37 °C in the incubation medium was 6.95 ± (SD) 0.01 with a range from 6.92 to 6.97. Five determinations were carried out in each case except where a greater number is shown in brackets.

adenine nucleotides, increasing inhibition being found from the mono to the tri phosphate. Again activity was more markedly inhibited when UMP was the substrate. CDP, CTP, UDP and UTP were also effective inhibitors. IMP and GMP were not inhibitors and contrary to Paglia and Valentine's (1975a) results with haemolysates, inosine and adenosine were ineffective as inhibitors. Despite repeated attempts to duplicate their results with these compounds, this was not achieved. When adenosine concentration was increased up to 4 mM with [2-$^{14}$C]CMP remaining at Ø.3 mM, no enzyme activity inhibition was evident either with purified enzyme or with crude haemolysate. This was the only finding that could not be duplicated with respect to the work presented by Paglia and Valentine (1975a).

It appeared that the major inhibitors of erythrocytic pyrimidine 5'-nucleotidase were the resultant products of the enzyme : inorganic phosphate and free pyrimidine nucleoside.

# 4. STUDIES OF NORMAL AND DEFICIENT LEVELS OF HUMAN ERYTHROCYTE PYRIMIDINE 5'-NUCLEOTIDASE

## Study of the First Case of Human Erythrocyte Pyrimidine 5'-Nucleotidase Deficiency Reported in South Africa

In 1977 Torrance, Karabus, Shnier, Meltzer, Katz and Jenkins (1977a) published a report describing the first known case of human erythrocyte pyrimidine 5'-nucleotidase deficiency to be discovered in South Africa. The patient was only 10 months old at the time of diagnosis. I quote his case history as described in the above paper.

> "The patient was the first-born son of healthy, unrelated Ashkenazi Jewish parents. There had been two previous pregnancies which ended in spontaneous abortions at about 3 months' gestation. His birth weight was 3.64 kg; the pregnancy had been uneventful and delivery was by caesarean section. He developed jaundice soon after birth and at 10 hours the total bilirubin level was 9.5 mg/100 ml. In spite of phototherapy the level rose steadily to 14 mg/100 ml on the fourth day but subsequently began to fall and by the sixth day was only 9.5 mg/100 ml. There was no evidence of fetomaternal incompatability and the jaundice was thought to

have been 'physiological' in nature. At the age of 10 months the child had an episode of gastro-enteritis and clinical examination at this time revealed pallor and 'bossing' of the forehead. The infant was first seen in Johannesburg at the age of 15 months. On clinical examination his weight was 9 kg, head circumference 48.5 cm, with pallor and frontal bossing. There was no hepatosplenomegaly or lymphadenopathy but the lower limbs were hypotonic and there was a moderate degree of kyphosis in the lumbar region. The milestones of motor development were somewhat delayed. A grade III ejection systolic murmur was considered to be functional in nature. Radiographs of the skull revealed thickened diploe. Laboratory investigations at this time showed a haemoglobin level of 7.6 g/dl, an MCH of 29 pg and an MCHC of 33 g/dl. The reticulocytes accounted for 10% of the total red cells and the striking feature of the peripheral blood film was the very marked basophilic stippling and diffuse basophilia. An osmotic fragility test was negative and the autohaemolysis test of Selwyn and Dacie showed a type I pattern, i.e. there was some correction of the haemolysis after 48 hours' incubation (5.96% without glucose and 5.74% with the addition of glucose). Haemoglobin electrophoresis did not reveal an abnormal component, either major or minor. HbF levels were normal for age and there

was no suggestion of HbH being present. Normal levels of glucose-6-phosphate dehydrogenase and pyruvate kinase were demonstrated while the level of pyrimidine 5'-nucleotidase in the red cells was markedly lowered (Table 3). The child has continued to show slow motor development which is thought to be unrelated to his anaemia. There is no intellectual impairment. At age 18 months, after an episode of otitis media, he was given a blood transfusion as preparation for a minor surgical procedure on the ear."

The patient's pyrimidine 5'-nucleotidase level was very low, 6 mU/g Hb. This hereditary deficiency of the enzyme, originally described by Valentine et al. (1974), had previously been reported in only 10 individuals from 8 different families (Valentine et al. 1972, Valentine et al. 1973,Valentine et al. 1974, Rochant et al. 1975, Ben-Basset et al. 1976, Oda and Tanaka 1976).

Enzyme Levels of Family of Enzyme Deficient Child

A study was made of erythrocyte pyrimidine 5'-nucleotidase levels of available family members in order to determine the feasibility of detecting the carrier state.

In 1977, 20 family members had been located and studies made of their enzyme levels. By 1979 11 additional

Table 3. Haematological data on the patient and his parents.

| | Patient | Mother | Father |
|---|---|---|---|
| Haemoglobin (g/dl) | 7.1 | 13.1 | 16.2 |
| PVC (%) | 22 | 38 | 47 |
| MCV (fl) | 81 | 85 | 92 |
| MCH (pg) | 27 | 30 | 33 |
| Reticulocytes (%) | 10.0 | 2.0 | 2.5 |
| 5'-Nucleotidase activity (mU/gHb) | 6 | 42 | 87 |
| Peripheral blood | Basophilic stippling | No basophilic stippling | No basophilic stippling |

family members had been located and studied. The enzyme levels in all available family members are shown in the pedigree (Figure 1Ø). As might be expected from a recessively inherited condition, two grandparents were also found to have low enzyme levels and are obvious heterozygotes, the paternal grandfather's level was 39 and the maternal grandmother's level was 5Ø mU/g Hb. However, detection of the heterozygote state was not an easy task. Those with less than 51 mU/g Hb appeared to be obvious heterozygotes, but the father, an obligatory carrier, had a level of 87 mU/g Hb. This level seemed reasonably close to normal enzyme levels previously described. This finding prompted the study of a normal population to establish a normal range of enzyme level.

## Linkage Studies

A range of gene marker studies was carried out on available family members. Bearing in mind the difficulty of assigning to normal homozygous or heterozygous status those family members within the 75-9Ø mU/gHb range the conclusions about linkage must be treated with reserve. In the matings and sibships in which there was little ambiguity about carrier status there were two informative matings for the MNSs blood group and red cell acid phosphatase systems, respectively, and only one for the ABO blood

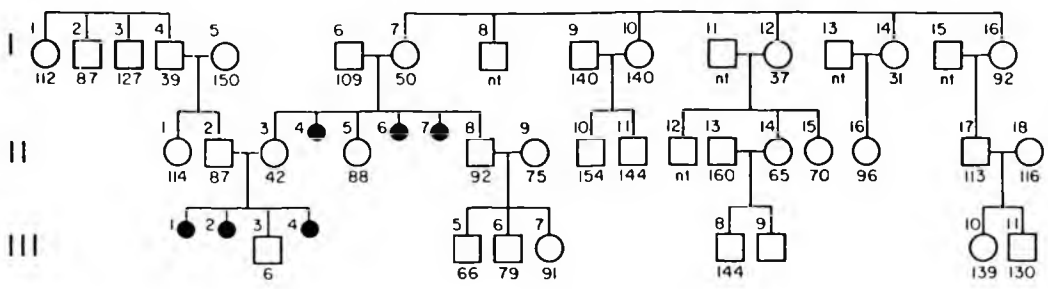



Figure 10. Pedigree of the family of the patient with pyrimidine 5'-nucleotidase deficiency. the enzyme levels in all members tested are shown in mU/gHb. The small closed circles indicated miscarriage. Heterozygotes are not shown because it was not possible to accurately determine which subjects were heterozygous.

group system. For none of these was a lod score obtained which demonstrated linkage between the loci and the locus for pyrimidine 5'-nucleotidase.

## Enzyme Levels Found in Normal Population Studies

Samples of normal blood, for determination of erythrocyte pyrimidine 5'-nucleotidase activity, were obtained from laboratory staff and from voluntary blood donors at two blood transfusion services in Johannesburg (South African Blood Transfusion Service and the Blood Transfusion Service of the South African Institute for Medical Research). Only samples from Caucasian adults were included in this study. In addition, samples were obtained from normal families as well as from families being studied for non-haematological genetic conditions. In all cases 6 ml of blood was collected into 1 ml ACD solution B in a vacutainer tube (Becton-Dickinson, New Jersey). The samples were kept at Ø-4$^{o}$C until assayed.

The enzyme assays were usually carried out on the day following collection but were on occasion carried out either on the same day or 2 days after collection. The method of assay was that of Torrance et al. (1977b).

The distribution of pyrimidine 5'-nucleotidase levels in 160 samples of fresh blood from adult Caucasians is shown in Figure 11. The two outlying subjects were excluded when statistical procedures were carried out

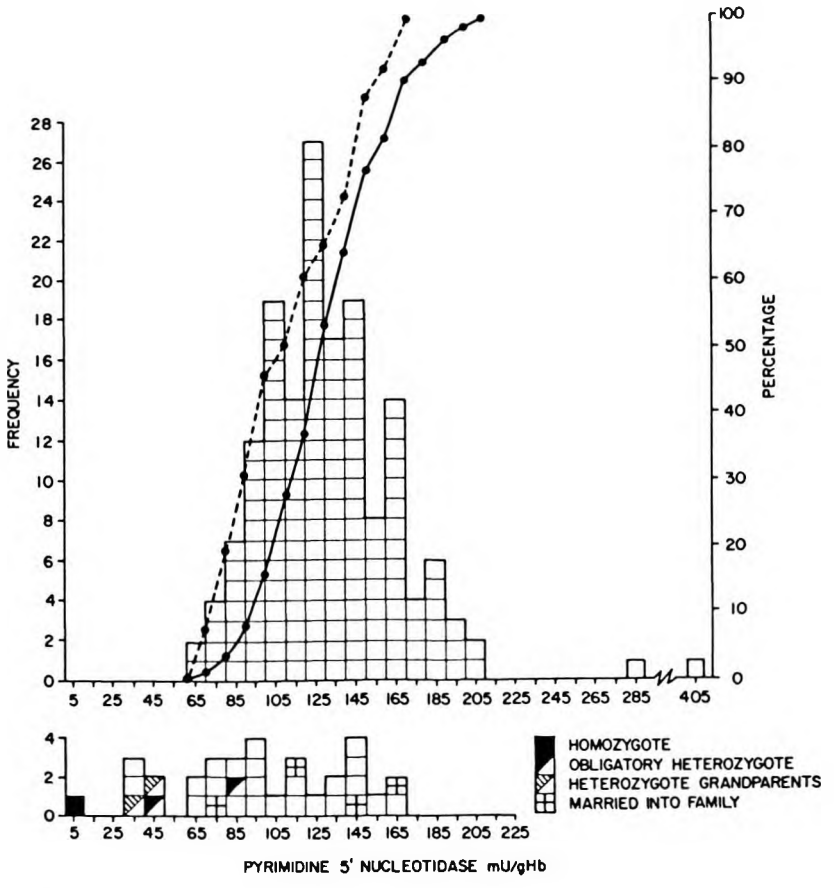



Figure 11. The upper histogram shows the distribution of erythrocyte pyrimidine 5'-nucleotidase activity in 160 normal subjects. The solid line shows the cumulative frequency polygon for the normal subjects (the two outlying subjects were omitted). The lower histogram shows the distribution of enzyme activity in erythrocytes from the deficient patient and members of his family. The cumulative frequency polygon for those members who are not obviously carriers of the deficient gene (enzyme levels above 60 mUgHb) is shown by the broken line in the upper figure. The presence of an obligatory carrier in this group indicates that some of the other members of this group may be heterozygotes. A consideration of the normal and family cumulative frequency polygons indicates that there may be as many as six heterozygotes in this group.

and when the cumulative polygon was drawn. The mean value for the remaining 158 cases was 130+SD 29.8 mU/gHb. There was no statistical difference (P>Ø.1) between the males, 128+SD 30.5 mU/gHb (N=58)and the females, 121+SD 26.6 mU/gHb (N=48)(the sex was not known in 52 cases).

Pyrimidine 5'-Nucleotidase Level in Frozen Blood

At the time of the first South African deficiency case study, there were nine families with previously reported cases, two of the families were black, two were reported with no mention made of ethnic or racial background, and the remaining five families were white. Two of the five families were of Jewish origin. Our case was also from a Jewish family. Two families were Ashkenazi Jews and the third was also probably Ashkenazi. Because of this a Jewish population was studied. 162 venous blood samples had been collected and frozen a year earlier for another study. These samples had been collected from young, healthy, gentile and Ashkenazi Jewish adults.

The samples had been collected into ACD and within 24 hours the plasma was removed and the packed cells mixed with an equal volume of glycerol preservative. The method has been described in Chapter 2. Before assay samples were thawed and the glycerol removed by overnight dialysis at $4^{\circ}C$ against physiological saline containing Ø.7 mM 2-mercaptoethanol and 2.7 mM

neutralized EDTA. The samples were then handled in the same way as the unfrozen samples. There was usually only a mild degree of haemolysis in these samples, but in 11 samples marked haemolysis was found after removal of the glycerol by dialysis. The washed cells from these samples were assayed but the results were excluded from the series, leaving 151 cases. Additional samples of venous blood frozen in this way were used to examine the effect of freezing.

To test the validity of using frozen blood for measuring erythrocyte pyrimidine 5'-nucleotidase levels, aliquots of a single sample of ACD blood were mixed with glycerol and stored at $-20^{\circ}C$. The initial enzyme level was determined on 20 aliquots of unfrozen blood in two separate sets of assays. Enzyme levels in frozen aliquots were measured on 20 separate occasions during the first month of storage and on 12 aliquots after a year. The mean enzyme activity in the unfrozen specimens was 133 $\pm$4.3 mU/gHb whilst the level in frozen stored blood was 137 $\pm$7.6 mU/gHb for specimens measured during the first month of storage and 144 $\pm$7.6 mU/gHb after storage for 1 year. Thus in this experiment there was a small but statistically significant ($P<0.05$) increase in the pyrimidine 5'-nucleotidase activity in frozen samples.

In another experiment, enzyme activity was determined on 10 specimens prior to freezing and on specimens

frozen for 1 month. The enzyme level in the frozen specimens was 114 ±15.4 mU/gHb and was not significantly different (P>Ø.5 paired t test) from the level in the same blood samples prior to freezing (122 ±7.4 mU/gHb).

The distribution of erythrocyte pyrimidine 5'-nucleotidase activity in 151 frozen blood samples is shown in Figure 12. Application of the two-sample Kolmogorov-Smirnov test showed that the distribution was statistically different (P<Ø.ØØ1) from that in fresh blood. Therefore it is not surprising that the mean enzyme activity of the frozen samples (153 ±44.7 mU/gHb) is significantly higher (P<Ø.ØØØ5) than that in fresh samples (130.Ø ±29.8 mU/gHb). These results differ from those obtained with fresh samples in that enzyme levels in erythrocytes from males (159 ±45.5 mU/gHb) were significantly higher (P<Ø.ØØØ5) than in erythrocyes from females (127 ±3Ø mU/gHb). But this value for frozen samples from females in not statistically different (P>Ø.1)from the value in fresh samples from females (121 ±26 mU/gHb). This means that the higher value obtained in frozen samples must be due only to samples from males. The male samples have a mean enzyme activity of 159 ±45.4 mU/gHb which is significantly higher (P<Ø.ØØØ5) than the value of 128 ±3Ø.5 mU/gHb obtained in fresh samples from males. When the values obtained from subjects of Ashkenazi Jewish origin were compared

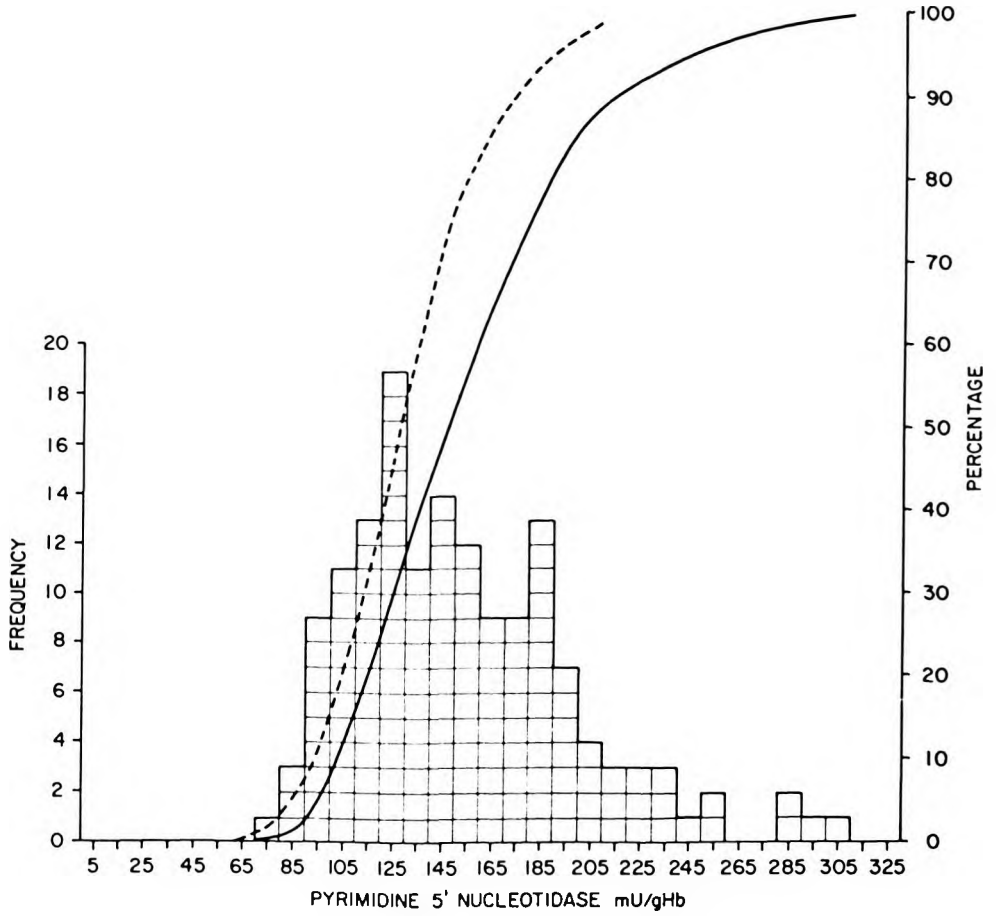



Figure 12. The histogram shows the distribution of erythrocyte pyrimidine 5'-nucleotidase activity in 151 samples of normal blood stored in glycerol at -20°C for longer than one year. The cumulative frequency polygon for the frozen samples is shown by the solid line and that for fresh samples ( not the same specimens) by the broken line. A two-sample Kolmogorov-Smirnov test shows that these distributions are statistically different ($P<0.001$).

with those from gentiles there was no significant difference ($P>0.1$) between the two groups. In both groups enzyme levels were higher in males than in females but the difference was statistically significant ($P<0.05$) only in the Jewish subjects.

## Variation in Pyrimidine 5'-Nucleotidase Levels in a Single Subject

Variation in erythrocyte pyrimidine 5'-nucleotidase activity in a single individual during an 18 month period is shown in Figure 13. The mean activity during this period was 147 $\pm$11.2 mU/gHb with values ranging from 128 to 169 mU/gHb. The fluctuations do not appear to be simple random ones since there appear to be periods of increasing or decreasing enzyme activity.

## Changes in Pyrimidine 5'-Nucleotidase with Cell Density

Enzyme levels were measured in haemolysates prepared from erythrocytes separated according to their density by the method of Danon and Marikovsky (1964) described previously. After separation, the heavy and light packed cells were haemolysed by the addition of 19 volumes of a 0.7 mM 2-mercaptoethanol and 2.7 mM EDTA solution. The haemolysates were then assayed according to Torrance *et al.* (1977b).

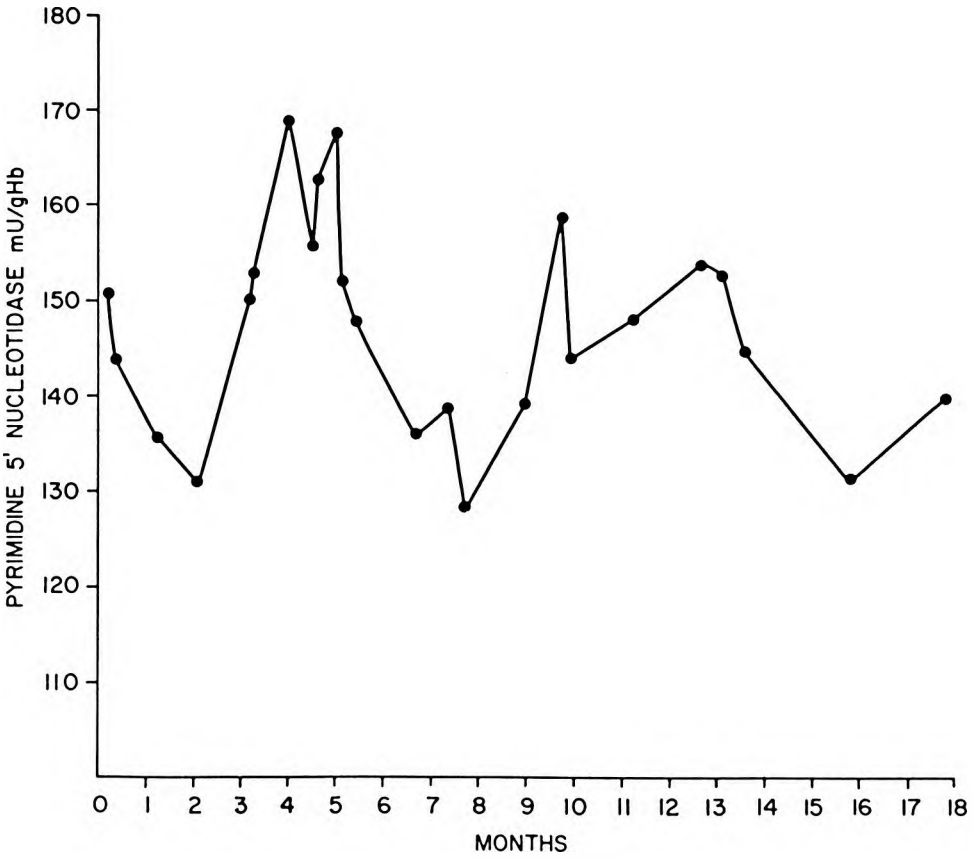



Figure 13. Changes in erythrocytic pyrimidine 5'-nucleotidase activity in a single subject during an 18 month period. The mean activity was 147±11.2 mU/gHb.

The level of pyrimidine 5'-nucleotidase in erythrocytes separated according to their densities is shown in Figure 14. Normal blood samples covering the range from high (163 mU/gHb) to low (83 mU/gHb) were examined. Because the erythrocyte density distribution varied considerably between individuals it was difficult to make accurate comparisons. Nevertheless, it was apparent that the least dense (youngest) erythrocytes have the highest enzyme level and that the level decreases progressively with increasing density (age). The results suggested that the youngest cells may start out with a level of between 300 and 400 mU/gHb and that the enzyme activity falls exponentially, reaching a level of 50 mU/gHb in the oldest erythrocytes. Exceptionally high levels of pyrimidine 5'-nucleotidase (1,500 mU/gHb) were found in the least dense erythrocytes of blood obtained from two pernicious anaemia patients who had reticulocyte counts of 16% and 8% whilst they were responding to treatment with vitamin $B_{12}$. They also showed a progressive fall in enzyme levels when more dense cells were examined. A similar pattern was observed in an abnormal case studied because of his unusually high enzyme level. He had previously been diagnosed as having polycythaemia vera but died before the diagnosis was verified. In the pyrimidine 5'-nucleotidase deficient case, the residual enzyme activity was confined to the least dense cells. Examination of the distribution of enzyme levels in the two obligatory heterozygotes showed a pattern similar to

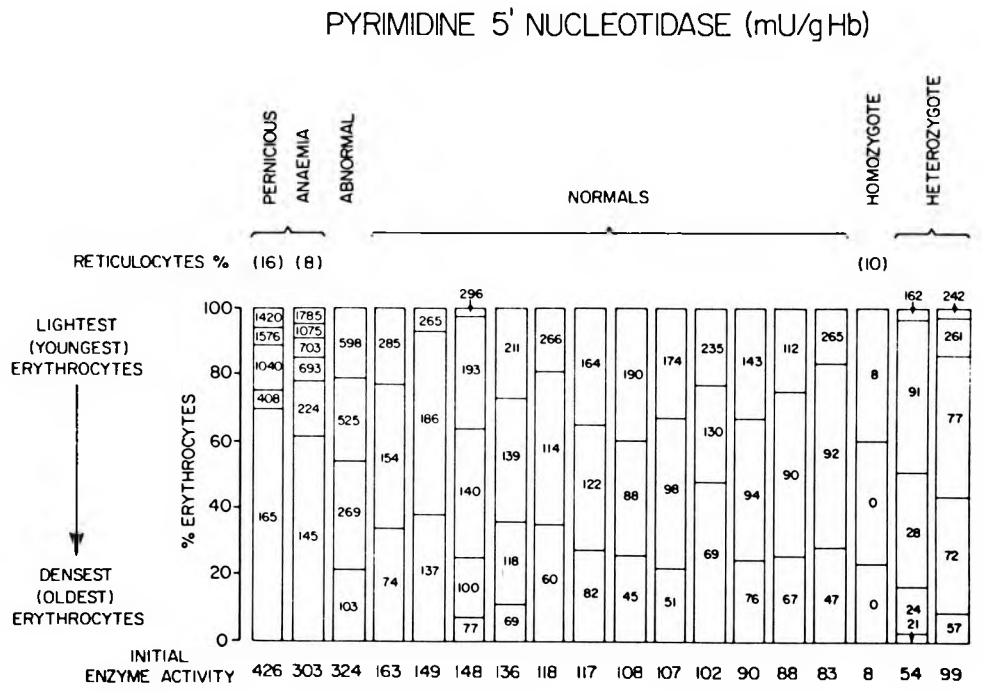



Figure 14. Pyrimidine 5'-nucleotidase levels (mU/gHb) in erythrocytes separated according to their density (age). The enzyme activity (mU/gHb) for each sample before density separation is shown at the bottom of each column. The normal samples were selected to cover the range from 163 to 83 mU/gHb; these are arranged in order of decreasing enzyme activity. Each column is divided into segments the length of which is proportional to the percentage of cells in that density range. The least dense (youngest) erythrocytes are at the top and the most dense (oldest) at the bottom. The enzyme activity (mU/gHb) of the erythrocytes in each density range is shown in the appropriate segment. The reticulocyte count was below 1.5% in all samples except those in which the level is shown.

that in normals with levels falling from highest in least dense to lowest in densest cells. The heterozygote with the lower level (the mother, 54 mU/gHb) had approximately half of the normal level in each density range whilst the distribution in the other heterozygote (the father, 99 mU/gHb) was in no way distinguishable from that in normals having a similar erythrocyte pyrimidine 5'-nucleotidase activity (Figure 14 page 74). Activity levels differ slightly from day to day (Figure 13 page 72). When the activity level of the deficient patient was first assayed it was found to be 6 mU/gHb, the mother's level was 42 mU/gHb and the father's level was 87 mU/gHb (Figure 10 page 64).

## Electrophoretic Studies

Electrophoretic studies were made of normal and enzyme deficient haemolysates to determine the possibility of identifying the carrier state. The method has already been described for Starch Gel Electrophoresis (Chapter 2 page 28).

Haemolysates were prepared for electrophoresis by freezing and thawing packed washed erythrocytes. The haemolysates were submitted to electrophoresis at 75 V for 17 h at 4$^{o}$C in starch-gel using a histidine-citrate buffer pH 7.0. The pyrimidine 5'-nucleotidase bands on the gel were specifically stained using the method of Anderson *et al.* (1974).

No difference in phenotype was evident between samples of high and low activity and one family member, considered to be heterozygous for the 'silent' allele (I-4), also gave a pattern indistinguishable from that found in normal individuals. A diagram of the patterns demonstrated on starch gel electrophoresis is shown in Figure 15. The photograph was of poor quality and is not presented. No extra band(s) or altered mobility was evident in the apparent 'silent' allele heterozygote but it must be conceded that the overall staining intensity of the usual bands was faint and a variant band might not have been discernible because of the low activity.

## Enzyme Kinetics in Heterozygotes and Normals

Table 4 shows the values of $K_M$ and $V_{max}$ measured in crude haemolysates prepared from two heterozygotes and two normals, one of whom was from the deficient family. The enzyme activity as measured in the standard assay is also compared with the value calculated from the Lineweaver-Burk plot at the substrate level used for the assay. It would seem that the $K_M$ of the enzyme in the heterozygotes is not markedly different from that in normals.

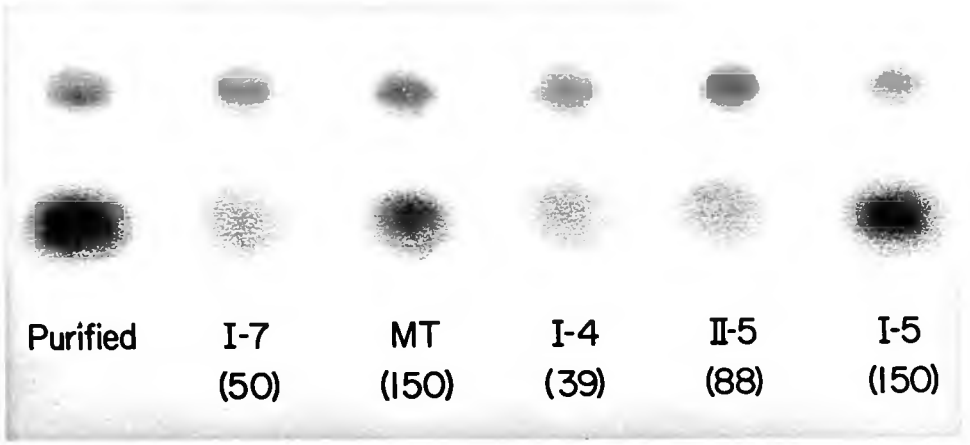



Figure 15. Comparison of the starch-gel pyrimidine 5'-nucleotidase pattern of the deficient family with a normal specimen and with a specimen which had been partially purified on DEAE cellulose. The family members can be identified from the pedigree ( Figure 1Ø page 64) and the enzyme levels are shown in mU/gHb. Two of the enzyme deficient child's grandparents (I-7) and (I-4) are clearly heterozygous for the defect whilst the other grandparent (I-5) has a normal enzyme level. The aunt (II-5) has an enzyme levelin the lower normal range and may be either normal or a heterozygote. The figure shows an artist's impression because it was not possible to obtain a satisfactory photograph of the gel which rapidly turned blue obscuring the enzyme pattern.

Table 4. Kinetic studies on the pyrimidine 5'-nucleotidase in haemolysates from two heterozygotes and two normals one of whom was from the deficient family. The $K_M$ and $V_{max}$ were determined from a Lineweaver-Burke plot. The value of V at the substrate concentration used in the routine assay $V_{Ø.3mM}$ was also determined from the plot and is shown for comparison with the assay value given in the first column.

| | Enzyme activity (mU/gHb) | $V_{Ø.3}$ (mU/gHb) | $V_{max}$ (mU/gHb) | $K_M$ (mM) |
|---|---|---|---|---|
| Family | | | | |
| Normal | 155 | 192 | 217 | Ø.Ø33 |
| Heterozygote | 5Ø | 127 | 155 | Ø.Ø66 |
| Heterozygote | 39 | 53 | 61 | Ø.Ø45 |
| Non-family normal | 12Ø | 151 | 174 | Ø.Ø46 |

## 5. DISTRIBUTION OF ERYTHROCYTE NUCLEOTIDES IN PYRIMIDINE 5'-NUCLEOTIDASE DEFICIENCY

The haemolytic anaemia resulting from a deficiency of the erythrocyte enzyme pyrimidine 5'-nucleotidase is characterized by marked basophilic stippling and the accumulation of high concentrations of nucleotides in the erythrocytes (Valentine *et al.* 1974). An attempt was made to define more precisely the composition of the nucleotide pool in pyrimidine 5'-nucleotidase deficiency. This information might well shed light on the basic cause of the haemolytic process as well as yielding information as to the source of the nucleotides. Two possible sources have been suggested.

Valentine *et al.* (1974) originally suggested that in pyrimidine 5'-nucleotidase dificiency the pyrimidine nucleotides arising from the catabolism of RNA during erythrocyte maturation were trapped in the erythrocytes. On the other hand, Harley *et al.* (1978) presented evidence that the mature erythrocyte actively synthesizes pyrimidine nucleotides which would then accumulate when pyrimidine 5'-nucleotidase was absent.

It was also possible that an examination of the erythrocyte nucleotide composition might provide a tool for identifying the heterozygote condition. Although

some heterozygotes were easily detected by having an erythrocyte pyrimidine 5'-nucleotidase activity of a third to a half of normal activity; one obligatory heterozygote, the father of the South African child with deficient enzyme, had an enzyme level of 87 mU/gHb which fell in the lowest one-tenth percentile of the normal range established in the previous study. The heterozygote might accumulate small amounts of the abnormal metabolites which although not evident from a spectral examination of an acid extract, might be apparent when the nucleotides were separated chromatographically.

Preparation of Perchloric Acid Extracts

The distribution of erythrocytic nucleotides was examined in four blood samples. The first was obtained from the enzyme deficient child. The second sample was obtained from the maternal grandmother of the child who was shown to be heterozygous for this condition by having an erythrocyte pyrimidine 5'-nucleotidase level of 50 mU/gHb. The remaining blood samples came from a normal subject and from a pernicious anaemia patient responding to vitamin $B_{12}$ treatment. This blood sample, which contained 16% reticulocytes, was used to determine whether the elevated reticulocyte count in the enzyme deficient blood (10% reticulocytes) altered the nucleotide distribution.

Blood samples from the antecubital vein were anticoagulated with heparin. The blood was centrifuged immediately after collection, at room temperature for 10 minutes at 3,000 x G. The plasma was removed and the packed cells, whose volume varied from 1.2 to 5 ml, were first haemolysed with an equal volume of distilled water and then extracted with 6% perchloric acid using the method given in Chapter 2 page 29. The extracts were stored at -20°C until chromatographed.

## Study of Whole Extracts Before Ion Exchange Chromatography

Before attempting to chromatograph the individual nucleotides present in the erythrocytes of the deficient child, the UV spectrum of a perchloric extract of erythrocytes from the patient and from a normal subject was studied. Valentine et al. (1974) findings of a shift in the UV spectrum was confirmed. Blood samples were obtained from the enzyme deficient child, both his parents and a normal subject for this study. Figure 16 gives the spectra obtained from these erythrocyte perchloric extracts. All were prepared in the same manner and the absorbance has been expressed at the erythrocytic concentration.

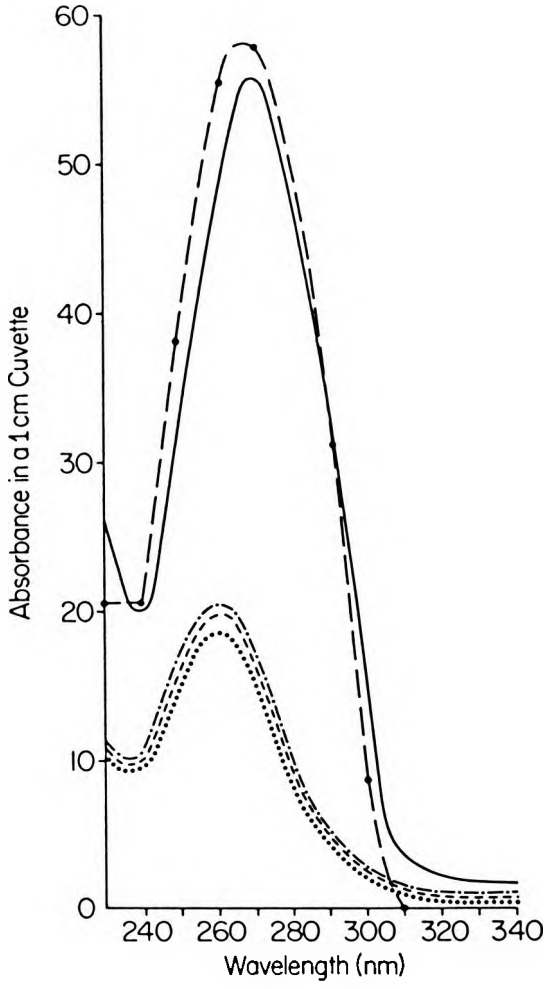



Figure 16. Spectral scan of a perchloric acid extract from a subject with pyrimidine 5'-nucleotidase deficiency (___,7 mU/gHb). A similar scan is shown for normal blood (....., 130 mU/gHb) and for both parents of the deficient subject (-.-.-, 90 mU/gHb)(----, 50 mU/gHb). The absorbance has been expressed at the erythrocytic concentration. The upper broken line (.----.) shows the UV spectrum calculated from the measured nucleotide levels in the enzyme deficient subject (Table 5 page 89) using published spectral scans (P.L. Biochemicals, 1973) of authentic ATP, CTP and UTP to obtain molar absorbance indexes at 10 nm intervals for adenine cytidine and uridine nucleotides respectively.

Ion Exchange Chromatography

Ion exchange chromatography (Chapter 2 page 30) was carried out on 1,0 X 30cm columns of Dowex 1 X 8 formate (Bartlett, 1968). The columns were eluted with 1 litre of a linear gradient of 1-5 M ammonium formate, pH3, produced by mixing 1 part of 5 M formic acid with four parts of 5 M ammonium formate. The elution positions of CMP, CDP, CTP, CDP-glucose, UMP, UDP, UTP, UDP-glucose, AMP, ADP and ATP in this chromatographic system were established using pure samples (Boehringer, Mannheim and Sigma, St Louis). To assist in the identification of pyrimidine nucleotides tracer amounts of [2-$^{14}$C]CMP and [2-$^{14}$C]UMP (Schwarz-Mann, Division of Becton, Dickson and Co., Orangeburg, New York) were added to the neutralized extract before chromatography. Both compounds had previously been purified on Dowex 1 X 8 $Cl^-$ columns. Fractions of 5 ml were collected and their phosphate content was estimated (Chapter 2 page 13) on aliquots from each fraction by the method of Bartlett (1959). A scan of the effluent at 260 nm was obtained with a double beam monitor (ISCO) but this was not sufficiently accurate to provide quantitative information. The optical density of each fraction was measured spectrophotometrically at 260 nm and at 280 nm. It was necessary to correct all the values for the linear increase in the optical density of the formate gradient. To calculate the concentration of nucleotides present it was assumed that the pH of the eluted

fractions was 3 throughout collection. Because the pH of the effluent solution only fell to 3 after 100 ml of effluent had been collected, this calculation underestimated cytidine nucleotide levels in the peaks collected between 60 and 90 ml and between 90 and 110 ml in the case of the deficient cells. The actual pH values in these two peaks were 3.6 and 3.2 respectively. In these cases the concentrations were recalculated from a spectrum of a sample after removing the formate as described below. The following millimolar absorption coefficients were used: cytidine nucleotides $E_{280}$ 13.0, $E_{260}$ 6.22; uridine nucleotides $E_{280}$ 3.9, $E_{260}$ 10.0; adenine nucleotides $E_{280}$ 3.07, $E_{260}$ 14.7. In this chromatographic system CDP and UMP eluted together. This was verified by co-chromatography of non-radioactive CDP with a tracer amount of [2-$^{14}$C]UMP. The radioactive UMP peak was coincident with the peak of CDP measured by its absorbance at 260 nm.

The various compounds in the eluted fractions were identified from their ultraviolet spectra at pH 2.7 and 10. Because the formate interfered with the spectrum this was removed from the sample in a vacuum oven at 80$^{o}$C and the residue redissolved in water. The ultraviolet spectrum was obtained in water, 0.02 N HCl and again after adding solid tris (hydroxymethyl) methylamine to bring the pH up to 10. The relative proportions of CDP and UMP in the erythrocyte extract were also estimated from the spectrum of the appropriate

fractions after removing formate. At pH 2 there was an isosbestic point for uridine and cytidine nucleotides at 267.5 nm with a millimolar absorption coefficient of 9.25.

Elution patterns:

Separation of the compounds present in the PCA extract of normal erythrocytes using ion exchange chromatography on Dowex formate (Figure 17) gave an elution pattern similar to that described previously (Bartlett, 1968). Chromatography of a similar extract from pyrimidine 5'-nucleotidase-deficient cells (Figure 18) showed the presence of at least eight nucleotides not present in normal erythrocytes. The concentrations of the various compounds are summarized in Table 5.

There appeared to be four uridine nucleotides present; the three 5'-phosphates UMP(108 nmol/ml cells), UDP (276 nmol/ml cells), UTP (220 nmol/ml cells) and a fourth uridine compound which eluted immediately before ADP in the position in which an authentic sample of UDP-glucose (Boehringer) was eluted in this chromatographic system. This compound was present in a concentration of 327 nmol/ml cells and appeared to be the major form of uridine nucleotide present in the cell. The cytidine nucleotide pool also appeared to consist of four compounds. Three of these compounds were identified; CMP (757 nmol/ml cells), CDP (278 nmol/ml cells)and CTP (831 nmol/ml cells).

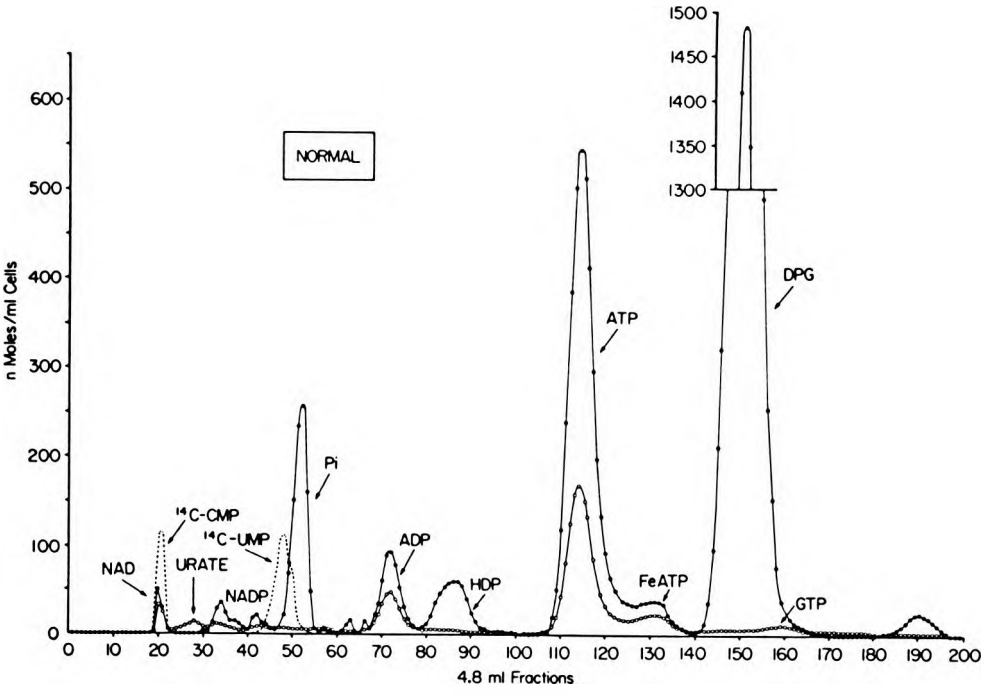



Figure 17. Phosphate compounds in normal erythrocytes. A neutralized perchloric acid extract was chromatographed on a 1 x 30 cm Dowex 1 x 8 formate column which was eluted at 1 ml/minute with 1000 ml of linear gradient 0-5 M ammonium formate (4 formic acid: 1 ammonium formate).●-●, Phosphorus; o-o, nucleotide concentration calculated from absorbance at 260 nm. -----, Tracer markers for [2-$^{14}$C]CMP and [2-$^{14}$C]UMP.

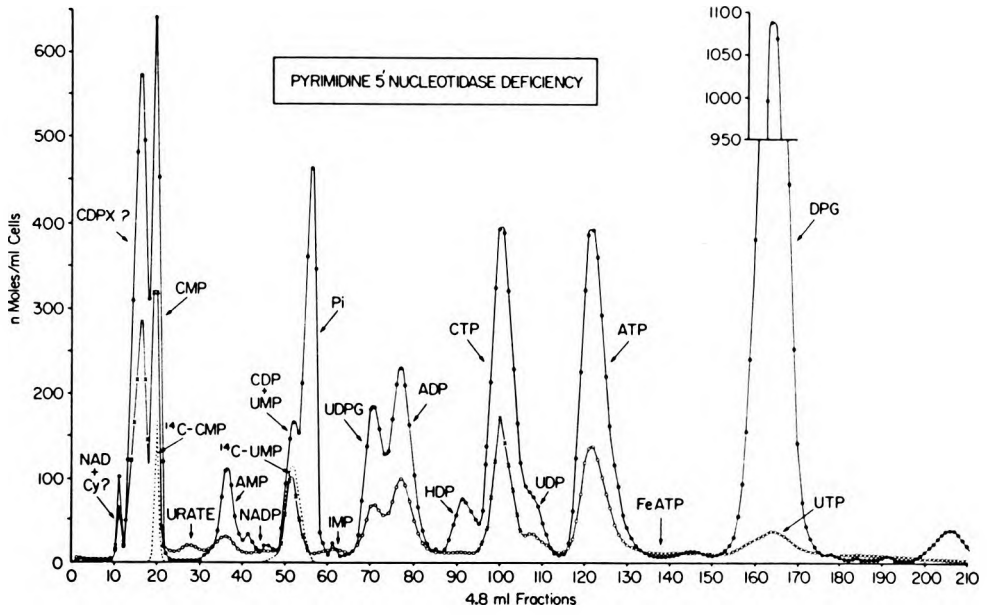



Figure 18. Phosphate compounds in pyrimidine 5'-nucleotidase deficient erythrocytes. A neutralized perchloric acid extract was chromatographed on a 1 x 30 cm Dowex 1 x 8 formate column which was eluted at 1 ml/minute with 1000 ml of linear gradient 0-5 M ammonium formate (4 formic acid: 1 ammonium formate).●-●, Phosphorus; o-o, nucleotide concentration calculated from absorbance at 260 nm. x-x, Nucleotide concentration calculated from absorbance at 280 nm (where absorbance at 280 nm was greater than at 260 nm. which is indicative of cytidine nucleotides). -----, Tracer markers for [2-$^{14}$C]CMP and [2-$^{14}$C]UMP.

Table 5. Concentration of acid-soluble phosphate compounds in erythrocytes from a pyrimidine 5'-nucleotidase deficient subject, a subject heterozygous for this condition, a normal subject, and a subject having 16% reticulocytes. The overall distribution between adenine, cytidine and uridine nucleotides is summarized at the bottom of the table. Nucleotide concentrations are calculated from UV absorbance. Phosphate levels were used to calculate concentrations of inorganic phosphate, hexosediphosphate and 2,3-DPG. Approximate elution positions are shown.

| *Pyrimidine 5'-nucleotidase (mu/g Hb)* | | *Deficient* 7 | *Normal* 122 | *Heterozygote* 50 | *Reticulocyte-rich (16%)* 426 |
|---|---|---|---|---|---|
| | *Elution position (ml)* | | *μM (nmol/ml erythrocytes)* | | |
| Unidentified | 55–60 | 105 | 0 | 0 | 0 |
| Cytidine compound | 60–90 | 1049 | 0 | 0 | 0 |
| CMP | 90–110 | 757 | 0 | 0 | ? |
| NADH | 100–105 | ? | 56 | 82 | 143 |
| Urate | 125–145 | 84 | 46 | 68 | 86 |
| AMP | 160–180 | 173 | 47 | 75 | 70 |
| NADP | 200–230 | ? | 50 | 168 | 50 |
| UMP | 240–280 | 108 | 0 | 0 | 0 |
| CDP | | 278 | 0 | 0 | 0 |
| Pi | 235–275 | 1517 | 943 | 947 | 952 |
| UDP glucose | 335–365 | 327 | 0 | 0 | 0 |
| ADP | 370–415 | 610 | 268 | 278 | 293 |
| HDP | 410–450 | 225 | 247 | 290 | 315 |
| CTP | 480–535 | 831 | 0 | 0 | 0 |
| UDP | 520–565 | 276 | 0 | 0 | 0 |
| ATP | 550–650 | 1063 | 1213 | 1010 | 1526 |
| FeATP | 650–690 | 0 | 196 | 135 | 240 |
| DPG | 725–825 | 4060 | 5083 | 4654 | 6175 |
| UTP | 765–865 | 220 | 0 | 0 | 0 |
| GTP | 750–900 | ? | 73 | ? | 70 |
| Adenine nucleotides | | 1846 (32%) | 1830 | 1748 | 2322 |
| Cytidine nucleotides | | 2915 (50%) | | | |
| Uridine nucleotides | | 931 (16%) | | | |
| Unidentified nucleotides | | 105 (2%) | | | |
| Total nucleotides | | 5797 (100%) | | | |

The unidentified compound was the most abundant, 1049 nmol/ml cells, and eluted from the ion-exchange column immediately before CMP, whose position was clearly identified by the radioactive tracer. This compound must therefore have been less polar than CMP. The UV spectrum clearly showed it to be a cytidine compound and the ratio of two phosphate to one nucleotide suggested the presence of a CDP compound. The CDP glucose complex was considered since this would be analogous to the UDP-glucose already shown to be present. Chromatography of an authentic sample (Sigma) showed that CDP-glucose eluted between CMP and CDP in a position in which no peak was obtained. Because the ratio of P to nucleotide in the CMP peak was 1.7:1 instead of the expected 1:1, the possibility that the unidentified peak was d-CMP was considered. Deoxy forms of the nucleotides tend to elute immediately before their parent nucleotides (Bartlett, 1968) but an authentic sample of d-CMP (Boehringer) eluted in the same position as labelled CMP marker. Thus the identity of the major cytidine peak remained unknown. There were other notable differences in the acid soluble components in the deficient erythrocytes. The erythrocytic concentration of inorganic phosphate was increased to 1517 nmol/ml cells which was about 50% above normal. Although the ATP level was normal (1063 nmol/ml cells), the concentrations of ADP (610 nmol/ml cells) and AMP (173 nmol/ml cells) were between 2 and 3 times normal, so that the distribution of adenine nucleotides was

abnormal with the lower energy forms being favoured. The Fe-ATP peak which eluted immediately after ATP itself (Bartlett, 1976a) was absent in the enzyme-deficient blood.

The accuracy of the nucleotide composition shown in Table 5 page 88 for the enzyme-deficient cells was checked by calculating the UV spectrum at 10 nm intervals using known molar absorbance values (P.L. Biochemicals, 1973). Figure 16 page 82 shows that the calculated curve was almost identical with that measured in the PCA extract of the cells.

A similar chromatographic separation was carried out on a PCA extract of erythrocytes obtained from a heterozygous subject whose pyrimidine 5'-nucleotidase level was 50 mU/gHb. None of the abnormal peaks found in the deficient subject were present (Table 5 page 88).

It was possible that at least some of the changes in the enzyme-deficient erythrocytes might have been due to the increased reticulocyte level. To exclude this possibility the distribution of acid soluble phosphate compounds in erythrocytes from a pernicious anaemia patient responding to vitamin $B_{12}$ therapy was examined. This blood contained 16% reticulocytes compared with 10% in the patient with pyrimidine 5'-nucleotidase deficiency. The level of pyrimidine 5'-nucleotidase in the reticulocyte rich blood was 426 mU/gHb which was

more than 3 times higher than that found in normal erythrocytes.

Density gradient separation of cells was carried out (Chapter 2 page 21) by the method of Danon & Marikovsky (1964) using mixtures of methyl phthalate and di-n-butyl (Miles Yeda, Rehovot, Israel). The enzyme activity was assayed for cells of differing densities (Figure 14 page 74). In the pernicious anaemia patient, the high level of enzyme activity in the erythrocyte pool was due entirely to an extremely high level of enzyme in the least dense erythrocytes. The enzyme level in the 20% of cells of lowest density was in excess of 1,300 mU/gHb. In contrast to this the level in the densest 65% of erythrocytes was 165 mU/gHb which was well within the range found in normal blood. The distribution of compounds in the PCA extract of this reticulocyte-rich blood was similar to that obtained with normal blood (Table 5 page 88) except that the level of 2,3-DPG was elevated 25% as would be expected in anaemia. The ATP level was also elevated to 1,526 nmol/ml cells.

# 6. DISCUSSION

## Background to Purification and Studies of the Properties of Pyrimidine 5'-Nucleotidase

Enzymes with 5'-nucleotidase activity have a wide distribution. They are found in bacteria, yeast, plants and in many animal tissues (Bodansky and Schwartz 1968, Heppel 1961, Drummond and Yamamoto 1971) . The characteristics of these enzymes vary considerably from enzyme to enzyme so that they have different substrate preferences and metal ion requirements. In addition, they exhibit different kinetic behaviour and are inhibited by different substances. Many of the 5'-nucleotidases are associated with membranes, whilst others are found in the cytosol. The 5'-nucleotidase of human erythrocytes is present in the cytoplasm. Its' presence was originally demonstrated in a haemolysate by Valentine et al. (1974) who showed that it differed from previously described enzymes with 5'-nucleotidase activity: it was specific for pyrimidine compounds (Paglia and Valentine 1975a). Their studies were carried out at pH 7.7-7.8 with haemolysates.

Purification of the Enzyme

Using classical purification procedures, a 250,000-fold purification of pyrimidine 5'-nucleotidase from human erythrocytes in 18% yield was achieved. This preparation was not homogeneous; on disc polyacrylamide gel electrophoresis two dense and two faint bands of protein were obtained. On starch gel it was possible to stain the enzyme (Anderson, Teng and Giblett 1974) and show that there were indeed two bands of enzyme with 5'-nucleotidase activity. Two bands of 5'-nucleotidase activity have also been observed on starch-gel electrophoresis by Anderson et al. (1974). Further purification was attempted by affinity chromatography using cytidine bound to Sepharose 4B, but this proved unsuccessful. The substituted agarose was able to bind the enzyme but recovery of the enzyme from the affinity column was not achieved. Indeed, it was surprising to note that while still bound to the cytidine column the enzyme appeared to retain its ability to hydrolyze [2-$^{14}$C]CMP.

The initial stepwise DEAE-cellulose procedure was included because it very rapidly removed most of the haemoglobin and other protein. This allowed the column DEAE-cellulose step to be carried out on a smaller column, which consistently gave better results than those obtained by applying the hemolysate directly to a large DEAE-cellulose column.

Inhibitors and Properties of Enzyme in Haemolysates and Purified Form

Effect of pH on enzyme activity:

This study confirmed the specificity of the erythrocyte enzyme for pyrimidine compounds at normal erythrocytic pH (7.2) and above. At this pH the enzyme activity with pyrimidine substrates was more than 10-fold that with purine substrates. Nevertheless, the results obtained with the purified enzyme suggested that there was weak purine 5'-nucleotidase activity with a pH optimum at 7.2. The purified enzyme also differed from the haemolysate in that it appeared to have a second pH optimum at 6.1 when AMP or GMP was substrate and between 6.0 and 6.5 when CMP or UMP was substrate. This was not always evident with CMP and was certainly absent when the radiometric assay with 0.3 mmol/l substrate was used. A similar double of pH optimum has been found with bull seminal plasma 5'-nucleotidase which had pH optima at 7.8 and 9.2 (Levin and Bodansky 1966). Polyacrylamide gel electrophoresis of both bull seminal plasma 5'-nucleotidase (Pilcher and Scott 1967) and our studies of human erythrocyte pyrimidine 5'-nucleotidase showed the presence of more than one active component. This heterogeneity may explain the double pH optima in both cases. It is also possible that the biphasic nature of the curve may have been due to pH-dependent instability of the enzyme during the 60 minute 37$^{o}$C incubation period. The purified enzyme was most stable

at pH 7 and lost as much as 50% of its activity after being incubated for 60 minutes at pH 5.5 or 8.0. However, the enzyme was protected, to some extent, from thermal inactivation by the presence of substrate (Paglia and Valentine 1975a). The overall pH activity curve must nevertheless have been influenced to some extent by thermal inactivation. In reviewing earlier literature Bodansky and Schwartz (1968) concluded that the form of the pH activity curve depended on the nature of the buffer used. This was also evident with the erythrocytic enzyme which was stimulated by glycine at pH 6.8.

Kinetic studies:

Investigation of the kinetic properties of the 5'-nucleotidase in erythrocyte haemolysates using the radiometric assay gave apparent $K_M$ constants of 30.0 and 33.0 µmol/l for CMP and UMP, respectively. These values were 5- to 10-fold lower than those reported by Paglia and Valentine (1975a), who measured inorganic phosphate liberated during a 2 hour incubation. The radiometric assay allowed activity to be estimated after a 5 or 10 minute incubation period giving a closer approximation to initial reaction rate. However, even using this short incubation period 10-20% of the substrate was converted to products. In spite of this there did not appear to be a marked difference between the results calculated from 5 and 10-minute incubations. When kinetic studies were carried out using the radiometric

assay the concentration range of 0.23-4.6 mmol/l used by Paglia and Valentine (1975a) gave a constant reaction rate. It was necessary to decrease the concentration range to 0.005-0.10 mmol/l to obtain satisfactory results. In the experiment shown in Figure 7 page 52 the $V_{max}$ for CMP and UMP was the same. This was not a consistent result as the $V_{max}$ was usually greater with UMP as substrate.

Kinetic studies with the purified enzyme showed that although the $V_{max}$ was greater with UMP rather than CMP as substrate, the $K_M$ for CMP (11.0 µmol/l) was lower than for UMP (16.6 µmol/l). The apparent $K_M$ values were similar to those for membrane bound 5'-nucleotidase from rat liver and rat heart (Drummond and Yamamoto 1971).

Inhibition studies:

The higher value of $K_M$ in the haemolysates suggested the presence of an inhibitor of the enzyme in the haemolysate. The presence of a dialysable inhibitor was indicated by the significantly higher ($p<0.005$ in a paired t test) enzyme activity in ten dialysed haemolysates (152.2 [SD 17.2] mU/g Hb) compared to their predialysis activities (139.5[SD 20.6] mU/g Hb).

It seemed possible that the dialysable inhibitor of the erythrocytic enzyme was inorganic phosphate which is present in normal red cells and has a concentration of 0.21-1.0 mmol/l. Figure 8 page 54 shows that the enzyme

was inhibited by inorganic phosphate. Kinetic experiments (Figure 9 page 55) suggested that the inhibition was non-competitive with $K_i$ being 1.5 mmol/l. Thus inhibition at physiological levels of inorganic phosphate would be weak and there would be little inhibition due to the phosphate formed during an assay in which 10% of 0.3 mmol/l substrate was converted. The non-competitive nature of the inhibition is in keeping with the non-reversible nature of the 5'-nucleotidase reaction. Thus phosphate must bind to the enzyme or the enzyme substrate complex at a separate site and so inhibit the reaction. It is also possible that inorganic phosphate may exert its inhibitory effect by altering the effective concentration of $Mg^{++}$ ions in solution. Swanson et al. (1983) reported binding of $Mg^{++}$ by nucleotide phosphates proportional to phosphate saturation. The pure enzyme displayed maximal activity at 0.05 mmol/l $Mg^{++}$ but in haemolysates this was achieved only at $Mg^{++}$ concentrations above 1.0 mmol/l (Paglia and Valentine 1975a). However, it seemed unlikely that this would give rise to the results shown in Figure 7 page 52.

Of the range of purine and pyrimidine nucleosides and nucleotides tested, only cytidine produced a marked inhibition of the purified enzyme when CMP was the substrate. Uridine was only a mild inhibitor. When UMP was substituted as substrate, cytidine remained a better inhibitor than uridine but surprisingly all the

nucleotides tested, both purine and pyrimidine, depressed the activity to between 73 and 78%. Only inosine and adenosine failed to inhibit activity. The difference between the results obtained with the two substrates placed some question on the reliability of the results obtained with UMP since the assay was less satisfactory with this substrate.

When haemolysate was used as a source of enzyme, cytidine and uridine were still effective inhibitors with both CMP and UMP as substrate. In both cases cytidine was the better inhibitor. In contradistinction to the results with purified enzyme, the haemolysate 5'-nucleotidase activity was inhibited by adenine nucleotides with the degree of inhibition increasing from AMP through ADP to ATP. This was particularly evident when UMP was the substrate. The di- and triphosphates of cytidine and uridine were also effective inhibitors but IMP and GMP as well as inosine and adenosine were ineffective as inhibitors. These results in haemolysates agreed with those of Paglia and Valentine (1975a) in that cytidine was a powerful inhibitor of the enzyme and uridine a less effective inhibitor with both CMP and UMP as substrate. However, it was not possible to confirm their observation that inosine and adenosine were more effective inhibitors of the 5'-nucleotidase activity than cytidine. With the radiometric assay these compounds did not appear to act as inhibitors. This was in agreement with the results

of an attempt to establish the kinetics of adenosine inhibition using purified enzyme. Addition of five levels of adenosine ranging from Ø.ØØ5 to Ø.2Ø mmol/l yielded a single line on a Lineweaver-Burk plot. Although equal concentrations of inhibitor and substrate were used by both Paglia and Valentine (1975a) and in this study, the concentration in their earlier assays was 2.2 mmol/l compared to Ø.3 mmol/l used in this assay. It seemed possible that this 7-fold concentration difference might be responsible for the discrepancy between the two studies. However, when the adenosine concentration was increased to 2 or even 4 mmol/l whilst the [2-$^{14}$C] CMP concentration remained at Ø.3 mmol, no inhibition of pyrimidine 5'-nucleotidase was evident with either haemolysate or purified enzyme.

Interpretation of the results of inhibition studies in haemolysates should be handled with caution because the haemolysate contains many enzymes which may interfere with the reaction under investigation. This has been confirmed by Torrance, Wulfsohn and Mills (1982). Thus addition of nucleoside triphosphates may have resulted in consumption of substrate via nucleoside monophosphokinase reactions whilst addition of ATP may have resulted in cytidine or uridine formed in the reaction being reconverted to their monophosphates by nucleoside kinase. It is also possible that during incubations with compounds like adenosine and inosine some of the phosphate liberated from the substrate may

have been diverted into 2,3-diphosphoglycerate (McManus and Borgese 1961; Deuticka et al. 1971; Torrance et al. 1982). However, the amount of free phosphate that could be lost in this way may be limited by a lack of oxidised NAD which is required for the glyceraldehyde 3-phosphate dehydrogenase reaction.

It would seem therefore that the major inhibitors of erythrocytic pyrimidine 5'-nucleotidase are the two products of the enzyme: the free pyrimidine nucleoside and inorganic phosphate.

## Distribution of Erythrocyte Pyrimidine 5'-Nucleotidase Levels in Normal Subjects

The scatter of erythrocyte pyrimidine 5'-nucleotidase levels found in the normal subjects is wide. Similar degrees of scatter have been found for other erythrocytic enzymes (Beutler 1975; Benohr & Waller 1975). Several possible causes of this wide distribution were studied.

To exclude the possibility that the wide range of enzyme activities was due to the method of expressing the result, a group of 16 subjects had cell counts, MCH and MCHC determinations carried out. This allowed the enzyme activity to be expressed in three units: which gave mean values (±SD) of 118 ±23.3 mU/gHb, 39 ±7.74 mU/ml cells or 36 ±7.3 mU/$10^{10}$ cells with coefficients

of variation of 19.7%, 19.7% and 20,4% respectively. Thus the scatter remained the same irrespective of the mode of expression. The variation due to differences in the reticulocyte concentration was also considered. Assuming the reticulocyte count in normals varied from 0.13% to 1.37% (mean 0.75 ±2.0) (Documenta Geigy), the reticulocyte contribution to the enzyme level would be 2.63 ±2.17 mU/gHb if 350 mU/gHb was taken as the reticulocyte level and 11.25 ±9.45 mU/gHb if the level of 1,500 mU/gHb found in treated pernicious anaemia was used. The latter value is probably falsely high because there is a release of premature reticulocytes in this situation. In either case, the contribution from reticulocytes would not be sufficient to explain the wide scatter of values obtained in normals. Another possible explanation for the variation in enzyme level is that the enzymes in the normals are all inhibited to a differing extent by subclinical levels of lead poisoning. The enzyme is extremely sensitive to lead (Paglia *et al.* 1975b, 1977; Buc & Kaplan 1978, unpublished studies by Torrance *et al.*) which is a common pollutant owing to its use as a petrol additive. It has been demonstrated that lead poisoning may occur from inhalation of fumes. Thus the levels of erythrocyte pyrimidine 5'-nucleotidase may simply be due to varying exposure to petrol fumes resulting in mild lead poisoning.

Finally, it is possible that the synthesis of the enzyme is determined by multiple alleles at a single genetic locus similar to the situation which exists for red cell acid phosphatase (Hopkinson et al. 1963). In this way there would be (at least) one allele responsible for the synthesis of an enzyme associated with high activity, one for low activity and one for intermediate activity. Provided all three alleles have significant frequencies in the population (thereby constituting a genetic polymorphism) a simple genetic theory would explain a Gaussian distribution.

The three alleles in the red cell acid phosphatase system can be recognised by the characteristic isozyme patterns they produce on starch gel electrophoresis, so one method of testing the multiple allele hypothesis as the explanation for pyrimidine 5'-nucleotidase levels was to examine the enzyme for qualitative heterogeneity. Preliminary starch gel electrophoresis of pyrimidine 5'-nucleotidase showed that two bands of activity were present in samples of high and low enzyme activity, and that these bands were of identical mobility in all subjects. These results were disappointing, but further studies need to be carried out along these lines. Polymorphism may be demonstrated by using different electrophoretic media or by the existence of differential inhibition characteristics. The use of quantitative differences alone would not appear to be

sufficiently sensitive to distinguish the different phenotypes.

## Distribution of Erythrocyte Pyrimidine 5'-Nucleotidase in the Deficient Family

Figure 11 page 66 shows the distribution of erythrocyte pyrimidine 5'-nucleotidase levels in normals and in the deficient family. Only the index case had a virtual absence of the enzyme. There was a group of five subjects having less than 50 mU/gHb or approximately one-third of the normal mean value. A further 12 individuals (including the father of the index case who is, by definition, an obligatory heterozygote) had values less than 100 mU/gHb; such values would only be found in 16% of normal subjects. The remaining 14 subjects had enzyme levels in excess of 100 mU/gHb and were probably not carriers of a deficient gene.

The family members with only one-third of normal activity were clearly heterozygous for the condition. However, the 12 subjects having less than 100 mU/gHb posed a problem since they included an obligatory carrier. It seemed likely that other members of this group may also have been carriers. If all subjects with less than 50 mU/gHb were excluded and the remaining 26 family members considered, 12 (46%) had values less than 100 mU/gHb compared with only 16% in the normal

population. If values below 90 mU/gHb were examined, then eight (31%) of the family fell in this group compared with 8% of the normal group. This suggested that six of the eight were carriers; however, it was possible that the family members who were not heterozygotes had low levels of the enzyme for other, as yet unexplained, reasons. This possibility was supported by family studies described later which showed that children of parents with low enzyme levels had similar low levels. The mother of the index case who was clearly a heterozygote with a level of 42 mU/gHb herself had a parent with a similar level (50 mU/gHb) and a parent with a level of 109 mU/gHb which was in the lower third of normal. Although the father of the index case also had a parent in the low heterozygote range (39 mU/gHb) his mother had a level of 150 mU/gHb which was in the highest third of the normal range. If the enzyme level was under simple genetic control then the surprisingly high level in the father, an obligatory heterozygote, may have been due to his having inherited a gene for high activity from his mother. This would mean that in the normal population there exists more than one gene for the enzyme, say A and A'of which A has a higher activity than A'. Three types of normal phenotype would then be possible, AA with a high activity (say 160 mU/gHb), A'A' with low activity (say 100 mU/gHb) and AA' with intermediate activity (130 mU/gHb). Two types of heterozygotes aA' and aA with levels of 50 and 80 mU/gHb would then be possible. It

would then not be possible to distinguish the heterozygote aA from the normal A'A'variant by measuring the erythrocyte enzyme activity.

To test this hypothesis, preliminary studies were carried out on 10 families. With the exception of one family, the enzyme levels in the children could be explained by assuming that the levels were the result of two alleles. The low levels (<115 mU/gHb) would have been due to A'A'; the medium levels (115-145 mU/gHb) to AA' and the high levels (>145 mU/gHb) to AA. Only one case in the 25 children could not have been explained on this basis. This was a child with a level of 153 (AA) from parents having levels of 140 (postulated AA') and 100 (postulated A'A') which would have made this a borderline case. Many more family studies will be needed to define clearly the mode of inheritance.

Use of Frozen Glycerolized Blood for Measurement of Pyrimidine 5'-Nucleotidase

Although the pyrimidine 5'-nucleotidase activity of 10 frozen glycerolized blood samples was not statistically different from the activity in fresh cells, a similar experiment in which multiple determinations were carried out on a single sample showed a statistically significant increase from 133 $\pm$4.3 mU/gHb to 144 $\pm$7.6 mU/gHb after 1 year's storage. An estimation of the precision of the assay from the duplicate determinations on 151 frozen and 160 fresh samples showed that the

coefficient of variation was 3.8% for both fresh and frozen samples, and the 95% confidence limits of the assay, when carried out in duplicate, included the mean ±7.6%. Thus the magnitude of the increase in enzyme activity in the frozen samples was of the same order as the methodologic error and was not of practical importance. These studies in which a comparison was made directly between fresh and frozen glycerolized blood indicated that although frozen blood might have given marginally elevated values it was satisfactory for measurement of pyrimidine 5'-nucleotidase activity. However, when the distribution of values in a batch of 151 frozen samples was examined, it appeared that there was a significant elevation in the level of the enzyme to 153 ±44.7 mU/gHb compared with 130 ±29.8 mU/gHb in the fresh samples. This increased enzyme level appeared to be confined to samples from males. The 11 blood samples excluded from the series because of marked haemolysis had a mean enzyme level of 201 ±28.9 mU/gHb which was significantly higher than that of any other group studied. It was of interest that all of these samples were from males. These observations suggested that high values were obtained when a significant proportion of the cells were haemolysed and that the elevated values found in the frozen glycerolized blood may have been due to a degree of haemolysis in some of the frozen samples. It is possible that the older (denser) cells with low levels of pyrimidine 5'-nucleotidase were more susceptible to haemolysis

leaving a younger cell population with higher enzyme activity. This would have had a marked effect because of the great difference in enzyme levels between the oldest and youngest cells (Figure 14 page 74). The results also suggest that the erythrocytes from males were more susceptible to haemolysis during freeze storage procedures than erythrocytes from females.

An alternative explanation for enhanced activity in the frozen samples was the possible disappearance of an inhibitor during storage. It seemed that there was an inhibitor present in the haemolysate because the $K_M$ of the enzyme in a haemolysate was 0.04 mM compared to 0.01 mM in a sample of the purified enzyme. The $K_M$ of the enzyme in a haemolysate prepared from a blood sample stored frozen for 18 months was 0.04 mM which was not different from values found in fresh haemolysates. Small increases in enzyme activity in ACD stored blood were also observed but this has not been studied sufficiently and the nature of the inhibitor has not been defined. Preliminary investigation of the effects of ATP and 2,3-diphosphoglycerate, both of which decrease during storage, suggested that these compounds were not responsible for the inhibition.

In spite of the higher enzyme levels found in the frozen glycerolized blood these samples should be satisfactory for screening a population for deficiency, but the fact that the values may be slightly higher should be kept in

mind. It is also apparent that in preparing samples for storage care should be exercised to minimize haemolysis.

At least two of the families previously reported and the family with pyrimidine 5'-nucleotidase deficiency reported here, were of Ashkenazi Jewish origin (Valentine et al. 1974; Ben-Basset et al. 1976). This suggested that the condition might be more common in the Jewish population. On this assumption, the distribution of the enzyme in Jewish and Gentile subjects was compared using frozen glycerolized blood samples. Although the Jewish group had one subject below 80 mU/gHb and two below 90 mU/gHb compared with none below 90 mU/gHb in the Gentile group, the cumulative frequency polygons showed the Jewish group to be displaced towards higher enzyme levels. However, a Karmogarov-Smirnov test showed no significant difference between the two distributions ($P>0.1$).

## Density Gradient Separation of Erythrocytes

An attempt was made to distinguish the heterozygote by separating the erythrocytes by density gradient centrifugation. As with other erythrocyte enzymes, the least dense cells, which are the youngest, had higher levels of pyrimidine 5'-nucleotidase than the denser cells. It seemed possible that the higher level in some heterozygotes might be due either to higher levels in the young cells or to a greater percentage of these

young cells. Thus an examination of the densest cells might have more clearly shown the heterozygote condition. Figure 14 (page 74) shows the enzyme levels in the erythrocytes of various densities from the enzyme deficient case as well as from both parents. For comparison, values in normal erythrocytes and two cases of pernicious anaemia during treatment with vitamin $B_{12}$ are shown. The distribution in one polycythaemic subject having an abnormally high level of pyrimidine 5'-nucleotidase is also shown. It was apparent that an examination of either the youngest or oldest cells was of no help in the identification of heterozygotes. The finding of 10 times normal levels in the reticulocytes of the treated pernicious anaemia patients along with normal levels in their denser cells suggested that the enzyme was present in high concentration in the premature erythrocyte and was lost very rapidly as the cell matured.

## Erythrocyte Nucleotides in Enzyme Deficiency and Normals

Spectroscopic examination of PCA extracts of erythrocytes:

Spectroscopic examination of the PCA extract of pyrimidine 5'-nucleotidase deficient erythrocytes (Figure 16 page 82) indicated that nucleotide levels were approximately threefold higher than in extracts from normal erythrocytes. The position of the

absorption maximum was shifted from 260 nm in the normal cells to 270 nm in the deficient cells. The maximum at 260 nm corresponded to that of adenine nucleotides which accounted for 96% of the nucleotides present in normal cells. The shift to 270 nm indicated the presence of abnormal nucleotides and suggested that a major part of the abnormal nucleotide pool consisted of cytidine nucleotides which have a maximum at 280 nm. These changes in the UV spectrum were the basis of a screening test for pyrimidine 5'-nucleotidase deficiency described by Valentine et al. (1974). Since this condition has an autosomal recessive mode of inheritance the parents of the index case were obligatory heterozygotes. In spite of this only one parent had a pyrimidine 5'-nucleotidase level which was clearly in the heterozygote range (42 mU/g Hb). The second parent had a level of 87 mU/g Hb which approached the lower end of the normal range: 129.5 ±29.7 (SD) mU/g Hb (N=158). In both parents the spectral test was no different from that found in normals and is therefore of no use in identifying heterozygotes. Valentine et al. (1974) also found the extracts from heterozygote erythrocytes had a normal UV spectrum.

Ion exchange chromatography of PCA extracts of erythrocytes:

Ion exchange chromatography of an acid extract of the enzyme-deficient erythrocytes confirmed the presence of large amounts of pyrimidine nucleotides. Adenine

nucleotides, which formed 96% of the nucleotide pool in normal erythrocytes, accounted for only 32% of the nucleotide pool in the deficient cells. In these cells cytidine nucleotides now dominated, contributing 50% of the nucleotide pool. Uridine compounds accounted for 16% and it was not possible to identify the remaining 2% of the nucleotide pool found in the deficient cells.

Study of abnormal nucleotide pool and recent identification of cytidine compound:

Abnormal nucleotides present in the enzyme-deficient cells included the 5' mono-, di- and tri-phosphates of uridine and cytidine as well as a compound which appeared to be UDP-glucose and an unidentified cytidine compound. This compound was later identified as CDP-choline by Swanson et al. (1983). Swanson used a different method employing $^{31}P$ NMR and $^{1}H$ NMR spectra to study distribution of metabolites in normal and pyrimidine 5'-nucleotidase deficient erythrocytes. His findings were in close agreement with those presented here. Paglia et al. (1983) suggested that CDP-choline could be expected to accumulate because of elevated levels of CMP present in the enzyme deficient cells. This excessive CMP could combine with membrane lecithin to reverse the choline phosphotransferase reaction and produce CDP-choline and 1,2-diacylglycerol. It had been expected that CMP and UMP, the two substrates for the deficient enzyme, would be the major contributors to the abnormal nucleotidase pool and that there would be a

smaller accumulation of the di and tri phosphates. In the case of the cytidine, the least polar compounds CMP (757 nmol/ml cells) and CDP-choline (1049 nmol/ml cells) predominated. The concentration of CTP was also high (831 nmol/ml cells) but that of CDP was lower (278 nmol/ml cells). The major contributors to the uridine pool were UDP glucose (327 nmol/ml cells) and UDP itself (276 nmol/ml cells), whilst the level of UTP was 220 nmol/ml cells and that of UMP was 108 nmol/ml cells. Surprisingly, UMP was the least rather than the most abundant uridine nucleotide present in the enzyme-deficient erythrocytes.

The fact that the combined amount of CTP and UTP was the same as that of ATP, the main energy source of the erythrocyte, may well have been the basic cause of the haemolytic process present in this condition. Both CTP and UTP may have replaced ATP as substrate for enzymes such as ATPase. If the rate of reaction with the abnormal substrates is different from that when ATP is substrate then the ionic balance of the cell may be upset causing haemolysis. Swanson et al. (1983) reported that the intracellular pH of normal cells was 7.2 while the enzyme deficient cells had an intracellular pH of 7.0 compared to an extracellular pH of 7.4 in both cases.

The enzyme-deficient cells also lacked the FeATP peak found in normals. As the function of this FeATP is at

present not known the significance of its absence is not clear. It seems possible that FeATP may be involved with the transportation of iron from membrane receptors to the sites of haemoglobin formation in the normoblast and reticulocyte and that the compound does not play an important part in the mature cell. The compound does in fact become labelled with radioactive-iron when reticylocytes are incubated with transferrin labelled with radioactive-iron (Torrance 1978, unpublished). It is also possible that the compound was an artefact formed only during the acid extraction procedure.

Apart from the abnormal nucleotide pool it was also evident from the elution pattern that the level of inorganic phosphate in the enzyme-deficient cells was about 50% above normal.

Heterozygote study:

The nucleotide composition of erythrocytes from the heterozygote was normal with no sign of even small amounts of the abnormal metabolites which accumulated in the enzyme-deficient cells. This was disappointing because it gives no help in detecting those heterozygotes who may have had enzyme levels overlapping the lower end of the normal range. Until it is possible to detect these heterozygotes accurately it will not be possible to define the gene frequency of this disorder.

High reticulocyte blood study:

None of the abnormal nucleotides found in the enzyme-deficient cells were present in the blood which had 16% reticulocytes. This indicated that the abnormal nucleotides were not due to the presence of an elevated reticulocyte count but were the direct result of the decreased level of pyrimidine 5'-nucleotidase. The elution pattern of the high reticulocyte blood differed from normal only in that the levels of 2,3-DPG and ATP were elevated as would be expected in anaemia. This virtually normal elution pattern was surprising because Bartlett (1976b) has shown that rat reticulocytes contain much higher concentrations of ATP, GTP, UTP and CTP than normal rat cells and also UDP-acetylhexosamine, and an unidentified cytidine compound not present in mature rat erythrocytes. However, Bartlett used blood with more than 90% reticulocytes and it is possible that to show these compounds clearly the proportion of reticulocytes must be greater than 16% which was the level in this study. It is also possible that the phenylhydrazine treatment used by Bartlett may have inhibited some erythrocyte enzymes resulting in changes in the intracellular composition or alternatively that these compounds are not present in human reticulocytes. The unidentified cytidine compound in rat reticulocytes was eluted after CTP and immediately before ATP. Because the chromatographic system used in this study was similar to that used by Bartlett, CDP-choline in the enzyme-deficient erythrocytes was different and less

polar than the unidentified cytidine compound in rat reticulocytes.

Source of abnormal nucleotides:

There is no evidence to support the suggestion that the abnormal pool of nucleotides in the enzyme-deficient erythrocytes arises from the degradation of RNA in the maturing reticulocyte. Indeed Harley et al. (1978) have calculated that the levels of pyrimidine nucleotides present in the enzyme-deficient cells would not be possible in the presence of the residual levels of enzyme found in the deficient subjects. In addition they showed that the mature erythrocyte was able to synthesize uridine nucleotides via the salvage pathway using either uridine or orotate as substrate. They suggested that the levels of circulating pyrimidine nucleoside precursors were sufficiently high to give rise to the observed erythrocytic nucleotide levels when only minimal levels of pyrimidine 5'-nucleotidase were present. The predominance of cytidine nucleotides would presumably be due either to a higher level of cytidine substrates or a more active cytidine kinase enzyme in the erythrocyte. These observations would mean that in normal erythrocytes there is an active turnover of pyrimidine nucleotides. It seems possible that this might be of functional importance with the mixed compounds of cytidine and uridine playing an important role, for example maintaining membrane integrity. An alternative suggestion was that the erythrocyte may

function as a vehicle for transporting pyrimidines in a similar manner to the mechanism proposed for purines (Murray 1971).

Possible causes of haemolytic anemia:

It appeared that in erythrocytic pyrimidine 5'-nucleotidase deficiency a large pool of abnormal nucleotides accumulated in the erythrocytes. These abnormal compounds may be the cause of the haemolytic anaemia found in this condition but the precise mechanism remains to be elucidated. In 1982 de Verdier et al. (1982) suggested that the haemolysis might be due to unbalanced lipid synthesis in erythrocyte membranes due to high concentrations of CDP-choline. Further studies will also be needed to establish whether the active cycling of pyrimidine compounds through erythrocytes has a functional significance or is a vestige of its metabolic requirements during its nucleated developmental stage.

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