PARTIAL ADENOSINE DEAMINASE DEFICIENCY WITHOUT IMMUNODEFICIENCY : BIOCHEMICAL AND GENETIC STUDIES

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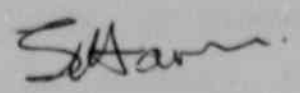
DECLARATION

I declare that this thesis is my own, unaided work and that it has not been submitted for a degree at any other University



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ABSTRACT

The adenosine deaminase enzyme from a Xhosa tribesman has been characterized. Red blood cell activity levels were 6-9% of normal whereas his white cell ADA levels were about 30% of normal. The enzyme's stability at 57°C was shown to be greatly reduced suggesting a mutation resulting in an enzyme with reduced stability *in vivo*. It was concluded that the discrepancy in ADA activity levels between red and white blood cells was due to the red cells being anucleate.

The proband's residual ADA was found to have a Michaelis Constant (K_m) for adenosine of 47.9 ± 15.8μM, a value which is not significantly different from that of normal ADA (51.7 ± 11.4μM).

Red cell deoxy-ATP levels were measured and found to be elevated two-to-three times over normal levels. Red cells from ADA-deficient patients with severe combined immunodeficiency (SCID) have been reported with deoxy-ATP levels elevated about 1 000 times. It was concluded that the slight elevation of deoxy-ATP levels in the proband were too low to have any noticeable effect on functions of his immune system.

Starch gel electrophoresis of red cell ADA from members of the proband's family in conjunction with red cell ADA activity levels suggested that both parents carried a gene for 'partial' ADA deficiency, both of

which had been inherited by the proband as well as one of his sibs. Isoelectric focusing studies suggested that the two, parental ADA partial deficiency genes were different from one another.

It was also found that another rare allele of ADA, possibly *ADA*^{5}, was segregating within the same family although this event appears to be unconnected with the ADA partial deficiency.

CONTENTS

1. INTRODUCTION

1.1. DISCOVERY OF ADENOSINE DEAMINASE DEFICIENCY

As is common with many scientific discoveries, the first enzyme deficiency associated with an immunodeficiency disease was found serendipitously. In the summer of 1972 some blood specimens arrived in the laboratory of Eloise Giblett for routine screening of genetic markers. The patient was a two year-old girl with severe combined immunodeficiency disease (SCID). One of the enzymes routinely tested for electrophoretic variants was adenosine deaminase (E.C.3.5.4.4), which Spencer *et al* (1968) had called ADA. To the surprise of Dr Giblett and her staff, ADA activity was undetectable in the red cells of the child. Her parents had the common ADA-1 electrophoretic phenotype but their ADA catalytic activity was about half normal. Within weeks blood from a second female patient with SCID as well as from her parents was tested. This patient's red cells also lacked ADA activity and her parents had lower than normal levels. Thus it seemed quite possible that the coexistence of these two very rare anomalies was related.

The most likely interpretation of these findings was that the parents of both patients were heterozygous for the common ADA^1 allele and a 'silent' allele at the same structural gene locus. The patients had inherited the 'silent' alleles from their parents and, as a consequence, had very little or no ADA catalytic activity.

In the autumn of 1973 a symposium on SCID was held in Albany, New York, and by this time no fewer than 12 of 22 patients with SCID had been found to be ADA-deficient. The existence of a causal relationship between the enzyme deficiency and SCID seemed highly probable although the mechanism was completely obscure.

Shortly before the Albany meeting the first example of ADA deficiency without immunodeficiency had been described (Jenkins 1973) which if not actually casting doubt upon the causal relationship hypothesis added another dimension to an increasingly intriguing field of research which has increased our understanding of the biochemistry of immunodevelopment and indeed our appreciation of its complexity.

1.2. SEVERE COMBINED IMMUNODEFICIENCY

1.2.1. Description

This syndrome usually presents in infants within the first few weeks of life; there are multiple infections of fungal, bacterial, viral and protozoal origin and these are severe and recurrent. They also suffer from diarrhoea and delayed growth and development. Candida infections are almost characteristically present. If untreated, death occurs within 3 years.

The children are lymphopoenic and show hypoplasia and dysplasia of the thymus. Both cell mediated and

antibody mediated immunity are impaired. T cells are markedly reduced, most patients with SCID having fewer than 5% E-rosetting T cells and often fewer than 2%. Tests for the capacity of cell-mediated immunity such as lymphocyte proliferative responses to antigens and mitogens (eg. phytohaemagglutinin(PHA) and to allogeneic lymphocytes (mixed lymphocyte response (MLR)) are profoundly depressed. They do not reject skin grafts nor do they mount a delayed hypersensitivity skin response either to antigens or after sensitization and challenge with dinitrochlorobenzene (DNCB). Most patients are severely hypogammaglobulinaemic with very low levels or even absence of IgG, IgA and IgE. Circulating IgM levels vary from complete absence to normal levels. Any circulating immunoglobulins that are found are often of maternal origin. Specific antibody responses are absent (Gelfand & Dosch 1983).

Some of the more common clinical and laboratory features of SCID are described above. The name 'SCID', however, represents a wide spectrum of disorders of complex heterogeneity but despite this patients all manifest a profound impairment of functional activity in T and B cells.

1.2.2. ADA deficiency and SCID

SCID can be inherited in an autosomal recessive or X-linked recessive fashion. ADA deficiency has been

found to be associated with one third to one half of SCID cases where the mode of inheritance is not obviously X-linked. A survey of 130 patients with SCID including both autosomal recessive and X-linked modes of inheritance (Hirschhorn *et al* 1979; Hirschhorn 1979; Ackeret *et al* 1976) revealed 22% with ADA deficiency.

The clinical presentation of the two cases reported by Giblett *et al* (1972) was not, however, exactly as described above. Both patients showed a clear impairment of cell mediated and humoral immunity but humoral immunity was not as severely affected as cell mediated immunity. In fact, some specific antibody responses were demonstrated and one patient remained healthy until the age of 2 years, older than any other child at presentation. Clinical manifestations usually develop when the child is aged 3-6 months but it appears that 10-15% of SCID cases due to ADA deficiency suffer from a less severe, more gradual, but nonetheless, if untreated lethal form of the disease. This seems to be due to normal or increased numbers of B cells although the antibody response reveals qualitative and quantitative abnormalities. Ig levels however, eventually fall. The clinical heterogeneity of ADA-deficient SCID could be due to genetic heterogeneity or random differences in exposure to foreign agents.

During the early studies on patients with ADA

deficiency it appeared that two features might discriminate between ADA-deficient and non-ADA-deficient SCID patients. The first of these were bony abnormalities, evident on physical examination, as prominence of the costochondral junctions similar to a rachitic rosary (Wolfson & Cross 1975; Cederbaum *et al* 1976). Cupping and flaring of the costochondral junctions are seen on X-ray examination as is a dysplastic pelvis. These changes are not, however, pathognomonic of ADA-deficient SCID because similar radiologic changes can be observed in non-immunodeficient patients who are severely malnourished as well as in ADA-normal immunodeficient patients (Hirschhorn, Vawter *et al* 1979). Despite the fact that these bony abnormalities are not specific, they have been clinically useful in alerting physicians to the possibility of ADA-deficient SCID. The second postulated specific feature of ADA-deficient SCID was a difference in thymic pathology; ADA-deficient patients retained some Hassall corpuscles (Huber & Kersey 1975), suggesting secondary atrophy of a previously differentiated thymus although subsequent studies have failed to find a correlation (Hirschhorn, Vawter *et al* 1979).

Neurological abnormalities have been reported in three cases of ADA-deficient SCID which seem to reflect the interaction of accumulated purines with

purinergic receptors on brain cells (Hirschhorn 1979; Polmar *et al* 1976; Hirschhorn *et al* 1983).

1.3. METABOLIC ROLE OF ADA

Adenosine deaminase is a key enzyme in the purine salvage pathway. It catalyzes the irreversible deamination of adenosine and deoxyadenosine by hydrolysis to inosine and deoxyinosine respectively, and occurs in all tissues where it has been looked for (Van der Weyden & Kelley 1976). The specific catalytic activity of ADA is highest in normal lymphoid tissues, particularly the thymus and spleen (Van der Weyden & Kelley 1976; Carson *et al* 1977; Martin & Gelfand 1981). In lymphoid cells the activity is higher in the T cells (Barton *et al* 1979) than in the B cells.

The processes of *de novo* synthesis of purines and pyrimidines generate inosine monophosphate and uridine monophosphate respectively, which are compounds with completed heterocyclic rings (Figs 1-2). The presence of the charged phosphates greatly hinders their transport across cell membranes. Mammalian tissues possess one or both kinds of nucleotidase, intracellular (Magnusson 1971; Fritzson 1978) and extracellular (De Pierre *et al* 1974; Fleit *et al* 1975) which remove the alpha-phosphates, thereby generating nucleosides that traverse cell membranes both by passive diffusion (Plagemann 1971) and facilitated transport (Plagemann 1971; Plagemann & Erbe 1974; Cohen, *et al* 1979).

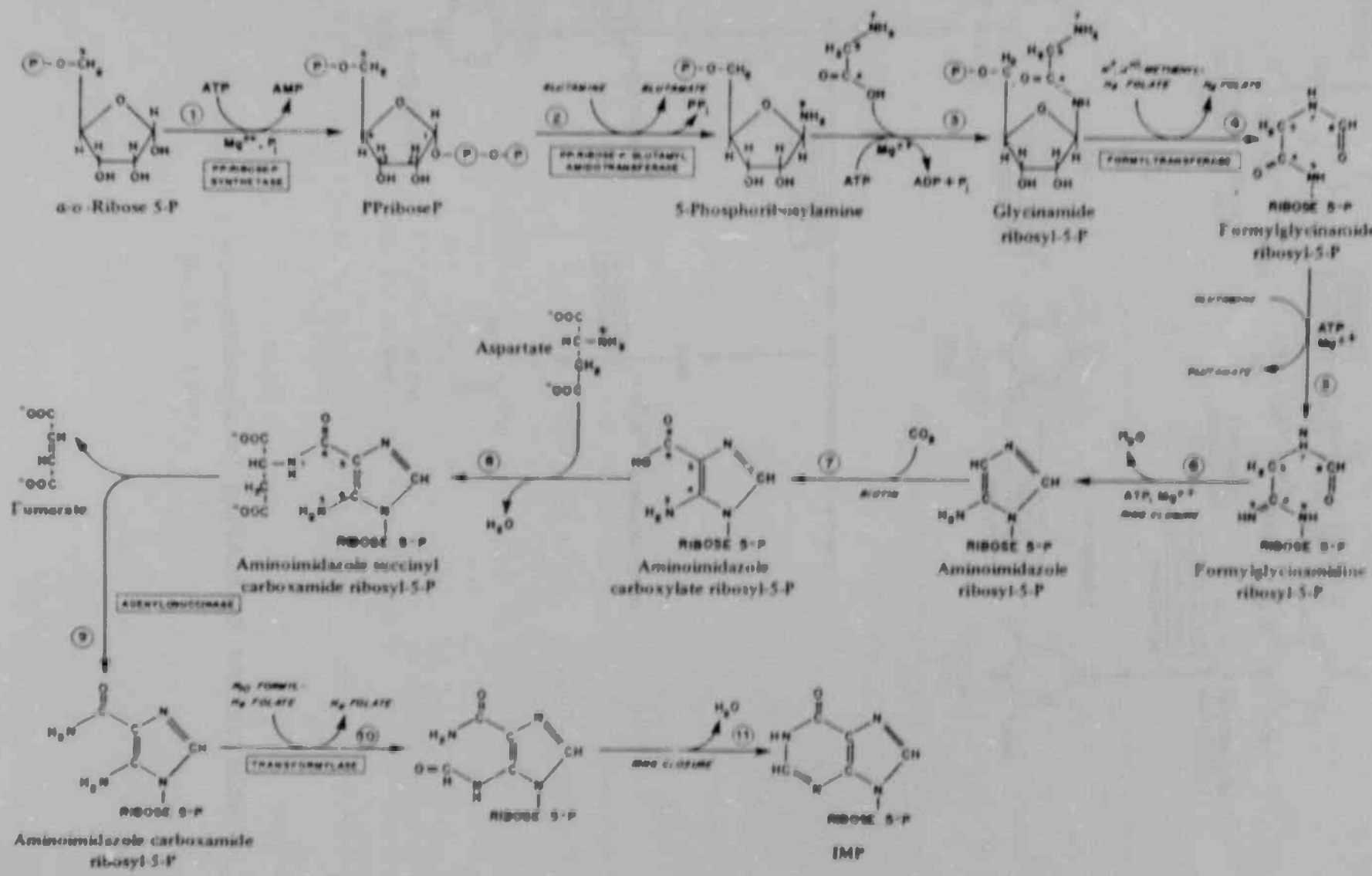


FIGURE 1 : Biosynthesis of purines (Martin 1981).

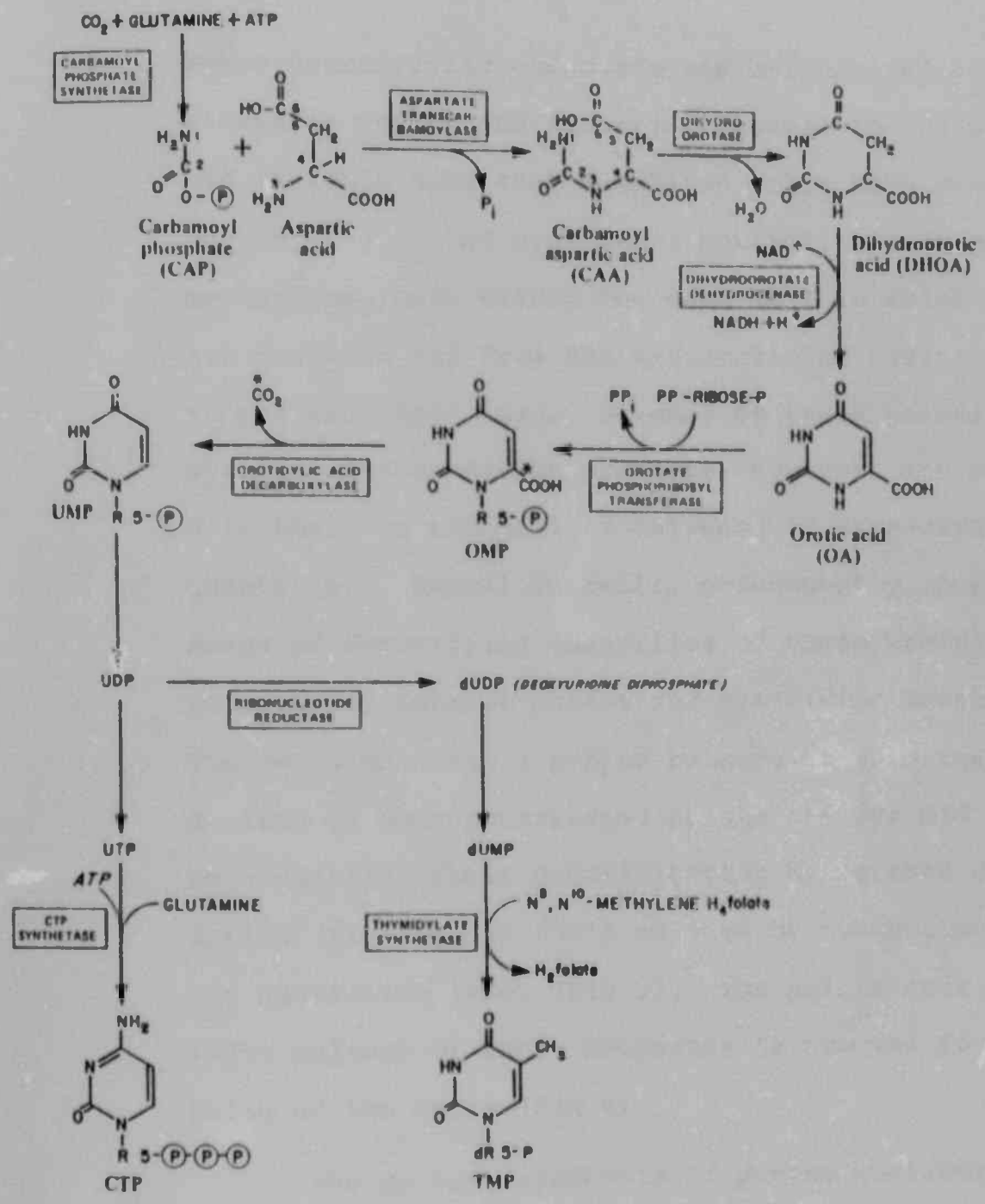


FIGURE 2 : Biosynthesis of pyrimidine nucleotides (Martin 1981).

These heterocyclic compounds are synthesized at considerable energy and substrate expense to the cells and it would seem that mammalian cells have evolved purine (Fig 3) and pyrimidine metabolite salvage mechanisms, both within the same cell in which they are produced and from the extracellular environment to prevent their loss. Several of these purine and pyrimidine degradation products, however, are potentially toxic to the cell if salvaged in excessive quantities. Mammalian cells, consequently, possess a means of detoxifying quantities of these useful but potentially harmful purine and pyrimidine metabolites. The cells maintain a proper balance between the production of dephosphorylated purine and pyrimidine metabolities, their detoxification by further degradation (Fig 4), and their salvage by metabolism back to the nucleotide level (Fig 5). The maintenance of the right balance of these processes is crucial to the well-being of the cells (Fig 6).

The *de novo* synthesis of purine nucleotides is controlled by feedback inhibition at several sites (Fig 1).

Firstly, feedback inhibition of 5-phosphoribosyl-1-pyrophosphate synthetase by the purine nucleotides AMP, GMP and IMP regulates the level of 5-phosphoribosyl-1-pyrophosphate (PRPP). The committed step in purine nucleotide biosynthesis is the conversion of PRPP into phosphoribosylamine by transfer of the side-chain amino

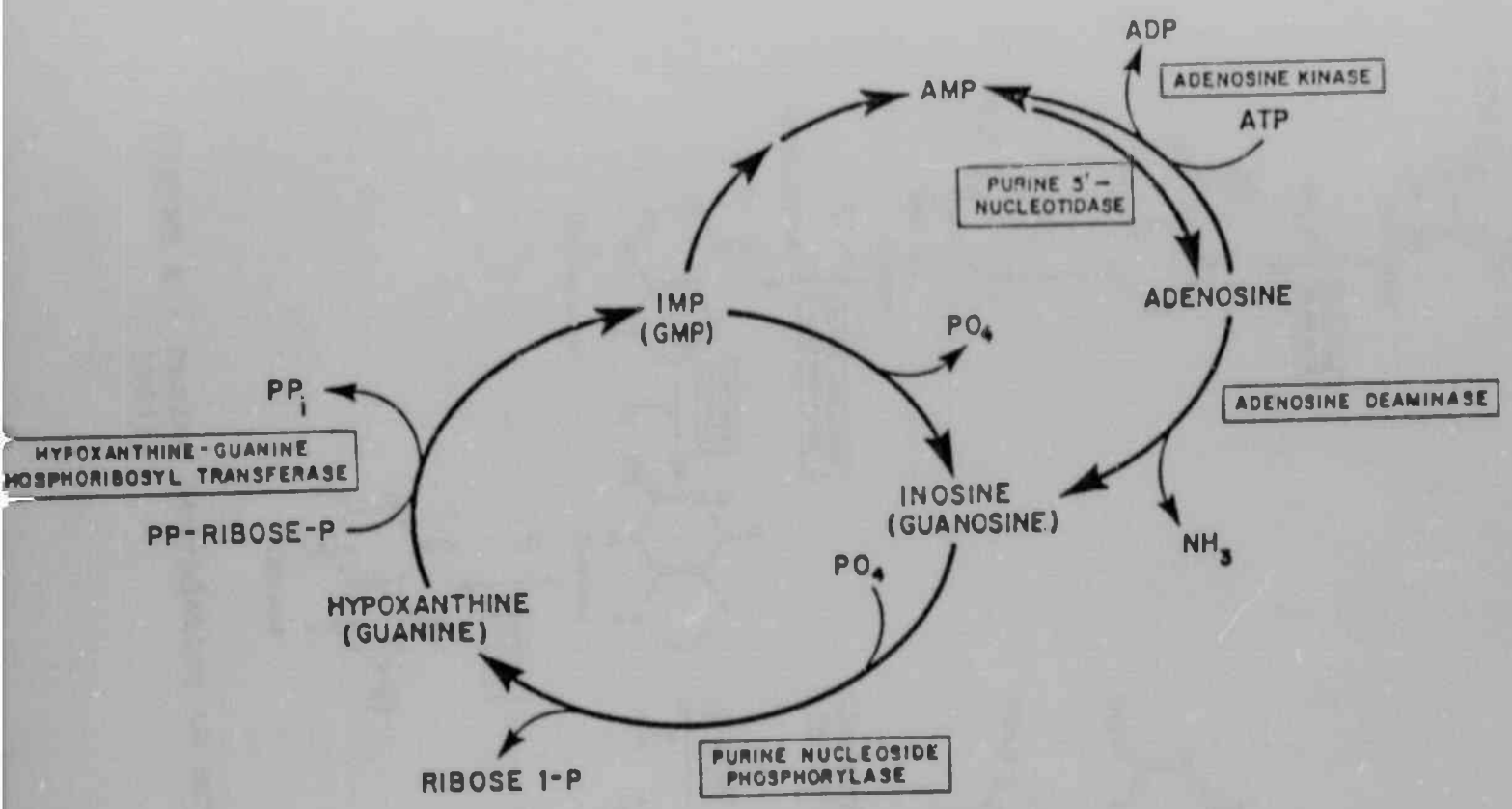
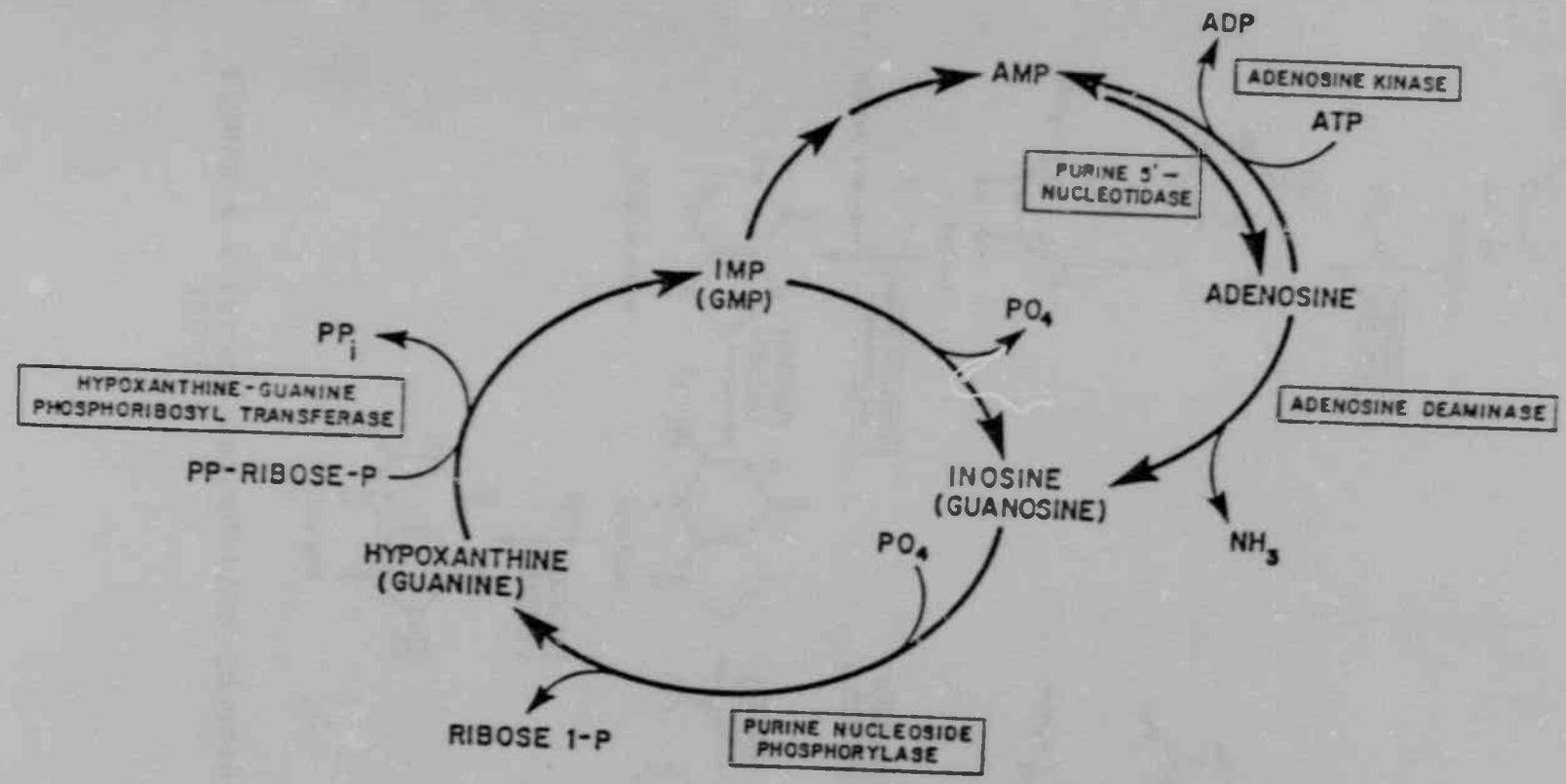


FIGURE 3 : The purine salvage cycles involving the interconversion of AMP, IMP and- to a lesser extent - GMP to their respective ribonucleosides and their eventual reconversion to purine ribonucleotides. Deoxyadenosine, deoxyinosine and deoxyguanosine share the same pathways except that deoxyadenosine and deoxyguanosine can be directly phosphorylated to deoxy AMP and deoxy GMP respectively (Martin 1981).



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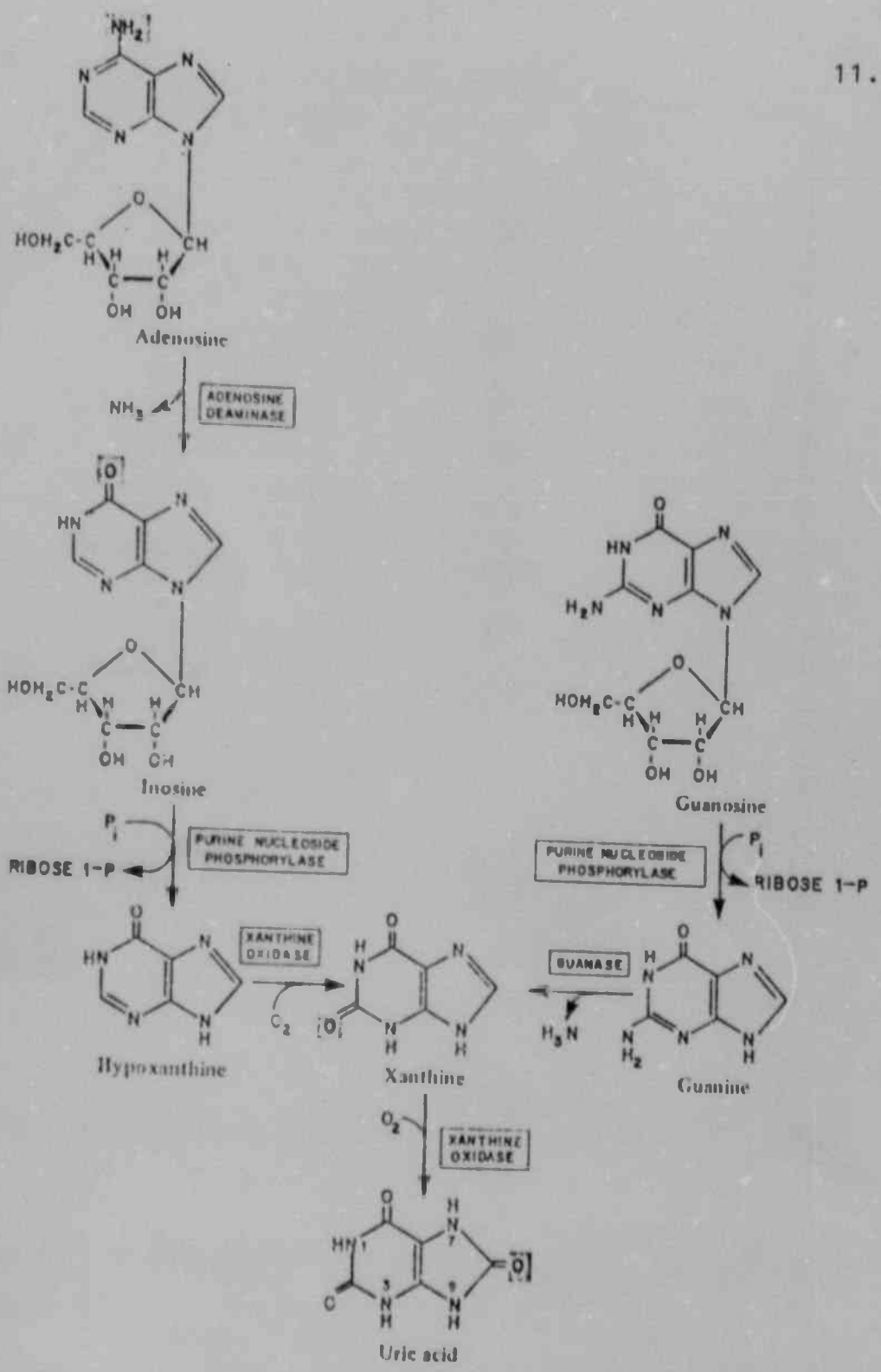


FIGURE 4 : Purine degradation to uric acid (Martin 1981).

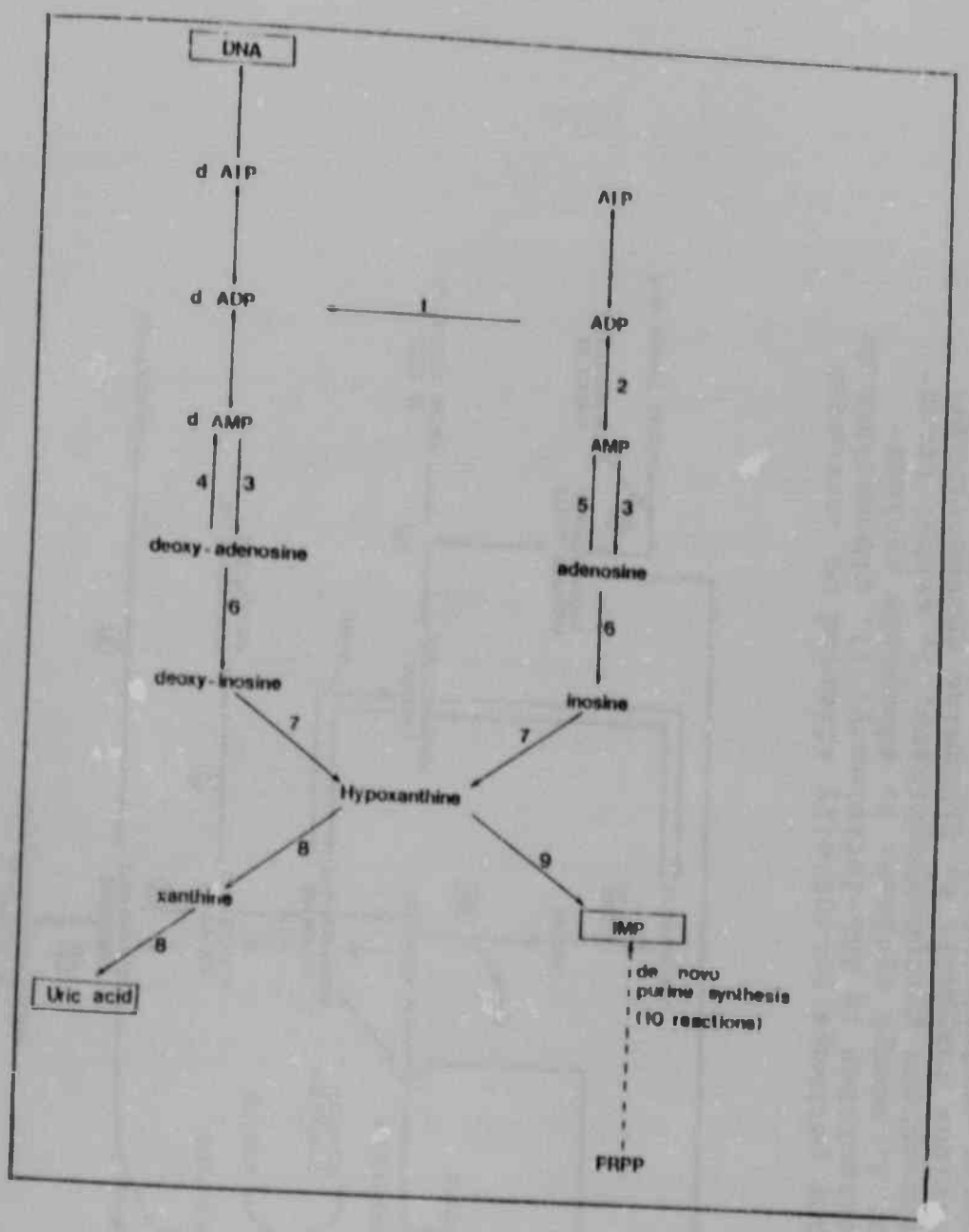


FIGURE 5 : Adenosine and deoxyadenosine metabolism (1, ribonucleotide reductase; 2, adenylate kinase; 3, 5'-nucleotidase; 4, deoxycytidine kinase; 5, adenosine kinase; 6, adenosine deaminase; 7, nucleoside phosphorylase; 8, xanthine oxidase; 9, hypoxanthine guanine phosphorybosyl transferase.

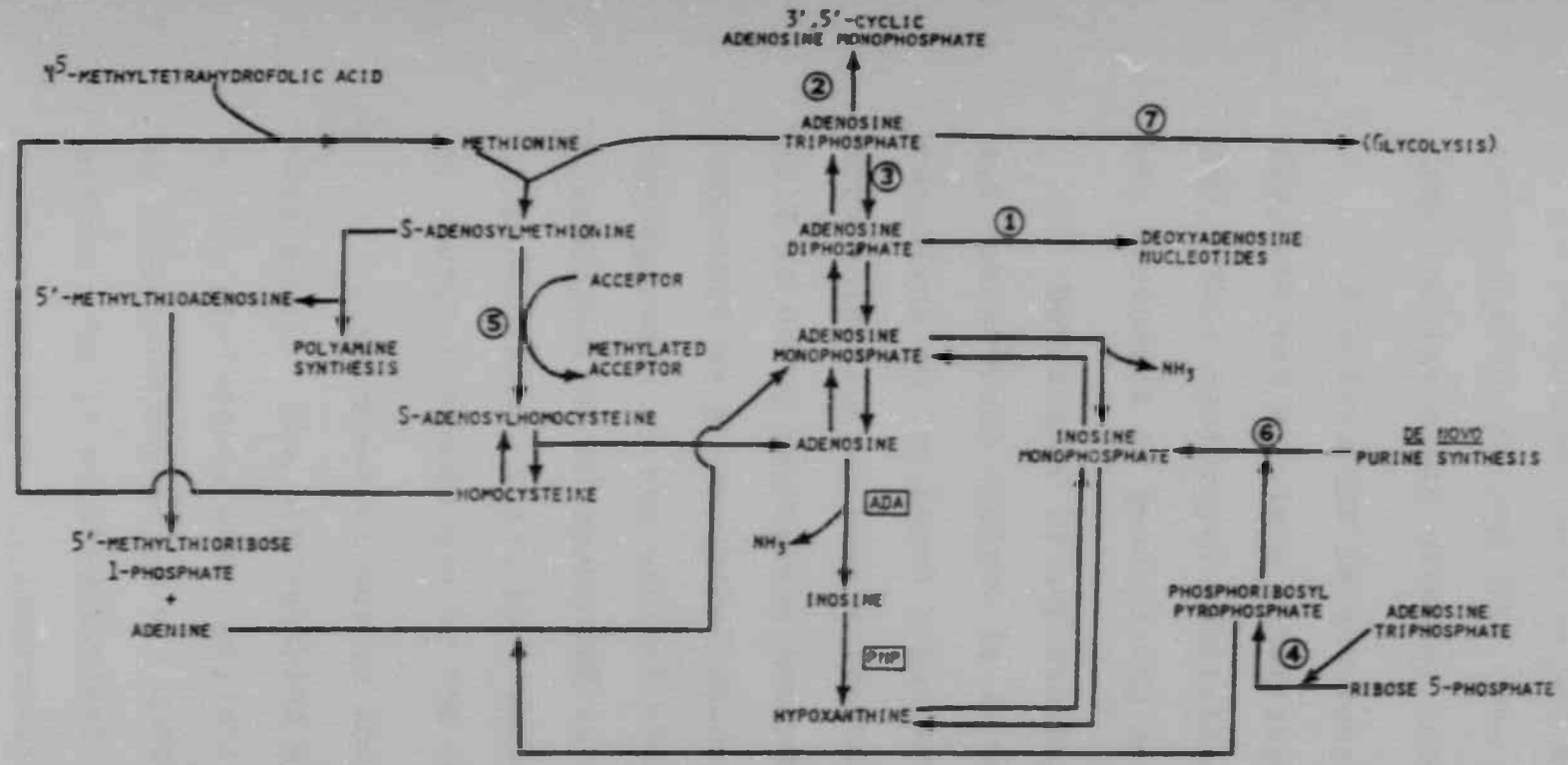
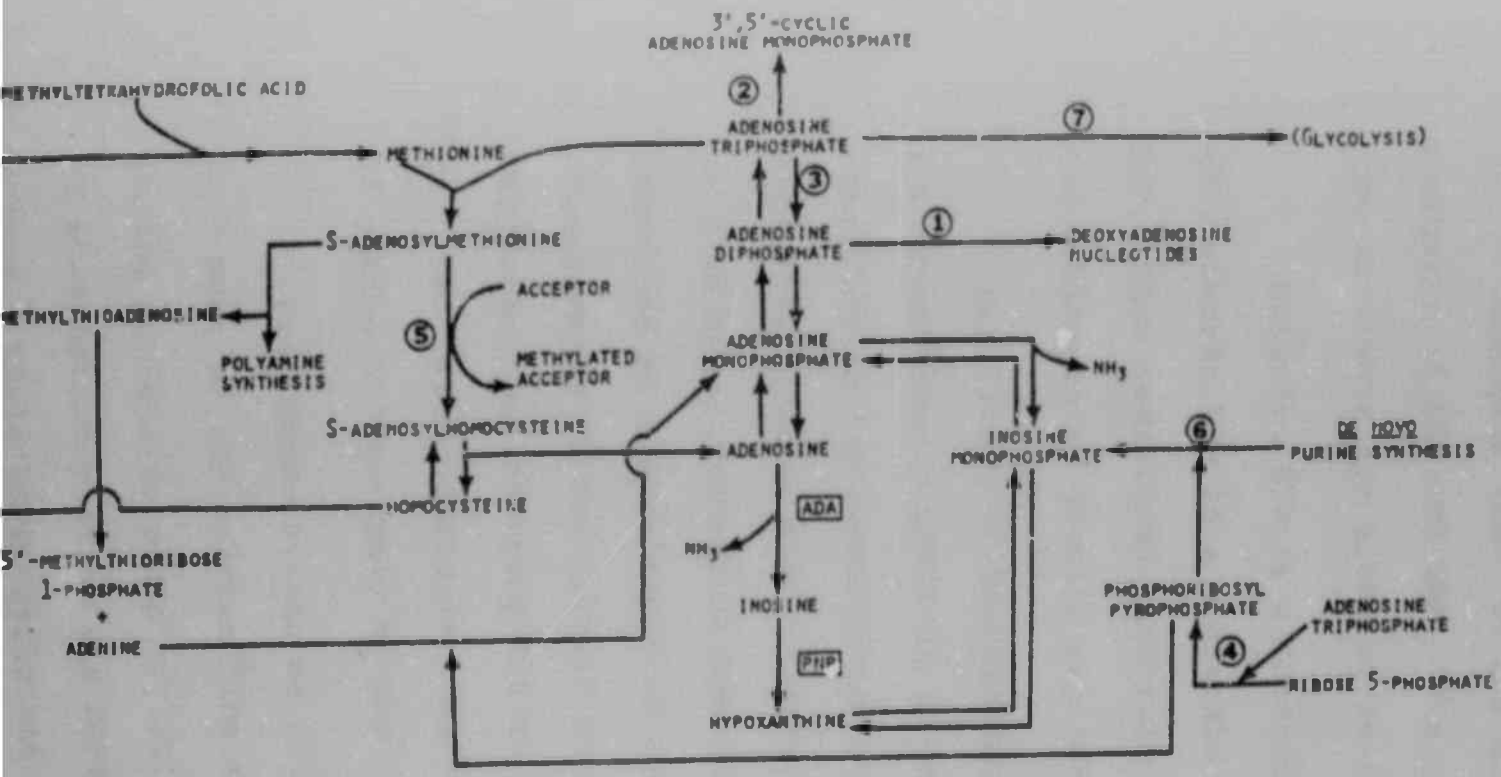


FIGURE 6 : Some purine pathways potentially affected by increased purine metabolites in ADA-deficiency: (1, ribonucleotide reductase; 2, adenyl cyclase; 3, adenylate cyclase; 4, ribosephosphate pyrophosphokinase; 5, methyl transferase (various enzymes); 6, glytamine phosphoribosyl pyrophosphate amidotransferase.



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group of glutamine. PRPP glutamyl aminotransferase is feedback inhibited by many purine ribonucleotides.

Inosinate (IMP) is the branching point in the synthesis of AMP and GMP. The reactions leading away from inosinate are sites of feedback inhibition.

Finally, GTP is a substrate in the synthesis of AMP, whereas ATP is a substrate in the synthesis of GMP. This reciprocal substrate relation tends to balance the synthesis of guanine and adanine ribonucleotides.

Deficiency of hypox[illegible]-guanine phosphoribosyl transferase (HGPRT) is [illegible] born error of metabolism resulting in the Lesch-Nyhan syndrome. Those affected with this disease have a tendency to self-mutilate as well as being aggressive towards others. The disease is inherited as a sex-linked recessive. The biochemical consequences of the virtual absence of HGPRT are an overproduction of urate and an elevated concentration of PRPP. Also, there is a marked increase in the rate of purine biosynthesis by the *de novo* pathway.

From observations on these patients it is apparent that most of the hypoxanthine and guanine produced by purine nucleoside phosphorylation, are normally salvaged by phosphoribosylation via HGPRT, a PRPP consuming reaction. While HGPRT-deficient Lesch-Nyhan patients with purine nucleoside phosphorylase deficiency cannot salvage hypoxanthine and guanine due to a deficiency of

these substrates for the salvage reaction. Accordingly, both Lesch-Nyhan patients and purine nucleoside phosphorylase deficient patients 'spare' PRPP, which in turn drives the *de novo* purine biosynthesis pathway to overproduction. The latter seems to account for the massive total purine overproduction in the patients with purine nucleoside phosphorylase deficiency and those with Lesch-Nyhan.

Adenosine or 2'-deoxyadenosine may be generated within the cell or they may enter the cell by one of several facilitated diffusion transport mechanisms (Fox & Kelley 1978, Fig 7). Adenosine and 2'-deoxyadenosine can be formed by the dephosphorylation of AMP or 2'-deoxy-ATP respectively. Ecto 5'-nucleotidase possess 5'-phosphomonoesterase activity and is localized in the external surface of the plasma membrane of lymphocytes (Webster *et al* 1974; De Pierre & Karnovksy 1974). This enzyme is important in allowing the uptake of adenosine from the extracellular AMP pool in human lymphocytes. Intracellular 5'-AMP is degraded to adenosine by a specific endo 5'-nucleotidase (E.C.3.1.1.3.5) or by non-specific phosphatases. The K_m of ecto 5'-nucleotidase has been reported as 8.9μM for AMP substrate (Webster *et al* 1979), and 67μM for endo 5'-nucleotidase (Fox & Kelley 1978) whereas the K_m of alkaline phosphatase, a non-specific phosphatase is 400μM for AMP (Fox & Kelley 1978) although of course, there are factors other than K_m which determine whether

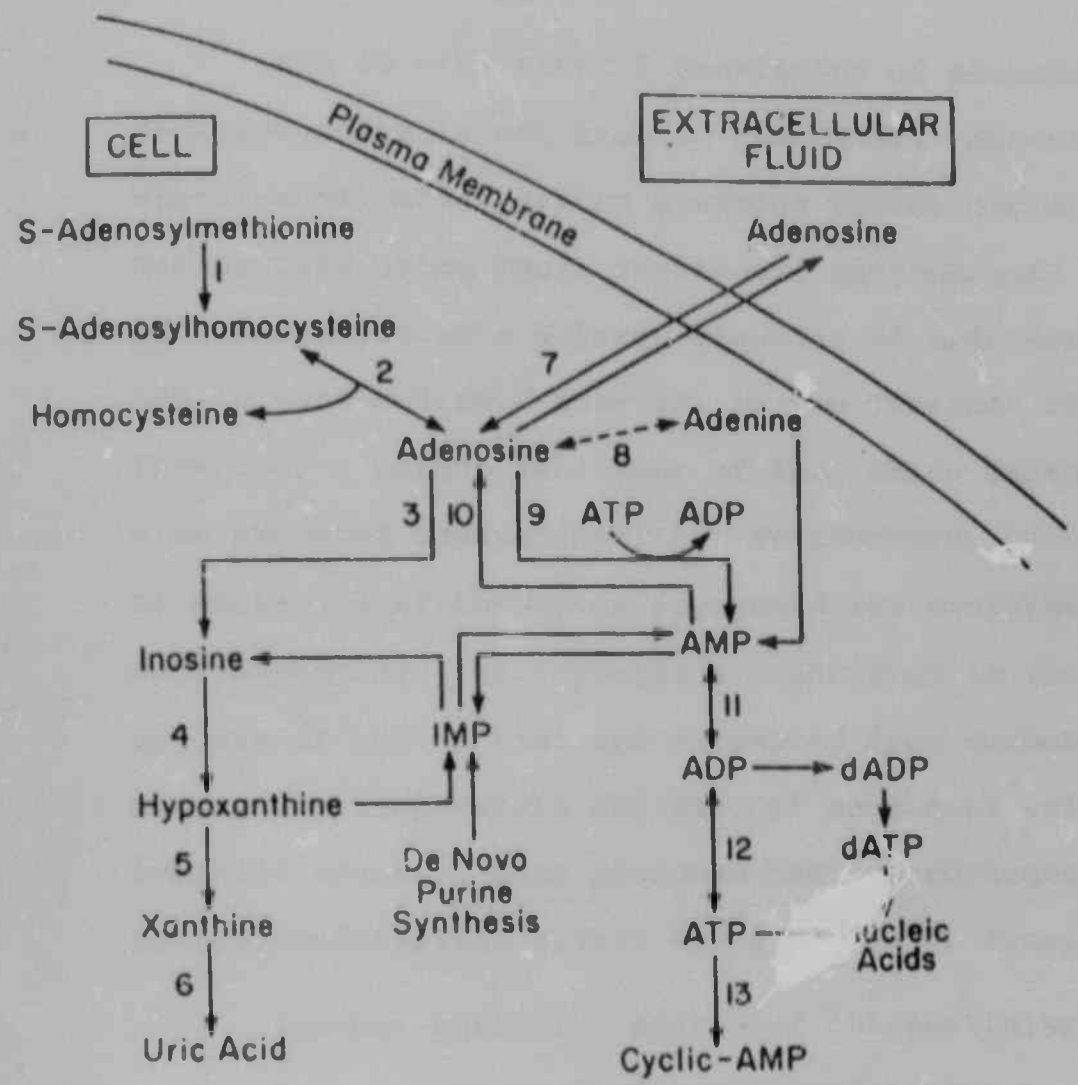

FIGURE 7 : Metabolism of adenosine - its formation and degradation within cells (Fox & Kelley 1978). (1, S-adenosylmethionine methyltransferases; 2. S-adenosylhomocyteine hydrolase; 3, adenosine deaminase; 4, purine nucleoside phosphorylase; 5 & 6, xanthine oxidase; 7, transport mechanisms; 8, adenosine phosphorylase (not establsihed); 9, adenosine kinase; 10, 5'-nucleotidase and non.specific phosphatase; 11, adenylate kinase; 12, nucleoside diphosphokinase; 13, adenylate cyclase.

one enzyme or another plays the more significant rôle in dephosphorylating AMP.

The *in vivo* site of generation of adenosine and deoxyadenosine is not known. Chan (1979) described an experimental model system studying purine excretion by macrophages using mouse peritoneal macrophages. Normally, macrophages excrete a large quantity of uric acid into the culture medium. However, in the presence of deoxycoformycin, a potent inhibitor of ADA, these macrophages also excreted deoxyadenosine. Furthermore, phagocytosis of nucleated erythrocytes augmented the excretion of deoxyadenosine. Macrophages are involved in the phagocytosis of nuclei that are extracted from normoblasts during erythrophoiesis and also of senescent cells in lymphoid organs. Chan proposed that macrophages of the reticuloendothelial system are a source of deoxyadenosine.

Another potential source of intracellular adenosine is the degradation of S-adenosylhomocysteine to adenosine and homocysteine (Fig 8). This hydrolysis reaction is catalyzed by the enzyme S-adenosylhomocysteine hydrolase (SAHH, E.C. 3.3.1.1) which in humans has its highest activity in liver, pancreas and kidney (Fox & Kelley 1978). The equilibrium of the reaction strongly favours formation of S-adenosylhomocysteine. The *in vivo* reaction favours degradation since the breakdown products, adenosine and homocysteine, are more easily removed by further degradation than S-adenosylhomocysteine (De la Haba &

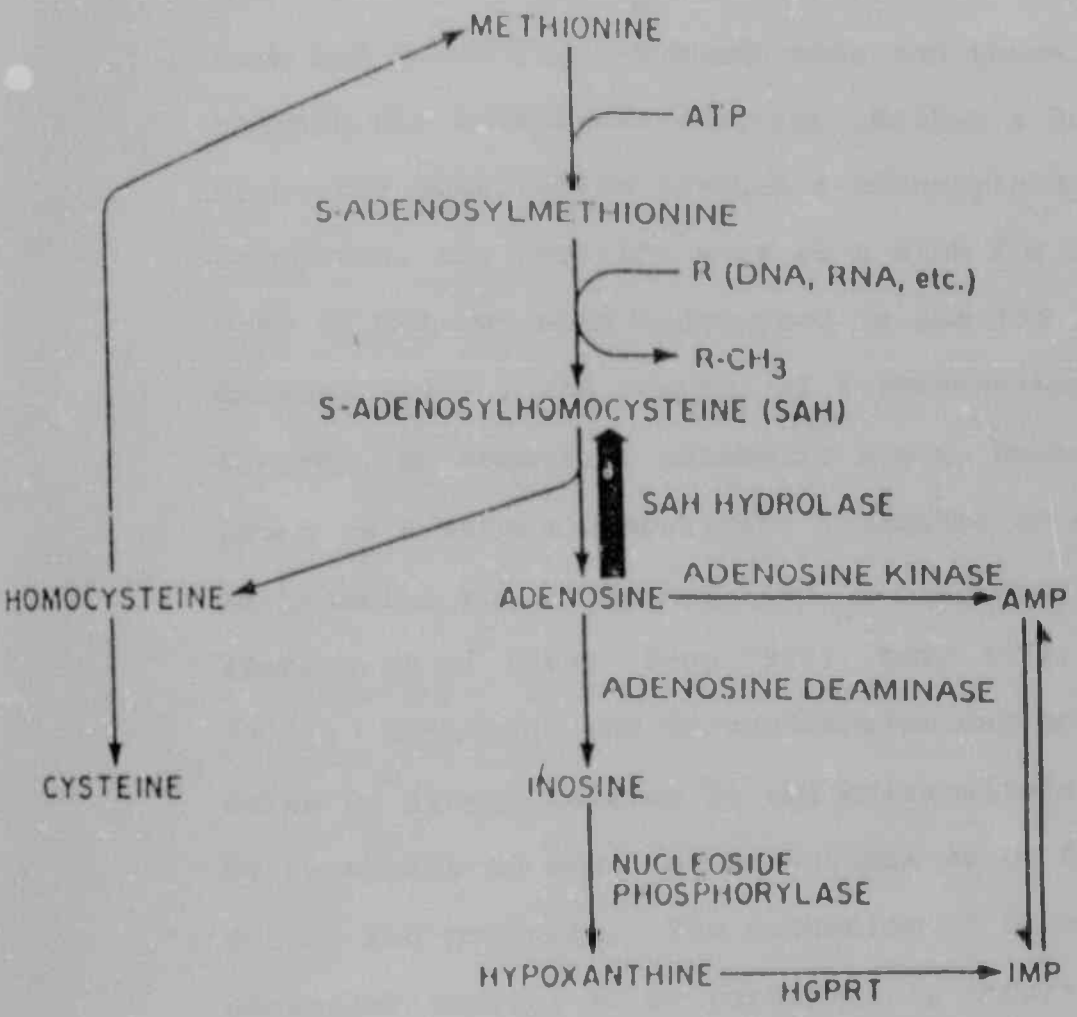

FIGURE 8 : Relationship of transmethylation to adenosine metabolism (Kredich *et al* 1978).

Cantoni 1959). The K_m of the enzyme is 1.5mM for adenosine and 4.5mM for L-homocysteine and these compounds inhibit the hydrolytic reaction (Walker & Duerre 1975). Since the equilibrium favours S-adenosylhomocysteine formation, the reaction acts as a sink for adenosine when it has not been hydrolysed by ADA (De la Haba & Cantoni 1959). The removal of S-adenosylhomocysteine is, however, an important metabolic event, because this compound is a strong competitive inhibitor of all transmethylation reactions mediated by S-adenosylmethionine (Zappia *et al* 1969; Pegg 1971; Kerr 1972; Glick *et al* 1975). Adenosine and deoxyadenosine may be removed from cells by direct release to the extracellular environment, by synthesis to adenine nucleotides or by degradation to purine end-products. The mechanism of direct release of adenosine appears to be particularly important in the liver suggesting that the liver acts as a potential source of purine substrate to other tissues (Lerner & Lowy 1974; Pritchard *et al* 1975). The mechanism of release is not known.

The degradation of adenosine and deoxyadenosine begins with their deamination by ADA to inosine and deoxyadenosine, respectively. Although the deamination of adenosine and deoxyadenosine is probably a necessary detoxification process, it is in humans a quantitatively minor contributor to the overall degradation of purines to the usual excreted form, uric acid. Those patients

with adenosine deaminase deficiency have normal levels of plasma and urinary urate (Mills *et al* 1978; Simmonds *et al* 1978).

The K_m of ADA in lymphocytes, fibroblasts and erythrocytes for adenosine and deoxyadenosine is about 40μM to 70μM for both (Dadonna & Kelley 1979; Hirschhorn *et al* 1976). The differences in the level of enzyme activity in various tissues appears to be related to the molecular form of the enzyme that predominates. There appear to be two soluble molecular forms of ADA with molecular weights of approximately 36 000 and 298 000 (Van der Weyden & Kelley 1976). These two forms are interconvertible in the presence of a protein that has an apparent molecular weight of 200 000 and has no adenosine deaminase activity (Van der Weyden & Kelley 1976). The small form of the enzyme predominates in tissue preparations exhibiting the higher specific enzyme activities such as spleen and stomach and which have no conversion activity whereas the large form of ADA predominates in tissue extracts exhibiting the lower enzyme activities and plentiful conversion activity such as kidney and lungs (Van der Weyden & Kelley 1976). An optimal level of ADA activity is essential for normal cell function in man since a deficiency of the enzyme is associated with an increased intracellular concentration of adenine mucleotides (Agarwal *et al* 1976; Polmar *et al* 1976) and an increased level of activity with the opposite effect

(Valentine *et al* 1977).

The removal of adenosine and 2'-deoxyadenosine by their conversion to nucleoside monophosphates is catalyzed by adenosine kinase (Fig 9) or, more importantly for deoxyadenosine, deoxycytidine kinase. Adenosine kinase occurs in all tissues but has it's highest activity in liver, spleen and lymph nodes (Krenitsky *et al* 1974).

The K_m value of adenosine kinase for adenosine (0.4-6.0uM) is significantly lower, by an order of magnitude, than that of ADA for adenosine whereas for deoxyadenosine the K_m of ADA is an order of magnitude lower than either adenosine kinase (400-1800μM) or deoxycytidine kinase (330-400μM) (Fox & Kelley 1978; Martin & Gelfand 1981; Ochs *et al* 1979; Carson & Kaye 1979).

When adenosine enters the cells, several factors determine whether it is to be converted to nucleotides or inosine. The utilization of adenosine is dependent upon the relative activities of ADA and adenosine kinase. At low concentrations adenosine is preferentially phosphorylated to AMP, but at concentrations above 3μM, degradation of adenosine to inosine and hypoxanthine is favoured (Schrader *et al* 1972). Deoxyadenosine will be preferentially deaminated by ADA at normal intracellular concentrations with phosphorylation occurring only at high concentrations, whether it be by adenosine kinase or deoxycytidine kinase (Carson *et al* 1979; Carson & Kaye 1979; Ullman *et al*1981).

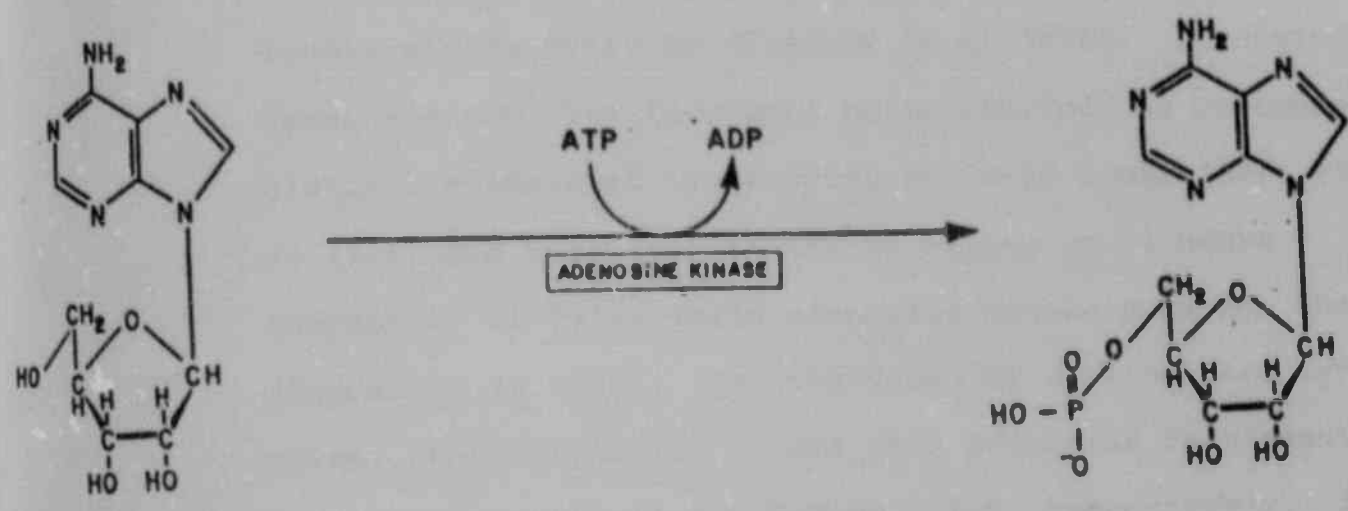

FIGURE 9 : Phophorylation of adenosine to AMP by adenosine kinase (Martin 1981).

Adenosine and deoxyadenosine metabolism may be altered during cell proliferation. Adenosine deaminase activity increases in regenerating mouse liver (Rothman *et al* 1971). Transformation of monocytes to macrophages is accompanied by a two-to-ninefold increase in the levels of ADA activity (Fischer *et al* 1976). Adenosine deaminase activity increases up to fourfold in phytohaemagglutinin-stimulated lymphocytes at 24-60 hours (Hovi *et al* 1976) and decreases to 44% of values at 72 hours (Snyder *et al* 1976) while adenosine kinase does not change (Snyder *et al* 1976). In stimulated or unstimulated lymphocytes, phosphorylation occurs when adenosine is present at a concentration below 5μM or 0.5μM, respectively. It seems possible that there is either an increased formation of adenosine or an increased entry of adenosine into these stimulated lymphocytes. Nucleoside transport rates have, indeed, been found to increase during cell proliferation (Berlin & Olivier 1975). Inhibition of ADA with or without the addition of adenosine or deoxyadenosine prevents the proliferation of monocytes and lymphocytes (Snyder *et al* 1976; Carson *et al* 1977; Fischer *et al* 1976; Hovi *et al* 1976; Carson & Seegmiller 1976; Fox *et al* 1975; Ullman *et al* 1976). Thus adenosine metabolism, modulated by ADA activity may be important during cell proliferation.

1.4. PURINE NUCLEOSIDE PHOSPHORYLASE (PNP)

Purine nucleoside phosphorylase catalyzes the conversion

of inosine and deoxyinosine, the products of ADA, to hypoxanthine and ribose 1-phosphate and deoxyribose-1-phosphate. The structural gene for purine nucleoside phosphorylase in humans has been assigned to the q13 band of chromosome 14 and codes for a 38 000 molecular weight subunit that combines with two other identical subunits to form the trimeric holoenzyme. This enzyme occurs throughout all human tissues but the peripheral blood lymphocytes, granulocytes and erythrocytes and the kidney contain the highest specific catalytic activities. Absence of this enzyme is associated with an immunodeficiency syndrome which is distinct from ADA-deficient associated SCID (Martin & Gelfand 1980).

Features of PNP-deficient immunodeficiency are severely depressed T cell functions as assayed by the response of lymphocytes to mitogens and allogeneic cells and marked reduction in T cell numbers. B cell immunity is normal or enhanced as evidenced by normal to elevated immunoglobulin levels, normal antibody response to antigens (even when T cell immunity is absent), normal numbers of B cells in the peripheral blood and normal numbers of plasma cells in lymphoid tissue. Biochemically the disorder is characterized as having normal to elevated adenosine deaminase levels, less than 1% of normal purine nucleoside phosphorylase activity, reduced serum uric acid levels, increased plasma levels of inosine, deoxy-

inosine, guanosine and deoxyguanosine, increased intracellular concentrations of deoxy-GTP and increased urinary excretion of inosine, guanosine, deoxyinosine and deoxyguanosine (Martin & Gelfand 1980).

In both ADA and PNP deficiency the primary target for metabolic damage appears to be the T cell, although ADA-deficient patients also develop B cell dysfunction at some stage of their immunodeficiency disease. The fact that B cell function is normal or nearly normal in PNP deficiency is likely to reflect a difference in the metabolic pathways between the two cell types. The nearly complete containment of cellular dysfunction to lymphoid cells in both diseases indicates the dependence of these cells on part of the purine salvage pathway not apparently necessary in other cells of the body (Martin & Gelfand 1980).

1.5. MECHANISMS RELATING ADA DEFICIENCY TO SCID

1.5.1. Adenosine-induced pyrimidine starvation (Figs 1 & 2)

This theory was based on the finding that abnormal concentrations of adenosine caused elevated levels of purine nucleotides and reduced levels of pyrimidine nucleotides (Green & Chan 1973). The growth rate of a cultured lymphoblastoid cell line established from blood cells of a patient with infectious mononucleosis was affected in this way when incubated with adenosine at concentrations higher than 1μM. This effect was prevented when uridine was provided as a source of extra pyrimidines indicating

that the adenosine induced toxicity was mediated by the lack of pyrimidines.

The addition of adenosine to cultured mammalian cells, also leads to an accumulation of orotic acid which appears to be due to a reduction in the activity of orotate phosphoribosyltransferase (OPRT) the enzyme that catalyzes the conversion of orotic acid to orotidine 5'-monophosphate (Green & Chan 1973; Fox & Kelley 1978).

Adenosine is capable of trapping P_i by phosphorylation to its nucleotides. Under physiological conditions the decrease of intracellular P_i concentration is accompanied by the inhibition of phosphoribosylpyrophosphate (PRPP) formation. Thus the accumulation of orotic acid might be explained by the ability of adenosine to limit the intracellular concentration of PRPP, a substrate for OPRT (Planet & Fox 1976). However, evidence that an adenosine kinase-deficient cell line was as sensitive to adenosine as the adenosine kinase-positive cell line suggested that adenosine does not need to be converted to its nucleotide in order to cause pyrimidine starvation (Hershfield *et al* 1977; Snyder *et al* 1977). There are several lines of experimental evidence which undermine the importance of adenosine-induced pyrimidine starvation leading to immune dysfunction.

i. Adenosine levels in plasma of ADA-deficient SCID patients were found to be insufficient to induce pyrimi-

dine starvation (Cohen *et al* 1978; Green & Chan 1973; Mills *et al* 1976).

ii. Pyrimidine nucleotides UTP, CTP, deoxy-TTP and deoxy-CTP were found to be elevated in an ADA-deficient SCID patient although this may be ascribed to erythrocyte transfusions given to this child (Hutton *et al* 1981; Schmalstieg *et al* 1977).

iii. ADA-deficient children do not excrete elevated amounts of orotic acid (Mills *et al* 1979).

iv. Despite demonstrable adenosine and deoxyadenosine mediated alterations of pyrimidine metabolism, these changes are not accompanied by marked interference of cell growth for ADA-deficient lymphoblasts (Parker *et al* 1982).

1.5.2. PRPP starvation (see Figs 1,2,3 & 6)

PRPP concentration is reduced in human lymphoblasts in the presence of adenosine due to inorganic phosphate being utilized to make phosphorylated adenosine derivatives. PRPP is an essential substrate not only for the *de novo* synthesis of pyrimidine but also for *de novo* purine biosynthesis, purine salvage pathways and pyrimidine nucleotide synthesis. It is possible that inhibition of pyrimidine synthesis as described above, is secondary to a decrease in the cellular concentration of PRPP. This is due to a decrease in the synthesis of PRPP when inorganic phosphate is limiting. A decrease in the intracellular

concentrations of this compound could therefore have profound metabolic effects. Further studies are required to determine the importance of PRPP mediated toxic effects on the immune system (Fox & Kelley 1978; Planet & Fox 1976; Snyder *et al* 1976).

1.5.2. Inhibition of S-adenosylhomocysteine hydrolase by adenosine and deoxyadenosine (Figs 8 & 10)

S-adenosylhomocysteine hydrolase (SAHH) catalyzes the reversible hydrolysis of S-adenosylhomocysteine to adenosine and homocysteine (De la Haba & Cantoni 1959). Physiological conditions favour this reaction although, kinetically speaking, the equilibrium position strongly favours the reverse reaction, because SAHH has a high binding affinity for adenosine. It was found that lymphoblast cell lines incubated with an ADA inhibitor such as deoxycoformycin (dCF) and adenosine show a striking elevation of S-adenosylhomocysteine (Kredich & Hershfield 1979). Deoxyadenosine does not act as a substrate for SAHH, but it binds to the enzyme irreversibly in such a way as to make the active site of the enzyme inaccessible resulting in a "suicide" inactivation of SAHH. S-adenosylhomocysteine then accumulates from both directions, as seen in Figure 8 (Hershfield 1979; Hershfield & Kredich 1979; Hershfield *et al* 1974). The hypothesis suggests that the accumulated S-adenosylhomocysteine inhibits the trans-methylation reactions of S-adenosylmethionine (SAM) many of which are physiologically

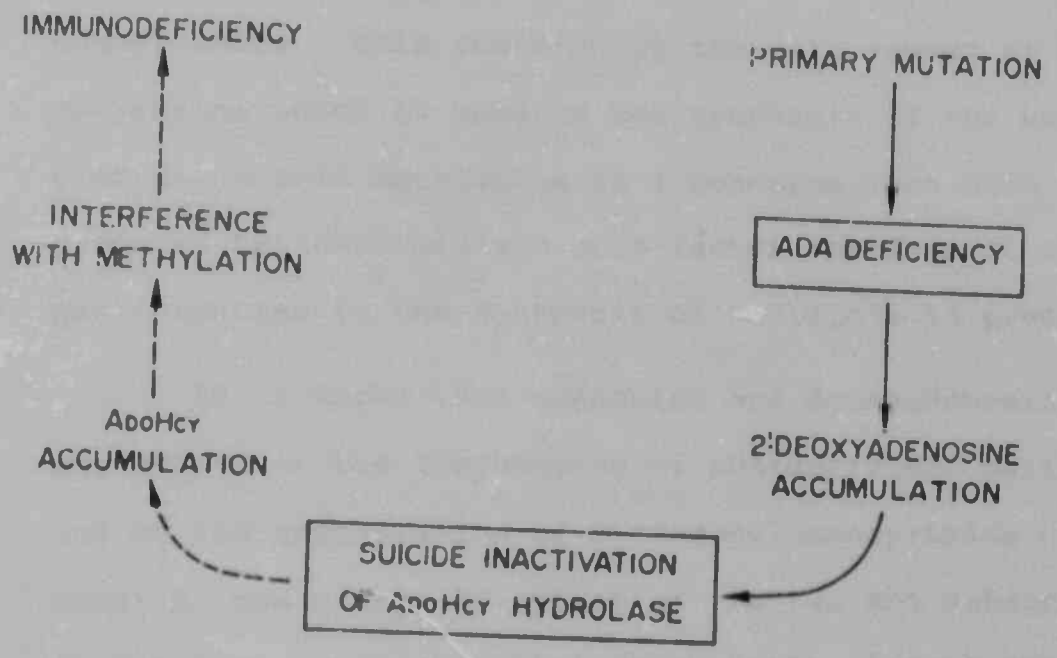

FIGURE 10 : Two enzyme deficiencies, one mutation Hershfield & Kredich 1978).

important. Another important result of the cleavage of S-adenosylhomocysteine by SAHH is the release of homocysteine. This reaction is the only source of homocysteine which is used in the synthesis of the essential amino acid methionine in a reaction that also produces tetrahydrofolate, a co-factor for several enzymes participating in the synthesis of nucleic acid precursors.

It is known that adenosine and deoxyadenosine accumulate in the lymphocytes of ADA-deficient patients and so the accumulation of S-adenosylhomocysteine could occur by the action of either of the two ADA substrates. It has been postulated that these toxic effects are not manifested until the lymphocytes are stimulated to divide. The cell is in a poor equilibrium position for methylation reactions as the concentration of S-adenosylhomocysteine is high, removal of it is blocked by the ADA deficiency causing an accumulation of adenosine favouring the reverse reaction and the accumulated deoxyadenosine is inhibiting the SAHH. The result of the extra burden of the immunostimulus is that things go from bad to worse and the cell "commits suicide" (Kredich 1974). Lymphocytes are long-lived cells which synthesise relatively small amounts of protein, so time allows the accumulation of S-adenosylhomocysteine whilst the low levels of protein production in the resting state do not impose too much stress on the impaired metabolism of the cells. ADA-deficient SCID patients have been reported to lack

SAHH activity in their erythrocytes due to *in vivo* inactivation by deoxyadenosine (Hershfield 1979; Hersnfield *et al* 1979). Data against this hypothesis includes experiments in human T and B lymphoblast. and in peripheral blood lymphocytes showing that no correlation could be found between inhibition of SAHH by deoxyadenosine and deoxyadenosine induced lymphocytotoxicity (Kefford *et al* 1982).

Interestingly, it has been shown that ADA and SAHH are syntenic on chromosome 20. This is a small chromosome containing only 2.4% of the total haploid autosomal length and so the chances of any one locus occurring in chromosome 20 is approximately one in forty. The occurrence of two functionally related loci together on this chromosome suggests the necessity for further enquiry rather than simply putting it down to chance (Hershfield & Francke 1982).

1.5.4. Inhibition of ribonucleotide reductase by deoxy-ATP (Fig 11)

Ribonucleotide reductase catalyzes the conversion of ribonucleotides at the diphosphate level to deoxyribonucleotides. This is the only route known for synthesis of deoxyribonucleotides in mammalian cells and so would appear to be essential for DNA synthesis (Moore & Hurlbert 1966; Reichard 1972). Using highly purified calf thymus ribonucleotide diphosphate, it was shown

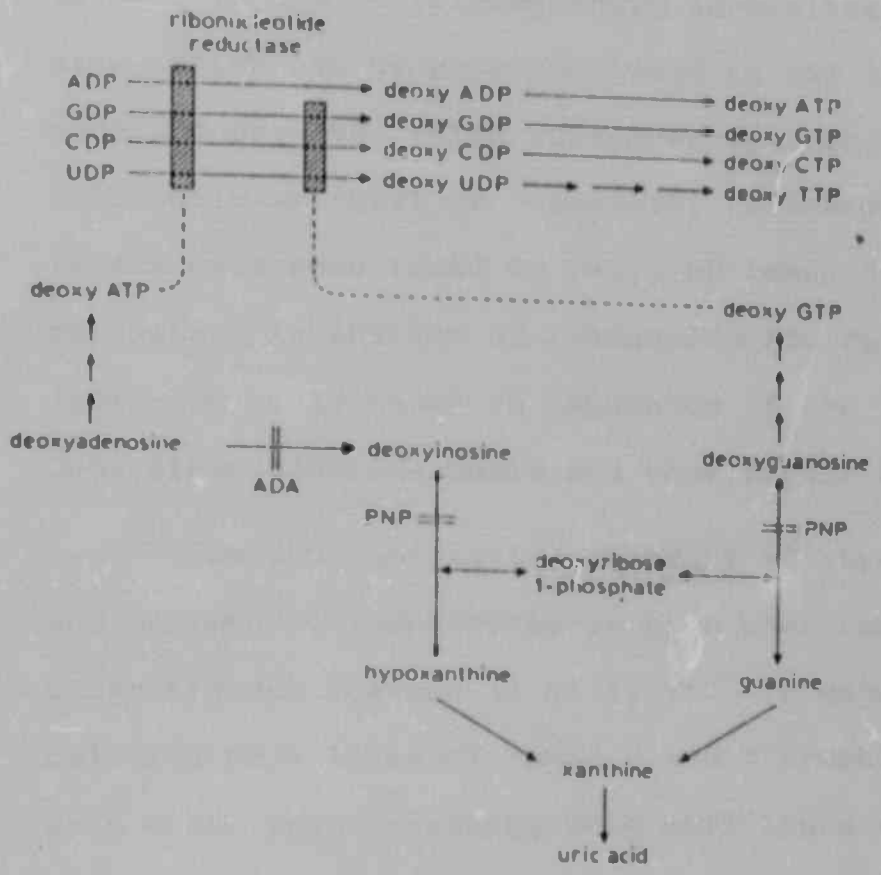

FIGURE 11 : Inhibition of ribonucleotide reductase by deoxy-ATP and deoxy-GTP in ADA and PNP deficiency.

that deoxy-ATP at a concentration of 5μM inhibits 50% of the ADP, GDP, CDP and UDP reactions (Eriksson *et al* 1979). In ADA deficiency there accumulates deoxyadenosine, which can be phosphorylated to the deoxyribonucleotides, deoxy-AMP, which subsequently increases the intracellular level of deoxy-ATP. Elevated deoxy-ATP levels have been found in cells of immunodeficient patients. Inhibition of ribonucleotide reductase by deoxy-ATP could cause an imbalance of the intracellular deoxyribonucleotide pools and thus impair DNA synthesis.

The phosphorylating capacity of thymus, spleen and peripheral lymphocytes is high when compared with other tissues (Carson *et al* 1977). In experiments with cultured cell lines of human B and T lymphoblasts it was seen that, when culturing both cell lines with deoxyadenosine, cell growth was more markedly inhibited in the T cell line than in the B cell line (Mitchell *et al* 1978). Together with this selective toxicity a selective accumulation of deoxy-ATP in the T lymphoblasts was observed (Mitchell *et al* 1978). This difference was shown to be not due to different rates of phosphorylation of deoxyadenosine since both cell types had a similar enzymatic capacity for catalyzing this reaction. Marked differences in the nucleotide degrading capacity, however, were noticed. 5'-purine nucleotidase (5'NT), the enzyme dephosphorylating deoxy-AMP and other mononucleotides to give the corresponding nucleosides, was found to have

much lower activity in T than in B cells (Thompson *et al* 1979; van Laarhoven and de Bruyn 1983; Wortmann *et al* 1979). Thus, the more rapid accumulation of deoxy-ATP might be considered to be the net result of both kinase and nucleotidase activity.

This mechanism is currently the most popular and it is now widely believed that deoxy-ATP inhibition of ribonucleotide reductase forms the biochemical basis of immunodeficiency in ADA-deficient SCID. This hypothesis has, however, been criticised on a number of scores. There are doubts about the selective toxicity towards T and B cells being explained on the basis of differences in activity of 5'-NT, an enzyme which has a considerable part of its catalytic activity located at the outer surface of the cell membrane (van Laarhoven & de Bruyn 1983).

Recent evidence suggests that the two mechanisms, inhibition of SAHH by deoxyadenosine and adenosine and inhibition of ribonucleotide reductase by deoxy-ATP may have differing degrees of importance in the production of toxicity in resting lymphocytes and proliferating lymphocytes (Lee *et al* 1982). In acute T cell leukaemia the lymphocytes are resting ie. there is little DNA synthesis. Treatment of this condition with deoxycoformycin (dCF), a powerful inhibitor of ADA, has often been found to bring about a state of complete remission (Prentice

et al 1980, 1981; Grever *et al* 1981). Deoxycoformycin treatment brings about a state similar to that in ADA-deficient SCID patients in terms of elevated metabolites. In acute T cell lymphocyte leukaemia, remission implies that the T cells have been destroyed. In this situation where the T cells are resting it is unlikely that inhibition of ribonucleotide reductase is going to be a major factor in their death. In resting lymphocytes treatment with hydroxyurea, an inhibitor of ribonucleotide reductase, failed to produce toxicity. Decreased SAHH activity was also seen, however, suggesting that in resting lymphocytes toxicity was mediated through inhibition of that enzyme or even by ATP depletion which is necessary for numerous metabolic reactions (Lee *et al* 1984).

In proliferating lymphocytes inhibition of ribonucleotide reductase is now the mechanism of choice for explaining the immune dysfunction. Deoxy-ATP concentrations are always highly elevated whilst SAHH activity is insignificantly altered. In fact, a B cell line despite almost complete inhibition of SAHH when rapidly proliferating, showed little toxic effects.

1.5.5. Adenosine induced elevation of intracellular cyclic AMP

The intracellular concentration of cyclic AMP is increased by adenosine in many tissues, including lymphocytes. Elevated levels of cyclic AMP may occur as a

direct result of the metabolic transformation of adenosine to cyclic AMP although a more important mechanism relates to the activation of plasma membrane adenylate cyclase by adenosine (Fox & Kelley 1978). As a result of elevated adenosine and deoxyadenosine levels in ADA deficiency, intracellular cyclic AMP levels in cells of the lymphoid system may be increased by either of the above mechanisms.

It is known that phytohaemagglutinin induced human lymphocyte blastogenesis is inhibited by cyclic AMP. Excretion of antibodies and lymphocyte mediated toxicity are also affected by this compound.

Whether the derivation of intracellular cyclic AMP plays an important role in the biochemical mechanisms leading to immune dysfunction in ADA deficiency is not yet clear. ADA-deficient leukocytes contained a cyclic AMP concentration (6mols/10^6 cells) about twice as high as control leukocytes (2.6mols/10^6 cells) (Schmalstieg *et al* 1977), however, the inhibitory effects of cyclic AMP described above were seen only at concentrations over 100μM. In addition T cell lymphosarcoma cell lines which were defective in some components of cyclic AMP action or metabolism, were resistant to increased cyclic AMP levels but sensitive to killing by adenosine and erythro-9-(2-hydroxy 3-nonyl) adenine (EHNA) (Ullman *et al* 1976).

These findings cast doubt on the importance of this mechanism in mediating the toxic effects of ADA deficiency.

1.6. BIOCHEMICAL BASIS OF THE IMMUNE DYSFUNCTION IN LYMPHOCYTES

Any hypothesis which tries to explain the association of ADA deficiency and immune dysfunction in terms of biochemical mechanisms must also take into account the preferential impairment of lymphocyte - particularly T lymphocyte - growth and development. Adenosine deaminase deficient children with immunodeficiency accumulate abnormally high concentrations of adenosine and deoxyadenosine in their plasma and urine but excrete normal quantities of total purines because, as explained above, adenosine and deoxyadenosine, in absolute quantitative terms, are a relatively minor component of the total purines excreted (Van der Weyden & Kelley 1976; Simmonds *et al* 1978; Mills *et al* 1978; Donofrio *et al* 1978; Mills *et al* 1976). The erythrocytes, lymphocytes and bone marrow cells from ADA-deficient patients exhibit markedly elevated concentrations of deoxy-ATP (Donofrio *et al* 1978; Cohen *et al* 1978; Coleman *et al* 1978; Chen *et al* 1978; Cohen, Gudas *et al* 1978) and deoxy-ADP (Coleman *et al* 1978; Chen *et al* 1978).

Observations on lymphoblast cell lines suggest that human T-lymphoblasts are far more susceptible to

deoxyadenosine toxicity than are B lymphoblasts and this susceptibility correlates with an increased ability of T cells to accumulate deoxy-ATP. T lymphoblast cell lines accumulate deoxy-ATP and are killed when cultured in the presence of deoxyadenosine during ADA inhibition whereas B lymphoblasts are resistant and do not accumulate deoxy-ADP (Mitchell *et al* 1978). Studies have shown that ADA inhibited T lymphoblasts accumulate deoxy-ATP at a rate of 20 to 45-fold faster than B lymphoblasts (Wortmann 1979).

The faster rate of accumulation in T cells of deoxy-ATP than B cells could be due either to an increased rate of phosphorylation of deoxyadenosine in T cells to form deoxyribonucleotide or a faster rate of dephosphorylation in B cells from the deoxyribonucleotide to the deoxyribonucleoside. It has been proposed that selective phosphorylation and trapping by T lymphocytes of deoxyadenosine mediated by lymphospecific deoxyribonucleoside kinase leads to an accumulation of toxic deoxyribonucleotides (Carson *et al* 1978). Human T lymphoblast cell lines were found to be more sensitive than human B cell lines to the growth inhibitory effects of deoxyadenosine. This differential sensitivity cound not be attributed solely to the relative amounts of deoxyadenosine phosphorylating activity but did correlate with the ability of the cells to accumulate deoxy-ATP. Therefore it seemed

possible that T and B cell lines might differ in their rate of deoxyribonucleotide breakdown (Carson *et al* 1979).

Deoxyadenosine phosphorylation can occur through adenosine kinase or deoxycytidine kinase for both of which, however, it is a poor substrate. At low but potentially significant deoxyadenosine concentrations, deoxycytidine kinase is the major route of phosphorylation. Deoxycytidine kinase is a lymphospecific enzyme. At higher deoxyadenosine concentrations, adenosine kinase, which occurs ubiquitously assumes the more important role in human lymphocytes (Carson *et al* 1977). Overall, phosphorylating activity of deoxyadenosine is only slightly higher in T cells than B cells and certainly not high enough to explain the discrepancy in deoxyribonucleotide levels (Carson *et al* 1981; Carson *et al* 1979; Wortmann 1979).

T cells have been demonstrated to breakdown deoxyribonucleotides far more slowly than B cells which is in accordance with the observation that T cells have much lower 5'-nucleotidase activity than B cells. Therefore, the combination of high deoxyribonucleoside phosphorylating activity and low deoxyribonucleotide dephosphorylating activity makes T lymphoblast cell lines inordinately susceptible to the toxic effects of deoxyribonucleosides and their analogues (Carson *et al* 1979). Enzyme activities in T cell lines are high for

ADA and deoxycytidine kinase and adenosine kinase and low for 5'-nucleotidase compared with B cells. In the absence of ADA, T cells accumulate phosphorylated derivatives of deoxyadenosine more rapidly than B cells along with the associated toxic effects. Studies of 5'-nucleotidase activity revealed that the membrane bound ecto-5'-nucleotidase activity levels were not related to the degree of deoxyadenosine toxicity. Rather, a soluble deoxynucleotidase activity occurring in T and B lymphoblastoid cell lines was inversely related to deoxyadenosine toxicity in ADA-inhibited cell lines. This soluble nucleotidase had different characteristics from the ecto-5'-nucleotidase (Carson *et al* 1981; Fritzson 1977).

1.7. ADENOSINE DEAMINASE (ADA)

1.7.1. Molecular heterogeneity and ADA binding protein

Studies of ADA from a variety of human tissues have provided evidence for molecular heterogeneity. In some tissues ADA exists exclusively as the small molecular form (molecular weight 33 000-38 000) while in other tissues the large molecular form of the enzyme predominates (molecular weight 220 000-298 000) (Madonna & Kelley 1979). It has been shown that under mildly denaturing conditions (50mM sodium succinate or 2.7M guanine hydrochloride) the large form of ADA can be converted to the small form of the enzyme (Akedo *et al* 1971; Van der

Weyden & Kelley 1976) and, conversely, that in the presence of appropriate tissue extracts, such as kidney, the small form of ADA can be converted to the large form of the enzyme. The interconversion is due to the enzyme molecule associating with a specific ADA binding protein (Van der Weyden & Kelley 1976; Nishara *et al* 1973; Hirschhorn 1975). Conversion of the small form to the large form of ADA occurs at 4°C, exhibits a pH optimum of 5.0 to 8.0, is associated with loss of binding protein activity of the cell extract and cannot be mimicked *in vitro* by other proteins, changes in ionic strength or by pH effects (Van der Weyden & Kelley 1976). Adenosine deaminase binding protein has been purified from human kidney which is the tissue with the highest binding protein activity. Analyses by SDS polyacrylamide gel electrophoresis and with Schiff staining suggest that the binding protein has a molecular weight of about 200 000 and that it is a dimeric glycoprotein consisting of two identical subunits each with a molecular weight of about 100 000 (Dadonna & Kelley 1977). Stoichiometric analysis suggests that the binding protein associates with ADA in the molecular ratio of 1 to 2 (Dadonna & Kelley 1977). This association is responsible for the appearance of the large form of ADA seen in lung and kidney tissue and skin fibroblasts. The association is reversible, hence the interconvertibility of the large and small species. The binding protein is located on the internal and

external cell surface attached to the cell membrane where it appears to act as an anchor for the ADA enzyme. The soluble cytoplasmic form of the binding protein appears to be generated during tissue disruption by proteolysis of the membrane form (Andy & Kornfeld 1982, 1984).

The function of the catalytically inactive ADA binding protein remains elusive. The large form of ADA appears to have increased heat stability suggesting that the binding protein may stabilize ADA *in vivo*. The specific activity of the large form, in addition, appears to be lower than the small form. Tissues with low ADA activity contain predominantly the large form of ADA and have high levels of binding protein.

The genetics of the ADA binding protein is interesting in that at least two loci, on chromosomes 2 and 6, are required for its expression (Koch & Shows 1978; Herbschleb-Voogt *et al* 1978), and it has been suggested that additional genes may be involved in the control and processing of ADA tissue isozymes (Koch & Shows 1978).

The small form of ADA has been purified from human erythrocytes, which are a readily available source of the enzyme (Dadonna & Kelley 1979: Dadonna & Kelley 1977; Schrader *et al* 1976; Osborne *et al* 1973). Purified erythrocyte ADA analysed by native polyacrylamide gel electrophoresis revealed three bands by Coomassie stain, each of which had ADA activity. Sodium dodecyl sulphate

(SDS) polyacrylamide gel electrophoresis of this preparation revealed only the major band of protein by Coomassie stain suggesting that the native enzyme existed as charge isomers rather than size isomers (Dadonna & Kelley 1979, 1977).

The native molecular weight of the purified enzyme was calculated to be 38 200 based on measurements of the Stokes radius of 24Å and the sedimentation coefficient, $S^{\circ}_{20,w}$ of 3.8×10^{-13}. The subunit molecular weight from SDS gel electrophoresis was estimated at 41 000 which suggests that human erythrocyte ADA is a single polypeptide chain. Staining with Schiff reagent suggested that the polypeptide chain is glycosylated (Dadonna & Kelley 1979, 1976; Schrader *et al* 1976).

1.7.2. Isozymes of adenosine deaminase (Figs 12 & 13)

A method for the detection of isozymes of ADA in human red cell haemolysates was first described by Spencer *et al* (1968). Three different types of isozyme pattern were observed. These patterns were found to be genetically inherited and occurred at polymorphic frequencies in population samples representing English, Negro and Indian peoples.

Each pattern consisted of at least three isozymes. In the type designated ADA-1 there were three regularly spaced components which exhibited decreasing staining intensity in order of their anodal electrophoretic

3.5.4.4—ADA

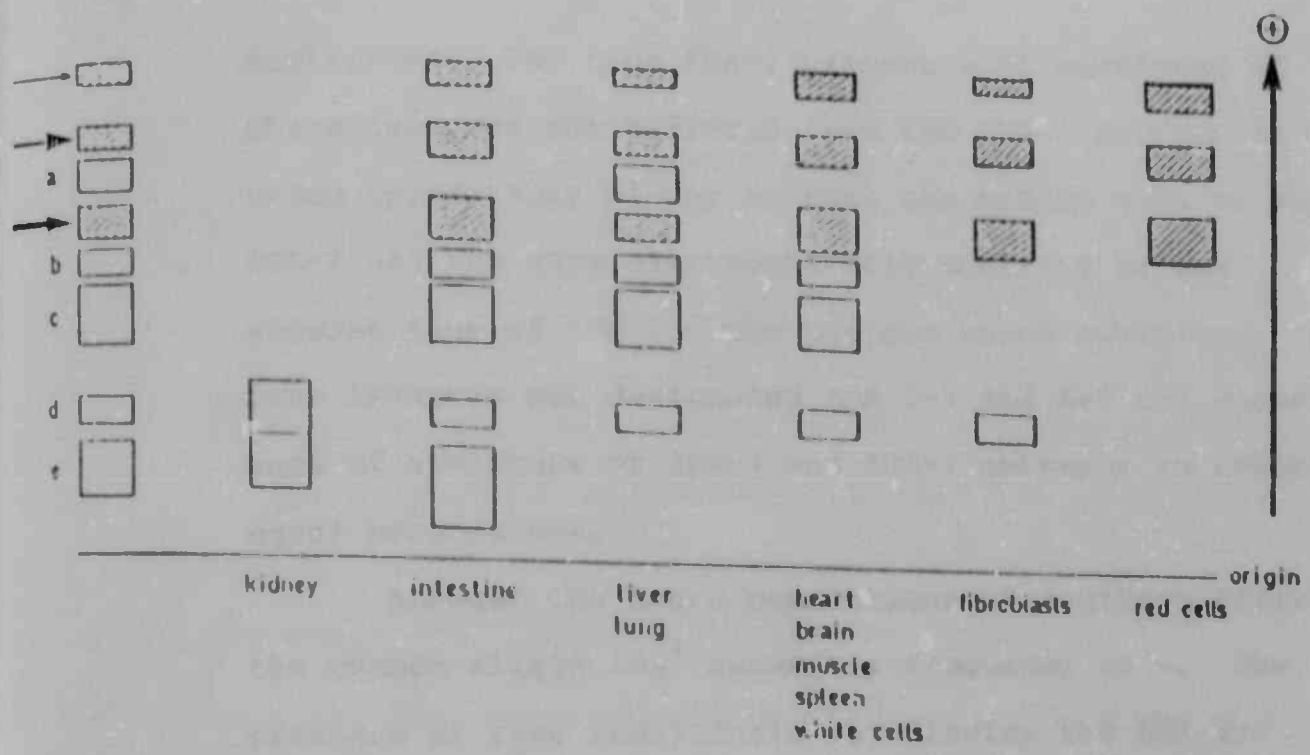

FIGURE 12 : Diagram showing ADA isozymes in different tissues. The arrows indicate the three isozymes (hatched) characteristically seen in red cells of the ADA 1 phenotype (Harris & Hopkinson 1976).

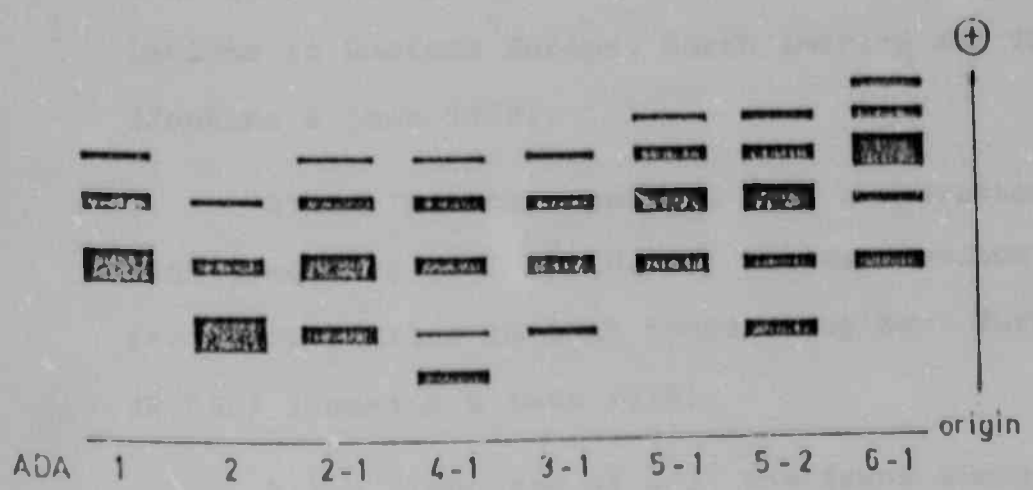

FIGURE 13 : Diagram of the red cell ADA isozyme patterns of various phenotypes (Harris & Hopkinson, 1976).

mobilities. The type ADA-2 pattern also consisted of three isozymes but differed from the ADA-1 pattern in being appreciably slower so that the middle zone of the ADA-2 had the same electrophoretic mobility as the slowest zone of ADA-1. The pattern which exhibited four isozymes was designated ADA 2-1 and had the appearance of a mixture of ADA-1 and ADA-2 patterns in roughly equal proportions.

Amongst the Negro populations of southern Africa the common allele ADA^1 assumes a frequency of 1. The presence of rare individuals who display the ADA 2-1 phenotype is assumed to have been a result of admixture with Caucasoid and 'Coloured' populations. The South African Caucasoid population carries the ADA^2 allele at a frequency comparable to that found in related populations in Western Europe, North America and Indian (Jenkins & Lane 1979).

Hindu Indians appear to have a characteristically high frequency of ADA^2 (0.107) whereas Moslems have a frequency similar to that found among West Europeans (0.066) (Jenkins & Lane 1979).

A low frequency of ADA^2 was found among the Afrikaaners in Windhoek. These people are the descendants of a relatively small number of ancestors, therefore, founder effect may well be a cause (Jenkins & Lane 1979).

An interesting effect of storage of red-cell

lysates at 4°C was noticed after several days. The relative staining intensities of the various isozymes changed progressively with time. In type ADA-1, for example, the slowest isozyme gradually became weaker and the intermediate and faster isozymes became stronger in staining intensity. A gradual increase in the staining intensity of the more anodal isozymes was also observed in ADA-2 and ADA 2-1 lysates, at the expense of the slowest components (Spencer *et al* 1968).

These storage effects can be minimized or reversed, however, by the addition of β-mercaptoethanol, a strong reducing agent, to the red-cell lysates at a final concentration of 20mM. The storage effects can also be accelerated by the addition of oxidized glutathione to red-cell lysates. These findings suggest that each of the isozymes contains at least one reactive sulphydryl group capable of undergoing an exchange reaction with oxidized glutathione which is known to accumulate in red cells on storage. The addition of a glutathione peptide to the isozyme molecule would increase the negative charge and hence its anodal electrophoretic mobility. Therefore the three banded isozyme of red-cell ADA arises from secondary modifications of a primary gene product by the addition of one or two more negative charges.

When tissues other than red cells were examined for ADA activity by starch gel electrophoresis, most

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When tissues other than red cells were examined for ADA activity by starch gel electrophoresis, most

tissue isozymes were found to be similar to the red-cell ADA isozyme but in many tissues additional isoenzyme forms were observed which differed in electrophoretic behaviour, in sulphydryl group activity and also in molecular size from the red-cell isozymes (Edwards *et al* 1971).

This second set of ADA isozymes did not exhibit the polymorphism of red-cell ADA although they were clearly heterogeneous in that their relative intensities varied between tissues in a tissue-specific way (Edwards *et al* 1971).

These data were initially interpreted as evidence for as many as four separate loci for ADA; a red-cell ADA locus and three tissue-ADA loci. This multiple locus hypothesis was effectively disproved when a child who was ADA-deficient and suffering from SCID was found to lack not only the red-cell isozymes of ADA but also all of the tissue-specific isozymes. Thus it appeared that in spite of the tissue-to-tissue variation in electrophoretic mobility, the different ADA enzyme activities were controlled by a single structural genetic locus (Hirschhorn *et al* 1973). The discovery of the ADA binding protein (Akedo *et al* 1972; Nishihara 1973) and the interconvertability of the large and small forms of ADA (Van der Weyden & Kelley 1976) provided a theoretical basis for the tissue-specific variation of ADA. A little later it was discovered that most of the miltiple forms of 'tissue'

ADA were glycoproteins which differed in their accessible sugar residues. This suggested that the binding protein was a single glycoprotein with heterogeneous carbohydrate content (Swallow *et al* 1977). By now the structural gene for ADA had been mapped to a single structural locus on chromosome 20 (Creagan *et al* 1973; Tischfield *et al* 1974) which was subsequently confirmed with the cloning of the structural gene for ADA (see later Orkin *et al* 1983).

In 1977, Champion and Shows reported that in fibroblasts from patients with mucolipidosis II (I-cell disease) and mucolipidosis III certain lysosomal enzymes as well as ADA, which is a non-lysosomal enzyme, showed altered electrophoretic mobilities that were, in part, attributable to the presence of excess sialic acid residues on the enzyme molecules. A similar observation was made on two sibs with sialidosis type I (Swallow *et al* 1979). The catalytic subunit of ADA contains little or no carbohydrate, however, the binding protein is glycosylated. Swallow *et al* (1981), after further electrophoretic analysis of 12 different patients with primary or secondary sialidase deficiency suggested that the increased electronegativity of a number of lysosomal enzymes, as well as ADA was due to the presence of excess sialic acid residues on the enzyme molecules and that sialidase played a role in the normal post-trans-

lational modification of glycoprotein enzymes by clipping off terminal sialic acid residues from the end of sugar chains after the completion of glycoprotein synthesis. The altered electrophoretic mobility of ADA implied that sialidase may act outside the lysosome as ADA is a soluble enzyme and also is over-sialylated in sialidase deficiency.

In summary, the red-cell, triple-banded ADA isozyme pattern is a consequence of secondary modification of a primary gene product at the sulphydryl sites. The tissue-specific isozymes are generated when the primary ADA gene product is bound by a tissue conversion factor of ADA binding protein, which is itself glycosylated in a tissue-specific manner.

1.7.3. The minor form of ADA: ADA_2

Early studies on the molecular heterogeneity of adenosine deaminase indicated the presence of small amounts of an 'intermediate' form of the enzyme, (molecular weight 110 000) in a variety of human tissues compared to the large and small forms with molecular weights of 298 000 and 38 000 respectively (Van der Weyden 1976). Analysis of splenic tissue from a patient with ADA deficiency and SCID, revealed in addition, that the molecular form of ADA present was exclusively the 'intermediate species' (Van der Weyden *et al* 1974). It was subsequently demonstrated by Schrader *et al* (1978)

that an 'aminohydrolase' intermediate in molecular weight between large- and small-form ADA existed in normal spleen as well as in splenic tissue of another patient with ADA deficiency and immune dysfunction. This splenic 'aminohydrolase' appeared to be different from normal ADA based on K_m value, immunoreactivity, pH optimum and insensitivity to the potent ADA inhibitor EHNA (Schrader *et al* 1978). Subsequently, it was demonstrated that an aminohydrolase was present in six normal and five ADA-deficient cell lines which appeared to be similar to the previously described splenic aminohydrolase. Analysis of the enzymatic, physical and immunoreactive properties of the lymphoblast aminohydrolase suggested that this enzyme was distinct from and unrelated to ADA (Dadonna & Kelley 1981) (see Table 1).

The minor form of ADA, sometimes referred to as ADA_2 (Hirschhorn *et al* 1979) differs from the major ADA in that it is active only at high substrate concentrations and is non-EHNA-inhibitible. Residual ADA activity found at high substrate concentrations in cells from ADA-deficient patients could represent in whole, or in part, this additional ADA_2 activity. Therefore, when investigating ADA from suspected ADA-deficient patients the enzyme should be assayed at low substrate concentration and only the EHNA-inhibitible ADA activity measured by performing assays in duplicate, with and without the inhibitor.

Table 1 : Characteristics of the molecular forms of adenosine deaminase activity in lymphocytes (Dadonna & Kelley 1981)

Property	EHNA insensitive aminohydrolase	Adenosine deaminase
Sedimentation coefficient ($1x10^{-13}$S)	7.46 ± 0,18 (11)	3.8 ± 0.02 (5)
Estimated molecular weight (from $S_{20,w}$)	110 000	38 000
pH optimum	5.5 - 6.0	6 - 8
K_m value		
adenosine	3.06mM	0.065mM
deoxyadenosine	0.72mM	0.075mM
Relative substrate specificity		
deoxyadenosine/adenosine	0.1	1.0

1.8. PARTIAL ADA DEFICIENCY

1.8.1. Discovery in the !Kung

Inherited deficiency of ADA usually results in, and appears to be causally associated with the condition of severe combined immunodeficiency disease (SCID) which is characterised by a lymphopoenia and lack of both cellular and humoral immunity. This usually proves fatal within three years unless treated by bone marrow transplantation. Shortly after the initial description of ADA-deficient SCID, an apparently healthy 10-year old !Kung child was described who lacked ADA in his erythrocytes (Jenkins 1973). His ADA deficiency had been discovered by starch gel electrophoresis whilst screening !Kung blood samples for a range of genetic markers including ADA. Electrophoresed haemolysates from this child were found to lack ADA activity whereas his father and mother demonstrated the ADA-1 isozyme pattern but with reduced intensity. His immunoglobulin levels were normal although there was evidence of a slight impairment of his cellular immunity. Absence of lymphopoenia was demonstrated with T and B cells in normal proportions. The haemoglobin and red cell counts were also normal. Adenosine deaminase activity was assayed and the propositus was found to have 2-3% of normal activity in his red cells, 10-15% of normal in his white cells and 33% of normal in cultured fibroblasts. Starch gel electrophoresis of ADA from fibroblast extracts showed

the ADA-2 isozyme pattern although, of weak intensity along with the normal pattern of ADA 'tissue' isozyme.

These data were interpreted as showing that the boy was homozygous for a rare ADA allele which was different from that involved in SCID, and that the level of ADA activity in the various tissues including lymphocytes was sufficient for normal immune function. Studies on the heat stability of white cell ADA showed that at 56°C the residual enzyme had a half life of 5 minutes compared with a control value of about 50 minutes. Therefore this rare ADA allele appeared to be responsible for an unstable form of the enzyme resulting in deficiency in the anucleated, mature red cells but with sufficient residual activity in the lymphoid cells to preserve immune function.

Population studies on the !Kung revealed that the gene coding for this unstable enzyme was present at polymorphic frequencies. This allele was designated ADA^{8}, and its frequency in the !Kung was 0.014, while ADA^{1} had a frequency of 0.896, and ADA^{2} was not encountered at all. A total of three homozygous !Kung individuals from 2 families were found (Jenkins 1973; Jenkins *et al* 1976,1979). Subsequently, four additional children from three families belonging to other populations have been found to have 'partial' ADA deficiency (Hirschhorn *et al* 1979).

1.8.2. Characteristics and possible mechanisms of partial ADA deficiency

These individuals had several features in common. All were deficient for ADA in their red cells but had substantial residual ADA activity in their peripheral blood mononuclear cells. They showed only slight, if any, accumulation of the toxic metabolite deoxy-ATP in their erythrocytes and excreted barely detectable amounts of deoxyadenosine in urine. This contrasts with the 500- 1000-fold elevation in erythrocyte deoxy-ATP content and urinary deoxyadenosine excretion by ADA-deficient SCID patients. Thus, total body adenosine deaminating activity was sufficient in these partially ADA-deficient children to prevent accumulation of deleterious nucleoside and nucleotide compounds (Hirschhorn *et al* 1983).

There are several different mechanisms which could result in genetic absence of ADA in erythrocytes but presence in lymphoid cells. One of these is the existence of two different ADA isozymes controlled by two separate loci in the two types of cells. This theory appears to be extremely unlikely since a single locus for ADA has been mapped to chromosome 20 and SCID patients show a profound lack of ADA activity in all tissues.

A second theory is that a mutation is involved at a regulatory locus controlling expression of ADA in erythrocytes. At the moment there is only slight sup-

portive evidence for the regulatory locus theory. For example, Valentine has reported autosomal, dominant inheritance of a 50-fold increase in ADA activity of erythrocytes but normal activity in lymphocytes and fibroblasts (Valentine *et al* 1977). Siciliano *et al* (1978) have reported expression of human ADA in hybrids between a human tumour line not expressing ADA and a murine cell line, suggesting a regulatory locus. Finally, a mutation at a structural locus coding for an unstable enzyme could result in absent or markedly diminished activity in erythrocytes because they are incapable of synthesizing new protein and have a relatively long life span during which time an unstable enzyme could be expected to diminish markedly in activity.

1.8.3. Genetic heterogeneity

Adenosine deaminase from four lymphoid cell lines derived from partially ADA-deficient individuals has been intensively studied and despite the common features described above there appears to be a high degree of heterogeneity in partial ADA deficiency to the degree that four different structural mutations appear to be involved (Hirschhorn *et al* 1983; Dadonna *et al* 1984).

One child had an enzyme which was unstable at 56°C, had a more basic than normal isoelectric point and a rate of enzyme degradation *in vivo* (B lymphoblast cell lines) which was one- and a-half times greater than normal. A

second child had an enzyme with normal heat stability, a more acidic isoelectric point, an increased rate of degradation of ADA *in vivo* and a reduced rate of enzyme synthesis, although mRNA levels were normal. The third child (the !Kung boy) had an enzyme with a normal isoelectric point but diminished stability at 56°C. The latter cell line was the only one of the four to show a two-fold elevation of ADA mRNA. Protein turnover studies demonstrated that ADA from the child was degraded at twice the normal rate suggesting a mutation in the protein which renders it more susceptible to proteolysis *in vivo*. The rate of synthesis, in addition was almost one- and a-half times as fast as normal. The fourth child had an enzyme with a normal isoelectric point and normal heat stability at 56°C which was initially taken as evidence for a mutation at the postulated regulatory locus. Protein turnover studies, however, showed a slower rate of synthesis and a rate of degradation approximately twice the normal rate, despite having normal mRNA levels. This additional evidence is more suggestive of a structural mutation than any regulatory fault (Hirschhorn *et al* 1983; Dadonna *et al* 1984).

1.9. RECENT DEVELOPMENTS

1.9.1. The ADA gene

Several groups have isolated sequences encoding

human ADA (Valerio *et al* 1983; Orkin *et al* 1983; Wiginton *et al* 1983). The availability of these clones has allowed studies of the structure and expression of the ADA gene in wild-type and ADA-deficient cell lines (Valerio *et al* 1984a; Dadonna *et al* 1984; Adrian *et al* 1984).

Northern blot analysis revealed a major ADA mRNA species of about 1.5 kilobases that has been shown to code for the ADA protein (Valerio *et al* 1983). Sequence analysis of a cDNA clone for human ADA revealed an open reading frame with the capacity to code for a protein with a molecular weight of 40 762 (Valerio *et al* 1984b). The complete gene was subsequently isolated and found to consist of 32kb of genomic DNA containing 12 exons.

The promoter region of ADA is unusual in that it does not appear to have the classical TATA box, which is typically located 20-30 bases 5' to the start of the coding sequence and has the consensus sequence $TATA^{A}_{T}A^{A}_{T}$ (Breathnach & Chambon 1981). The so-called CAAT box which is often found 80bp 5' to the start of the first exon of eukaryotic genes (Benoist *et al* 1980) is also absent. The promoter region is, however, rich in G/C content, a feature of some other gene promoter regions (Valerio *et al* 1985).

1.9.2. Molecular basis of immunodeficiency

The molecular basis of ADA deficiency has also

been investigated using ADA cDNA clones. In most ADA-deficient cell lines, ADA mRNA is present in normal or supranormal amounts (Dadonna *et al* 1984; Valerio *et al* 1984; Dadonna *et al* 1985) but enzyme activity and immunologically detectable protein levels are greatly diminished (Dadonna *et al* 1980; Wiginton & Hutton 1982). The majority of the mutations are therefore thought to lie within the protein coding sequence. Because the ADA mRNA in ADA-deficient cell lines can often be translated *in vitro* into normal sized ADA protein (Valerio *et al* 1984; Adrian *et al* 1983), it can be inferred that the mutant ADA proteins are sometimes unstable *in vivo*. S1 nuclease digests single-stranded RNA and DNA. Hybridization between complimentary strands of DNA or DNA and RNA protects the molecules from this enzyme. If the hybridization is incomplete, the region of imperfect pairing will also be digested which will be revealed by subsequent strand separation by denaturation and electrophoresis. S1 nuclease analysis of mRNA from seven ADA-deficient cell lines revealed four with patterns indistinguishable from those of normal cells and three with structural aberrations in the mRNA (Adrian *et al* 1984). These results suggested that point mutations are primarily responsible for most, though not all cases of ADA deficiency. In one particular cell line, GM-1715, derived from a patient with ADA deficiency and immunodeficiency, sequence analysis of the cloned ADA DNA revealed a point

mutation at base number 302, codon 101, which predicts a glutamine residue to be substituted for arginine in the ADA protein (Bonthron *et al* 1985), which appeared to be solely responsible for the loss of function of this allele. Restriction fragment length analysis, however, suggested that this cell line was heterozygous for two different ADA deficiency alleles (Bonthron *et al* 1985). It can be confidently predicted that in the near future, further sequence analysis of other mutant alleles will permit the detailed mapping of sites in the ADA protein that are critical to enzyme function and stability.

2. MATERIALS AND METHODS

2.1. Subjects

The proband, an apparently healthy Bantu-speaking black male in his early thirties, was discovered during routine ADA phenotyping by starch gel electrophoresis. He and his family lived in the Eastern Cape around Port Elizabeth. All of them, including the proband, his parents, two sisters, wife and child, consented to give blood which was collected at the South African Institute for Medical Research, Port Elizabeth laboratories. These samples were then flown to Johannesburg on the first available flight. Subsequently ACD blood samples from 68 random Xhosa blood donors were also flown to our laboratory from Port Elizabeth for a population study on the incidence of partial ADA deficiency.

2.1.1 Sample preparation

The venous blood samples collected into acid-citrate-dextrose (ACD) anticoagulant 'Vac-U-Test' tubes were received in the laboratory in good condition within twenty-four hours of collection. Plasma was removed and the red cells washed three times in 0.9% saline with removal of the white cell buffy coat after each centrifugation. The red cells were preserved by the addition of an equal volume of a glycine based preserving fluid (Tripotassium citrate ($K_3C_6H_5O_7.H_2O$) 0.06 moles/ℓ, sodium

dihydrogen phosphate ($NaH_2PO_4.2H_2O$) 0.02 moles/ℓ dissolved in 600mℓs of deionised water and 400mℓs glycerol), and stored at -20°C. When a sample was required the preserving fluid was removed by overnight dialysis against 0.9% saline at 4°C. The red cells were then washed in 0.9% saline and haemolysed by freezing and thawing or by sonication. Alternatively, haemolysates were prepared from undialysed, preserved red cells by sonication and from washed, fresh red cells by freezing and thawing or by sonication.

2.2. PREPARATION OF WHITE CELL LYSATES

Two volumes of 3 percent w/v Dextran in 0.9 percent w/v saline were added to a measured volume of whole blood in a measuring cylinder, inverted to ensure thorough mixing and then allowed to stand at room temperature for 30 minutes. Most red cells settled down allowing the supernatant to be removed. This was centrifuged at 600g for five minutes to spin down the white cells and any remaining red cells. The supernatant was then discarded and the cell pellet cooled on ice. The remaining red cells were lysed by adding 3mℓ of chilled water and mixed for 70 seconds with a 5mℓ automatic-adjustable-pipette. This solution was then mixed well with 1mℓ of 3.6 percent w/v sodium chloride and centrifuged at 600g for five minutes. The supernatant containing haemoglobin and lysed red cell ghosts was discarded. This procedure

for lysing red cells was repeated if necessary until the cell pellet consisted of white cells only. This pellet was washed twice with 4mℓ of cold 0.9 percent w/v sodium chloride and finally stored frozen in the pellet form at -70°C.

2.3. HAEMOGLOBIN AND PROTEIN ESTIMATION IN LYSATES

Haemoglobin concentration was measured by adding 20ul of haemolysate diluted 1:5 with water to 3mℓ of ferricyanide-cyanide reagent (Drabkins solution). The pigment is converted to cyanmethaemoglobin and the optical density read at 540nm after fifteen minutes incubation at room temperature (Beutler 1975). The Drabkins solution was made by dissolving 200mg $K_3Fe(CN)_6$, 50mg KCN and 1g $NaHCO_3$ in 1ℓ distilled water. The spectrophotometer was calibrated for haemoglobin estimation and found to give an optical density reading of 0.248 when the haemoglobin concentration was known to be 1mg Hb per 3mℓ Drabkins.

Protein estimations in white cell lysates were made by the method of Lowry *et al* (1951). A series of protein standards was prepared by dissolving known amounts of bovine serum albumin (Sigma) in distilled water in the range 30µg/mℓ to 180µg/mℓ. One millilitre of a 2 percent w/v potassium sodium tartrate solution was added to 1mℓ of 1 percent w/v copper sulphate and 98mℓs of a solution which consisted of 2 percent w/v Na_2CO_3 and 0.1M NaOH,

and well mixed. Four millilitres of this resulting solution were added to a series of tubes which contained either 1mℓ of the protein standard or 1mℓ of the white cell lysate diluted 1:50. Four millilitres of the same solution were added to 1mℓ of water to provide the 'blank'. All tubes were prepared in duplicate and then allowed to stand at room temperature for ten minutes. During this time a working solution of Folin-Ciocalteu reagent (Merck) was prepared by diluting it in the ratio of 1mℓ Folin's reagent to 1.36mℓ of water. At the appropriate time 0.5mℓ of the diluted Folin's reagent was added to each tube and given an instant, vigorous mixing. The mixtures were then allowed to stand at room temperature for a further thirty minutes after which the optical density at a wavelength of 660nm of each solution was measured on a Perkin-Elmer 550 spectrophotometer. The known protein standards were used to plot a standard curve from which the protein levels in the test samples were determined.

2.4. STARCH GEL ELECTROPHORESIS OF ADA

The bridge buffer for electrophoresis contained citric acid monohydrate, 0.41M and sodium hydroxide 1.21M and had a pH of 6.5. The gel buffer consisted of 0.005M L-histidine monohydrochloride, which had been adjusted with a dilute solution of NaOH to pH 6.5. Ten percent starch gels were prepared and samples were applied

on Whatman No 17 filter paper rectangles which had been saturated with the lysates. Electrophoresis was carried out horizontally for 16 to 17 hours at +4°C with an applied potential difference of 3.5 V/cm constant voltage. The gels were then sliced and the cut surfaces stained for adenosine deaminase using an agar overlay technique. The staining mixture contained 150mg sodium arsenate, 40mg adenosine, 6mℓ nitro blue tetrazolium salt solution (100mg NBT/10mℓ water), 2mℓ phenazine methosulphate solution (100mg PMS/20mℓ H_2O), 40µl of a suspension of xanthine oxidase in an ammonium sulphate solution (Boehringer) equivalent to about 0.8 units and 40µl of a suspension of nucleoside phosphorylase (Boehringer) also equivalent to about 0.8 units, dissolved in 20mℓ Tris HCℓ buffer, 0,05M pH 8. One gramme of agar (Difco Bacto-agar) was dissolved by boiling in about 10mℓ water and added to the staining mixture. Incubation was carried out at 37°C for about two hours.

The reaction proceeds as shown in Fig 14.

$$\underline{\text{Adenosine}} \xrightarrow{\underline{\text{ADA}}} \text{Inosine} \xrightarrow{\underline{\text{NP}}} \text{Hypoxanthine} \xrightarrow[\underline{\text{PMS}}]{\underline{\text{XOD}}} \text{xanthine}$$

$$\text{Formazan} \leftarrow \underline{\text{NBT}}$$

Figure 14

The underlined components are contained in the reaction mixture applied to the gel. NP, nucleoside phosphorylase; XOD, xanthine oxidase; PMS, phenazine methosulphate; NBT, nitroblue tetrazolium. ADA, adenosine deaminase.

The inosine produced at the sites of adenosine deaminase activity is converted to hypoxanthine in the presence of nucleoside phosphorylase. The hypoxanthine is then oxidized by the action of xanthine oxidase and during this reaction the tetrazolium salt NBT is reduced in the presence of phenazine methosulphate to blue insoluble formazan.

2.5. ADENOSINE DEAMINASE ACTIVITY MEASUREMENT

2.5.1. Spectrophotometric method

This method used to measure adenosine deaminase activity is very similar to that described by Hopkinson *et al* (1969). Haemolysates and white cell lysates were prepared as described above and the adenosine deaminase measured spectrophotometrically with adenosine or deoxyadenosine as substrate at pH 7.5. The red cell lysates were diluted 1:5 with water and 50µℓ of this dilute haemolysate was added to 3.0mℓ phosphate buffer, 0.05M, pH 7.5 containing 0.306µmoles substrate (adenosine or deoxyadenosine) 0.3µmoles EDTA, 3µmoles β-mercaptoethanol and 0.2 units of xanthine oxidase (Boehringer) in a final volume of 3.06mℓ and the rate of increase in optical density at 293nm was measured in a Perkin-Elmer 550 Spectrophotometer. The reaction was carried out at 37°C and the blank cuvette was identical to the test cuvette except that adenosine was omitted. Inosine, the product of adenosine deaminase activity, is converted by nucleoside phosphorylase present in red cell lysates to hypoxanthine; the latter is then converted to xanthine

and uric acid by the action of the added xanthine oxidase. Thus in the presence of excess nucleoside phosphorylase and xanthine oxidase the rate of the increase in optical density at 293nm resulting from the conversion of adenosine to uric acid gives a measure of adenosine deaminase activity.

White cell lysate adenosine deaminase activity was measured in essentially the same way as for haemolysates. White cell lysates were prepared as described under the methods for sample preparation and used without diluting. The reaction mixture in the test cuvette was the same as that for red cell measurements except for the addition of 0.2U of nucleoside phosphorylase. This is necessary because there is insufficient endogenous nucleoside phosphorylase present in white cell lysates for the reaction to proceed optimally. Blanks were constituted in the same way as the reaction mixture except that 50µℓ water was added instead of white cell lysate.

Activity measurements were expressed in terms of haemoglobin concentrations for red cell lysates and protein concentrations for white cell lysates.

2.5.2. Radiochemical method

This method of measuring adenosine deaminase activity was used in addition to the spectrophotometric method because of its greater sensitivity, especially for

measuring the low adenosine deaminase levels found in the red cells of partially deficient subjects.

The method used was the same as that described by Hirschhorn *et al* (1979), which was based on a similar method described by Coleman and Hutton (1975). The principle of this radiometric assay lies in measuring the conversion of (^{14}C) adenosine to (^{14}C) inosine; these two substrates being separated by descending paper chromatography prior to their quantitation.

Red cell lysates were prepared as described for starch gel electrophoresis. The haemolysates from individuals known to have normal adenosine deaminase activity were diluted fourty-one times with 1mM Tris, pH 7.5, whereas haemolysates from individuals known to be partially deficient for adenosine deaminase were diluted sixteen times with the same Tris buffer.

The red cell lysates were incubated with water, (U^{14}C) adenosine (Amersham 500-600mCi/mmol) or deoxyadenosine (Amersham >450mCi/mmol) and a Tris EDTA buffer, pH 7.5 such that the final concentrations of each component in the reaction mixture was as follows:

(^{14}C) adenosine or deoxyadenosine, 0.09mM, 1μCi/μmol Tris, 24mM; EDTA, 2.4mM; EHNA, 40μM in a final volume of 100μℓ. The amount of water and lysate added could be varied so that between 10 and 30 percent of the adenosine was converted to inosine during the incubation period.

The incubation was carried out at 37°C for thirty minutes and the reaction terminated by the addition of 20µℓ of 2.1M perchloric acid to the reaction mixture. After a brief centrifugation in a bench centrifuge and subsequent neutralisation of a 60µℓ aliquot of the supernatant with 10µℓ of 2.21M NaOH, 20µℓ aliquots were co-chromatographed with 3mM internal standards on DE81 paper, with 1mM ammonium formate as solvent, by descending paper chromatography. The areas containing purines were revealed under UV light by their defluorescence, cut and the radioactivity of these sections determined. Adenosine and deoxyadenosine were widely separated from hypoxanthine, inosine and deoxyinosine. Activities were calculated by determining the percentage of the total counts converted from substrate to product, knowing the initial substrate concentration and period of inactivation.

2.6. MICHAELIS CONSTANTS

Method

The adenosine deaminase activities of samples from the subject and controls were measured by the radiochemical method, as previously described, at final substrate concentrations of 50µM, 75µM, 100µM, 125µM, 150µM and 175µM. The method of least squares was used to draw Lineweaver-Burke plots representing the data and Michaelis constant estimates obtained from these by calculating reciprocals of the x-axis intercepts.