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STUDIES OF HUMAN INOSINE TRIPHOSPHATASE AND ITP METABOLISM

City University of New York PH.D. 1979

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STUDIES OF HUMAN INOSINE TRIPHOSPHATASE AND ITP METABOLISM

by

DEBRA LYNNE FREEDENBERG

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

STUDIES OF HUMAN INOSINE TRIPHOSPHATASE

AND ITP METABOLISM

by

DEBRA LYNNE FREEDENBERG

Advisor: Dr. Kurt Hirschhorn

A high pressure liquid chromatography (HPLC) method has been developed for assay of Inosine Triphosphatase (E.C. 3.6.1.19) in human erythrocyte and lymphocyte extracts. Under conditions developed the assay is extremely sensitive (capable of detecting 5 picamoles of product), reproducible, and specific for ITPase. The assay is linear for thirty minutes with increasing protein ranges using extracts of both erythrocytes and lymphoid lines.

A long term lymphoid line established from an individual reported to have no detectable ITPase activity in erythrocytes was found to have approximately 25% residual activity by three methods: measurement of the rate of conversion of ITP to IMP, measurement of the rate of release of pyrophosphate, and starch gel electrophoresis subsequent to serial dilutions of lymphoid lysates. The variant ITPase was found to be mildly heat labile in comparison to the normal ITPase lymphoid enzyme when extracts were incubated at 56°C and 50°C for up to one hour. No isozymes

of ITPase were detected on starch gel electrophoresis in either the variant or normal lymphoid lines.

ITPase was found to be a pyrophosphatase in both normal lymphoid cells and the partially deficient ITPase variant. When 8-^{14}C ITP was used as a substrate, increases only in radio-labeled IMP were detected. A sedimentable phosphatase was also found to hydrolyze 8-^{14}C ITP. The variant had normal levels of activity of this enzyme. This nonspecific phosphatase activity was eliminated by centrifugation of lysates at 27,000 x g for thirty minutes.

Metabolic studies demonstrated that intact normal lymphocytes have the ability to synthesize and accumulate ITP. However, the maximal level of accumulation of ITP (0.144 nanomoles/10^6 cells) in the variant was one hundred times greater than the maximal level found in normal cells (0.001 nanomoles/10^6 cells) under any conditions.

The influence of various conditions on the accumulation of ITP was investigated. The absence of glutamine in the culture media was found to be a predisposing factor to the accumulation of ITP.

In the variant ITPase lymphoid line, the accumulation of ITP was found to vary inversely with the specific activity found in GTP (-0.98, $p = 0.0001$), to be positively correlated with the ratio of IMP/AMP (0.91, $p = 0.0001$) and to be

positively correlated with the amount of IDP present (0.96, $p = 0.0001$). In addition, the ratio of ATP/GTP was significantly diminished (due to decreased ATP) in the variant line from normal lines under conditions of ITP synthesis. The decrease in ATP did not stoichiometrically account for the increase of ITP. No significant difference was found in ATP/GTP ratios if ITP was not accumulated. A greater percentage (92% vs. 75%) of total radioactive nucleotides was found in IMP under conditions of ITP synthesis. In the normal lines ITP accumulation also showed an inverse correlation with the specific activity of GTP (-0.92, $p = 0.0001$).

Lymphoid lines were also found to have the ability to synthesize XDP and XTP. The conditions of XDP and XTP synthesis were not determined.

Acknowledgments

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And, of course, I would like to thank my family and friends for their continual encouragement and love--without whom my graduate education would not have been possible.

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Nucleotides

AMP, ADP, ATP	Adenosine Mono, Di, and Tri Phosphate
CMP, CDP, CTP	Cytosine Mono, Di, and Tri Phosphate
dITP	Deoxyinosine Triphosphate
GMP, GDP, GTP	Guanosine Mono, Di, and Tri Phosphate
IMP, IDP, ITP	Inosine Mono, Di, and Tri Phosphate
NAD	Nicotine Adenine Dinucleotide
NADP	Nicotine Adenine Dinucleotide Phosphate
UMP, UDP, UTP	Uridine Mono, Di, Tri Phosphate
XMP, XDP, XTP	Xanthosine Mono, Di, and Tri Phosphate

Nucleosides and Bases

Aden	Adenine
AdenR	Adenosine
Cyt	Cytidine
dAdenR	Deoxyadenosine
DIno	Deoxyinosine
GuanR	Guanosine
Hx	Hypoxanthine
Ino	Inosine
Thym	Thymidine
Urd	Uridine
Xan	Xanthine
XR	Xanthosine

Enzymes

ADA	Adenosine Deaminase
APRT	Adenine Phosphoribosyltransferase
HGPRT	Hypoxanthine Guanine Phosphoribosyl-transferase
PNP,NP	Purine Nucleoside Phosphorylase

Other

ACD	Acid Citrate Dextrose
AIR	5-Aminoimidazole Riboside
F6P	Fructose 6 Phosphate
HMS	Hexose Monophosphate Shunt
HPLC	High Pressure Liquid Chromatography
PRPP	Phosphoribosyl Pyrophosphate
PP_i	Pyrophosphate
P_i	Inorganic Phosphate
R5P, Ribose 5P	Ribose 5 Phosphate

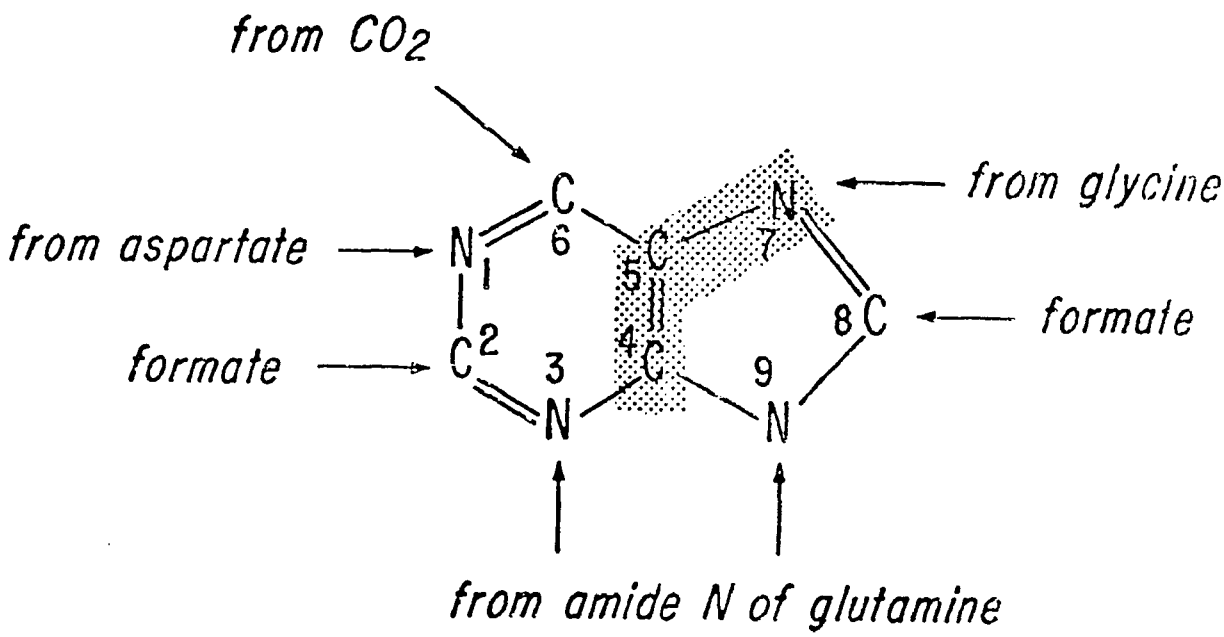
History

Excessive quantities of uric acid in blood is perhaps the oldest known metabolic abnormality in man. Uric acid concentrations in blood were shown to be increased in patients with gouty arthritis in 1854. Uric acid was demonstrated to be a purine compound in 1898 by Fischer. With the advent of radio-labeled compounds, the origin of individual members of the purine ring was defined. In 1943 Barnes and Schoenheimer fed pigeons ^{15}N ammonium citrate and found that uric acid was labeled. They also demonstrated the failure of ^{15}N urea histidine and arginine to be incorporated into uric acid (1). In 1947 Shemin and Rittenberg showed ^{15}N glycine was incorporated into uric acid. Studies with isotopes by Buchanan and Greenberg around 1950 identified the other precursors of the purine ring. Carbon atoms 2 and 8 are donated by formate (via tetrahydrofolate), carbon 4 and 5 from glycine, carbon 6 from CO_2, nitrogen atom 1 from aspartic acid, and nitrogen atoms 3 and 9 from glutamine (Figure 1). The present concept of purine synthesis de novo in humans is illustrated in Figure 2.

Purine nucleotides may be synthesized de novo or formed from free nucleosides and bases. De novo synthesis results in the production of inosine monophosphate which may be converted to adenosine monophosphate and guanosine monophosphate. The

Figure 1

Sources of Atoms from the Purine Ring, From Astrin (22)



resulting mononucleotides may be converted to di and tri nucleotides, deoxyribonucleotides, or catabolized to bases and nucleosides. Many interconversions exist at the base and nucleoside level. The final degradative product of purine metabolism is uric acid. Once uric acid is formed, there is no possibility of reutilizing the purine base.

Purine nucleotides participate in many cellular functions. Purines are the precursors of DNA and RNA, and nucleotides serve as an energy source for many reactions. Purine nucleotides may also serve as co-factors in many reactions.

A. Purine Biosynthesis--De Novo

The first committed reaction of purine biosynthesis is the condensation of phosphoribosyl pyrophosphate and glutamine in the presence of magnesium to form B-phosphoribosylamine, glutamic acid, and pyrophosphate. PRPP is an active intermediary in purine metabolism. The synthesis of PRPP is mediated by the enzyme phosphoribosyl pyrophosphate synthetase which catalyzes the reaction ribose-5-phosphate and ATP to form PRPP and AMP. PRPP synthetase is under several control mechanisms. Inorganic phosphate acts as an allosteric activator of PRPP synthetase (2). Removal of phosphate leads to complete loss of activity of PRPP synthetase (3). The K_m of PRPP synthetase for ATP is 1.4×10^{-5} (3), 3.3×10^{-4} for R5P (3), and 3.3×10^{-3} for P_i.

PRPP synthetase is reported to undergo aggregational changes in the presence of saturating concentrations of ATP-Mg The small molecular weight form is a dimer with two subunits of 30,000 each. In the presence of Mg-ATP two higher molecular weight forms(720,000 and 1,200,000) are found, and these appear to be the active form of the enzyme (4).

Alterations of metabolism which increase the availability of ribose-5-P are reported to increase levels of PRPP (and hence purine biosynthesis). Ribose-5-P is synthesized via the hexose monophosphate shunt of glucose metabolism (5) or non-oxidative degradation of fructose 6-P (6). There is also a report of synthesis via uronic cycle, but this is believed to be of little importance in synthesizing R-5-P in human tissues (7). Methylene blue, a dye which affects oxidative metabolism, is believed to increase PRPP levels by increasing the availability of R-5-P. Glutathione reductase has also been implicated in controlling levels of PRPP by increasing the availability of R-5-P via production of NADP, a cofactor in reactions of hexose monophosphate shunt (8).

End product inhibition is another mode of regulation of PRPP synthetase. ADP is a competitive inhibitor, competing with ATP with a K_i 1 x 10^{-5}. Plots of substrate versus velocity change from hyperbolic to sigmoidal in the presence of ADP and increasing concentrations of P_i. PRPP and 2,3 DPG

compete with ribose-5-P with a K_i of 5×10^{-5} and 5.3×10^{-3} respectively. As pointed out by Wynngarden and Kelley, only the K_i of 2,3 DPG approaches the concentration found physiologically in some tissue types (9).

PRPP synthetase is also inhibited by heterogeneous metabolic pool inhibition (10). This type of inhibition is noncompetitive and nonspecific. The amount of inhibition depends on the amount of total nucleotide pool with ITP, IDP, GDP, GTP, TDP, NAD, NADPH, AMP, ADP, and FAD among the reported contributors (3). Inhibition is nonsynergistic. This type of regulation allows for finely modulated control of the enzyme with a continuum of varying degrees of inhibition possible.

Glutamine, the other substrate for phosphoribosylpyrophosphate amidotransferase, is synthesized by glutamine synthetase. Glutamine synthetase isolated from E. coli (11) is activated by adenylation by ATP. The enzyme is subject to cumulative end product inhibition. AMP inhibits glutamine synthetase. For complete inhibition of glutamine synthetase, saturating concentrations of CTP, AMP, carbamylphosphate, alanine, tryptophan, histidine, and glucosamine 6 phosphate are needed. It has also been suggested that abnormalities in the degradation of glutamine would cause either increased purine biosynthesis (decreased degradation) (12) or decreased

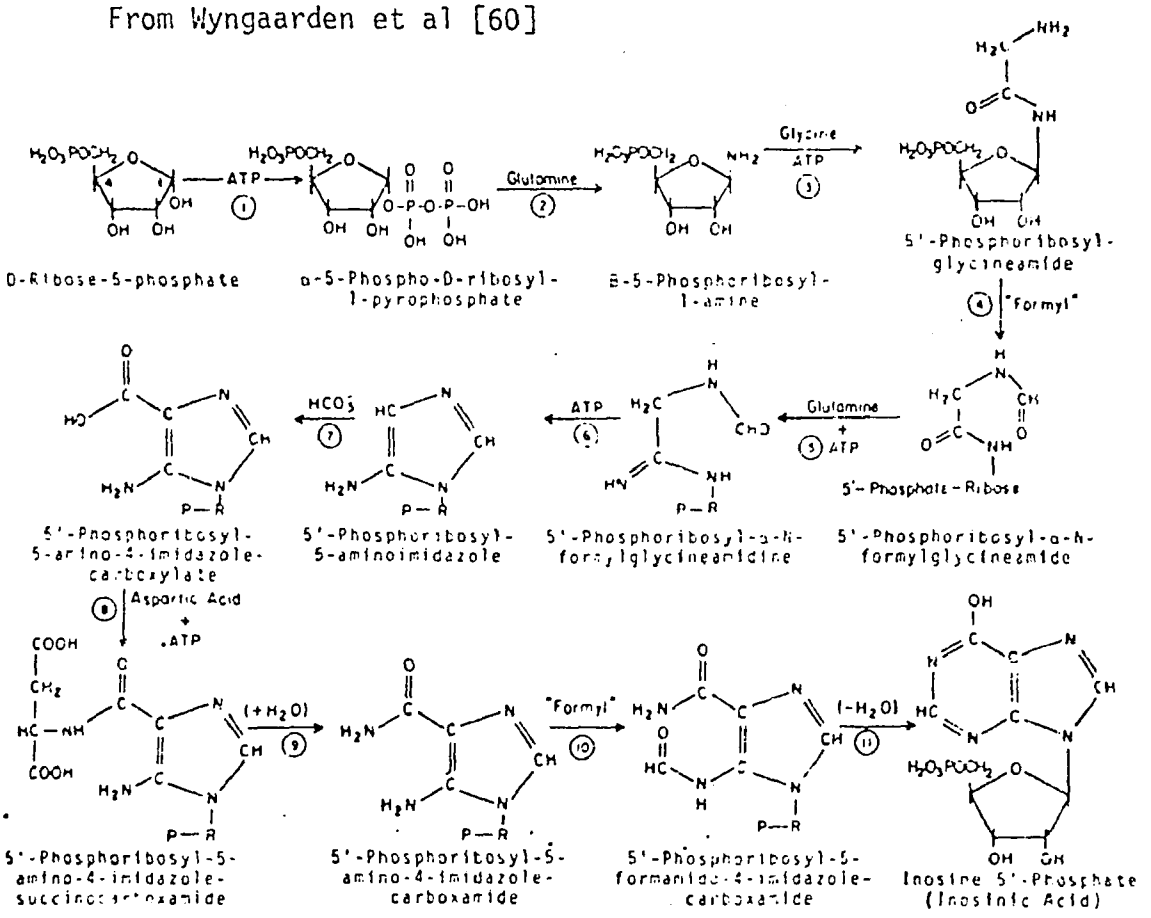
purine biosynthesis (increased degradation of glutamine or increased utilization of glutamine in another pathway). Glutamine concentration has been shown to be rate limiting to purine synthesis in human fibroblasts (13).

Amidophosphoribosyltransferase, the enzyme which catalyzes the first committed step of purine biosynthesis de novo, is also under several types of controls. The K_m for PRPP is 2.5×10^{-4} in human lymphoblasts and 1.6×10^{-3} for glutamine. Amidophosphoribosyltransferase from human placenta is reported to bind PRPP first, undergo a conformational change, then bind glutamine (14). In the presence of PRPP, the placental enzyme is in an active low molecular weight form 133,000 (15). In the presence of purine ribonucleotides the enzyme is present in a large inactive form (270,000) (15). Inhibition of this enzyme is equally effective by adenosine and guanosine nucleotides. Synergistic inhibition is produced by the combination of 6 amino and 6 hydroxy (ex: AMP and IMP) (14,15) which can be completely overcome by PRPP in the placental enzyme. Inhibition in lymphoid cells by 6 hydroxy and 6 amino nucleotides is reported to be additive (16).

The other enzymes of purine biosynthesis de novo are shown in Figure 2. It is not generally believed that there are other control points prior to the synthesis of IMP.

Figure 2

Purine Biosynthesis De Novo
From Wyngaarden et al [60]



Enzymes of Purine Biosynthesis De Novo

1 PRPP Sythetase
2 PRPP Amidotransferase
3 GAR Synthetase
4 GAR Formyl transferase
5 FGAM Synthetase
6 AIR Cycloligase
7 AIR Carboxylase
8 S-AICR Synthetase
9 Adenylosuccintate Lyase
10 FAICAR Transferase
11 Inosinate cyclohydrolase

B. Interconversions of Nucleotides

Conversion of IMP to AMP and GMP

Conversion of IMP to AMP. Conversion of IMP to AMP is mediated by two enzymes, adenylosuccinate synthetase and adenylosuccinate lyase. The reaction proceeds as shown in Figure 3. AMP and GDP inhibit adenylsuccinate synthetase with AMP a stronger inhibitor (17). GTP is an energy source for this reaction. Aspartate concentration has been reported to be rate limiting in the conversion of IMP to AMP (18,19). Increased formation of ^{14}C AMP from ^{14}C hypoxanthine was reported if aspartate was added to incubation media of human fibroblasts (20) and Erlich ascites cells (19).

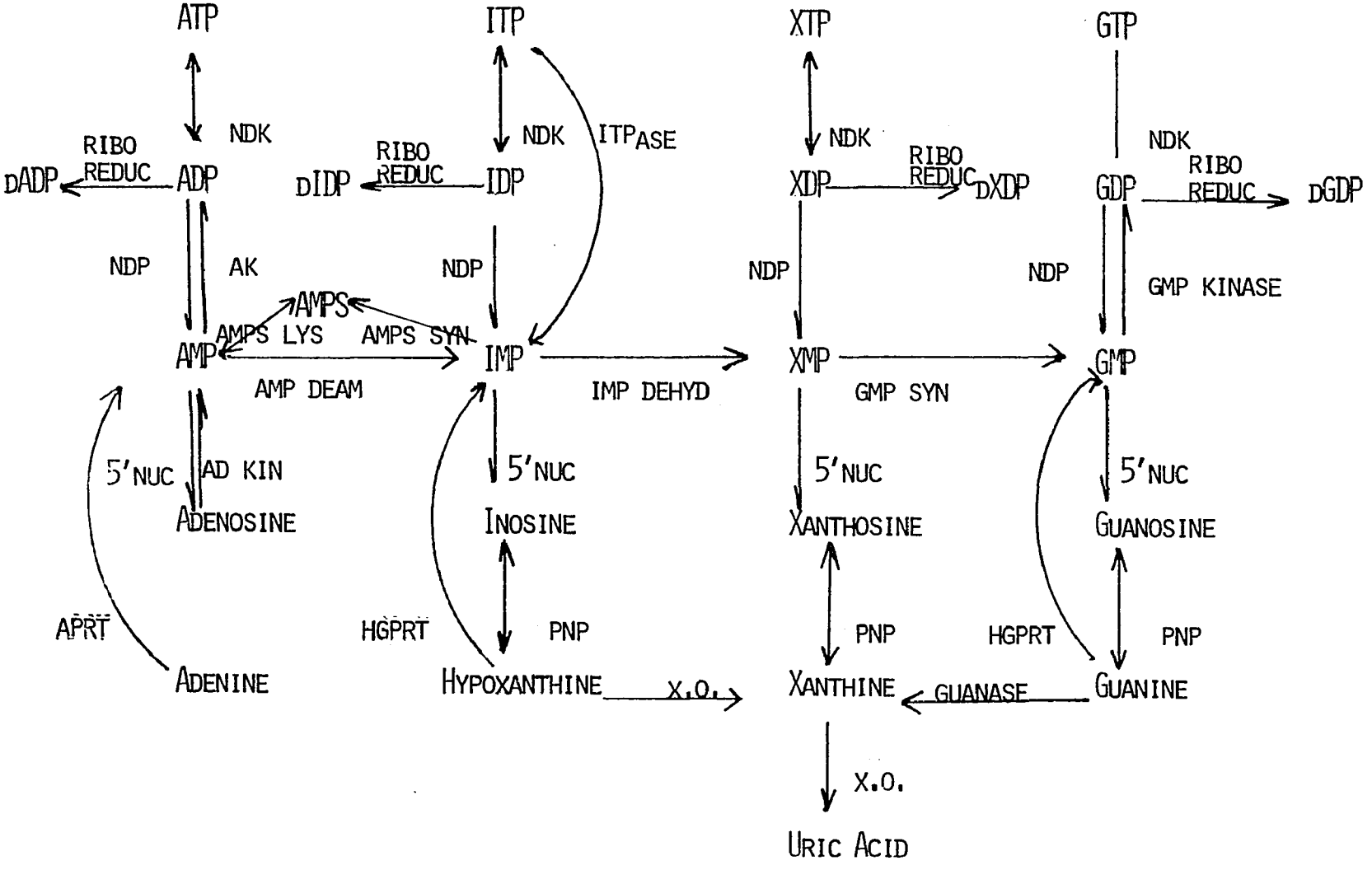
Conversion of IMP to GMP. Conversion of IMP to GMP takes place as shown in Figure 3. It is a two-step reaction. IMP dehydrogenase, the enzyme of purine metabolism that requires NAD as a cofactor, catalyzes IMP to XMP. GTP has been reported as an inhibitor of IMP dehydrogenase (21,22). XMP and IDP have also been reported as inhibitors of IMP dehydrogenase in lymphoid cells (22), although not at physiologic concentrations.

The next reaction involves amination of XMP to GMP. Glutamine and ATP are required. Relatively little has been studied in mammalian systems on the controls of GMP synthetase. Glutamine concentration has been demonstrated to be

Figure 3

Purine Nucleotide Interconversion

Figure 3


ATP
ITP
XTP
GTP
NDK
RIBO REDUC
dADP
ADP
dIDP
IDP
ITPASE
XDP
dXDP
GDP
dGDP
NDP
AK
AMPS
AMPS LYS
AMPS SYN
AMP
IMP
XMP
GMP
AMP DEAM
IMP DEHYD
GMP SYN
GMP KINASE
5'NUC
AD KIN
ADENOSINE
INOSINE
XANTHOSINE
GUANOSINE
APRT
ADENINE
HGPRT
PNP
HYPOXANTHINE
X.O.
XANTHINE
GUANASE
GUANINE
URIC ACID

rate limiting in the synthesis of guanine nucleotides (XMP → GMP) (23, 24, 25).

Convergence of AMP and GMP to IMP

In human cells AMP is converted to IMP by adenylate deaminase. Regulation of the enzyme has been reported in which ATP stimulates the deamination and GTP inhibits the reaction (21, 26). ATP is also reported to reduce the K_m for AMP and change sigmoid kinetics to hyperbolic. ATP can overcome inhibition by GTP, but GTP will prevent the stimulation of the enzyme by ATP. The K_m of adenylate deaminase for ADP is $0.4 - 4 \times 10^{-3}$ (22, 27).

Conversion of GMP to IMP by GMP reductase has been reported to occur in human erythrocytes (28). ATP is reported to inhibit to action of GMP reductase (29). The level of activity of GMP reductase is reported to be low in human erythrocytes (28).

Conversion of Mononucleotides to Dinucleotides and Trinucleotides by Phosphorylation

AMP is phosphorylated to ADP by a specific monophosphate kinase, adenylate kinase (30). In general, monophosphate kinases tend to be very specific, while diphosphate kinases tend to have broad specificity. GMP is phosphorylated to GDP by guanylate kinase, which is also reported to utilize IMP at about 1% of the rate of GMP. Several isozymes of guanylate kinase have been reported, but no variation in their distribution has been

found in humans (31). A fuller discussion of IMP as a substrate for guanylate kinase will be reported in the section on ITP metabolism.

ADP, GDP, and IDP are all converted to their respective triphosphates by nucleoside diphosphokinase. This enzyme is probably a series of enzymes with varying pH optimas (32) and slightly varying substrate affinity for various diphosphates. Control mechanisms have not been worked out in human cells.

ADP, GDP, and IDP may also be converted to the deoxynucleotides by ribonucleotide reductase. Controls of ribonucleotide reductase are rather complex and have been reported only in bacterial systems. Deoxy ATP and deoxy GTP inhibit ribonucleotide reductase in human cells. The allosteric regulation of ribonucleotide reductase has been reported (33).

C. Salvage of Purine Bases

Salvage reactions of purine bases are reactions in which preformed purine rings are condensed with PRPP to form nucleosides, or a preformed nucleoside is phosphorylated.

PRPP Reactions

APRT. Adenine and PRPP condense to form AMP and PP_i. The enzyme has an apparent K_m for adenine

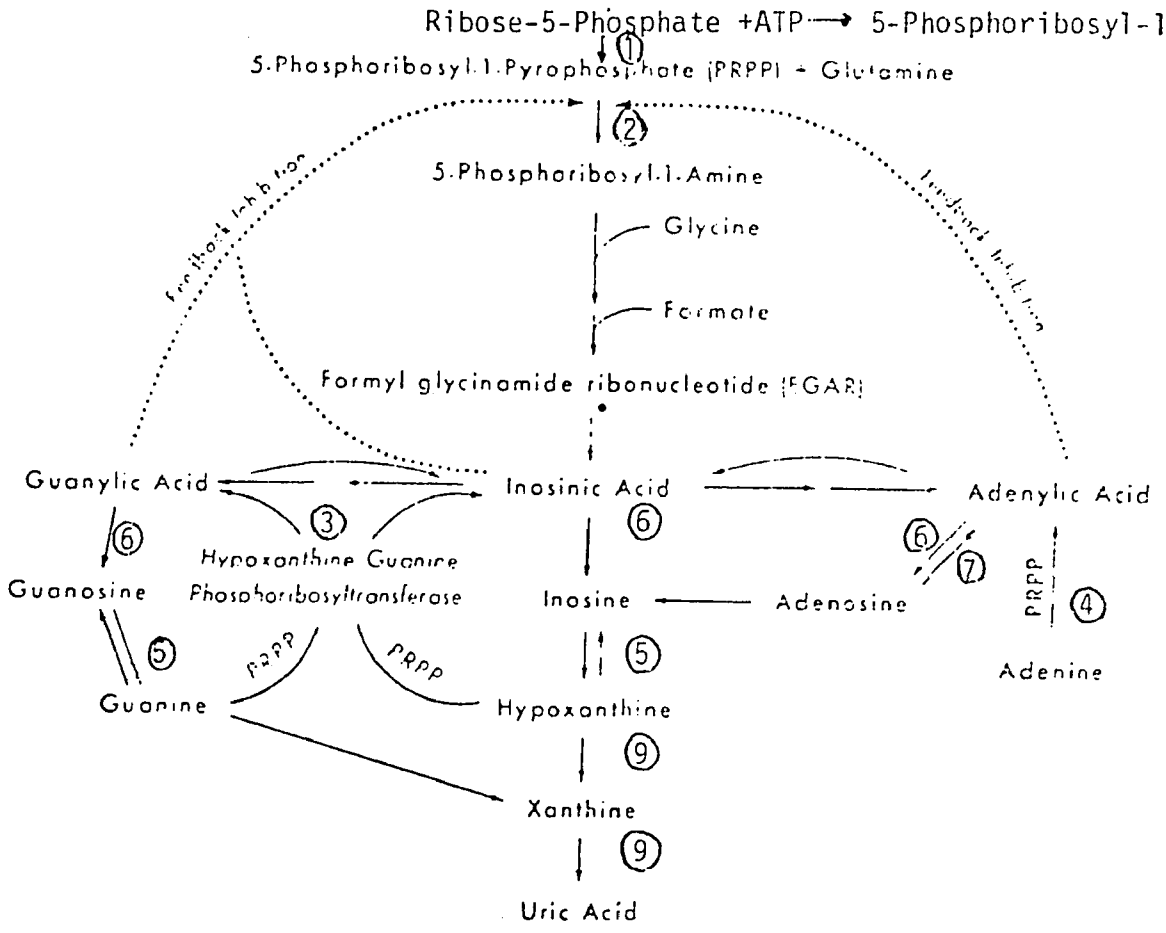
of 1 x 10^{-6}, and a K_m of 6.6 x 10^{-6} M for PRPP. The enzyme is inhibited by AMP, which is competitive with PRPP for substrate binding site (34). The equilibrium constant of the reaction is 290, which makesit irreversible for AMP formation. ADP and ATP are reported to inhibit the enzyme as well as GMP (35). K_i values are 1.18 x 10^{-6}, 2.19 x 10^{-6}, and 3.95 x 10^{-4} for GMP, AMP, and ATP respectively. APRT is reported to be stabilized by PRPP. AIC, an intermediary, in purine biosynthesis de novo is also a substrate for APRT (36). Although human erythrocytes lack the capacity to synthesize purines de novo, they do have some of the enzymes involved in purine biosynthesis. The ribosyl derivative of AIC can be phosphorylated by a kinase. Both this compound and the compound resulting from APRT action on AIC (see Figure2), a normal intermediary in purine biosynthesis, can undergo ring closure and methylation to form IMP by the usual pathway.

HGPRT. HGPRT catalyzes the reaction of hypoxanthine, guanine, and xanthine with PRPP. Xanthine has a maximal rate of 0.3% that of guanine (37). K_m value of hypoxanthine is 1.7 x 10^{-6}, 5 x 10^{-6} for guanine, and 2.5 x 10^{-4} for PRPP. The enzyme requires magnesium (38), and regulation of the enzyme has been reported to depend on

Figure 4

Purine Interconversion and Salvage

Modified from Wood et al. [43]



Enzymes of Purine Interconversion and Salvage

1 PRPP Synthetase
2 PRPP Amidotransferase
3 Hypoxanthine-Guanine Phosphoribosyl Transferase [HGPRT]
4 Adenine Phosphoribosyl Transferase [APRT]
5 Purine Nucleoside Phosphroylase [PNP]
6 5' Nucleotidase
7 Adenosine Kinase
8 Adenosine Deaminase [ADA]
9 Xanthine Oxidase [XO]

Mg^{++} to PRPP ratios. Under conditions of low magnesium to PRPP ratios the enzyme exhibits sigmoidal kinetics, while in high Mg/PRPP the enzyme kinetics are hyperbolic. IMP and GMP are feed back inhibitors (in Erlich ascites cells GMP is a stronger inhibitor), as well as GDP and GTP in human erythocytes (37). Inhibition by AMP and GMP is formally competitive with PRPP but involves mutually exclusive binding of the two substances.

Phosphorylation of Preformed Nucleosides

Adenosine Kinase. Adenosine kinase phosphorylates adenosine to AMP. The reaction is controlled by the level of adenosine. Adenosine kinase in human erythrocytes is reported to be inhibited by ADP, GDP, IMP, AMP, and GMP with K_i values of 4×10^{-4}, 1.4×10^{-3}, 2×10^{-3}, 8×10^{-3}, and 2×10^{-3} respectively (38). If adenosine is present in low concentrations, it will be phosphorylated; in higher concentrations it will be deaminated by adenosine deaminase (39).

Inosine Kinase. Reports of inosine kinase activity in human cells have been sketchy. IMP was reported to be formed in HGPRT-deficient liver and leukocytes when incubated with inosine (40,41). However, other investigators (42, 43) were unable to confirm these findings in fibroblasts and lymphocytes.

Guanosine Kinase. Guanosine kinase activity has been reported in human liver but not in human fibroblasts (44).

D. Degradative Enzymes

5' Nucleotidase

5' nucleotidase catalyzes the reaction of the nucleoside monophosphate to the nucleoside. ATP, GTP, UTP, and CTP are all reported to be inhibitors of the enzyme. Inhibition varies with the substrate, with 12 uM ATP inhibiting AMP hydrolysis by 50% and 240 uM ATP inhibiting IMP hydrolysis by 50% (45).

Nucleoside Phosphorylase

Nucleoside phorphorylase catalyzes the reaction of base ribose and P_i to a base and ribose 1 phosphate. The reaction is freely reversible, and the direction depends on the concentration of substrate and product. Guanosine and inosine are substrates for purine nucleoside phosphorylase, with xanthosine utilized to a lesser extent (46). Adenosine is cleaved by NP of liver (47). Adenine is not reported to be utilized as a substrate in human erythrocytes.

Adenosine Deaminase

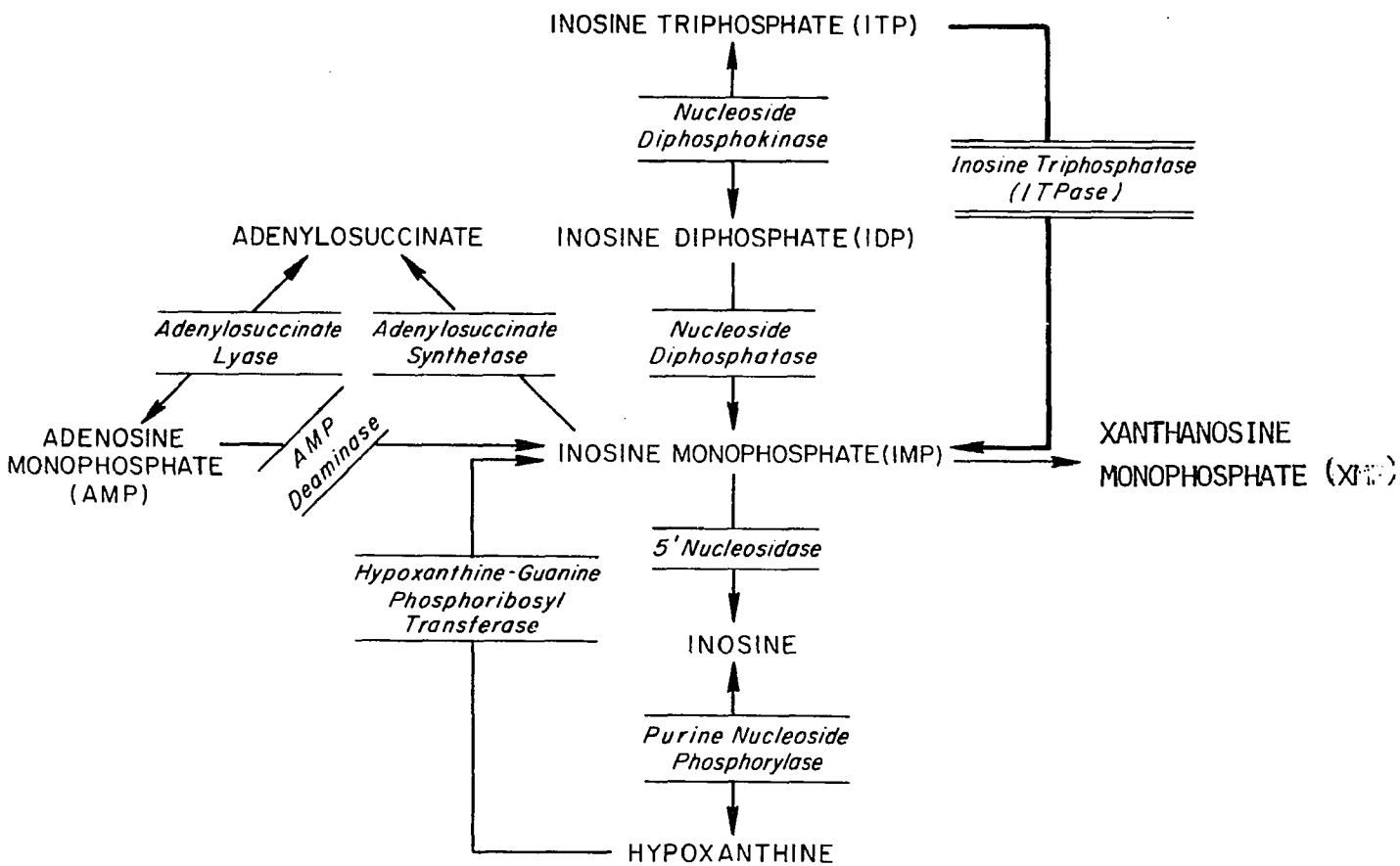
Adenosine deaminase catalyzes the deamination of adenosine to inosine The enzyme is present in a low molecular weight catalytically active form. The low

molecular weight form combined with a higher molecular weight enzymatically inactive protein gives rise to tissue isozyme (48). The enzyme has an apparent K_m for adenosine of 2×10^{-5} M and does not distinguish between deoxyadenosine and adenosine (49).

While each of the enzyme reactions has been discussed individually, it should be remembered that many of the compounds have alternate pathways and that several enzymes with varying K_m's vie for them as substrates. Figure 5 shows the alternate pathways for IMP. AMP also has a multitude of choices, as shown in Figure 3.

Figure 5

Inosine Nucleotide Interconversion


INOSINE TRIPHOSPHATE (ITP)
Nucleoside Diphosphokinase
Inosine Triphosphatase (ITPase)
ADENYLOSUCCINATE
INOSINE DIPHOSPHATE (IDP)
Adenylosuccinate Lyase
Adenylosuccinate Synthetase
Nucleoside Diphosphatase
ADENOSINE MONOPHOSPHATE (AMP)
AMP Deaminase
INOSINE MONOPHOSPHATE (IMP)
XANTHANOSINE MONOPHOSPHATE (XMP)
5' Nucleosidase
Hypoxanthine-Guanine Phosphoribosyl Transferase
INOSINE
Purine Nucleoside Phosphorylase
HYPOXANTHINE

Defects in Human Purine Metabolism

A. Increased Activity of Purine Enzymes

1. PRPP Synthetase (ATP + R5P → AMP + PRPP)

In 1972 Sperling et al. reported an accelerated rate of PRPP synthesis in two gouty brothers with marked overproduction of urate (50). Erythrocytic PRPP synthetase from these patients was shown to have reduced sensitivity to feedback inhibition of GDP and other regulatory nucleotides. In addition, reduced sensitivity to feedback inhibition by 2,3,DPG was present (51). Other variants of PRPP synthetase have been reported resulting in overproduction of PRPP. Becker et al. reported on a variant PRPP synthetase that had an altered catalytic unit. The enzyme had an increased specific activity, with normal feedback inhibition, normal affinity for substrate, and normal amounts of enzyme present (52). This patient was hyperuricemic, had increased urinary uric acid, and gout. A third mutation was reported in which there was increased affinity for R-5-P, a substrate of PRPP synthetase (53).

2. Adenosine Deaminase (Adenosine → Inosine)

Valentine has demonstrated increased red cell adenosine deaminase activity in 12 members of a family with hemolytic anemia. The enzyme was found to have

properties similar to the normal enzyme with respect to K_m, specific activity, heat stability, and K_i of guanylurea sulfate (54). Valentine et al. postulated that the increased activity of adenosine deaminase in this family was due to an excess of enzyme molecules (54). However, Valentine also reported a 2-3 fold increase of pyrimidine 5' nucleotidase activity in these patients, the significance of which is unclear.

B. Decreased or Absent Activity of Purine Enzymes

1. HGPRT Deficiency (Hypoxanthine + PRPP ⟶ IMP)
(Guanosine + PRPP ⟶ GMP)

Seegmiller et al. in 1967 described the gross deficiencies of the enzyme HGPRT in erythrocyte lysates of three patients with Lesch-Nyhan Syndrome. The deficiencies of HGPRT were subsequently demonstrated in all tissue studies of patients with Lesch-Nyhan Syndrome (55). Clinical features of this syndrome include choreoathetosis, mental retardation, spastic cerebral palsy, uric acid urinary stones, and compulsive self-mutilation (56).

Purine metabolism in patients deficient in HGPRT activity has been extensively studied. The most marked metabolic abnormalities are the inability to convert hypoxanthine and guanine to IMP and GMP respectively and excessive production of uric acid due to increased purine biosynthesis de novo (57). The rate of ^{14}C

formate incorporation into FGAR (an intermediate in purine biosynthesis de novo) in the presence of azaserine was increased four to six fold over normal fibroblasts (58,59). PRPP, a substrate for HGPRT has been implicated in the regulation of purine biosynthesis de novo. As previously reviewed, PRPP is also a substrate for PRPP amidotransferase, the rate limiting step of purine biosynthesis de novo. Elevated levels of PRPP have been reported in tissues from patients with HGPRT deficiency. Since PRPP is normally present in amounts well beneath the K_m of PRPP amidotransferase, the increased availability of PRPP was postulated to be the basis of increased de novo synthesis of purines (60). In addition, PRPP converts PRPP amidotransferase to a more active form (60). Hence, alterations in the level of PRPP have been postulated to affect purine biosynthesis. However, several investigators have reported conditions where PRPP levels are not rate limiting (59,61).

Purine nucleotides inhibit the activity of PRPP amidotransferase. Lack of inhibitory nucleotides was postulated as an alternative explanation for increased purine biosynthesis de novo (61). Thompson et al. (59) have presented evidence that hypoxanthine normally inhibits de novo synthesis, while HGPRT deficient

lymphoblasts and fibroblasts are resistant to the inhibition produced by hypoxanthine.

Several abnormalities in purine metabolic enzymes secondary to the deficiency of HGPRT have been reported. PRPP synthetase activity in cultured fibroblasts and lymphocytes from patients deficient in HGPRT is increased 2-4 times. However, PRPP synthetase activity is normal in erythrocytes of patients with HGPRT deficiency (60).

APRT is increased in both activity and amount in erythrocytes from patients with HGPRT deficiency (60), but activity is normal in cultured fibroblasts and lymphoblasts from these patients (62). PRPP has been reported to stabilize APRT (63).

IMP dehydrogenase activity has been reported elevated in erythrocytes of patients with HGPRT deficiency but normal in leukocytes, fibroblasts, and muscles of these patients (64). It has been proposed that the increased activity of IMP dehydrogenase in erythrocytes of patients with HGPRT deficiency is due to the insensitivity to inhibition by a dialyzable inhibitor (65).

Mutations in the HGPRT enzyme exhibit a great degree of heterogeneity. Mutations have been reported resulting in reduced to absent activity, increased sensitivity to inhibition by products (66), decreased sensitivity to

inhibition by end products (67), increased heat stability (68), decreased heat stability (66,67,68), altered electrophoretic mobility (66,69-71), altered substrate specificity, altered K_m (72,73), altered stability in vivo (74), CRM^+ mutants, and CRM^- mutants (75,76).

Partial deficiency of HGPRT is associated with gout (77). Clinically, patients with partial deficiency of HGPRT may have acute gouty arthritis, renal stones, crystalluria, neurologic dysfunction, and high serum urate levels (76). As mentioned above, there is a great deal of heterogeneity in enzyme activity, and values of up to 30% of normal have been reported in documented cases of partial HGPRT activity. In addition, in one patient with clinical features consistent with partial HGPRT activity no HGPRT activity was found in erythrocytes, lymphocytes, or fibroblasts (78).

Defects in purine metabolism associated with total deficiencies of HGPRT have been reported present in some of the partially deficient patients. Increased activity of PRPP synthetase in fibroblasts, increased stabilization of APRT in erythrocytes, and increased activity of IMP dehydrogenase in erythrocytes were reported present in some of the partial deficients. Excess serum urate was reported in partial deficients, as was increased

^{14}C glycine incorporation into uric acid. Because of the heterogeneity, it is difficult to evaluate the abnormality of purine metabolism in partial deficients.

Studies of enzyme activity in vitro do not always give an accurate assessment of enzyme activity in vivo or of alterations caused by dysfunction of an enzyme. Hence, metabolic studies of the conversion of a precursor to product in intact cells is informative. Conversion of hypoxanthine to inosine monophosphate did not occur in fibroblasts totally deficient in HGPRT. However, a partial deficient (20% of normal activity as determined by an assay of fibroblast lysates, no activity in erythrocytes) was able to convert ^{14}C hypoxanthine to ^{14}C IMP in intact cells at a rate comparable to normal fibroblasts (79). In addition, in this particular patient's leukocytes 14% of normal activity was recorded in assay of lysates, with 100% of normal activity in intact cells. The enzyme was found to be thermolabile in comparison to enzyme from normal leukocytes. Clinically, this patient presented with only mild neurologic symptoms. A first cousin, clinically normal, was found to have the same variant enzyme activity (80).

Since cells deficient in HGPRT are unable to convert

hypoxanthine to IMP, a unique opportunity is provided to investigate alternate pathways of IMP synthesis. Incubation of intact leukocytes deficient in HGPRT with ^{14}C inosine did not result in the production of radio-labeled IMP to a considerable extent (41) providing evidence against the existence of an inosine kinase in these cells. As previously mentioned, incubation of erythrocytes deficient in HGPRT with a radio-labeled intermediary of purine biosynthesis de novo resulted in the formation of radio-labeled IMP (36).

2. APRT Deficiency (Adenine + PRPP $\longrightarrow$ AMP + RIP)

Reduced APRT activity in humans was first reported by Kelley et al. in 1968 (81). Total deficiency of erythrocytic APRT has been reported in several families (82,83). Clinically, total deficiency of erythrocytic APRT manifested as urinary lithiasis in two patients. Calculi were composed of 2,8 dihydroxyadenine (83). However, a sibling with the same degree of APRT deficiency was totally healthy (83). Reports of partial deficiency of APRT were probably due to heterozygosity. A heat stable enzyme allele has a frequency of 85% and the heat labile enzyme allele has frequency of 15% (84). No disease was associated with allelic forms of APRT (84). APRT deficiency was found to have the same incidence in a normal population and a gouty population (85). A long

lymphoid line deficient in APRT (selected in vitro by resistance to 2,8 diaminopurine or 8 azaserine) did not overproduce purines and had normal levels of PRPP synthetase (85). The line showed a gross inability to convert ^{14}C adenine to ^{14}C AMP (85). From this limited data it is difficult to interpret the effect of reduced APRT activity on purine metabolism. However, it is clear that deficiency of APRT results in very different alterations of purine metabolism than deficiency of HGPRT.

3. Adenosine Deaminase (Adenosine $\rightarrow$ Inosine)

In 1972 Giblett et al. reported deficiency of adenosine deaminase in two patients with severe combined immunodeficiency. The metabolic basis of the immune dysfunction was first proposed to be an increase of cAMP, a known immunosuppressant, secondary to increased levels of adenosine (86). Pyrimidine starvation was proposed by Green et al. (87) to be the basis of the immune dysfunction, since in cultured cells adenosine toxicity could be overcome by addition of uridine. Pyrimidine starvation was theorized to be due to depletion of PRPP by adenine or decreased de novo pyrimidine synthesis. However, addition of uridine does not restore immune function in ADA deficient

peripheral leukocytes. Addition of exogenous ADA restored function. Levels of cAMP and pyrimidine nucleotides have been reported to be normal in peripheral leukocytes from ADA deficient patients (88).

Next, adenosine was postulated to be toxic to B and T lymphocytes. Excess adenosine was postulated to be converted to S-adenosylhomocysteine, a potent inhibitor of DNA methylation (89). However, cultured lymphocytes from patients deficient in ADA or normal lymphocytes in the presence of an ADA inhibitor were demonstrated to be no more sensitive to adenosine than normal lymphocytes (90).

The current hypothesis is that deoxyadenosine, a substrate of adenosine deaminase, accumulates in tissues of patients lacking ADA. ADA deficient leukocytes were reported to be 100 times more sensitive to the toxic effects of deoxyadenosine than normal leukocytes (91). Deoxy ATP, a metabolite of deoxyadenosine, was demonstrated to accumulate in erythrocytes of immunodeficient ADA deficient patients but not in immunocompetent patients. Experiments with cultured T lymphoma cells by Ulman et al. demonstrated that deoxy ATP accumulated if these cells were incubated with deoxyadenosine in the presence of an inhibitor of ADA (92). Deoxy ATP, a potent inhibitor of ribonucleotide reductase, prevents

the conversion of ribonucleotides to deoxyribonucleotides, particularly CDP to dCDP (92). Thus, Ulman et al. demonstrated that the toxicity of deoxyadenosine could be overcome by the addition of deoxycytidine to the culture media (92).

The immune system was theorized to be particularly susceptible to the toxic effects of deoxy ATP since it is composed of rapidly dividing cells. In addition, it has been noted that T lymphocytes have the capacity to synthesize dATP at approximately 20-45 times that of of B lymphocytes. This difference is believed to be due to differential activity of 5' nucleotidase in these two cell types (93). B lymphocytes are reported to have 44 fold more 5' nucleotidase activity than T lymphocytes. 5' nucleotidase would compete for dAMP, thereby reducing the amount of dAMP available for conversion to dADP and dATP.

Metabolic studies have indicated the rate of purine biosynthesis de novo is normal in ADA deficient fibroblasts (59). Incubation of ADA deficient erythrocytes with 8-^{14}C inosine resulted in the formation of IMP and in some patients the formation of ITP (94). As previously mentioned, incubation of ADA deficient lymphocytes with 8-^{14}C adenosine resulted in the increased accumulation of ATP.

Evidence of genetic heterogeneity in ADA deficiency is found in those individuals who are ADA deficient in erythrocytes but possess 20-30% of normal activity in lymphocytes (95). As can be expected from the above discussion, these individuals were found not to accumulate dATP. Further metabolic studies of these partial deficients in terms of abnormalities of purine metabolism have yet to be conducted. Further evidence of heterogeneity was found in ADA deficient individual who was found to have more CRM^+ enzyme than could be accounted for by residual activity present, indicating the presence of an inactive enzyme in this patient (96).

4. Purine Nucleoside Phosphorylase (Inosine → Hypoxanthine)
(Guanosine ⇢ Guanine)

Deficiency of purine nucleoside phosphorylase was reported by Giblett et al. (97) in a patient with a T cell defect. The basis of the immune deficiency is believed to be accumulation of deoxyguanosine, a substrate for purine nucleoside phosphorylase. Deoxyguanosine is phosphorylated to deoxy GTP in T cells. Deoxy GTP inhibits ribonucleotide reductase from catalyzing the reduction of GDP to dGDP and CDP to dCDP (98). Support of this hypothesis is found in that deoxyguanosine was observed to be elevated in erythrocytes from two patients with PNP deficiency. In addition, in a model T cell

system double mutants deficient in the ability to either phosphorylate deoxyguansine or transport deoxyguanosine and deficient in purine nucleoside phosphorylase activity were resistant to the toxic effects of deoxyguanosine (99). Other substrates of purine nucleoside phosphorylase deoxyinosine, inosine, and guanosine were not reported to be toxic to T cells. All of the substrates have been reported to be excreted in the urine of PNP deficient children (100). The differential toxicity of deoxyguanosine in T cells and B cells is believed to be due to differential ability to phosphorylate deoxyguanosine. Thymus has the highest ability to phosphorylate deoxyguanosine of all human tissues studied (101).

Purine biosynthesis de novo was reported to be elevated in patients with purine nucleoside phosphorylase deficiency but normal in cultured fibroblasts (100). However, Thompson et al. presented evidence that the rate of purine biosynthesis de novo of PNP deficient fibroblasts in media containing hypoxanthine is normal but accelerated in purine free media. The observed normal rate of purine biosynthesis de novo was proposed to be due to the inhibition of purine biosynthesis de novo by hypoxanthine present in the media (59).

Genetic heterogeneity has been well documented in

purine nucleoside phorphorylase deficients. Variants include an electrophoretically abnormal unstable enzyme, electrophoretically abnormal inactive stable enzyme, and an unstable enzyme with increased affinity for substrate. Two brothers with partial deficiency of purine nucleoside phosphorylase had milder clinical abnormalities than patients with total deficiency (102). These children had almost normal serum and urinary uric acid levels (103).

5. 5' Nucleotidase (IMP $\longrightarrow$ Inosine + P_i)
(GMP $\longrightarrow$ Guanosine + P_i)

Deficiency of 5' nucleotidase was reported by Johnson et al. in lymphocytes of patients with common variable agammaglobulinemia (104). Edwards et al. have found normal 5' nucleotidase activity in these patients but reduced ecto 5' nucleotidase activity in lymphocytes of patient with x linked agammaglobulinemia (105). Schwarber et al. failed to confirm the findings of Edwards et al. (106).

6. Adenylate Kinase--2ADP $\longrightarrow$ ATP + AMP

Deficiency of adenylate kinase 1 (red cell isozyme) is associated with hemolytic syndromes resembling pyruvate kinase deficiency (107).

Deficiency of adenylate kinase (muscle isozyme) was reported by Schmidtt et al. in the mother and sister

of two children who had died of malignant hyperpyrexia after halothane anesthesia (108). Red cell AMPwas normal. No information on other aspects of purine metabolism is available, nor is it certain if the occurrence of malignant hyperpyrexia and adenylate kinase deficiency in this family was not merely coincidental.

7. ATPase (ATP $\longrightarrow$ ADP)

There have been several unconfirmed reports of ATPase deficiency associated with hemolytic anemia (109,111). No further details are available.

8. AMP Deaminase (Muscle Isozyme)

Deficiency of the muscle isozyme of AMP deaminase has been reported in five families (111). The clinical symptoms include muscle weakness and fatigue after exercise. No information is available on alterations of purine metabolites in these individuals.

Metabolic Effects of Partial Enzyme Deficiencies

The metabolic effects ascribed to partial enzyme deficiencies vary. In part this variance may be due to the method used to ascertain the metabolic abnormality. Hence, a variant of HGPRT with an unstable enzyme showed no activity in erythrocyte lysates, reduced activity in leukocyte lysates, but normal activity in intact lymphoid cells. There were no alterations of metabolism in intact leukocytes. Predictably, the clinical consequences of this sort of variant was less

severe than total deficiency of HGPRT.

In contrast, other variants with partial HGPRT deficiency, while showing milder clinical courses than total HGPRT deficients, exhibited some of the same metabolic abnormalities found in total deficiency. Increased activity of PRPP synthetase in fibroblasts, increased stabilization of APRT in erythrocytes, increased de novo biosynthesis, and excess serum urate have all been reported to be present in partial HGPRT deficients.

Leukocytes from a patient partially deficient in ADA were demonstrated not to accumulate deoxy ATP in contrast to leukocytes from patients totally deficient in ADA. The partially ADA deficient individual was immunocompetent.

Thus, it becomes apparent that the metabolic effects of a partial deficiency will depend on the functioning of the residual enzyme in vivo. If sufficient enzyme activity is present in tissues that manifest the dysfunction to metabolize substrates at physiologic concentrations, no alterations of metabolism will be observed in vivo. However, tissue culture conditions which stress these cells may reveal metabolic abnormalities in partial deficients. Thus, in the case of the HGPRT variant, if initial studies had been carried out in intact cells, the patient would never have been classified as a partial deficient.

These examples demonstrate that the metabolic abnormality exhibited in a partial deficient will depend on the functioning of the enzyme in vivo and the in vitro method utilized to ascertain these alterations.

It is not possible to correlate the severity of the metabolic abnormality solely with the amount of residual activity established in vitro. In addition, different enzymes will need different amounts of residual activity to catabolize the amount of substrate present physiologically. The level of activity sufficient to catabolize the amount of substrate present may vary from tissue to tissue.

HPLC Background

High pressure liquid chromatography is the chromatographic separation of compounds under high inlet pressures on small diameter columns packed with small size particles (10 microns). As in conventional chromatography, high pressure chromatography can be liquid-liquid in which the sample is partitioned between the mobile and stationary liquids (partition chromatography), liquid-solid chromatography (adsorption chromatography), ion exchange chromatography, or exclusion chromatography. Reverse phase chromatography is the situation in which the stationary support is nonpolar, while mobile phase is polar. Paired ion chromatography is chromatography in which a polar particle is "paired" with another polar particle to produce a nonpolar compound, which can then be analyzed by reverse phase chromatography.

Advantages of liquid chromatography include high sensitivity, good resolution, quantitative recovery of sample, and small sample size. Chromatograms are reliable and reproducible. The method is versatile in that many parameters can be changed to produce desired separations.

Among the parameters that can be varied are pH of solvent, ionic strength, flow rate, column packing and volume of sample injected and gradient length and shape (linear, concave, etc.).

Detection of compounds in the case of nucleotides and

nucleosides is accomplished by passing the sample through a highly sensitive U.V. monitor (flow cell of 8 ul). A fraction collector can be connected to the outflow for collection of fractions and monitoring radioactivity if a radioactive compound has been injected. Although the standard wavelength of detection of nucleotides is 254, a variable wavelength U.V. detector can be employed for greater sensitivity in the detection of the compound of interest. Variable wavelength recorders also help in the identification of unknown compounds.

Identification of compounds can be accomplished through several means. Comparison of the elution time of a standard with an unknown peak is helpful as a first step. However, in cell extracts the pH may vary, or other compounds affecting the retention time may be present. In this case coinjection of the standard and unknown compound (use of an internal standard) is helpful. It should be noted that two different compounds can co-elute with the same elution time.

Variable wavelength recorders are of use in allowing the comparison of ratios of absorption of a compound at different wavelengths (for example, a protein might absorb more at 280 nanometers than a nucleotide; adenosine would absorb more at 260 than 250, etc.). In some instruments, called dual wavelength recorders, a chromatogram can be traced at two wavelengths at once. However, the wavelengths are usually set (254 and 280), thus

losing the versatility of a variable wavelength recorder.

Compounds collected from the chromatogram can be characterized by either physical or chemical methods. If enough of a substance is present, it can be subjected to conventional scanning spectroscopy by a Gilford or Carie Spectrophotometer. In the case of a nucleotide, it can be physically hydrolyzed (boiled in 1M perchloric acid) (112) to bases and nucleosides and then reinjected on a column which analyzes bases and nucleosides.

Perhaps the most elegant method of identification is the enzymatic peak shift, first described by Brown et al. (113). The biochemical specificity of the enzyme is utilized. For example, a purine mononucleotide can be identified by treatment with 5' nucleotidase which will result in formation of the corresponding nucleoside if the mononucleotide is a substrate for 5' nucleotidase. The resultant compound can then be analyzed on a nucleoside/base analyzing system. The mixture can further be treated with purine nucleoside phosphorylase resulting in base formation from the nucleoside, if the nucleoside is a substrate for purine nucleoside phosphorylase (as an example, XMP + 5' nucleotidase $\rightarrow$ xanthosine, xanthosine + PNP $\rightarrow$ xanthine). As pointed out by Brown (113), the enzymatic peak shift can be used to "unmask" the chromatogram. If a compound is present in large quantities, it can be shifted

away from the area of interest by treatment with an enzyme. The classical example is hexokinase used to shift ATP to ADP (113). A note of caution should be added though, since it is possible that the substrate specificity of the enzyme might be such that it will also shift compounds buried under the major peak (in the case of hexokinase ITP, which elutes near ATP, is also a substrate for hexokinase) (114).

Another enzyme that is particularly useful in the identification of purine nucleosides is adenosine deaminase, used to shift adenosine to inosine. Since both of these compounds are easily observable in the chromatogram, inosine and adenosine can both be positively identified. An interesting finding by use of peak shift methodology involving adenosine deaminase is that different forms of the enzyme have different substrate specificities with modified bases (115).

In summary, high pressure liquid chromatography provides a convenient, relatively rapid system for the analysis of nucleotides and nucleosides. Among its advantages are high sensitivity, good reliability, and good reproducibility. The ability to produce a full nucleotide or nucleoside profile of a cell extract in a single analysis is of great advantage. Among the disadvantages are the ability to analyze only one sample at a time and the possibility of co-elution of two compounds. Another disadvantage is the time involved in

maintenance of columns and pumps, although this is relatively minor when compared to the benefits.

Use of Long-Term Lymphoid Lines for Studies in Purine Metabolism

Long-term lymphoid lines are long-term cultures established from B lymphocytes. The first long-term cultures of human lymphoid cells was established by Epstein and Barr from Burkitt's lymphoma cells (116). Establishment of lymphoid cultures from normal individuals was reported in 1967 (117).

Long-term lymphoid lines are especially suited to investigation of defects in purine metabolism. The lines grow permanently in cell culture and retain their diploid karotype. Many enzyme defects, as well as alterations of metabolism, demonstrated in fresh tissues can also be demonstrated in lymphoid lines. Cells with rare enzyme defects can be grown in large numbers and kept available for future investigation. In addition, lymphoid cells express the enzymes necessary for both de novo and salvage pathways of purine metabolism.

These cultures provide an excellent model to investigate alterations of purine metabolism.

Tissue culture, however, does present some limitations. Cells are grown in artificial media which very often does not possess physiologic levels of compounds. Alterations in the composition of media may have profound affects on the metabolism of the cell. One such example is the presence of hypoxanthine in media constituted from undialyzed fetal calf serum, in concentrations sufficient to inhibit purine biosynthesis de novo (59). Another limitation is that all

enzymes of a pathway may not be operative in a single tissue type, but other tissues in the body may express these enzymes. The pathology associated with a defect of the enzyme may not be present in the lymphocyte. For example, partial deficiency of HGPRT results in purine overproduction with deposition of urate in joints (gout). However, B lymphocytes lack xanthine oxidase, the enzyme which degrades purine bases to urate. These cells instead excrete hypoxanthine into the media. Sole use of a B cell in vitro model for studying partial HGPRT deficiency would in no way predict or explain the excess urate found in these patients.

In contrast, lymphoid cells are uniquely sensitive to deficiency of another enzyme of purine metabolism, adenosine deaminase (118). While the primary enzyme defect is present in all tissue types, lymphoid cells manifest the severest dysfunction.

Another objection to the use of lymphoid lines is the possibility that the transformation necessary for the cells to grow indefinitely alters cellular metabolism and is not representative of the same tissue type in vivo. However, many studies have demonstrated that the same metabolic abnormality manifest in lymphoid lines was also present in fresh tissues from the same individual.

Thus, while studies with long-term lymphoid cultures present many attractive opportunities, a note of caution must

be introduced. From the data available lymphoid lines present an excellent model for defects in purine metabolism.

Base and Nucleoside Transport

Since metabolic studies to be discussed later depend on the transport of radio-labeled nucleosides and bases across intact cell membranes, a brief review of the factors affecting purine transport will be discussed. Purine bases are hydrophilic in nature. Transport across membranes depends on facilitated diffusion (119). The carrier mediated system is believed to be shared by purine and pyrimidine bases. Hypoxanthine is reported to have the highest affinity for the transport carrier with uptake of purine bases highest at low cell densities (119).

Nucleoside transport is also believed to be a carrier mediated process. Whittan et al. have reported that erythrocytes are freely permeable to purine nucleosides (120). In polymorphonuclear leukocytes adenosine and thymidine have been demonstrated to share a saturable system. Among the factors reported to affect transport of nucleosides are: the stage of cell growth, density of cell population, availability of serum factors, and the level of cAMP (119).

Transport of nucleosides and bases are increased from the G1 to S stage. The level of transport drops after G2 (119). Transport of nucleosides has been demonstrated to vary

inversely with the density of cell population. However, virally transformed cells do not show this density dependence (119). Increases in cAMP have been demonstrated to decrease the transport of nucleosides across the membrane (119). Transport of purine bases across cell membranes is readily inhibited by a series of competitive and noncompetitive inhibitors (119).

In human fibroblasts Cohen et al. have provided evidence for the uptake of inosine by two transport systems. At low concentrations of inosine (10 uM inosine) transport of inosine is independent of purine nucleoside phosphorylase activity. At high concentrations of inosine (100 uM) human fibroblasts lacking in purine nucleoside phosphorylase can transport a reduced amount of inosine. Thus, in human fibroblasts two transport mechanisms are operative (121).

A clone of mouse lymphoma cells deficient in the ability to transport adenosine was demonstrated to be deficient in the ability to transport all other purine and pyrimidine nucleosides tested (122). Transport of bases was found to be intact. This observation lends credence to the existence of two separate transport systems for nucleosides and bases. Kinetic studies indicate that the same carrier is involved in the facilitated transport of both purine and pyrimidine nucleosides (122).

Table 1

Characteristics of ITPase

Chief Investigator	Liakopoulou	Vanderheiden	Wang	Holmes	Vanderheiden	Hershko	Chern	Wang
enzyme source	human RBC	human RBC	human RBC	human RBC	human RBC	rabbit RBC	rabbit RBC	rabbit liver
fold purification	--	1	1153	1	2,300	100	2,000	668
K_m	1×10^{-4}	6×10^{-4}	3.46×10^{-5}	7×10^{-5}	1.3×10^{-5}	3×10^{-5}		
pH optima	7.5-8.5 Broad	8.5 9.5 + DTT			8.6 tris/HCl 9.6 glycine	8.7	7-8 tris 9.75 B-alanine	9.75
buffer	tris	tris	B-alanine	tris	glycine	tris	B-alanine	B-alanine
ion required	Mg^{++} 50 mM	Mg^{++}	Mg^{++} 1 mM	Mg^{++} 20 mM	Mg^{++}		Mg^{++} 10 mM no rxn Ca^{++}, Fe^{++}, Zn^{++}, Cu^{++}	Mg^{++} 1mM
subcellular localization	not stroma bound			cytosol lymphoid cells		soluble = PP_i stroma bound = P_i		
reaction products	ITP IDP + P_i	ITP IMP + PP_i				ITP IMP + PP_i not reversible no PP_{32} exchange		
assay method	P_i	^{32}P ITP		P_i	^{14}C ITP	P_i	Dowex-1 P_i	

Table 1 (Continued)

Chief Investigator	Liakopoulou	Vanderheiden	Wang	Holmes	Vanderheiden	Hershko	Chern	Wang
ITP conc. max.	10 mM	2 mM			2 mM		0.5 mM	
substrate inhibition		yes		no	no			
SH requirement		yes			no	no	yes	
inhibitors				IDP K_i 1.2×10^{-5}	pH MB		IDP	
purification scheme			(1) $CaPO_4$ gel elution (2) CM Sephadex (3) DEAE-Sephadex ion filtration		(1) Biorad-extract (2) Ammonium sulfate (3) DEAE (4) Iso-electric focusing	(1) DEAE cellulose (2) Ammonium sulfate	(1) Sephadex G-100 gel filtration (2) DEAE cellulose	(1) Heat denature (2) Ammonium sulfate (3) DEAE
substrate specificity			30x more active ITP than GTP					
dITP	--	--	--	108	--	--	--	--
ITP	--	--	--	100	100	100	100	100
XTP	--	--	--	58	--	--	--	--
UTP	--	--	--	18	1.4	4	12	9
GTP	--	--	--	1	0.8	4.6	10	8
ATP	--	--	--	5	0	0	10	0.3
CTP	--	--	--	3	0.3	--	0.5	1.1
IDP	--	--	--	8	0	0	0	2.6
IMP	--	--	--	1	--	--	--	--

Characterization of ITPase

Products of the ITPase Reaction

Inosine triphosphatase activity [E.C. 3.6.1.19] was first reported by Liakopuolou and Alivaistos (123) in the course of a study on ATP:NMN adenyl transferase in acetone powders of human erythrocytes. They reported the enzyme reaction proceeded from ITP $\rightarrow$IDP + P_i with IDPase activity repressed by 50 mM Mg^{++}. In 1969 Chern et al. reported on an enzyme with similar properties in rabbit red blood cells. However, Chern et al. reported that the reaction proceeded as a pyrophosphatase (from ITP$\rightarrow$IMP + PP_i) (124). This conclusion was based on their observation that detection of ITP hydrolysis in hemolysates heated at 85°C for 5 minutes was dependent on the addition of pyrophosphatase and the chromatographic detection of IMP on Dowex-1 columns. In studies with the purified enzyme, Hershko et al. also investigated the properties of rabbit red blood cell inosine triphosphatase. These investigators also concluded that the enzyme was a pyrophosphohydrolase. By subjecting the hemolysates to heating at 57°C for five minutes, the endogenous pyrophosphatase was inactivated, leaving PP_i in the reaction mixture. The purified enzyme was found to have no IDPase activity. The reaction was

reported to be irreversible based on the finding of no $^{32}PP_i$ exchange (129). Vanderheiden, in studies with human erythrocytes, found the enzyme to be a pyrophosphatase based on experiments which utilized $\gamma B^{32}PP$ ITP. Utilizing this labeled compound as a substrate, Vanderheiden found no ^{32}P-IDP was produced, with $^{32}PP_i$ being liberated. In these studies the concentration of $^{32}PP_i$ increased from zero to ten minutes of incubation time. After ten minutes of incubation a gradual decrease of $^{32}PP_i$ was noted, and accounted for by the activity of an inorganic pyrophosphatase splitting the ^{32}PP to $^{32}2P_i$ (128).

Wang and Morris, in studies on rabbit liver, found the enzyme to be a pyrophosphatase in this tissue also. The reaction products were analyzed by Dowex-1 chromatography. IMP was detected, but no IDP (130) was detected.

$\underline{K_m}$

The apparent K_m of ITPase for ITP has been reported to be 1×10^{-4} (123), 6×10^{-4} (125), and 7×10^{-5} (127) in human hemolysates. Purified ITPase from human hemolysates has yielded estimates of the K_m as 3.46×10^{-5} (enzyme purified 1,153 fold)(126) and 1.3×10^{-5} (128)(enzyme purified 2,300 fold). ITPase from rabbit red blood cells purified 100 fold was reported to have a K_m of 3×10^{-5} (129).

pH Optima

pH optima for ITPase has been reported to vary with the buffer system used. In a tris/HCl buffer system, the pH optimum has been reported to be broad pH 7.5-8.5, 8.5 and 9.5 in the presence of dithiothreitol (125) in human erythrocytes. Human ITPase was reported to have a pH optimum of 9.6 in glycine buffer (128). ITPase in rabbit erythrocytes has been reported to have a pH optima of 8.7 in tris (129), and 9.75 in B-alanine (124). The pH optimum was reported in rabbit liver to be 9.75 utilizing a B-alanine buffer (130).

Metal Ion Requirements

Mg^{++} has been reported to be necessary for optimal ITPase activity (1-50 mM)(123-130). ITPase was reported to have no activity if other divalent cations (Ca^{++},Fe^{++},Zn^{++}, Cu^{++}) were substituted for Mg^{++} (124). An exception to this was Mn^{++} which served as a cofactor in the ITPase reaction, but less enzyme activity was present than if Mg^{++} was the cofactor (124). Monovalent cations are reported to produce inhibition of ITP hydrolysis (128).

pI

The isoelectric point of ITPase was reported to be between 4.8 and 5.1 (128) in human erythrocytes.

Purification

Chern et al. partially purified rabbit red cell ITPase by use of ammonium sulfate fractionation (40-70%), Sephadex G100 gel filtration, and DEAE chromatography. The purification was approximately 2,000 fold. Hershko et al. have also purified rabbit red cell ITPase by use of DEAE cellulose and ammonium sulfate precipitation (38-62% saturation) starting with stroma-free hemolysates (approximately 100 fold purification).

Purification of rabbit liver ITPase was achieved by first a high speed centrifugation (143,000 g for 2 minutes), heating the supernatant at 65°C for 5 minutes, reverse ammonium sulfate fractionation, and DEAE Cellex chromatography (126,130). Purification was 668 fold.

A purification scheme resulting in 1,153 fold purification of human erythrocytic ITPase has been reported (126). Calcium phosphate gel elution, CM Sephadex, and DEAE Sephadex ion filtration were utilized to purify the enzyme. Recently, Vanderheiden has reported on further purification of ITPase from human erythrocytes. Utilizing DEAE cellulose (Cellex D), ammonium sulfate precipitation (40-80%), DEAE cellulose (DE-32), an 85% cut of ammonium sulfate, and isoelectric focusing, the

purification achieved was 2,300 fold.

Maximal Substrate Concentration

Maximal substrate concentration was reported to be 10 mM (123), 2 mM with substrate inhibition occurring at higher ITP concentrations (125), and 2 mM with no substrate inhibition occurring at higher substrate concentrations (128). The maximal substrate concentration in partially purified ITPase from rabbit erythrocytes was 0.5 mM (124). Holmes et al. suggested that the initial finding of substrate inhibition was due to the non-enzymatic degradation of the substrate ITP to IDP. IDP is an inhibitor of ITPase (127).

Inhibition of ITPase

IDP has been reported to inhibit ITPase (K_i 1.2 x 10^{-5}) (127). p-hydroxymercuribenzoate has also been reported to inhibit ITPase activity. Recently Vanderheiden has reported that reaction mixtures containing 5 mM $CaCl_2$ and 1.2 mM $MgCl_2$ completely inhibits ITPase activity (128). Increasing concentrations of hemolysate have also been reported to inhibit ITPase activity (124,125,127).

Sulfhydryl Requirement

Early reports of ITPase activity found a thiol requirement for activation of ITPase activity in both

human and rabbit red blood cells (124,125). However, later reports on partially purified enzyme did not find this (128,129).

Molecular Weight

The molecular weight of ITPase from human erythrocytes was estimated to be 37,000 by sucrose density centrifugation (126). The same value was obtained for ITPase isolated from rat liver by sucrose density centrifugation (130).

Substrate Specificity

Partially purified rabbit erythrocyte ITPase was found to cleave dITP as effectively as ITP. XTP was cleaved at approximately 71% the rate of ITP. GTP and UTP were cleaved at 10% and 12% the rate of ITP cleavage respectively. ATP was cleaved at 10% the rate of ITP (124). Another investigation of rabbit red cell ITPase found ITPase to catalyze the hydrolysis of GTP, UTP, CTP, and ATP to the corresponding monophosphates and PP_i at 4.5, 4.0, 0.5, and 0.0% respectively of the rate of ITP breakdown. GTP was reported to have a K_m of 8×10^{-4}, with GTP hydrolysis inhibited in a non-competitive manner by IMP with a K_i of 3×10^{-4} (129).

Human erythrocytic ITPase was found to catalyze the hydrolysis of dITP as rapidly as ITP, XTP at 58% of the rate of ITP; UTP, GTP, and ATP were hydrolyzed at 18%, 9%, and

5% the rate of ITP hydrolysis (127) respectively. Highly purified human erythrocytic ITPase (2,300 fold) was found to catalyze the hydrolysis of UTP at 1.4% the rate of ITP and the hydrolysis of GTP at 0.8% the rate of ITP (128). ATP was not hydrolyzed. Utilizing starch gel electrophoresis XTP was as active as ITP, with no activity reported for GTP and ATP (127). Purified rabbit liver ITPase was found to catalyze the hydrolysis of XTP and ITP as effectively as ITP, GTP at 8% of the rate of hydrolysis of ITP, and UTP at 9% (124).

Subcellular Localization

In rabbit red blood cells ITP phosphohydrolase was localized to the soluble cytoplasmic portion of cell lysates (129). Stroma-bound nucleoside triphosphatase activity was found to result in P_i formation with ATP, ITP, and GTP as substrates (ITP $\longrightarrow$ IDP + P_i) (129). Subcellular localization of ITPase activity in human long-term lymphoid lines was reported by Holmes et al. (127). Their finding was that ITPase was cytoplasmic and not a residual activity of a GTPase or ATPase, since these activities were enriched in different fractions.

In rat liver, Nodikoff and Heus (131) reported that less than 10% of the ITP dephosphorylating activity was

present in the cytosol. However, as pointed out by Vanderheiden, the assay used was not optimal for ITPase activity. Irie et al. have reported a mitochondrial ITPase in rat liver, as well as a cytosol ITPase. Mitochondrial ITPase was reported to have copurified with PPase activity on Sephadex G-100, DEAE Sephadex A-50, and migrated with the same mobility on disc gel electrophoresis (132). Vanderheiden found that ITP phosphohydrolase activity was localized in the cytosol (133).

Tissue Specificity

ITPase has been found to be a ubiquitous enzyme. In rabbits ITPase activity was found in brain, pancreas, kidney, thymus, spleen, lung, heart, muscle, skeletal muscle, bone marrow and liver (130). Brain and liver had the highest specific activity.

A tissue survey in rats found the enzyme to be present in erythrocytes, brain, heart, kidney, liver, lung, muscle, ovary, spleen, testes, and thymus (134). In human tissues enzymatic activity has been found in erythrocytes, peripheral leukocytes, long-term lymphoid cultures, fibroblast cell cultures, fetal and adult liver, and brain tissue (127).

Isozymes

No isozymes of ITPase have been reported. In all

human tissues surveyed, the enzyme migrated as a single band with the same electrophoretic mobility on starch gel electrophoresis (127). Hopkinson and Edwards (134) in a survey of "many" individuals found one electrophoretic variant on starch gel electrophoresis of human hemolysates.

On polyacrylamide gel electrophoresis, Wang and Morris reported that human and rabbit erythrocyte ITPase migrated as one band, while rabbit liver "ITPase" exhibited two bands of activity. However, one band of the rabbit liver was not substrate specific staining with ATP, GTP, and glucose-1-phosphate. This band was not thought to represent ITPase (130).

Exponential Decay of ITPase Activity in Human Erythrocytes

In human red blood cells ITPase activity was reported to decay exponentially with erythrocyte age. The half-life was reported to be 35 days (127).

Physiologic Role of ITPase

The physiologic role of ITPase at present remains unclear. Hershko et al. proposed that the physiologic role of ITPase was actually a GTPase with a high K_m to help regulate pool sizes. Subsequent studies with highly purified ITPase in human erythrocytes, however, have shown the GTP hydrolyzing activity of ITPase to be

under one percent. Wang and Morris have proposed that the physiologic role of ITPase is to prevent ITP and XTP from being incorporated into RNA and DNA. Vanderheiden has proposed that the physiologic role of ITPase is to prevent degradation of ATP. Postulating that IMP is pyrophosphorylated (ATP + IMP $\longrightarrow$ ITP + AMP), he then suggests that ITPase may be the rate limiting step in a series of reactions. The series would be ATP + IMP $\longrightarrow$ ITP + AMP, ITP $\longrightarrow$ IMP + PP_i, and $PP_i \longrightarrow 2P_i$. The net reaction of this sequence would be ATP $\longrightarrow$ AMP + $2P_i$. If ITPase were the rate limiting step, it would then regulate ATP degradation.

Correlation Between ITPase Activity and ITP Accumulation

Correlation between accumulation of ITP in erythocytes incubated with radioactive inosine or radioactive hypoxanthine and ITPase activity has been reported by Vanderheiden (135), Soder et al. (136), and Henderson (137). ITP accumulation in erythrocytes follows an inverse relationship with ITPase activity. Vanderheiden identified individuals whose erythrocytes contained low levels of ITP but accumulated ITP on incubation with inosine, individuals whose erythrocytes contained 20-25 fold higher concentrations of ITP than normal erythrocytes, and individuals whose erythrocytes did not accumulate ITP when incubated with radio-labeled inosine. Vanderheiden

classified individuals on the basis of ITP accumulation into high ITP accumulators (ratio of cpm in ^{14}C ITP/^{14}C IMP of .14-.21), intermediate ITP accumulators (ratio of cpm in ^{14}C ITP/^{14}C IMP of .01-.03), and low ITP accumulators (ratio of cpm in ^{14}C ITP/^{14}C IMP of .001-.003). Soder et al. reported a continuous spectrum of ITPase activity. The K_m for ITP of individual samples did not correlate with the level of ITPase activity. Variations of total nucleotide synthesis did not correlate with the amount of ITP synthesis. Soder et al. also conducted mixing experiments with partially purified ITPase from pooled outdated blood and mixed it with lysates prepared from individuals with high ITPase activity, low ITPase activity, and medium ITPase activity. The resultant activity was the sum of what would be expected from each of the individual lysates, ruling out an inhibitor present in the ITPase lysates.

Familial and Population Studies

ITPase activity from families of individuals with low ITPase activity showed a bimodal distribution. Families of individuals with high ITPase activity showed a unimodal distribution. A random population of 213 individuals showed a bimodal distribution with fewer at the lower range of activity. Vanderheiden postulated an autosomal mode of inheritance with a heterozygous-homozygous distribution in families of ITP are deficient individuals. ITPase was

postulated to have a codominant mode of transmission. ITPase has subsequently been mapped to the human autosome 20 (138,139).

Population studies reported by Vanderheiden (135) reported the incidence of ITPase deficiency to be 0.35% in the normal population, 0.67% in an institutionalized psychiatric population, and 6.40% (140) in a population of patients diagnosed as paranoid schizophrenics. Fraser et al. found that 5% of the normal population's red cells accumulated ITP when incubated with radioactive hypoxanthine, while the incidence of this finding was 16% in a mentally retarded population (31). Studies by Holmes et al. indicate an incidence of ITPase deficiency in a random population of 150 individuals of 5%, 0% in a paranoid schizophrenic population (n = 30), and a skewedness toward high ITPase activity in a mentally retarded population (127). No correlation was found by Holmes et al. between ITPase activity and sex, racial group, origin, age, or central nervous system dysfunction. These investigators reported on eight individuals who were classified as ITPase deficient (based on the definition of ITPase deficiency being those individuals having ITPase activity of less than 60 enzyme units). Of these eight individuals, two individuals showed enzyme activity of 15 enzyme units and no activity by starch gel electrophoresis, while the others with activity of less than 60 enzyme units showed a faint band on starch gel electrophoresis. This band had the same electrophoretic

mobility as the enzyme with normal activity. Thus, there is some evidence of genetic heterogeneity in ITPase "deficiency."

Correlation of ITPase Activity in Erythrocytes and Other Cell Types

Although ITPase has been reported in various tissue types (125,126,127), as previously reviewed, there is only one report of a correlation between levels of ITPase activity in red cells and another tissue type (peripheral leukocytes) (127). It was reported that there is a statistically significant correlation (0.57) between the level of ITPase activity in erythrocytes whose enzyme activity is under 200 enzyme units and enzyme activity in leukocytes. Lack of correlation between erythrocytes and peripheral leukocytes was reported in those individuals whose erythrocytes showed greater than 200 units of enzyme activity (127). Fraser et al. have reported that mixed leukocytes from an individual whose erythrocytes accumulated ITP on incubation with ^{14}C hypoxanthine did not accumulate ITP. Leukocytes from one individual were found to be lacking in ITPase activity (141). It is not possible to determine from the published data the level of ITPase activity in this individual's erythrocytes.

Metabolic Studies and ITP Accumulation

Metabolic studies of ITP accumulation have been sparse.

The first observation of ITP accumulation in erythrocytes was made in 1965 on the erythrocytes of two siblings (142) (presumably ITPase deficient). Erythrocytes stored in acid citrate dextrose for four weeks and subsequently incubated with inosine were reported to accumulate ITP (143), as were erythrocytes incubated with hypoxanthine (144). Other investigators found that erythrocytes stored for periods of two to six weeks in a medium containing inosine pyruvate and phosphate accumulated ITP (144). No IDP was observed. However, studies on nucleotide levels in erythrocytes stored at $4^{o}C$ with inosine (145,146,147) and studies on erythrocytes stored in acid citrate dextrose (with and without inosine incubation) (148) by several other investigators failed to detect any ITP. Among the first reports of ITP accumulation in "normal erythrocytes" was that made by Fraser et al. (31) in which 2% suspensions of erythrocytes were incubated in modified Fisher's medium containing 100 uM ^{14}C hypoxanthine for two hours at $37^{o}C$. In these studies a distribution of the amount of ^{14}C ITP accumulating in hemolysates from various individuals was obtained. Values above 70 nanomoles ITP/10^{10} cells in 2 hours were considered high ITP accumulators. Another investigation of ITP accumulation in red cells under similar incubation conditions (50 uM hypoxanthine or inosine, 25 mM phosphate, and 50 mM glucose) found that after 24 hours of

incubation the concentration of ITP had reached 0.4 mM (149). IDP was also present in lesser concentrations. No radioactivity was reported in adenine or guanine nucleotides when incubated with inosine (31,149), and no ITP accumulated when erythrocytes were incubated with adenine or guanine (31). Henderson et al. (150) and Henderson (137) also reported ITP accumulating when normal erythrocytes were incubated with hypoxanthine. A continuous distribution of the amount of ^{14}C ITP accumulating in a population was obtained (136,137). Since the early reports of ITP accumulation were from blood stored in acid citrate dextrose, the effects of storage on ITP accumulation were investigated. Various reports were published that found the effect of storage on ITP accumulation varied. In one report the effect of storage on ITP accumulation in erythrocytes was equivocal (Fraser et al.). In another, ITP accumulation increased by storage of whole blood as a 10% solution in acid citrate dextrose (150). Yet another reported that five times as much ITP accumulated in a dialyzed hemolysate when whole blood was previously stored for 24 hours in ACD (Vanderheiden) (128). ITP accumulation was maximal after one day of storage at 22°C and increased over controls from 3-7 days by storage at 4°C (150). ITPase activity was reported constant during the period of storage.

Phosphate concentration and pH was reported to affect ITP accumulation with maximal ITP accumulation occurring at 50 mM phosphate at pH 7.4 (137). Among the factors reported to have no effect on the amount of ^{14}C ITP accumulating were concentration of DPG, variation in the rate of synthesis of IMP, 10 mM Na_2SO_4 or 10 mM $NaSO_3$, anerobic conditions, the presence or absence of pyruvate, and the amount of total nucleotide synthesis. While the above mentioned factors had no effect on ITP accumulation, there was evidence that several of them affected the amount of IMP synthesized from nucleoside precursors. Thus, PO_4 and pH increased IMP synthesis, while DPG and anerobic conditions diminished IMP synthesis. The alterations were thought to be secondary to PRPP alterations. Phosphate and pH would increase PRPP levels, while DPG and anerobic conditions would decrease PRPP (DPG inhibits PRPP synthetase; anerobic conditions would increases DPG formation) (137).

Time of incubation was the most crucial factor mentioned by these investigators for ITP synthesis. IMP synthesis lagged from 0-2 hours after which the rate was linear for up to 24 hours under conditions used by Henderson et al. (150). In contrast, the rate of ITP accumulation increased disproportionately after two hours. Thus at 24 hours there was one hundred times as much ITP as at 2 hours. ITPase activity remained constant up to

24 hours. To explain these findings, these investigators proposed that in order to accumulate ITP a crucial concentration of IMP must be reached first. By implication, Henderson et al. are proposing that the rate controlling step in ITP accumulation in red cells is phosphorylation of IMP to IDP, by pyrophosphorylation of IMP to ITP. This would then mean that variations of "IMP kinase" would be responsible for ITP accumulation and not the level of activity of ITPase. Along these lines, variations in isozymes of guanylate kinase, the only known enzyme that has the ability to phosphorylate IMP (145,146) was investigated (Fraser et al.) (31). GMP kinase has the ability to phosphorylate IMP with a V max 0.2% that of GTP and has a K_m about twenty times higher than that for GMP (151,31). Different isozymes of GMP kinase vary in their ability to use IMP as substrate, with maximal activity at 1% the activity of GMP as substrate (31). No individual variation of guanylate kinase isozyme distribution was found in erythrocytes from three hundred individuals (31). Another report of ITP accumulation was made by Agarwal et al. in two patients with adenosine deaminase deficiency (94). In this study erythrocytes from two patients with adenosine deaminase deficiency who had previously been transplanted with bone marrow or fetal liver accumulated ITP on incubation with inosine. Erythrocytes

from two other patients with adenosine deaminase deficiency did not accumulate ITP under these conditions, nor did any heterozygotes or normals. When this study was repeated with erythrocytes from one patient, ten and fifteen months later no ITP accumulation occurred. It is of interest to note that both ITPase and ADA are located on chromosome twenty. ITP accumulation has also been reported to be independent of the ability to accumulate 6 thio ITP from 6 mercapto-purine ribonucleoside (149) and 6 methylmercaptopurine ribonucleoside triphosphate from 6 methylmercaptopurine ribonucleoside.

In summary, ITP accumulation in erythrocytes has been inversely correlated with levels of ITPase activity. It has been reported to occur in erythrocytes stored in acid citrate dextrose subsequently incubated with inosine and in blood stored in inosine pyruvate and phosphate. ITP accumulation has also been reported to occur in "normal" red cells under certain incubation conditions. The accumulation of ITP was increased by incubation at pH 7.4 in 50 mM phosphate and increased dis-proportionately with time of incubation. Factors not influencing ITP accumulation include variation in the rate of synthesis of IMP, concentration of DPG, anaerobic conditions, presence or absence of pyruvate, and 10 mM Na_2SO_4, or 10 mM $NaSO_3$. ITP accumulation is not related to the ability to phosphorylate 6 mercaptopurine ribonucleoside.

The mode of synthesis of ITP has not been conclusively proven, although there is some suggestive evidence of IMP being phosphorylated to IDP (enzyme unknown) (133). IDP can be phosphorylated to ITP by nucleoside diphosphokinase. IDP can also be converted to deoxy IDP by ribonucleotide reductase. IDP, in addition, can undergo a phosphate exchange with ATP to form ITP in cardiac sarcoplasmic reticulum of dogs. There is a report of direct pyrophosphorylation of IMP to ITP (133), as well as a suggestion of irreversible dismutation of IDP (2 IDP $\longrightarrow$IMP + ITP) (133).

In an effort to determine the mode of synthesis of ITP, Vanderheiden (128) incubated whole blood (stored overnight at 4°C in NaEDTA) from patients deficient in ITPase with $8^{14}C$ inosine. The specific activity of IMP, IDP, and ITP which accumulated in extracts of the whole blood over a period of 6 hours was monitored. As has been previously mentioned, there has been some postulation that guanylate kinase is responsible for the phosphorylation of IMP to IDP, so Vanderheiden incubated blood from normals with guanosine following the same protocol. In theory, if the same enzymes were responsible for the synthesis of ITP and GTP, the same pattern of specific activity and relative rate of synthesis would be obtained between mono-, di-, and trinucleotides. His results indicated that the pattern of synthesis of ITP in whole blood was inosine $\rightarrow$ hypoxanthine $\rightarrow$

IMP→ITP→IDP (pyrophosphorylation of IMP). However, the result he obtained for synthesis of GTP was guanosine → guanine → GDP (or GTP) → GTP (or GDP) → GMP in opposition to the accepted dogma of guanosine→guanine →GMP →GDP → GTP. To explain these findings, it was postulated that in some of the steps required for GTP synthesis, an enzyme nucleotide complex was formed (enzyme-GMP) with the dissociation of the nucleotide from enzyme or protein being the rate limiting step. Blood from an ITPase deficient patient was used in the inosine incorporation studies, while blood from normal subjects was used in the guanosine incubation studies. In short, the mode of synthesis of ITP in whole blood remains unclear.

To further investigate the mode of synthesis of ITP, Vanderheiden then utilized a system which included "inhibitors of ITPase activity," 5 mM $CaCl_2$ and 1.2 mM $MgCl_2$ in the assay of the lysates. Although Vanderheiden reports that $CaCl_2$, Co, and Mn have differential effects on the synthesis of ITP and GTP, it is difficult to evaluate these results, since measurements of ITP accumulation were made using dialyzed fresh human hemolysates incubated for one hour or stored human hemolysates incubated for five hours as an enzyme source, while measurements of GTP were made utilizing cat brain cortex cytosol incubated for 15 minutes or dialyzed

stored human hemolysate incubated for ten minutes as enzyme sources. In any event, under the conditions chosen by Vanderheiden synthesis of GTP and GDP were linear with time and protein, while synthesis of IDP and ITP were not linear at early time points (before 30 minutes), or low protein concentrations. Again, it should be noted that ITP and GTP synthesis were measured in two different systems; ITP and IDP accumulation measured in ox brain cytosol incubated for one hour, while GTP and GDP accumulation were measured in dialyzed fresh human hemolysate incubated for 15 minutes.

Perhaps because of these differences, an "ITPase free" hemolysate (prepared by passing hemolysate over DEAE-32 column) was compared with respect to ITP and GTP synthesis in the presence of varying concentration of Mg^{++} and Ca^{++}. However, this is not a valid comparison, since time of incubation varied (ITP 3 hours, GTP 15 minutes), as well as protein concentration (five times more protein in the preparations used for ITP synthesis than GTP synthesis). In addition, since ITP and GTP synthesis were measured in a fraction eluted from a column, it is possible that other enzymes involved in the synthesis of ITP or GTP were not present.

Nucleotide metabolism in red cells is rather unique in that all enzymes required for de novo synthesis are not present. IMP is not converted to AMP (no adenylosuccinate synthetase)

and IMP is converted to GMP at only very low rates. It is of tremendous interest to show ITP synthesis in a cell that has all purine biosynthetic pathways functioning.

Vanderheiden has recently reported a system in which ITP synthesis was demonstrated in cell lysates of human leukocytes, lymphocytes, platelets, and brain. ITP synthesis was also demonstrated in lysates of rat brain, heart, kidney, adrenal gland, muscle, ovary, testes, and thymus. No ITP was reported in the liver, lung, and spleen. In order to demonstrate synthesis of ITP in cell extracts, it was reported necessary to inhibit ITPase activity by use of Ca^{++} ion concentration of 5 mM, 2.2 mM magnesium, 5 mM ATP, and 1 mM IMP. IDP was reported present in heart, liver, muscle, and erythrocytes.

ITP can be substituted for the ATP requirement of yeast hexokinase (114), phosphofructokinase (152), and adenylate kinase 3(134) (not present in red cells).

A recent report indicates that ITP is inhibitory to L-glutamic acid decarboxylase (glutamic acid to γ aminobutyric acid) with a K_i of 3.5×10^{-1}. GTP has a K_i of 3.0×10^{-1} and ATP K_i 5×10^{-1} under the same conditions. The inhibition was reported to be linear and noncompetitive (153).

Although there is no solid evidence, Vanderheiden has postulated that ITPase deficiency may be contributory

to paranoid schizophrenia by virtue of its inhibition of L-glutamic acid hydrolase. Reduced L-glutamic acid decarboxylase activity has been reported in the brains of schizophrenics (154). In comparing another mental disease associated with derangements of nucleotide metabolism (HGPRT deficiency) to ITPase deficiency, it has been suggested that both enzyme deficiencies involve reduced availability of IMP (153). However, in ITPase deficient erythrocytes the rate of synthesis of IMP has been shown to be comparable to normal erythrocytes (31). In addition, an ITPase deficient cell would still have two pathways available for synthesis of IMP, both de novo and salvage, as opposed to an HGPRT deficient cell which would rely only on de novo synthesis. Thus, in cell types in which no de novo pathway exists, a cell deficient in HGPRT would have no way of synthesizing IMP.

Materials

Fetal calf serum, Penicillin/Streptomycin, 0.4% trypan blue in saline, and RPMI 1640 media were purchased from GIBCO. Flasks (25 cm) for cell culture were purchased from Falcon Plastics. All nucleotides and nucleosides were purchased from either Sigma or P&L Laboratories. Dithiothreitol (infra red grade) and n-octylamine were purchased from Aldrich. Methanol (U.V. grade, glass double distilled) was purchased from Burdick Jackson. Aqueous Counting Scintillant was purchased from Amersham, as was radio-labeled inosine (8-^{14}C inosine 60 mCi/m mole), radio-labeled inosine triphosphate (8-^{14}C inosine triphosphate 50 mCi/m mole), and 8-^{14}C hypoxanthine (55 mCi/m mole). Freon 112 was purchased from Matheson Gas Co. Extra dry nitrogen gas was supplied by either Smith or Ohio Medical Products. Inorganic yeast pyrophosphatase was purchased from Boehringer-Mannheim, as was purine nucleoside phosphorylase and calf intestine adenosine deaminase. Sigma was the vendor for snake venom 5' nucleotidase. Whatman Inc. was the vendor for Whatman 3MM paper, as well as the HPLC SAX ion exchange column. Monobasic potassium phosphate was purchased from Mallinkrodt. PEI cellulose plates were purchased from Brinkman. Unsubstituted thin layer chromatography cellulose plates were purchased from Eastman-Kodak, as was Blue Brand X-Ray film and Kodak no screen X-Ray film. A C-18 u Bondpak HPLC

column was purchased from Waters Associates. The rest of the laboratory reagents were purchased from Fisher.

Cell Cultures

GM 1619 is a long-term lymphoid line supplied by the Human Genetic Mutant Cell Repository, Camden, New Jersey, submitted by Dr. B. Vanderheiden. The line was established from a twenty-nine year old white female whose erythrocytes were deficient in Inosine Triphosphatase /E.C. 3.6.1.19/ (135). The line produced IgM and Kappa chains IgG was weakly positive.

Metabolic Experiments

Lines utilized as controls for incubation studies were established from healthy individuals who are heterozygous carriers for various disorders. GM 2133 was derived from a 65 year old white male who was a "latent carrier for acute intermittent porphyria," GM 2150 from a healthy 28 year old white female at risk for Huntingtons Chorea, and NB 82, a line established by Dr. N. Beratis at the Mount Sinai Hospital from a heterozygote with familial type II hypercholesterolemia. EBV 21, a line derived from an individual homozygous for homocystinuria, was used in a single incorporation experiment. These lines were assayed for ITPase activity by two different methods (see below).

Lines utilized for ITPase assay on HPLC in addition to

the above included GM 2148, a line established from a healthy, normal 54 year old white female (mother of GM 2150), GM 1488, a line established from a 23 year old white male with schizophrenia, GM 2135, a line from a 21 year old white male with acute intermittent porphyria (son of GM 2133), NB 83, NB 59, and NB 79 lines established by Dr. N. Beratis from a patient homozygous for homocystinuria, a heterozygote for type II familial hypercholesterolemia, and a female heterozygote for type II hypercholesterolemia respectively.

High Pressure Liquid Chromatography Equipment

High pressure liquid chromatography was performed on equipment manufactured by Laboratory Data Control. The gradient capacity of the system was provided by Gradient Master I, Model 1601 electronics unit. Pumps capable of withstanding 5,000 psi were also manufactured by Laboratory Data Control (Constametric I and Constametric II). A variable wavelength spectrophotometer was attached to the system (spectro monitor I). A LDC chart recorder was connected to the U.V. monitor.

Columns used for high pressure liquid chromatography were the SAX anion exchange column for nucleotide analysis. A guard column also supplied by Whatman was attached to the SAX column. Reverse phase uBondpak C18 was used for nucleoside analysis (Water Associates). Buffers were initially

prepared fresh daily and degassed by passage through a 20u filter (Millipore Inc.)

Subsequently, it was found that buffers would last for a week if stored at 4°C. pH of the buffers were checked daily and buffers degassed by passage through a 20u filter under vacuum immediately prior to use. A full discussion of HPLC will be included in the background section.

Methods

1. Cell Culture

Lymphoid cultures were maintained in 16% heat inactivated fetal calf serum, RPMI 1640 supplemented with 2mM glutamine and 10 U/ml penicillin, 10 ug/ml streptomycin. Cultures grown in glutamine-free media were supplemented with Pen/Strep only. Cultures grown in glutamine and aspartate-free media were grown in RPMI 1640 reconstituted from powdered form from GIBCO's selectamine kit. Cell cultures were routinely tested for bacterial and mycoplasma infections and consistently found to be negative. A chromosomal karyotype of the lymphoid line GM 1619 was performed, and the line was found to be euploid (46XX).

The cultures were carried by diluting into fresh media (one to one dilution) of complete RPMI 1640 once a week. Viability studies of the cultures were performed by a one-to-one dilution of an aliquot of the cell culture with 0.4%

trypan blue (155). Cell counts were then performed on the diluted aliquot of cell culture by use of hemocytometer.

Lines were also seeded at 2 x 10^{-5}, fed four days later, and cell counts performed at daily intervals. Under these conditions cells entered log growth phase. Incorporation studies were performed with cells under various growth conditions as indicated.

2. ITPase Assay

1. Preparation of enzyme for assay

A. Red Blood Cells

Blood was collected by venipuncture into 1.2% heparin (1,000 U/ml). The whole blood was centrifuged for ten minutes at 2,000 x g, then the plasma and buffy coat removed and discarded. The packed erythrocytes were washed two times in normal saline. Lysis of the packed erythrocytes was accomplished by adding four volumes distilled water. The hemolysate was centrifuged for thirty minutes at 27,000 x g at 4°C to remove stroma. Hemoglobin determinations were performed on the hemolysates by the method of Austin and Drabkin (156).

B. Lymphoid Cells

Lymphoid cells were centrifuged for ten minutes

at 2,000 x g, then washed two times in normal saline. The cells were then lysed (approximately 2.5 x 10^7 cells) in either 0.3 ml distilled water (for phosphate assay) or 1.0 ml distilled water (for HPLC assay), and sonicated in a Bronson Sonifier for 3 x 10 seconds at 33% power output. The lysates were then centrifuged at 4°C for 30 minutes (27,000 x g). Protein was determined by the method of Lowry (157) on the supernatant.

Inosine Triphosphatase Activity As Measured by Release of Inorganic Phosphate

Assay of inosine triphosphatase (E.C. 3.6.1.19) was accomplished by use of a modification of the assay reported by Holmes et al. (127). The assay was as follows: In a final volume of 0.8 ml 100 mM tris/HCl pH 8.5, 100 mM $MgCl_2$, 1 mM dithiothreitol, 1.25 mM ITP, 1 unit inorganic yeast pyrophosphatase, and 0.025-0.1 ml enzyme preparation were added. The enzyme preparation contained 1-2 mg/ml protein for lymphoid cell lysates and 20-40 mg/ml hemoglobin for hemolysates. The reaction mixture was routinely incubated for ten minutes at 37°C. Longer incubation periods (up to 30 minutes) were utilized where indicated.

The reaction was stopped by addition of 0.2 ml ice cold TCA (20% W/V). Alternatively the reaction was stopped by addition of 0.16 ml 2.1 N perchloric acid and neutralized

with 0.16 ml of 2.21N KOH. The tubes were centrifuged and the supernatant removed for inorganic phosphate determination by the method of Rathbun and Betlach (158).

Phosphate Determination

800 ul of the supernatant was transferred to a fresh tube. To a final concentration of 1M acetic acid buffer (3M acetic acid and 3M sodium acetate), 0.75% formaldehyde, 0.06% ammonium molybdate (from 2% ammonium molybdate stock), 0.45 mM stannous chloride (made as a 7 mM stock solution in which 100 ul glacial acetic acid was added to 3 ml H_2O containing 38 mg stannous chloride, then the solution was brought to a final volume of 25 ml). The mixture was incubated for 15 minutes at 25°C; then absorbance at 720 nanometers was determined in a Gilford 301 Spectrophotometer. It was found important to add the acetic acid/formaldehyde buffer first, then the molybdate, and finally stannous chloride in the proper order. The mixture must be vortexed immediately after adding stannous chloride to obtain reliable results. Monobasic potassium phosphate was used as a reference standard for phosphate determination.

Several types of controls for the enzyme assay were performed. First, a full reaction mixture, minus the substrate ITP was incubated for 37°C for 10 minutes, then stopped, and phosphate determined as above (to determine the amount of

phosphate in all lysates). More routinely, an incubation mixture, missing the enzyme preparation, was incubated for 10 minutes at 37°C. The enzyme preparation was then added while the tube was on ice just before the TCA was added. The phosphate value obtained in this blank was then subtracted from the value obtained for the full incubated reaction mixture. In some experiments a substrate blank was obtained by incubating the entire reaction mixture, minus the enzyme preparation for ten minutes, and then determining phosphate content.

3. Inosine Triphosphatase Assay by High Pressure Liquid Chromatography--Chromatographic Determination of Inosine Monophosphate

A High Pressure Liquid Chromatography assay was developed as follows: Enzyme preparations were made as described above. The reaction mixture for the HPLC ITPase assay contained the following: 100 mM tris/HCl pH 8.5, 100 mM $MgCl_2$, 1.25 mM ITP, and 25-100 ul enzyme preparation. The mixture was incubated at 37°C for ten minutes and stopped by the addition of 160 ul 2.1N PCA, neutralized with 160 ul 2.21N KOH. The reaction mixture was then centrifuged for one minute in a microfuge (Brinkman) at 4°C. Controls were performed by incubation of the reaction mixture, minus the cell lysate,

at 37°C for the appropriate time interval. The cell lysates were added after the 2.1N PCA had been added to the tube. 25-100 ul of the assay mixture was then analyzed by HPLC.

Analysis by HPLC was accomplished using a buffer system consisting of: Buffer A = 0.007M KH_2PO_4, 0.007M KCl pH 4.0, Buffer B = 0.25M KH_2PO_4, 0.50M KCl pH 5.0. Separation of ITP/IDP/IMP was accomplished utilizing a ten-minute linear gradient of zero to 100% Buffer B, followed by a five-minute period of 100% Buffer B run isocratically. A flow rate of 1.5 ml/min was maintained. U.V. absorbance was monitored by an LDC variable wavelength Spectro Monitor I, set at 248 nanometers. A Laboratory Data Control chart recorder was connected to the Spectro Monitor I Spectrophotometer. The retention time of ITP, IDP, and IMP were compared to the retention time of standards, although this proved to be unnecessary, since the only compounds present in measurable amounts were ITP, IDP, and IMP.

In assays of ITPase which utilized 8-^{14}C ITP, 10 uCi of ITP was added to a total of ten ml of the incubation buffer as described above. An appropriate reduction was made in the amount of unlabeled ITP added to the incubation mixture to keep the final concentration of

ITP at 1.25 mM. A fraction collector was connected to the outflow of the HPLC system, and fractions were collected at 0.4 minute intervals. The fractions were counted in ACS, an aqueous accepting liquid scintillant.

3. Starch Gel Electrophoresis

Starch gel electrophoresis was performed in accordance with the Holmes modification(127) of Hopkinson and Edwards' method (134). The bridge buffer was a 0.1M tris/0.1M maleate/0.01 media / 0.1M $MgCl_2$ pH 7.4. The gel buffer was a 1:10 dilution of the bridge buffer. A 12% starch gel was prepared with B mercaptoethanol (0.75 ml/240 ml starch) added after heating and just before degassing. The electrophoresis was run at 120 V across the gel (1.5 V/cm) between cooling plates at 4°C for 17 hours. The staining method was a two-stage procedure. Stage one: filter paper saturated with a solution containing 0.05M $MgCl_2$ 2 mM ITP, 0.1M tris/HCl was overlayed on a cut gel surface. The gel was incubated at 37°C for one to two hours. The filter paper was then removed, and a mixture of 20 ml 2% acid molybdate, 1 gram ascorbic acid, and 20 ml 2% agar was poured over gel. Areas of enzyme activity were visualized as blue bands on a yellow background which appear within 30 minutes to 2 hours.

4. Incorporation Studies

An aliquot of cell culture was removed for viability

studies and cell counts. Incorporation studies were performed at various times of subculture. Aliquots containing approximately 2.5 x 10^7 cells were centrifuged (1000 x g for 10 minutes) and washed two times in phosphate buffered saline (Dulbecco's, with no $CaSO_4$ added). An aliquot was extracted and designated as absolute zero time of the sample.

For incorporation studies the remaining cells were resuspended in an incubation buffer of 10 mM glucose, 50 mM potassium phosphate pH 7.5, 2 mM $MgSO_4$, 75 mM sodium chloride, and with 1 u penicillin/ml and 10 ug streptomycin/ml (94). The cells were resuspended at approximately 10^7 cells per 200 ul incubation buffer. The cells suspended in the incorporation mixture were pre-incubated for 30 minutes at 37°C with shaking. At this time an aliquot of the incubation mixture was extracted and considered a "glucose zero." Inosine or hypoxanthine was added to the incubation mix to a final concentration of 50 uMolar (25 ul of 1.6 mM inosine plus 300 ul of 8-^{14}C inosine per 500 ul of incubation mix).

The cells were incubated in the presence and absence of nucleosides with shaking for one and two hours. Aliquots of 250 ul were removed, centrifuged, and the

pellets extracted at appropriate time points as described below.

The extraction procedure was as follows: One ml of 70% MeOH was added to the cell pellet and vortexed vigorously. The methanol extract was allowed to stand at -20°C for at least twenty-four hours. The extract was centrifuged in a microfuge (Beckman) for one minute. The supernatant was transferred to a fresh tube and evaporated to dryness under a stream of extra dry nitrogen gas. The powder was resuspended in 100-200 ul of water and frozen at -20°C. Cell extracts prepared in this way were stable for at least one week. The pellets from the methanol extract were solubilized with 0.5 ml of 1N NAOH/1% SDS at 37°C. 25-50 ul of this solution was used for protein determination by method Lowry (155). In experiments in which radioactive label was utilized, 250 ul of the solubilized pellet was added to 15 ml of an aqueous accepting scintillation fluid (ACS, Amersham), allowed to stand for two to four hours and radioactivity determined.

4. Red cell incubation

Red cells were incubated with inosine as described by Agarwal (94). Whole blood obtained by venipuncture of healthy individuals was collected in heparinized

syringes (1000 u/ml). The whole blood was centrifuged and washed two times in phosphate buffered saline. Packed erythrocytes were resuspended to a twenty percent suspension in the same incubation medium used for lymphoid lines described above. The suspensions were incubated at 37°C for 30 minutes at which time 50 uM inosine was added. 200 ul aliquots were added to 100 ul cold 12% perchloric acid. The acid extracts were neutralized either by adding an equal volume of 2.21N KOH followed by centrifugation to remove the $KClO_4$ precipitate or by extracting the acid in 0.5M n-octylamine in freon (Kyhm) (159).

5. Analysis of nucleosides, nucleotides, and bases

In initial studies analysis of nucleosides and nucleotides were accomplished by the method of Crabtree and Henderson (24). Nucleotides were analyzed by thin layer chromatography on PEI cellulose sheets. The sheets were placed in 4 molar sodium formate for five hours and left overnight in methanol:water (1:1). Standards were spotted on each channel (5 ul of a 15 umol/ml solution). A wick of 3MM Whatman paper was applied to the top of the sheet. Samples were then spotted on each channel (20-30 ul of neutralized cell extract in 5 ul aliquots). The sheet was left standing overnight in

methanol:water, and the paper wick was removed. The chromatogram was developed in 0.5M sodium formate with the solvent front rising to 2.5 cms. above origin, transferred to 2.0M sodium formate without drying and the solvent front allowed to rise to 7 cms origin, and finally to 4M sodium formate within the solvent front allowed to rise to the top of the sheet. Nucleotides were visualized under short wave U.V. In experiments in which a labeled precursor had been used, an autoradiogram of the TLC sheet was developed. The sensitivity was increased by use of a 7% PPO/ether flur solution poured on the TLC sheet (160). A blue sensitive X-ray film was used for development of autoradiogram. The autoradiogram was left in a dark freezer for approximately one week before being developed. Areas of radioactivity were cut out of the PEI cellulose sheets, eluted with 0.1N HCl and counted in ACS.

Nucleosides and bases were separated by two-dimensional thin layer chromatography (Crabtree and Henderson) (24) on cellulose sheets. Standards and neutralized cell extract (20 ul) were spotted at origin, developed in the first direction in a solvent system consisting of acetonitrile:ammonium acetate 0.1M pH 7.0:ammonia (60:30:10). The sheet was air dried. One centimeter of cellulose is scraped off at the top and bottom of the plates. The

plate was rotated so that the sample was in the lower right corner and developed two times in 1-butanol:methanol:water: ammonia (60:20:20:1). Nucleosides and bases are visualized under short wave U.V. light. Radioautography was performed as described for nucleotides.

In addition, high pressure liquid chromatography was utilized for analysis of nucleotides and nucleosides. For analysis of nucleotides a Whatman SAX anion exchange column was used. Fifty to one hundred microliters of cell extract were injected onto the column. Buffer conditions were slightly modified from the method of McKeag and Brown (161). Buffer A was 0.007M KH_2PO_4, 0.007M KCl, pH 4.0; Buffer B was 0.25M KH_2PO_4, 0.5M KCl pH 5.0. Flow rate was a constant 1.5 ml/minute. Buffer A was run isocratically for 5 minutes at which time a twenty-minute linear gradient (0 to 100% Buffer B) was run. Buffer B was run isocratically for twenty minutes for elution of trinucleotides following the gradient. Reequilibration time was kept constant at 15 minutes. During analysis of samples, in which radioactivity was present, a fraction collector (LKB) was connected to the outlet of the U.V. detector. All fractions were collected in 0.4 minute intervals directly into counting vials (Biovials-Beckman). 3.5 ml of ACS Scintillant was added to each vial, and the fractions were counted in a liquid scintillation counter (Hewlett Packard Isocap 300). Approximately 120 fractions

were collected per. sample.

Nucleoside analysis on HPLC was accomplished by use of a Waters Assoc. C_{18} micobondpak column. Buffer conditions were as follows: Buffer A 0.01M $NH_4H_2PO_4$ pH 5.5, Buffer B 40% methanol in Buffer A. A linear gradient (0-50% B) was run for thirty minutes, a modification of the method of Hartwick and Brown (162) Flow rate was 1.5 ml/min. Reequilibration time was 15 minutes.

Quantification of the amount of nucleotide or nucleoside present was made by a comparison of the area under a peak compared to the area of a standard of known concentration. Area was measured manually and was calculated by multiplying height times width at one half of the peak height.

Identification of compounds present in the chromatogram utilized several methods. Initially, the retention time of an unknown peak was compared to that of a standard. The standard was then coinjected with a cell extract and was watched for co-elution with the unknown peak. On peaks of particular interest additional methods were employed. Inosine triphosphate was positively identified in extracts made from cells that had been incubated with radioactive inosine. From these cell extracts the peak suspected of being ITP was collected as it was eluted from the SAX column. The eluant was then subjected to boiling in 1M perchloric acid for 30 minutes (112). The acid hydrolyzed

sample was neutralized with 5N KOH and concentrated by blowing a stream of nitrogen over the sample. The concentrate was rechromatographed on a Waters C_{18} microbondpak column for nucleoside and base analysis as described above. The peak eluting as hypoxanthine was collected and counted in ACS. Radioactivity was recovered in a peak co-eluting with hypoxanthine.

XMP was identified by treating the suspected compound (collected as above from cell extracts that had been incubated with radio-labeled inosine) with 5' nucleotidase (10 ul 5' nucleotidase /250 u/0.6 ml/ plus 100 ul cell extract) and reanalyzing for xanthosine on a nucleoside column. Fractions eluting from the column were collected and counted in ACS. The radioactivity was found in the xanthosine peak with an additional unidentified minor contaminant present (5% of recovered radioactivity). An aliquot of the "XMP" was treated with 5' nucleotidase and purine nucleoside phosphorylase (10 ul yeast nucleoside phosphorylase + 10 ul 1M $NaPO_4$ + 100 ul cell extract) and reanalyzed on a nucleoside column for xanthine.

In compounds which were present in large enough concentrations the eluant from the HPLC system was collected and subjected to a U.V. scan by a scanning Gilford spectrophotometer.

Adenosine, inosine, and hypoxanthine were positively

identified by use of the enzymatic peak shift (Brown) (113). An aliquot of the cell lysate was incubated with calf intestinal adenosine deaminase /10 ul of 1:10 dilution of ADA + 100 ul cell extract/ for one hour at 37°C. 50 ul of this incubation mix was reanalyzed on the nucleoside column. The disappearance of the tentatively identified adenosine peak and the appearance of a greater amount of the tentatively identified inosine peak was observed. The remaining 50 ul of the cell extract was then incubated with purine nucleoside phosphorylase (10 ul PNP and 50 ul cell extract), and a shift from inosine to hypoxanthine was identified on the chromatogram.

RESULTS

A. Phosphate Assay

The standard assay for inosine triphosphatase has relied on a linked enzyme system. In this assay system ITP is converted to IMP + PP_i by the enzyme preparation. The PP_i is split to $2P_i$ by pyrophosphatase. Inorganic phosphate is then determined. The assay thus relies on a phosphate determination for levels of activity of ITPase. ITPase activity in lymphoid lines, as measured by production of phosphate, was linear with increasing protein concentrations, tested from 100 to 500 ug of protein per ml of reaction mixture. Activity fell off above 500 ug of protein per ml of reaction mixture (Figure 6). ITPase activity in human erythrocytes, as measured by production of phosphate, was linear from 13 to 100 ug hemoglobin per 1 ml reaction mixture with a fall off of activity occurring above 100 ug hemoglobin per ml reaction mixture (Figure 7). Maximal enzyme activity was obtained at a concentration of 1.25 mM ITP (Figure 8).

Since detection of enzyme activity in theory should require splitting of pyrophosphate to inorganic phosphate ($PP_i \longrightarrow 2P_i$), activity was determined with and without the addition of exogenous pyrophosphatase. Figure 9

Figure 6

Linearity of ITPase Assay with Protein Concentration As Measured by Phosphate Determination in Lymphoid Cells

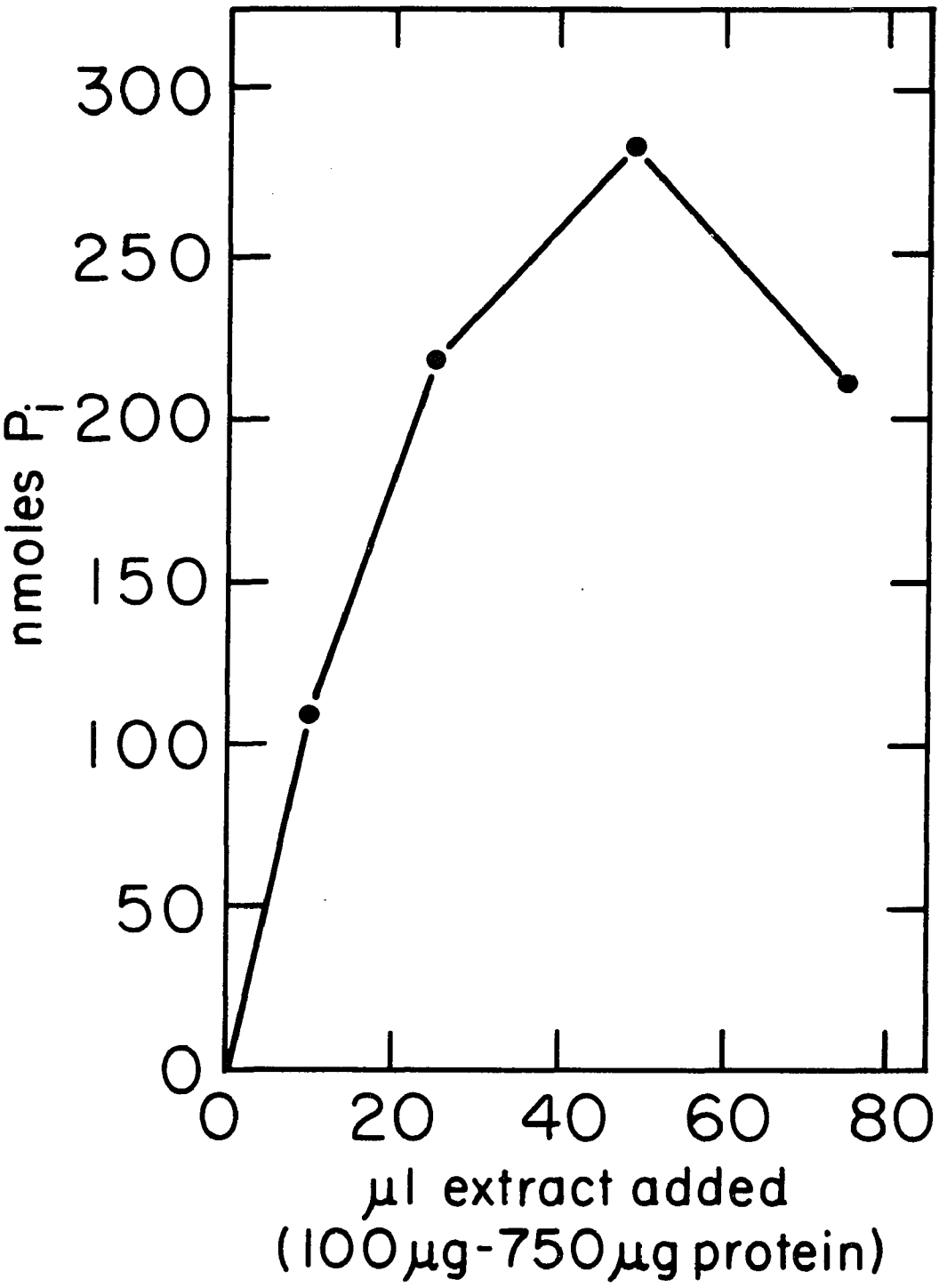

300
250
200
150
100
50
0
nmoles P_i
0
20
40
60
80
µl extract added
(100µg-750µg protein)

Figure 7

Linearity of ITPase Assay with Protein Concentration As Measured by Phosphate Determination in Erythrocytes

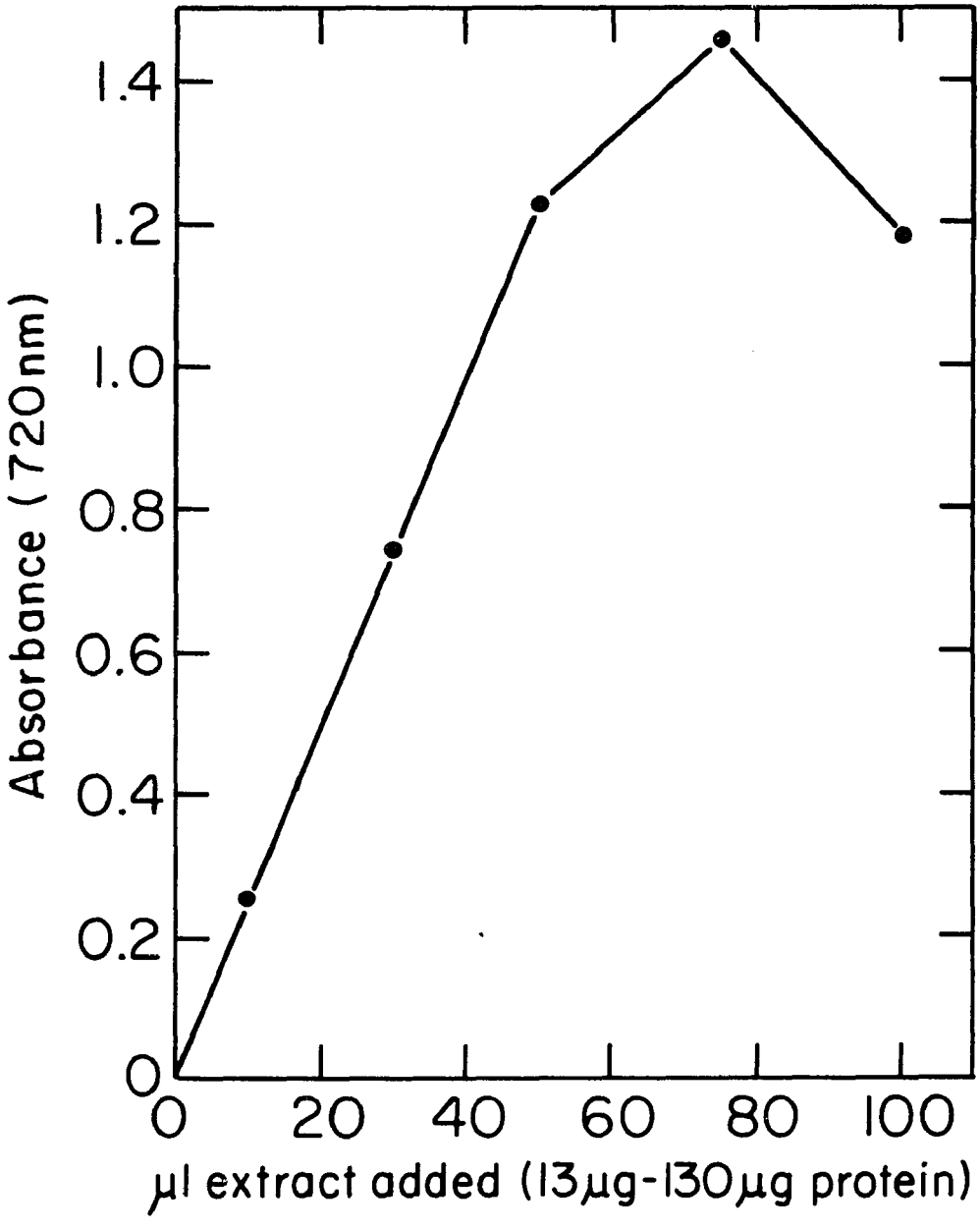
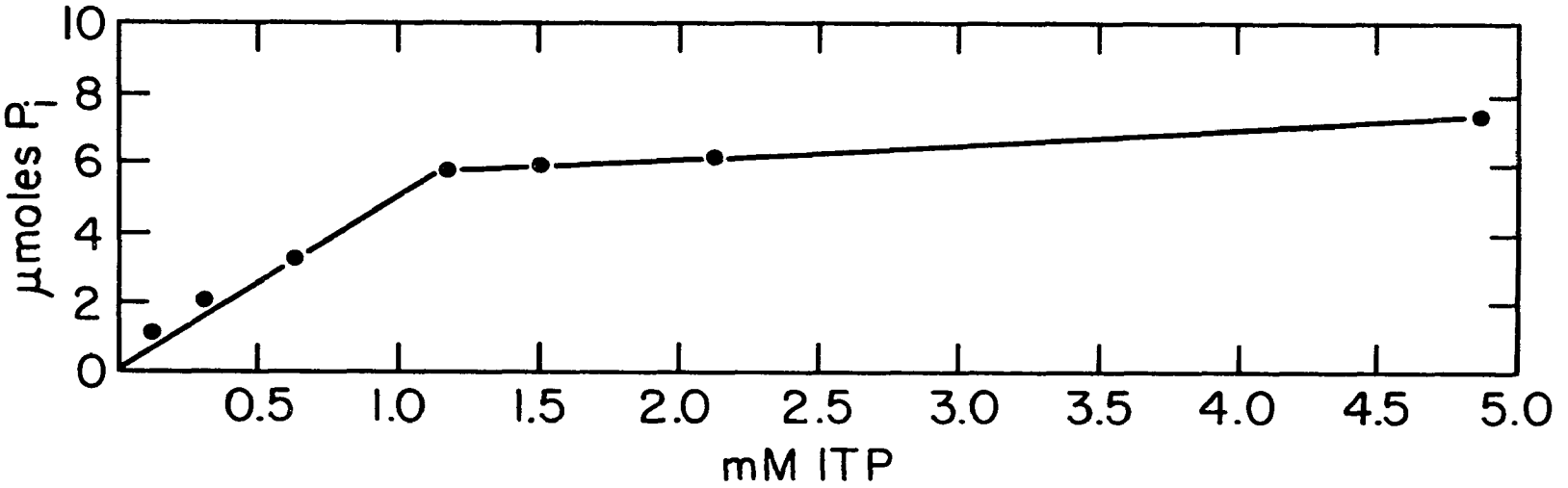

Absorbance (720nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
µl extract added (13µg-130µg protein)

Figure 8

Maximal ITP Concentration

for ITPase Activity


10
8
6
4
2
0
µmoles P_i
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
mM ITP

indicates that the addition of exogenous pyrophosphase had no effect on the detection of ITPase activity in human erythrocytes when assayed at pH 9.2. Protein concentration was shown to have no effect on the action of the endogenous pyrophosphatase under conditions used to assay ITPase. Table 2 indicates that the presence or absence of exogenous pyrophosphatase had only a very slight effect on the detection of ITPase activity as measured by phosphate production either in red cells or lymphocytes. Indeed, in lymphocyte preparations there appears to be a slight quenching of P_i detection in the presence of exogenous pyrophosphatase. Therefore, the endogenous pyrophosphatase activity under conditions utilized for assay of ITPase activity was investigated.

Data presented in Table 2 indicate that the presence or absence of exogenous pyrophosphatase had little effect on the cleavage of pyrophosphate under conditions used for ITPase assay in human hemolysates. Investigation of the effects of pH showed that the endogenous pyrophosphate was active at pH 8.5, pH 8.7, and pH 9.5 in human hemolysates. In lysates from human lymphoid lines the addition of exogenous pyrophosphatase had a slight effect on the cleavage of pyrophosphate. This data would indicate that in lysates of human lymphoid lines

Figure 9

Effect of Addition of Exogenous Pyrophosphatase on Detection of ITPase Activity by Phosphate Production

---------- no exogenous pyrophosphatase added

_____ exogenous pyrophosphatase added

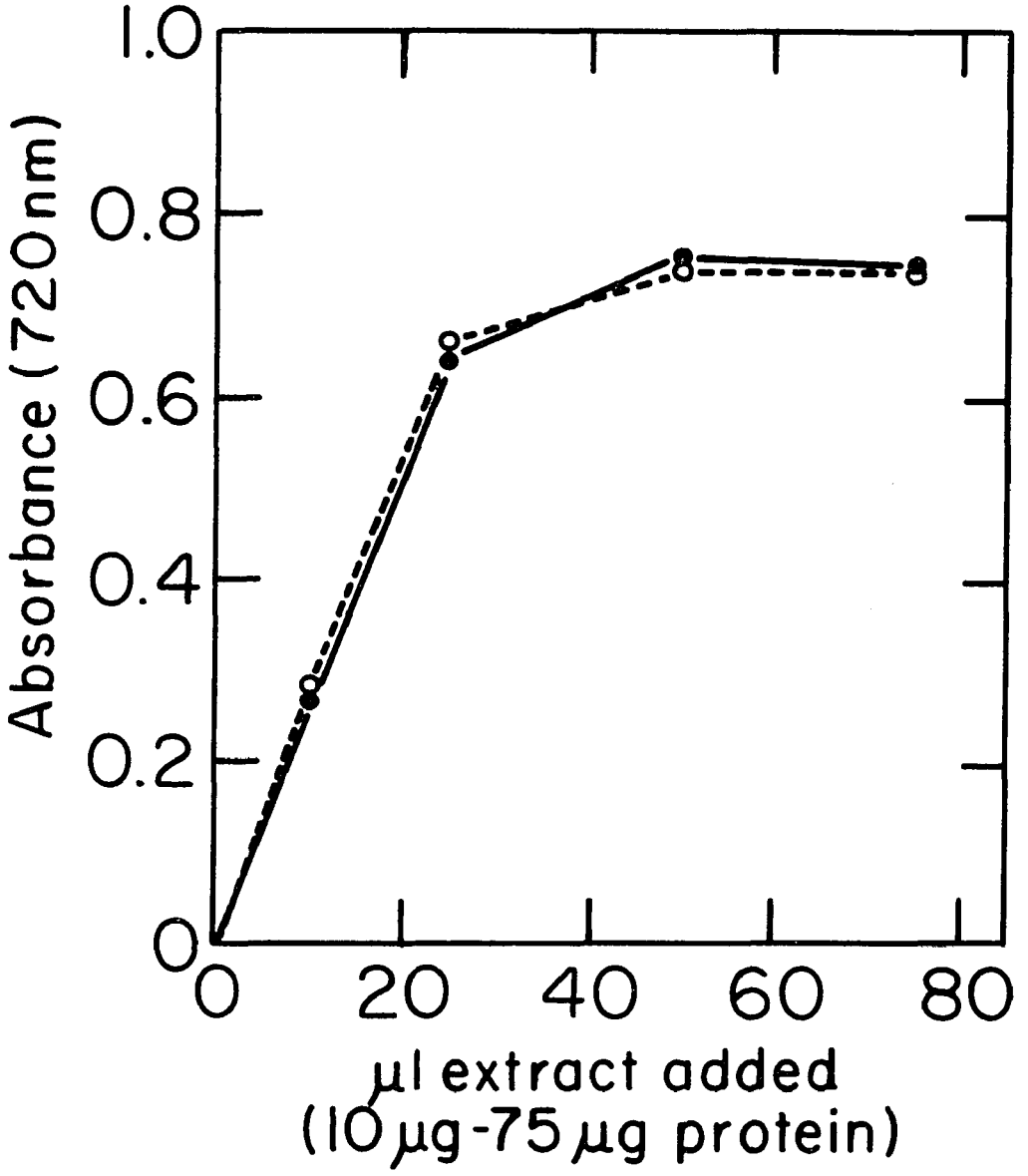

1.0
0.8
0.6
0.4
0.2
0
Absorbance (720nm)
0
20
40
60
80
µl extract added
(10µg-75µg protein)

Table 2

Endogenous Pyrophosphatase Activity--Phosphate Assay+

	A. ITP Substrate*	B. ITP Substrate and Exogenous PPase**	% Difference
Lymphoid			
normal	1.281	1.200	-6
ITPase variant*	1.008	0.979	-3
Erythrocytes			
normal	1.336	1.452	+8
heat inactivated 5' 55°C	1.235	1.237	0.16

	C. Pyrophosphate Substrate	D. Pyrophosphate Substrate and Exogenous PPase	% Difference
Lymphoid			
normal	0.903	1.032	+13
ITPase variant	0.929	1.135	+18
Erythrocytes			
pH 8.5	0.940	0.947	+1
pH 8.7	0.890	0.897	+1
pH 9.5**	0.783	0.779	+1

*Approximately four times as much protein was used for ITPase variant. All other proteins are approximately equal.

**All assays were conducted in a tris/HCl buffer of pH 8.5, unless noted. The assay at pH 9.5 was conducted with B-alanine as buffer.

+Activity measured by absorbance at 720 nanometers.

and human hemolysates the amount of pyrophosphate formed by the action of ITPase can be totally degraded to inorganic phosphate by the action of an endogenous pyrophosphatase.

In addition, data are presented (Table 2) showing that incubating human hemolysates at 55°C for five minutes did not inactivate the endogenous pyrophosphatase, as was reported for rabbit hemolysates (124).

A lymphoid line variant in ITPase activity (to be discussed later) was shown not to be deficient in the ability to cleave pyrophosphate in the presence or absence of ITP.

Results shown in Table 3 indicate that the mean value of ITPase activity in human hemolysates was 289 $\pm$ 170 umoles PP_i/hr/gram Hb (n = 10) with a range from 198 to 510 umoles PP_i/hr/gram Hb.

Determination of ITPase activity in lysates from human lymphoid lines indicates a mean value of 15,687 $\pm$ 750 umoles PP_i/hr/gram protein. The range was found to be from 14,700 to 16,578 umoles PP_i/hr/gram protein (n = 4).

The level of ITPase activity in a lymphoid line established from an individual whose red cells were reported to be totally deficient in ITPase activity (135)

Table 3

Comparison of Levels of Activity

of Inosine Triphosphatase

	Assay Method	
	Phosphate*	HPLC**
Lymphoid Lines		
Normal	15,687 ± 750	10,188 ± 2928
Sample Size	4	12
# determinations/sample	2-4	1-3
SEM	375	845
Variant***	3,114 ± 912	2,289 ± 268
Sample Size	1	1
# determinations/sample	6	6
SEM	372	109
Erythrocytes		
Normal	289 ± 170	124
Sample size	10	1
# determinations/sample	1-4	2
SEM	55	--

*Values expressed as umoles PP_i/hr/gram protein.

**Values expressed as umoles IMP/hr/gram protein.

***Standard deviation given for the number of determinations, not for sample size.

was found to have partial ITPase activity, with a mean of 3,114 ± 912 (6 determinations), approximately 20% of the mean for lymphoid lines. This value fell beneath two standard deviations from the mean (Table 3).

Under conditions utilized for the ITPase assay IDP is degraded at about 4% the rate of ITP. IMP and ATP are not utilized as substrates under standard assay conditions (Table 4). However, it was found that, if a high-speed centrifugation spin of the lysate (27,000 x g) was omitted, ATP was also utilized as a substrate (Table 4).

B. HPLC Assay

An HPLC assay of ITPase was developed that measures direct conversion of ITP to IMP. As shown in Figure 10, ITP, IDP, IMP, and inosine are visible as widely separated U.V. absorbing peaks. Hypoxanthine co-elutes with inosine. As little as five picamoles of IMP can be detected.

As shown in Figure 11, the assay of ITPase by HPLC was linear from 10 to 40 ul of a 1:5 dilution of packed erythrocytes per ml of reaction mixture. The assay was linear for twenty minutes (Figure 12).

The HPLC assay of human lymphoid lines for ITPase was linear in the range of 5-75 ug protein per ml reaction mixture (Figure 13) and linear for twenty minutes (Figures 14, 15).

The data presented in Table 5 show that the assay

Table 4

Ability of Cell Extracts

to Degrade IDP, IMP, and ATP under ITPase Assay Conditions

27,000 x g supernatant	% Activity of ITP As Substrate		
	Normal Lymphoid*	ITPase Variant* Lymphoid	Human Hemolysate**
ITP	100	100	100
IDP	4.1	5.0	4.4
IMP	0	0	0
ATP	0	0	0
Crude Homogenates			
ATP	120	100	--

*Assay by phosphate determination.

**Assay by HPLC.

Figure 10

HPLC Assay of ITPase

Absorbance at 248 nanometers

full scale IMP .08

full scale IDP .08 normal and blank

.16 variant

full scale ITP .64

Note full scale switched during analysis.

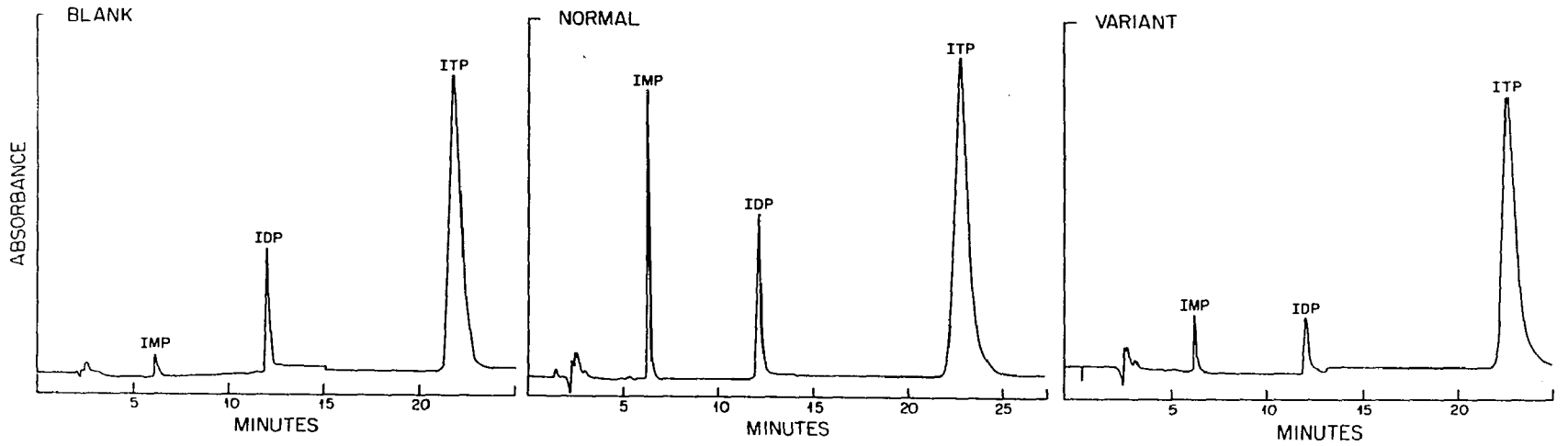

BLANK
NORMAL
VARIANT
ABSORBANCE
IMP
IDP
ITP
5
10
15
20
25
MINUTES

Figure 11

Linearity of HPLC Assay

with Protein Concentration in Erythrocytes

% conversion of ITP to IMP

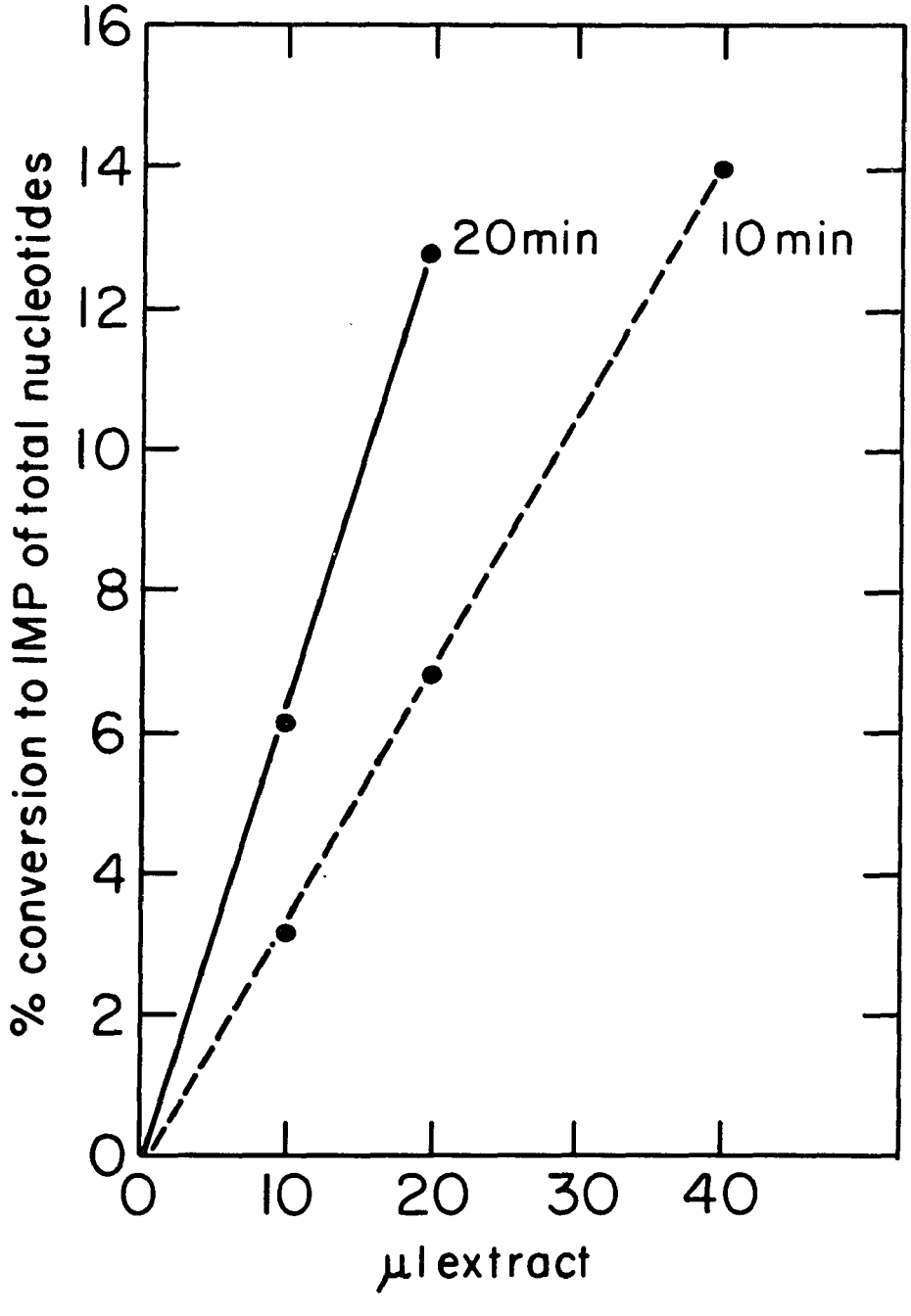

16
14
12
10
8
6
4
2
0
% conversion to IMP of total nucleotides
20min
10min
0
10
20
30
40
µl extract

Figure 12

Linearity of HPLC ITPase

Assay with Time in Erythrocytes

% conversion of ITP to IMP

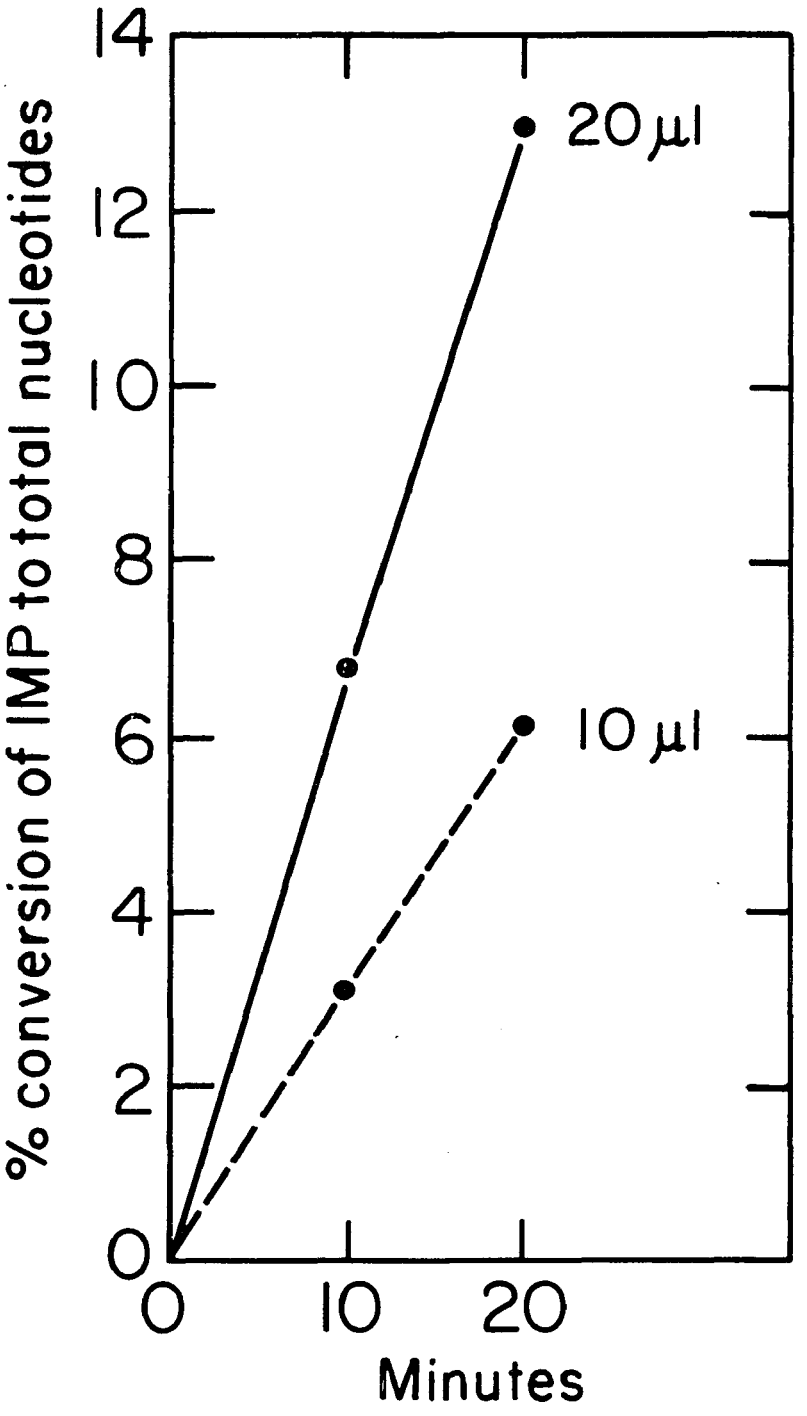

% conversion of IMP to total nucleotides
0
2
4
6
8
10
12
14
0
10
20
Minutes
20 µl
10 µl

Figure 13

Linearity of HPLC ITPase

Assay with Protein Concentration in

Lymphoid Lines

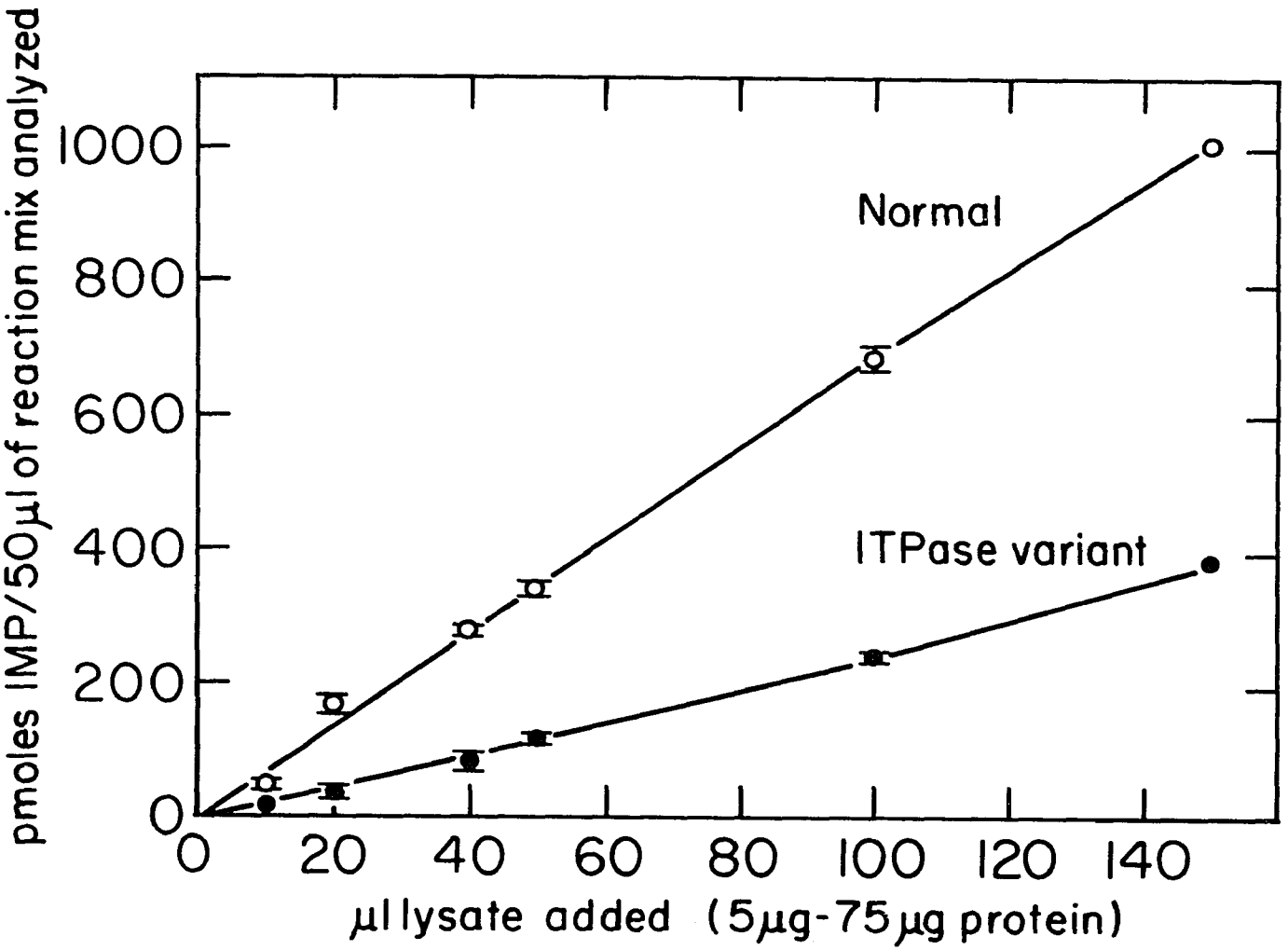

pmoles IMP/50µl of reaction mix analyzed
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
µl lysate added (5µg-75µg protein)
Normal
ITPase variant

Figure 14

Linearity of ITPase Assay by HPLC

with Time in Lymphoid Lines

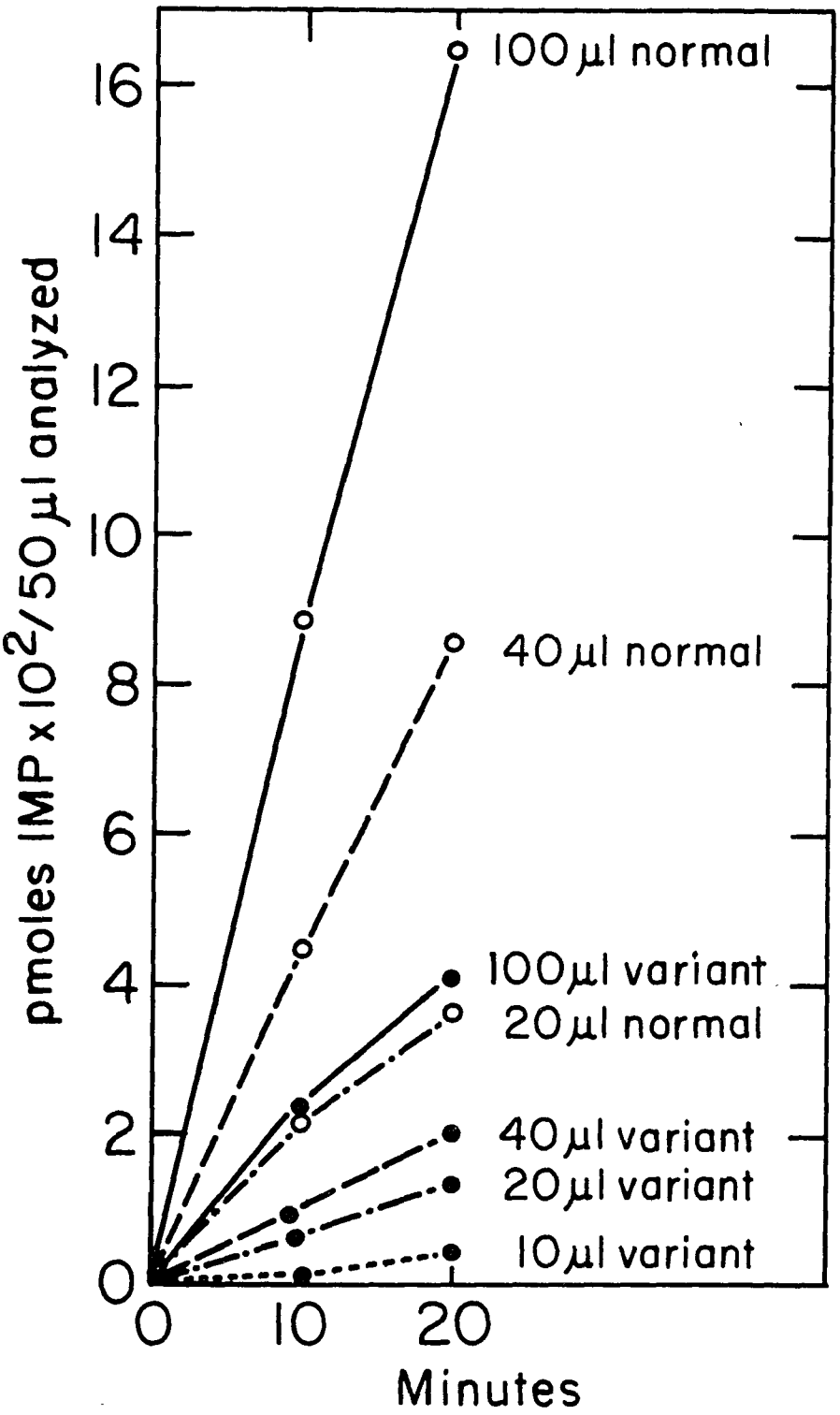

100 µl normal
40 µl normal
100µl variant
20µl normal
40µl variant
20µl variant
10µl variant
pmoles IMP x 10²/50 µl analyzed
0
2
4
6
8
10
12
14
16
0
10
20
Minutes

Figure 15

Linearity of HPLC ITPase Assay

with Time and Protein

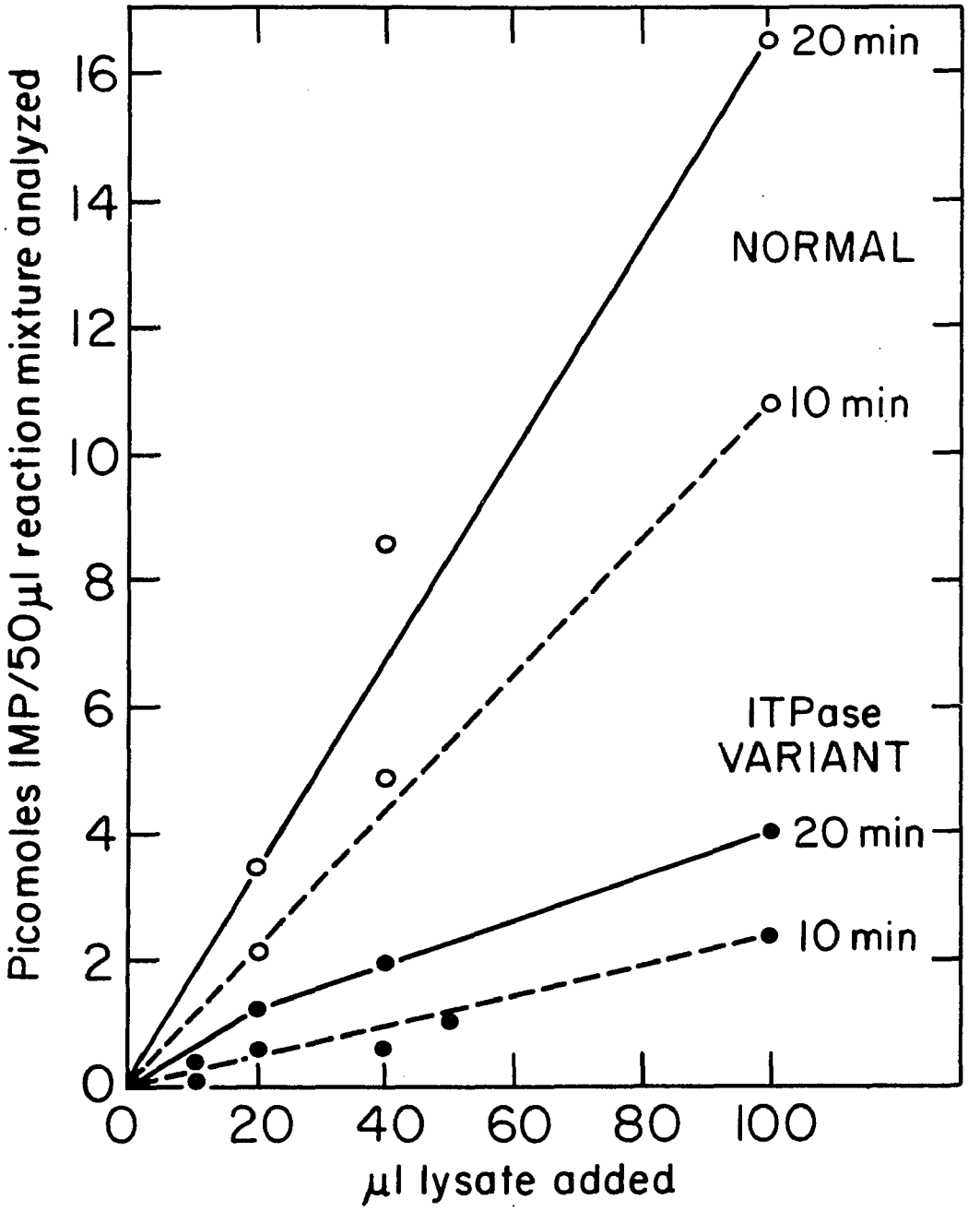

Picomoles IMP/50µl reaction mixture analyzed
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
µl lysate added
20 min
NORMAL
10 min
ITPase
VARIANT
20 min
10 min

Table 5

Reproducibility of HPLC ITPase Assay

Replicates of Same Sample picomoles IMP/hr/mg/50 ul analyzed	Effect of Storage at -20°C of Neutralized Reaction Mixture before Analysis on HPLC picomoles IMP/hr/mg/50 ul analyzed		Reproducibility of Same Lymphoid Line Assayed Over a Period of Several Months picomoles/hr/mg/50 ul analyzed	
			(ITPase variant)	
			Line 1	Line 2
217.65	day 0	170.2	229.31	43.80
216.00	day 1	166.4	225.77	56.32
208.00	day 2	192.0	227.35	45.27
	day 3	159.4		47.77
	day 4	160		44.30
	day 8	240		55.71
mean = 213.88	mean = 181.33		mean = 227.48	mean = 48.861
SD = 5.16	SD = 31.11		SD = 17.74	SD = 5.709
n = 3	n = 6		n = 4	n = 6
SEM = 2.97	SEM = 12.70		SEM = .88	SEM = 2.33

is highly reproducible for replicates of the same sample (213.88 ± 5.16, n = 3). In addition, two lymphoid lines freshly assayed over a period of months showed little variation in the amount of ITPase activity present. Line one had a mean of 227.478 ± 1.774 umoles IMP/hr/gram protein/50 ul analyzed (n = 3). Line two (the ITPase variant) had a mean of 48.861 ± 5.709 umoles IMP/hr/gram protein/50 ul analyzed (n = 6).

Since the assay is basically a two-step assay, the effect of storage of the neutralized reaction mixture (step 1) at -20°C before analysis on HPLC (step 2) was investigated. The results presented in Table 5 indicate that the neutralized reaction mixture is stable to freezing for a minimum of four days. ITP is unstable, and it is possible that the slight increase in IMP seen on day eight was due to non-enzymatic degradation of ITP.

The mean of ITPase activity found in lymphoid lines was 10,188 ± 2928 (n = 12). The range was from 5,906 umoles IMP/hr/gram protein to 13,917 umoles IMP/hr/gram protein (Figure 16, Table 6). ITPase activity in a lymphoid line established from an individual reported to have no ITPase activity in red cells was found to be 2,289 ± 268 in six determinations, approximately 22.4% of mean. This value was more than two standard

Table 6

ITPase Activity in Lymphoid Lines by HPLC Assay

uMoles IMP/hr/gram protein

Lymphoid Line	Value	# of Different Protein Concentrations	# of Times Assayed
1	7705	3	1
2	5906	2	3
3	8907	3	1
4	13,824	3	2
5	5805	2	1
6	13,917	2	3
7	12,635	2	2
8	10,274	2	1
9	11,914	1	1
10	12,200	3	1
11	8176	2	1
12	10,200	1	2

Mean 10,188 $\pm$ 2928

SEM = 845

Table 7

ITPase Activity in a Line with Variant ITPase Activity

Determination #	uMoles IMP/hr/ Gram Protein	# of Different Protein Concentrations
1	2054	3
2	2640	3
3	2120	3
4	2240	3
5	2077	3
6	2613	2

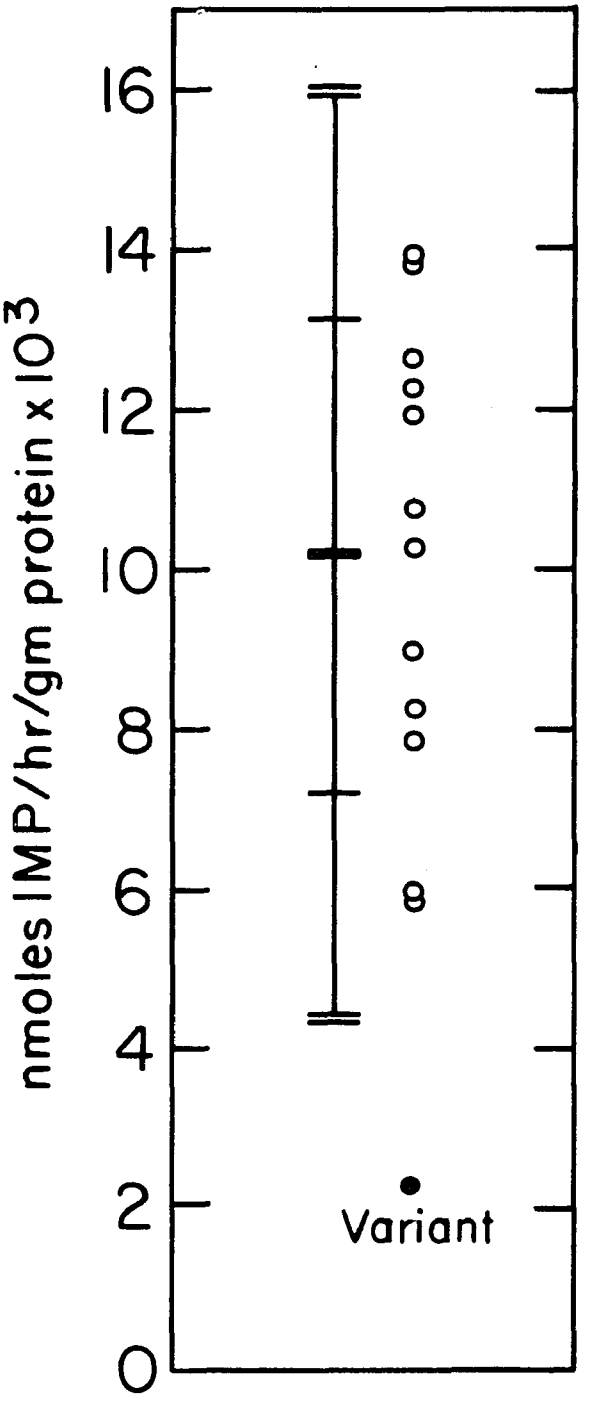
mean 2.289 $\pm$ 268

SEM = 56

Figure 16

Distribution of ITPase Activity in Human Lymphoid Lines by HPLC Assay

DISTRIBUTION OF ITPase ACTIVITY IN HUMAN LYMPHOID LINES



deviations from the mean (Figure 16, Table 7).

C. Products of the ITPase Reaction

The enzyme activity was found to be pyrophosphorlytic in both normal lymphoid lines and the variant lymphoid line. Evidence of this was obtained by incubating cell lysates with ^{14}C ITP. The ^{14}C ITP utilized in these experiments was found to be contaminated with 5% IDP, .18% IMP, and 1% hypoxanthine/inosine. When conversion of ^{14}C ITP to ^{14}C IMP was monitored by HPLC, no radioactivity above the levels present in the radio-labeled substrate was found in IDP, hypoxanthine, or inosine (if the lysates utilized as an enzyme source had been centrifuged at 27,000 x g) (Figure 17, Table 8).

However, in crude homogenates (lysates were not centrifuged at 27,000 x g) total hydrolysis of ITP was found to be approximately two times as great as that in a lysate which had been centrifuged at 27,000 x g. In contrast to the studies with lysates that had been centrifuged at 27,000 x g, the reaction appeared to proceed from ITP $\rightarrow$ IDP + P_i $\rightarrow$ IMP + P_i since radioactivity was found in IDP (Table 8).

The line variant in ITPase activity was found to have comparable activity to a normal line in this sedimentable ITPase, while exhibiting 25% of normal

ITPase Activity as Monitored by the Conversion of ^{14}C ITP to ^{14}C IMP by HPLC

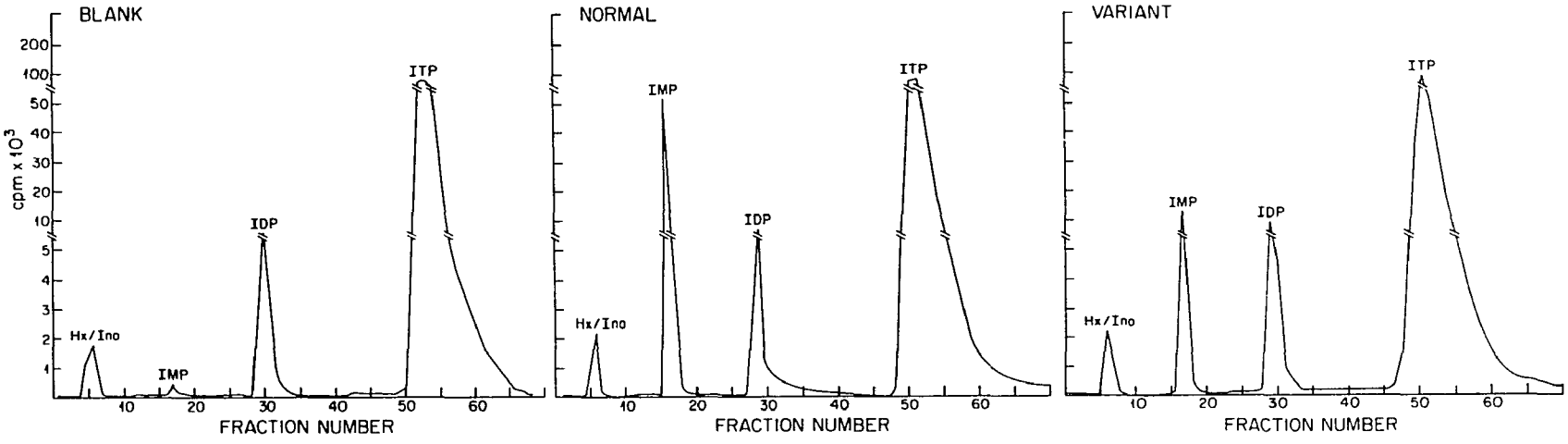

BLANK
NORMAL
VARIANT
cpm x 10³
200
100
50
40
30
20
10
5
4
3
2
1
Hx/Ino
IMP
IDP
ITP
10
20
30
40
50
60
FRACTION NUMBER

Table 8

Conversion of ^{14}C ITP to ^{14}C IMP Monitored by HPLC Assay

% Total Counts

	Control	Normal Lymphoid 27,000 x g Supernatant	ITPase Variant (Supernatant)	Normal Lymphoid (Crude)	ITPase Variant (Crude)
Hx/Ino	1.00	1.00	1.00	1.00	2.00
IMP	0.17	18.00	4.50	28.00	24.00
IDP	4.67	5.00	5.00	20.00	20.00
IDP	94.33	76.00	89.00	49.00	55.00

activity in the cytosol ITPase (Figure 17, Table 8). The 25% residual activity of the cytosol ITPase in the variant line was not due to the activity of the particulate-bound ITPase, since the mechanism of the two reactions appears to differ.

Studies conducted in stroma-free red cell hemolysates indicated that, under conditions of ITPase assay, IMP and ATP were not degraded enzymatically, while IDP was degraded at approximately 4% the rate of ITP in agreement with the data obtained from lymphoid lines by phosphate determination (Table 4).

D. Comparison of HPLC Assay and Phosphate Assay

Comparison of the values obtained by HPLC assay and phosphate production assay were in fairly close agreement. The mean value obtained by HPLC assay of lymphoid lines was 10,188 $\pm$ 2928 umoles IMP/hr/gram protein. The mean value obtained by phosphate determination of ITPase activity in lymphoid lines was 15,687 $\pm$ 750 umoles PP_i/hr/gram protein. The reaction is known to proceed stoichiometrically as ITP $\rightarrow$ IMP + PP_i. The difference between the means can easily be explained by the small sample sizes (n = 4 for phosphate assay, n = 12 for HPLC assay), the large population variance, and the use of different lymphoid lines in a population sample.

The mean value obtained of ITPase activity in a

lymphoid line established from an individual reported to be deficient in ITPase activity in red cells was 2,289 ± 268 umoles IMP/hr/gram protein as assayed by HPLC (Table 7). That obtained by measurement of phosphate production was 3,114 ± 912 umoles IMP/hr/gram protein for the same lymphoid line.

E. Heat Inactivation Studies

Heat inactivation studies of normal lymphoid lines and the line with reduced ITPase activity found that the mutant line showed slight instability to heat. No significant difference was found in heat denaturation studies at 50°C between the variant line and a normal line when lysates were incubated for 15 minutes, while longer times of incubation (up to one hour) showed a significantly decreased level of activity in the variant line (Figure 18). Preincubation of a normal and the variant line at 56°C showed significantly reduced activity at all time points (Figure 19).

F. Starch Gel Electrophoresis

Detection of ITPase activity on starch gel is based on phosphate production. ITP is cleaved to IMP + PP_i by ITPase immobilized in the starch gel after electrophoresis. The PP_i is cleaved non-enzymatically by the acid pH of the acid molybdate overlay.

Figure 18

Heat Inactivation of ITPase in Lymphoid Lysates Incubated at 50°C

ITPase Activity Determined by HPLC Assay

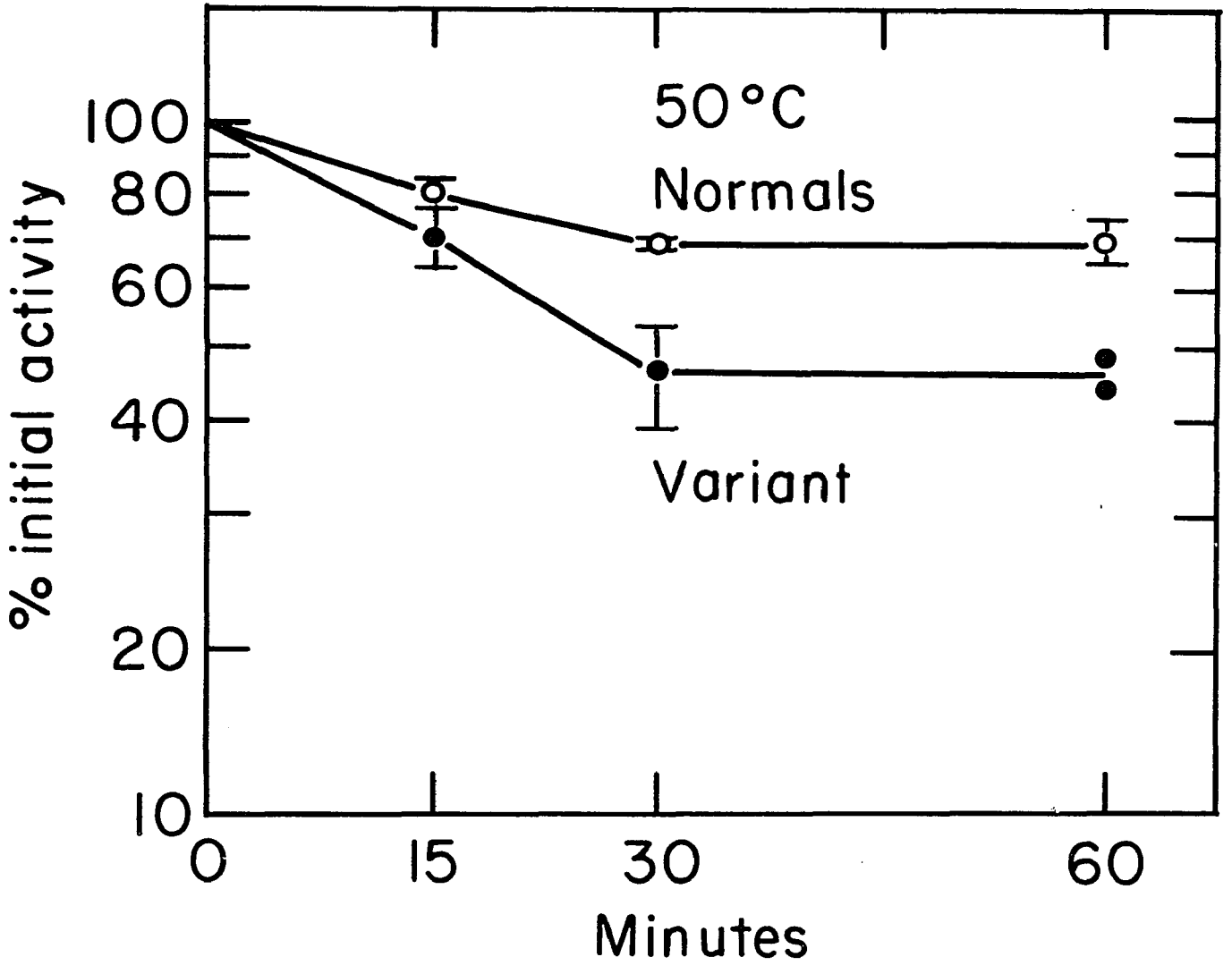
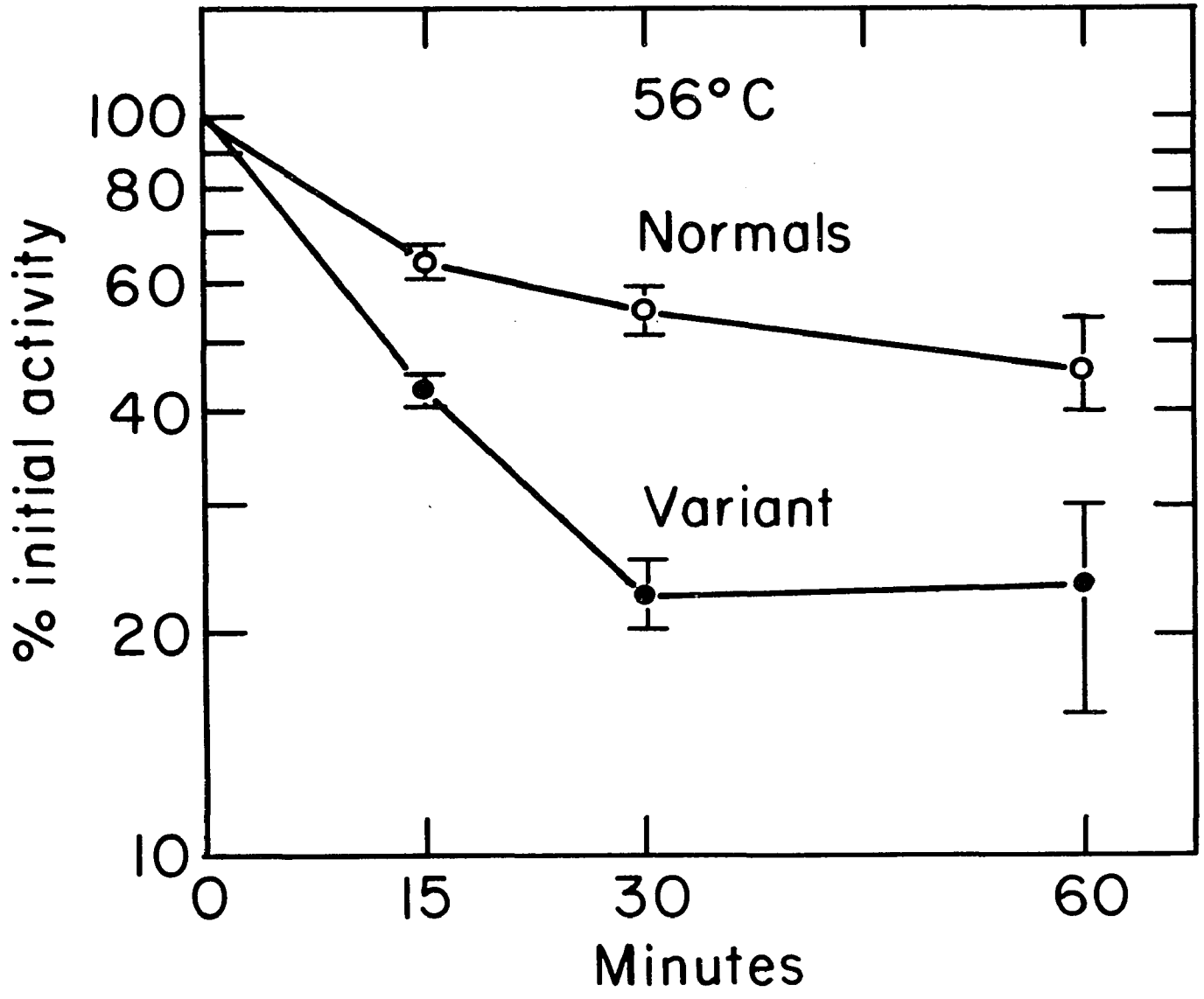

50°C
Normals
Variant
100
80
60
40
20
10
% initial activity
0
15
30
60
Minutes

Figure 19

Heat Inactivation of ITPase in Lymphoid Lysates Incubated at 56°C

ITPase Activity Determined by HPLC Assay


56°C
Normals
Variant
% initial activity
100
80
60
40
20
10
0
15
30
60
Minutes

The enzyme preparations were prepared by serial dilutions of a lysate. As can be seen in Figure 20, the lysate of a normal lymphoid line diluted 1 to 4 stained with approximately the same intensity as an undiluted lysate of the ITPase variant at the same protein concentration.

On starch gel electrophoresis the variant ITPase line was shown to have approximately 25% of the activity of a normal line. The activity of ITPase in this variant line was approximately the same utilizing XTP, dITP, or ITP as a substrate by starch gel (Figure 20).

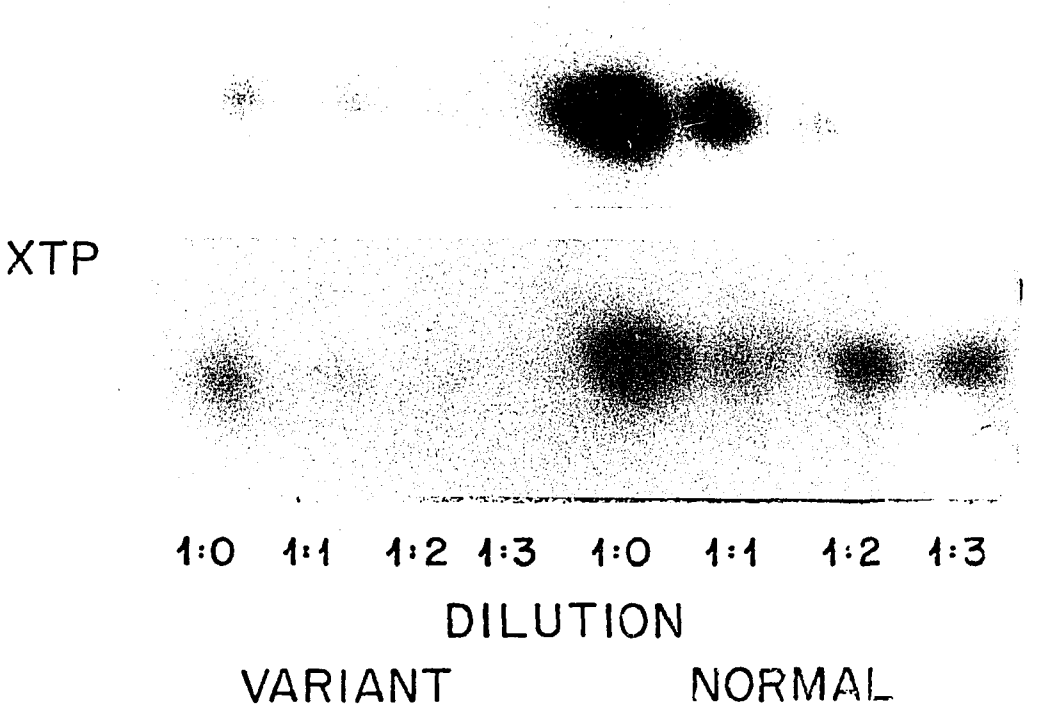
Figure 20

Starch Gel Electrophoresis

Protein concentrations of normal and variant were the same.

The same concentration of ITP and XTP was utilized for detection of ITPase activity.

STARCH GEL ELECTROPHORESIS



Metabolic Studies in Red Cells

Analysis by Thin Layer Chromatography

Since ITP accumulation was initially reported in red cells, human erythrocytes were chosen for preliminary studies of ITP metabolism. Red cells were incubated with radio-labeled inosine for one to two hours under conditions that were reported to result in ITP accumulation in some individuals. Aliquots of the reaction mixture were spotted on PEI cellulose sheets as previously described. The amount of nucleotide compounds present in an aliquot of cell extract was beneath the level needed to visualize the nucleotide on PEI cellulose by U.V. light. Therefore, radio-labeled precursors were utilized and carrier standards added to each channel. Auto-radiograms of the thin layer chromatogram were developed to localize areas of radioactivity. These areas were then eluted from the chromatogram.

When red cells were incubated for two hours under the stated incubation conditions, the radioactivity was present in a band that comigrated with authentic AMP/IMP. Lesser amounts of radioactivity were found in a band that comigrated with ATP. An area of radioactivity was barely visible in the region of ADP (Table 9). Analysis of nucleo-sides revealed that the majority of radio-labeled base or nucleoside present was hypoxanthine (Table 9).

Table 9

Radio-Labeled Compounds Present in Human Erythrocytes Incubated for Two Hours with 8-^{14}C Inosine Analyzed by Thin Layer Chromatography

% Total cpm

Compound	Sample 1	Sample 2
GTP	0	0
ITP	0	0
ATP	4	4
IDP	0	0
ADP	1	1
GMP	0	0
AMP/IMP	69	71
hypoxanthine	26	23
inosine	1	1
xanthine	0	0
guanosine	0	0
adenine	0	0
adenosine	0	0
uric acid	0	0

Analysis by High Pressure Liquid Chromatography

It became apparent that greater sensitivity was required for the detection of ITP. Therefore, nucleotides were analyzed by high pressure liquid chromatography. Initial analysis of nucleotides by HPLC utilizing published methods did not result in adequate separation of inosine and adenosine nucleotides. Hence, development of a high pressure liquid chromatography system particularly suited for the analysis of inosine nucleotides and nucleosides was initiated. The advantage of this analysis system was that unlabeled compounds could be quantitated as well as labeled compounds. A typical separation of purine and pyrimidine base and nucleoside standards is shown in Figure 21. Separation of purine and pyrimidine nucleotides is shown in Figure 22. The retention times of standard compoundswere initially determined individually in these systems. The retention time of the purine and pyrimidine nucleotides on a SAX anion exchange column, under stated buffer conditions, is shown in Table 10. The retention time of purine and pyrimidine bases and nucleosides on a C_{18} microbondpak column is shown in Table 11. Absolute retention times varied somewhat in cell extracts; therefore, relative retention times which remained constant were also monitored.

Human erythrocytes incubated with 50 uM inosine showed a thirty fold increase in the amount of IMP (Table 12).

Figure 21

Separation of Nucleoside Standards by HPLC conditions as described in methods section

Absorbance at 248 nanometers

Absorbance full scale = .01

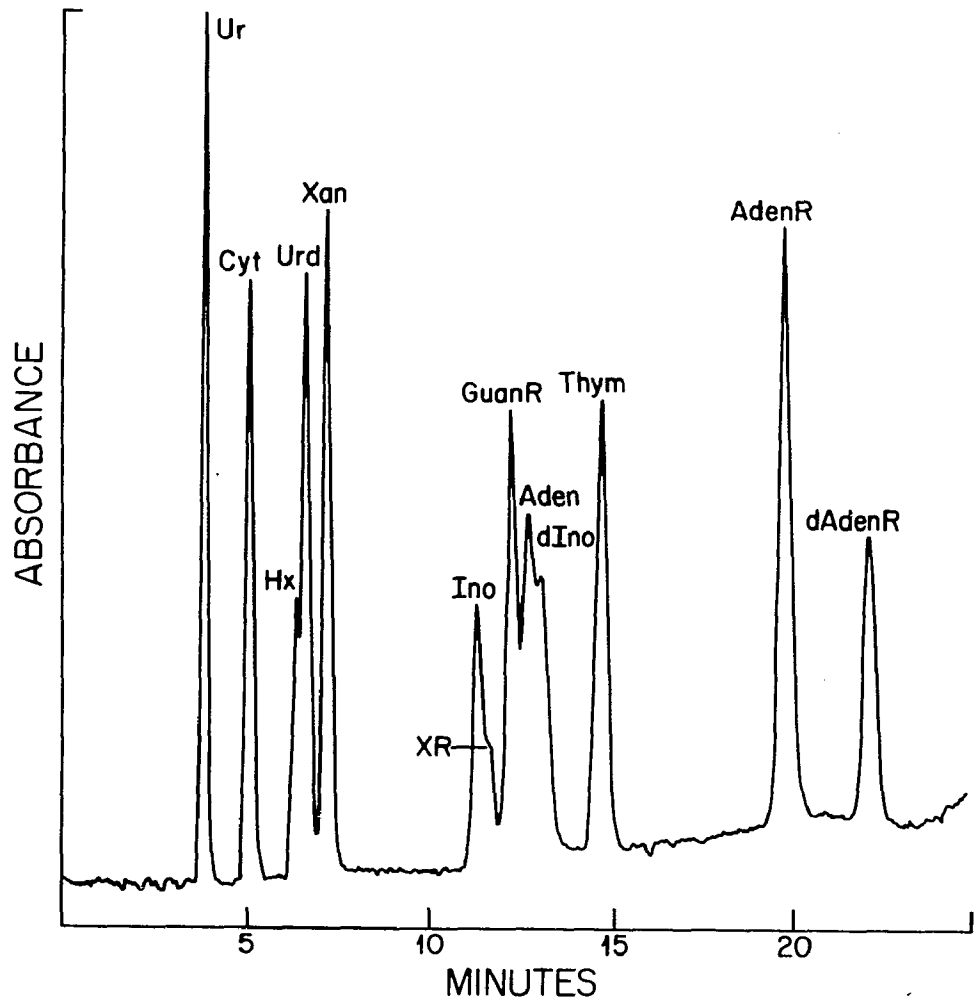

Ur
Cyt
Urd
Xan
AdenR
GuanR
Thym
Aden
dIno
dAdenR
Hx
Ino
XR
ABSORBANCE
5
10
15
20
MINUTES

Figure 22

Separation of Nucleotide Standards by HPLC

conditions as described in methods section

Absorbance at 248 nanometers

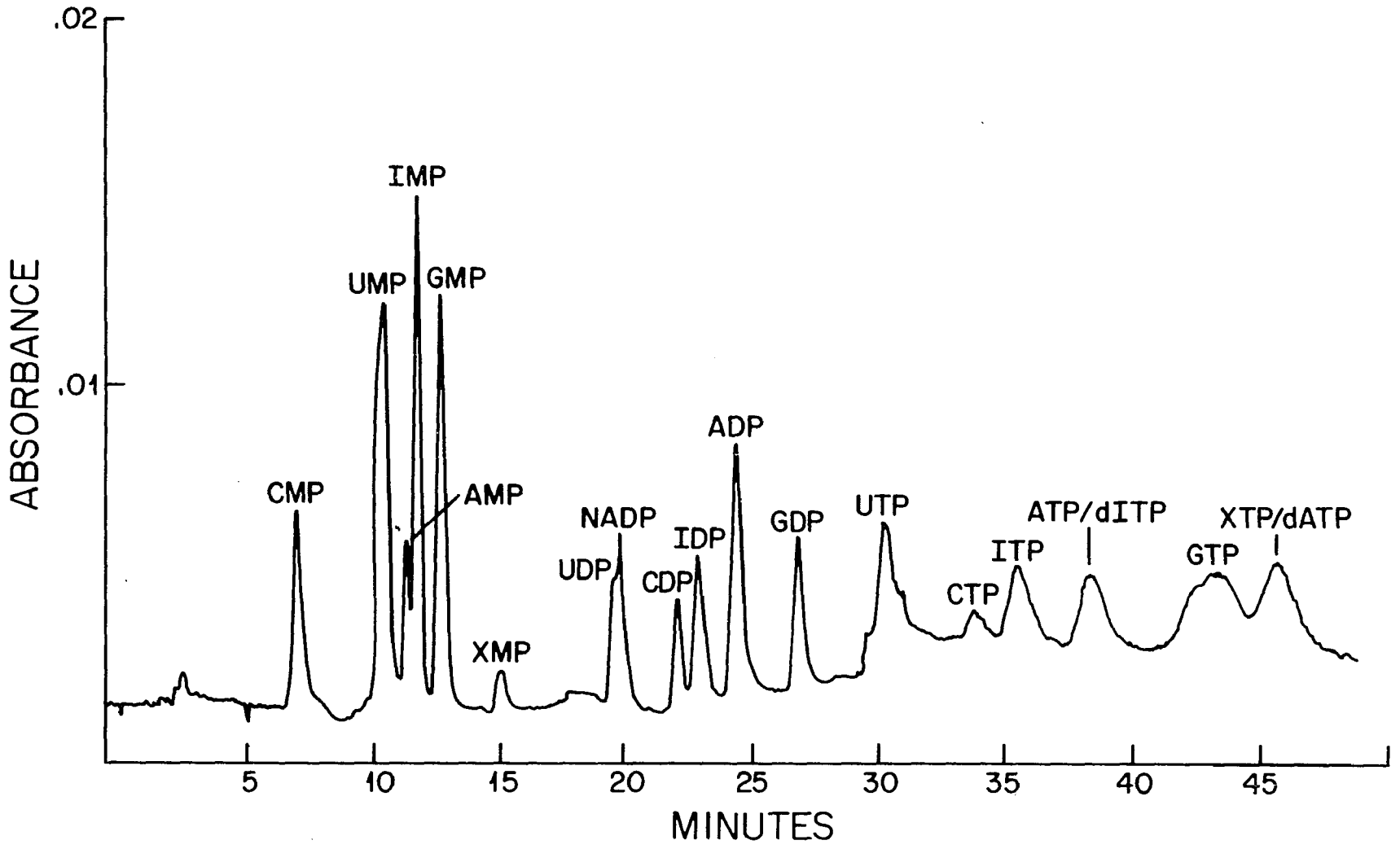

.02
.01
ABSORBANCE
IMP
UMP
GMP
AMP
CMP
XMP
NADP
UDP
IDP
CDP
ADP
GDP
UTP
CTP
ITP
ATP/dITP
GTP
XTP/dATP
5
10
15
20
25
30
35
40
45
MINUTES

Table 10

Retention Time of Nucleotide Standards

	Retention Time (in Minutes)	s.d.	Number of Runs
CMP	7.83	0.04	16
NAD	7.88	0.32	5
UMP	8.51	0.20	11
AMP	9.88	0.05	17
IMP	10.50	0.10	18
GMP	14.13	0.22	12
UDPG	14.65		1
XMP	15.00	0.26	4
UDP	18.10	0.34	12
NADP	19.12	0.65	3
CDP	19.51	0.31	8
IDP	20.38	0.09	20
ADP	22.50	0.20	18
dIDP	23.10		1
GDP	24.60	0.34	12
XDP	26.25	0.08	5
UTP	29.25	0.26	16
CTP	30.88	0.17	16
ITP	32.75	0.09	28
ATP	36.50	0.16	20
dITP	36.53		2
GTP	40.25	0.08	17
XTP	43.64	0.34	4
dATP	43.64		1

Table 11

Retention Time of Nucleoside Standards

	Retention Time (in minutes)	s.d.	Number of Runs
Uracil	3.92	0.54	15
Cytidine	5.12	0.84	14
Hypoxanthine	6.44	0.10	15
Uridine	6.72	0.05	7
Deoxycytidine	7.25	0.13	3
Xanthine	7.28	0.12	9
Inosine	11.43	0.14	12
Xanthosine	11.76	0.27	15
Guanosine	12.33	0.11	9
Adenine	12.83	0.15	15
Deoxyinosine	13.14	0.18	5
Deoxyguanosin	14.50		1
Tymidine	14.66	0.10	7
Adenosine	19.73	0.26	12
Deoxyadenosine	22.02	0.13	4

Table 12

Response of Human Erythrocytes to Incubation with Inosine

nanomoles/10^6 cells

	0 time	1 hr.		2 hr.	
		-Inosine	+Inosine	-Inosine	+Inosine
IMP	0.06	0.15	1.50	0.5	1.5
AMP	0.24	0.18	0.29	0.35	0.26
GMP	0.16	0.18	0.13	0.12	0.12
IDP	--	--	--	--	--
ADP	2.92	1.98	1.60	1.49	1.9
GDP	0.56	0.44	0.46	0.32	0.47
ITP	--	--	--	--	--
GTP	2.41	2.46	2.08	2.93	1.89
ATP	18.0	27.6	24.6	17.1	28.9
CTP	0.06	0.28	0.28	0.28	0.18
UTP	0.06	0.15	0.15	0.07	0.14

On incubation alone there was a ten fold increase of IMP. There was no corresponding increase in GTP, GDP, GMP, ADP, or AMP. While the amount of CTP and UTP shows a slight increase, the increase was present if the cells were incubated in the incubation medium alone. Thus the increase of CTP and UTP was due to the effects of the incubation medium and not due to incubation with inosine. No ITP was present under these conditions (Figure 23). It was possible with the large amount of ATP present that ITP might have been buried under the ATP peak, so standard ITP, IDP, and IMP were cochromatographed with the cell extract (Figure 24). The limit of detection was 25 picamoles of ITP relative to 289 nanomoles of ATP. Figure 24 illustrates the position of ITP relative to the position of ATP in the cell extracts. Figure 25 shows the separation of nucleosides and bases present in the cell extract. Under these incubation conditions normal red cells from three individuals did not show ITP accumulation.

At two hours of incubation no inosine or deoxyinosine was detectable. However, there was an increase in hypoxanthine. Figure 26 indicated that deoxyinosine and inosine would have been visible if present.

Extraction of Free Nucleotides

Earlier studies indicated a high level of nonspecific phosphatase activity in lymphoid lines. Investigation of extraction procedures was initiated in the hope of minimizing

Figure 23

Free Nucleotide Content in Human Erythrocytes

Absorbance at 248 nanometers

Conditions as described in methods section

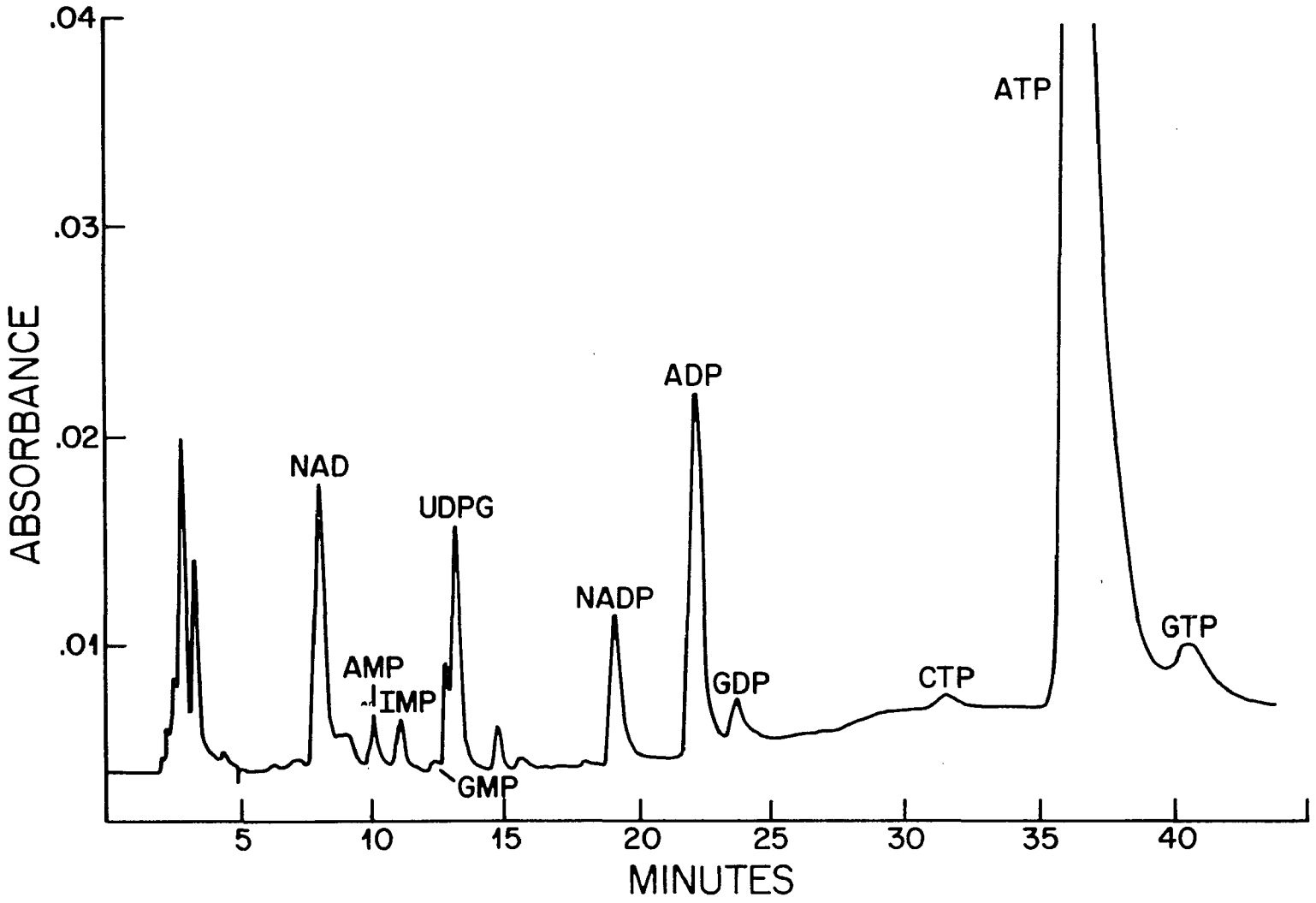

.04
.03
.02
.01
ABSORBANCE
NAD
AMP
IMP
UDPG
GMP
NADP
ADP
GDP
CTP
ATP
GTP
5
10
15
20
25
30
35
40
MINUTES

Figure 24

Free Nucleotide Content in Human Erythrocytes

plus internal standards of IMP, IDP, and ITP

Absorbance at 248 nanometers

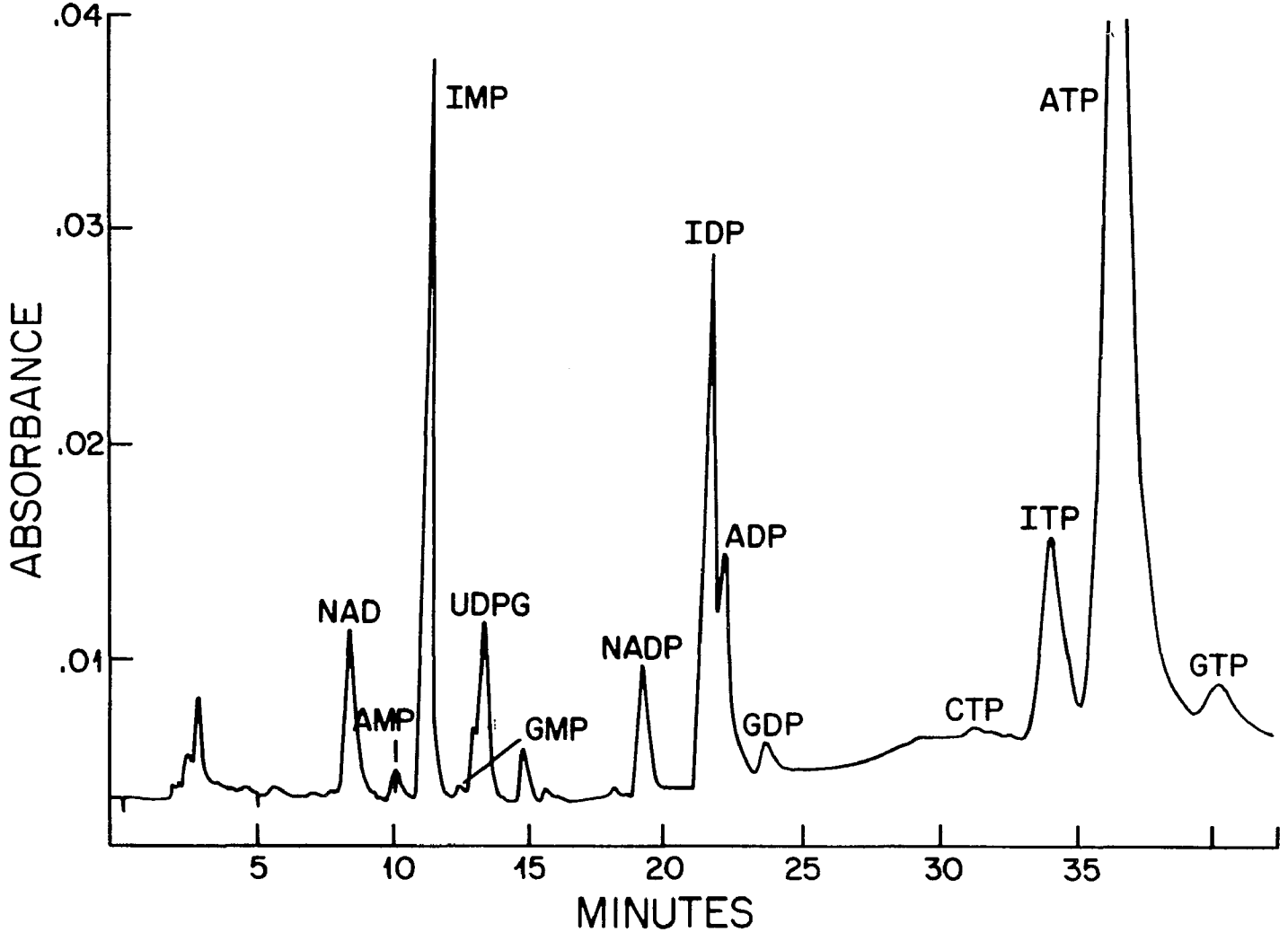

.04
.03
.02
.01
ABSORBANCE
IMP
IDP
ATP
NAD
AMP
UDPG
GMP
NADP
ADP
GDP
CTP
ITP
GTP
5
10
15
20
25
30
35
MINUTES

Figure 25

Nucleoside and Base Content in a Human Erythrocyte Extract

Absorbance at 248 nanometers

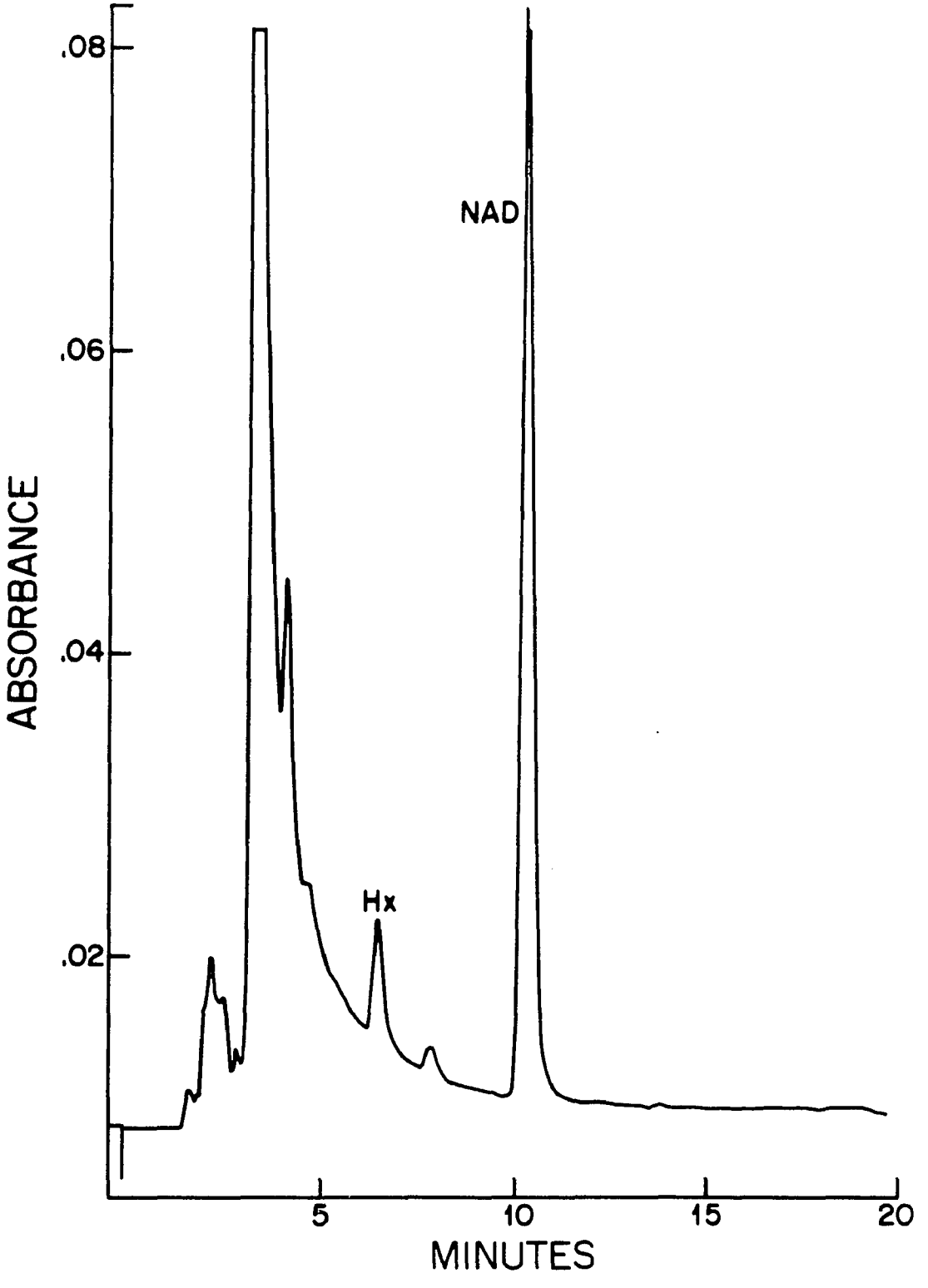

.08
.06
.04
.02
ABSORBANCE
NAD
Hx
5
10
15
20
MINUTES

Figure 26

Nucleoside and Base Content in Human Erythrocyte Extracts plus internal standards of hypoxanthine, inosine, deoxyinosine, adenosine, and deoxyadenosine

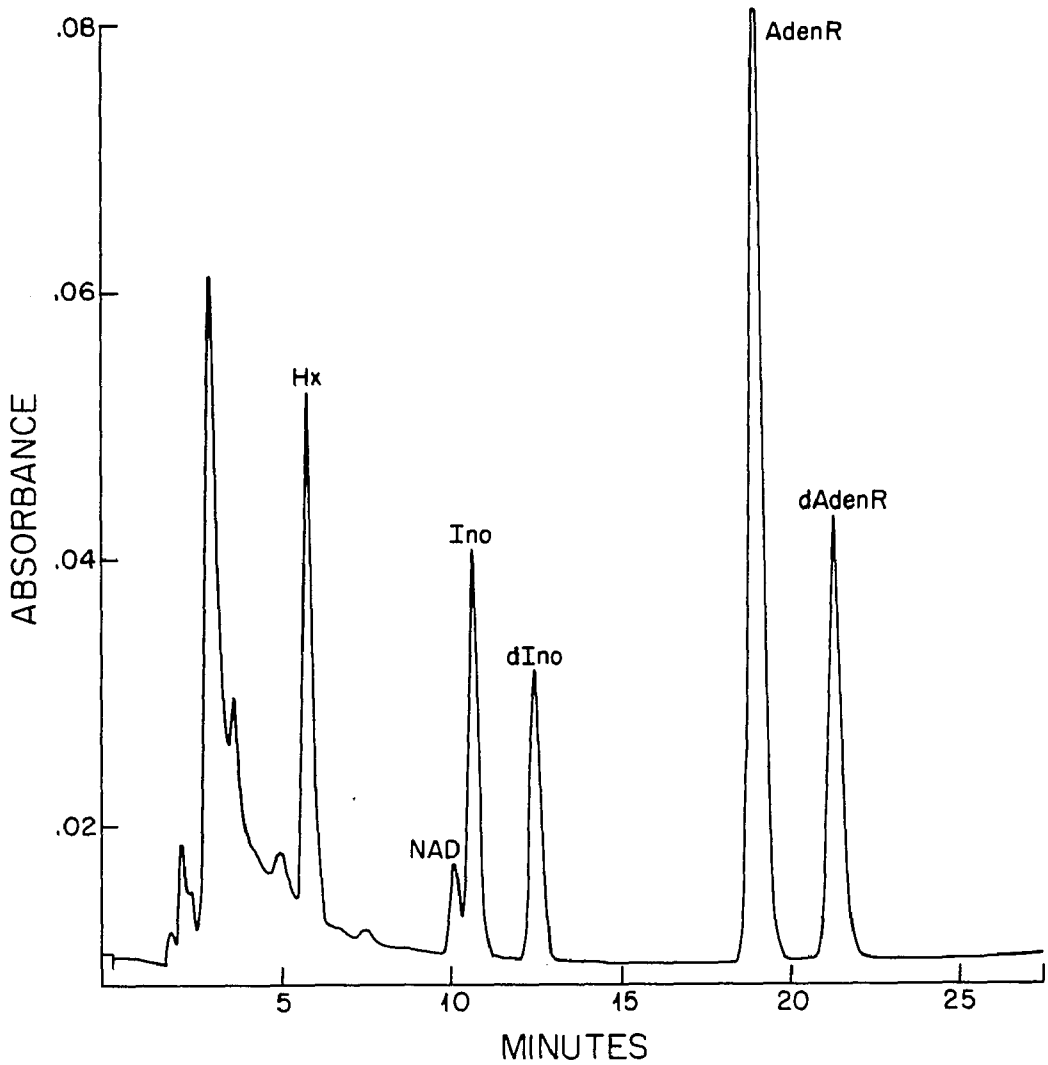

.08
.06
.04
.02
ABSORBANCE
Hx
Ino
dIno
NAD
AdenR
dAdenR
5
10
15
20
25
MINUTES

Table 13

Investigation of Extraction Procedures

Standards--% Recovery of Unextracted Standard

Method	Inosine	ITP
PCA + KOH	78	65
PCA + Freon Amine	79	70
70%* Methanol	86	81
70%** Methanol		75

*ITP added after methanol was added.

**ITP added to cell mixture prior to the addition of Methanol.

the exposure of ITP to nonspecific nucleotidase.

In addition, ITP is a compound that is particularly acid labile. Extraction of free nucleotides is classically performed using perchloric acid, followed by neutralization of the extract. Therefore, various methods of neutralization were investigated. It was found that ITP was more stable to freon-amine neutralization than to neutralization with KOH. However, both of these methods depend on the skill of the investigator, depending on the speed with which the extract is neutralized after PCA addition. Extraction with 70% methanol does not subject the nucleotides to acid conditions. Therefore, extraction with methanol was compared to the two other methods. ITP was added after extraction with perchloric acid or methanol. The methanol extracts were evaporated to dryness and reconstituted. The PCA extracts were neutralized with either KOH or freon amine. As shown in Table 13, ITP is more stable to methanol than to either of the methods using perchloric acid.

In addition, it was found that, if ITP was added to a cell mixture prior to extraction by 70% methanol, approximately 75% of the ITP was recovered. This would rule out substantial degradation of ITP by a nucleotidase present in the cell extract.

Metabolic Studies--Lymphoid Lines

ITP accumulation in intact cells other than erythrocytes has not been previously demonstrated. It is presumed that the

ITPase activity in lymphoid tissue is sufficient to degrade all of the ITP synthesized or that this tissue lacks the ability to synthesize ITP. The discovery of an individual with reduced activity of ITPase in lymphoid cells presented a unique opportunity to investigate parameters affecting ITP accumulation.

Because of the level of residual enzyme activity (20%) in the ITPase variant it was uncertain if any metabolic abnormalities would exist in these lymphoid cells. An initial investigation of a growing cell culture revealed that the ITPase variant line contained 0.144 nanomole/10^6 cells of ITP. No ITP was detected in the normal control. Furthermore, on incubation in a glucose medium ITP was also present in the variant line. When 8-^{14}C inosine (conditions as described in the methods section) was added to the incubation medium, these cells synthesized ITP at a rate that was linear for two hours (0.031 nanomoles/10^6 cells at one hour and 0.064 nanomoles/10^6 cells at two hours). In contrast, the normal cell line had no ITP present in the glucose incubation extract, and a small amount of ITP present at one hour of incubation with inosine, but no detectable ITP at two hours (not detectable either by cpm or U.V. absorbing material). IDP was present in detectable amounts when ITP was present. Radio-labeled IDP was present in the ITPase variant. ITP was

positively identified by collecting the peak, hydrolyzing with acid to the base, and determining that both the U.V. absorbing material and radioactivity now eluted with the expected retention time of hypoxanthine (see methods section). When the inosine incorporation experiment was repeated, ITP again accumulated but in a slightly different pattern. In this experiment no ITP was present in the free nucleotides of the ITPase variant grown in complete culture media (Figure 27). When these cells were switched to a glucose phosphate media for twenty-five minutes, there was still no ITP present. However, on addition of inosine these cells synthesized 0.31 nanomoles ITP/10^6 cells in one hour, and 0.064 nanomoles ITP/10^6 in two hours (Figure 29). In contrast, no ITP was detected in the normals at zero time or two hours of incubation (Figures 28 and 30). At one hour a small amount of ITP, one fiftieth of that in the variant, was accumulated (0.001 nanomoles ITP/10^6 cells).

Further investigation was centered on determining the conditions of cell culture which were permissive for ITP accumulation. Since the ITPase variant was found to have a mildly unstable enzyme, it was possible that the cells possessed enough residual ITPase activity to degrade all of the ITP synthesized while actively synthesizing protein in log phase, but at stationary phase lacked enough residual activity to degrade all the ITP synthesized.

Figure 27

Free Nucleotide Content in a Lymphoid Lysate of ITPase Variant

Absorbance at 248 nanometers

Full scale .08 to XMP

.01 to CDP

.04 ADP and GDP

.02 UTP

.01 CTP

.08 ATP and GTP

Note switch in full scale during analysis

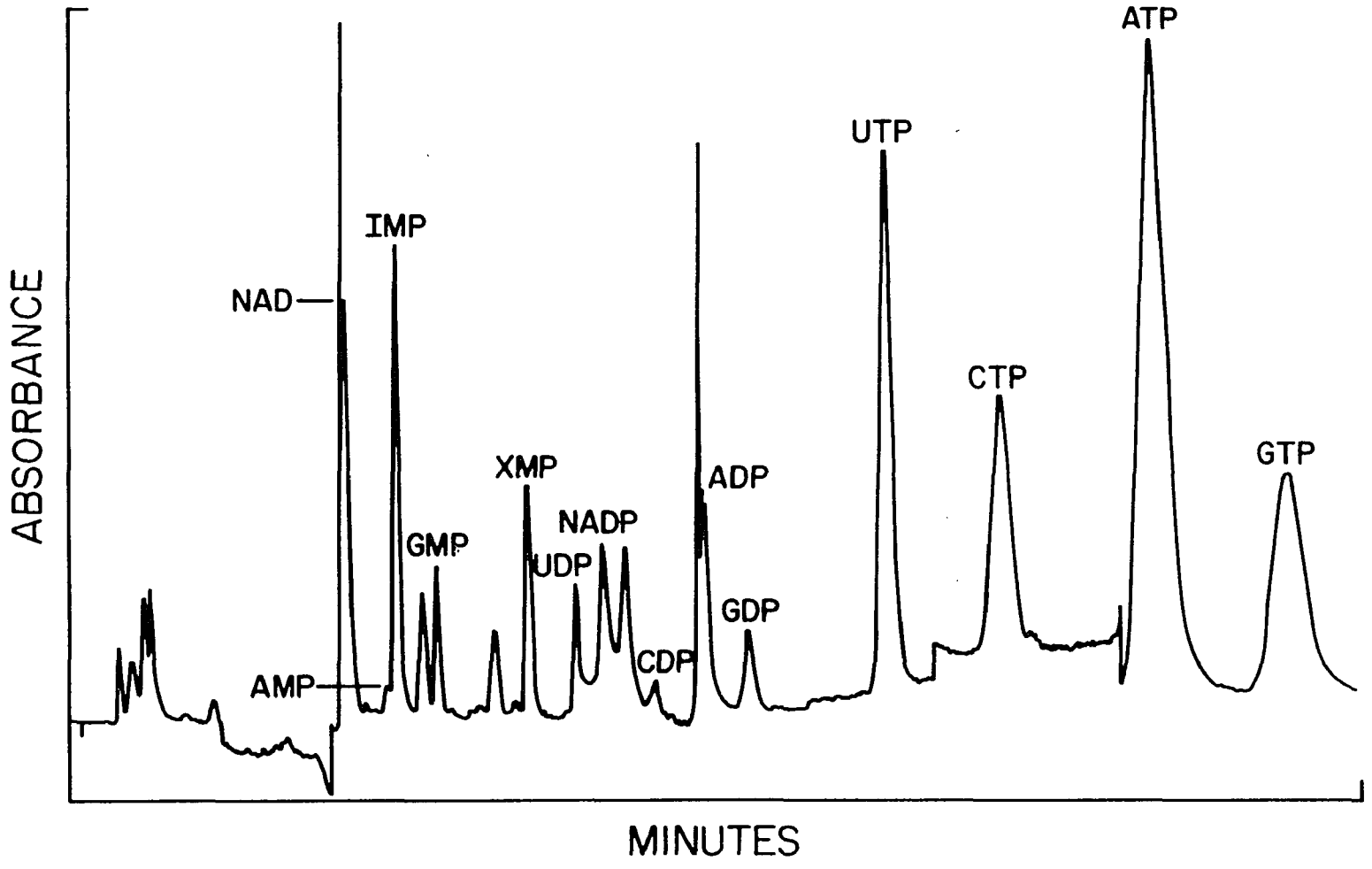

ABSORBANCE
MINUTES
NAD
AMP
IMP
GMP
XMP
UDP
NADP
CDP
ADP
GDP
UTP
CTP
ATP
GTP

Figure 28

Free Nucleotide Content in a Lymphoid Lysate of ITPase Normal Cells

Absorbance at 248 nanometers

Full scale .08 to XMP

.01 to CDP

.04 ADP and GDP

.02 UTP

.01 CTP

.02 ATP

.04 GTP

Not change in full scale during analysis

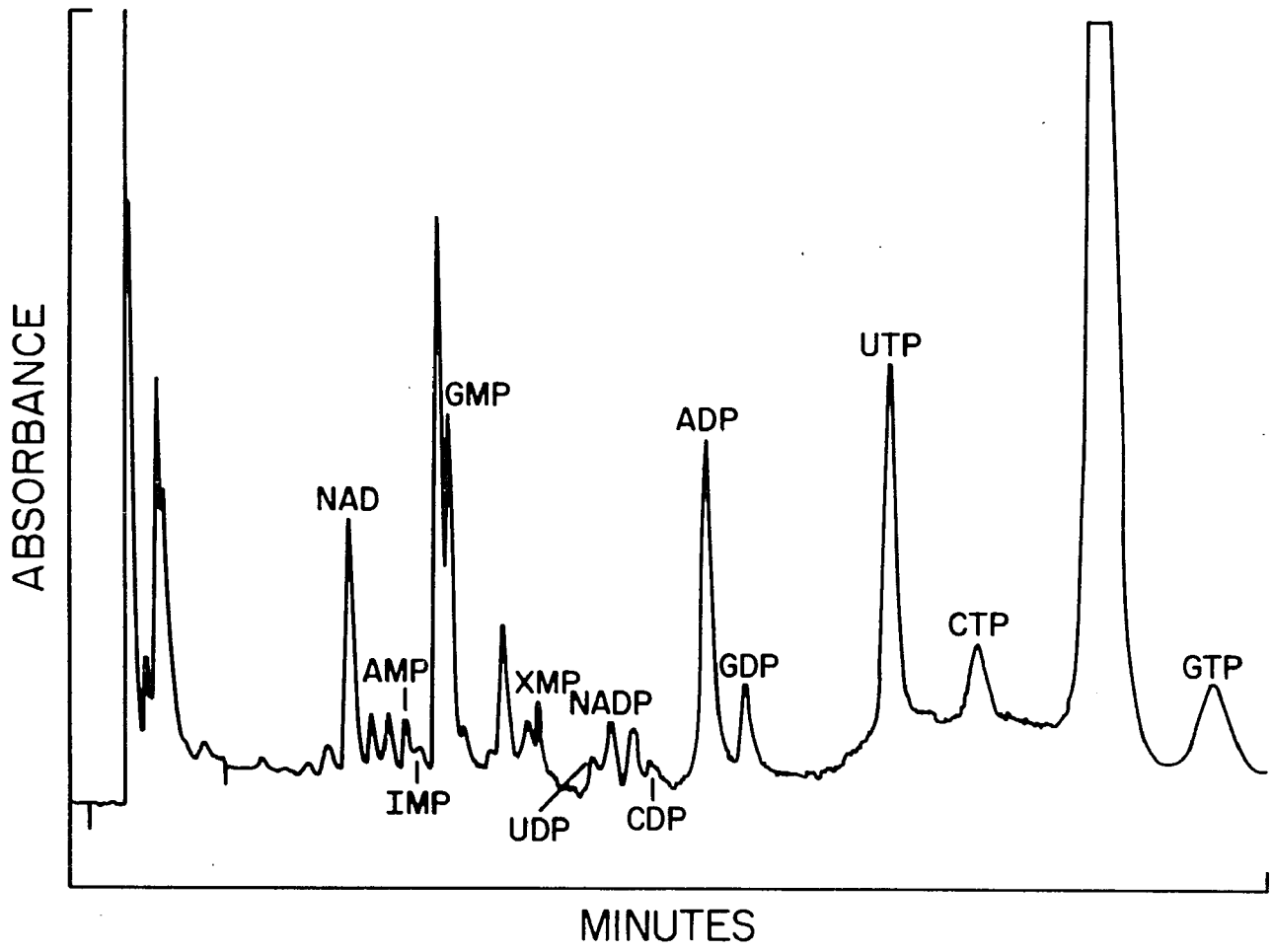

ABSORBANCE
NAD
AMP
IMP
GMP
XMP
UDP
NADP
CDP
ADP
GDP
UTP
CTP
GTP
MINUTES

Figure 29

Free Nucleotide Content and Radioactivity

Present in ITPase Variant Lymphoid Lines

Incubated for Two Hours with 8-^{14}C Inosine

Analyzed by HPLC

Absorbance at 248 nanometers

Full scale .08 to XMP

.01 to ITP

.02 ATP and GTP

Note change in full scale during analysis

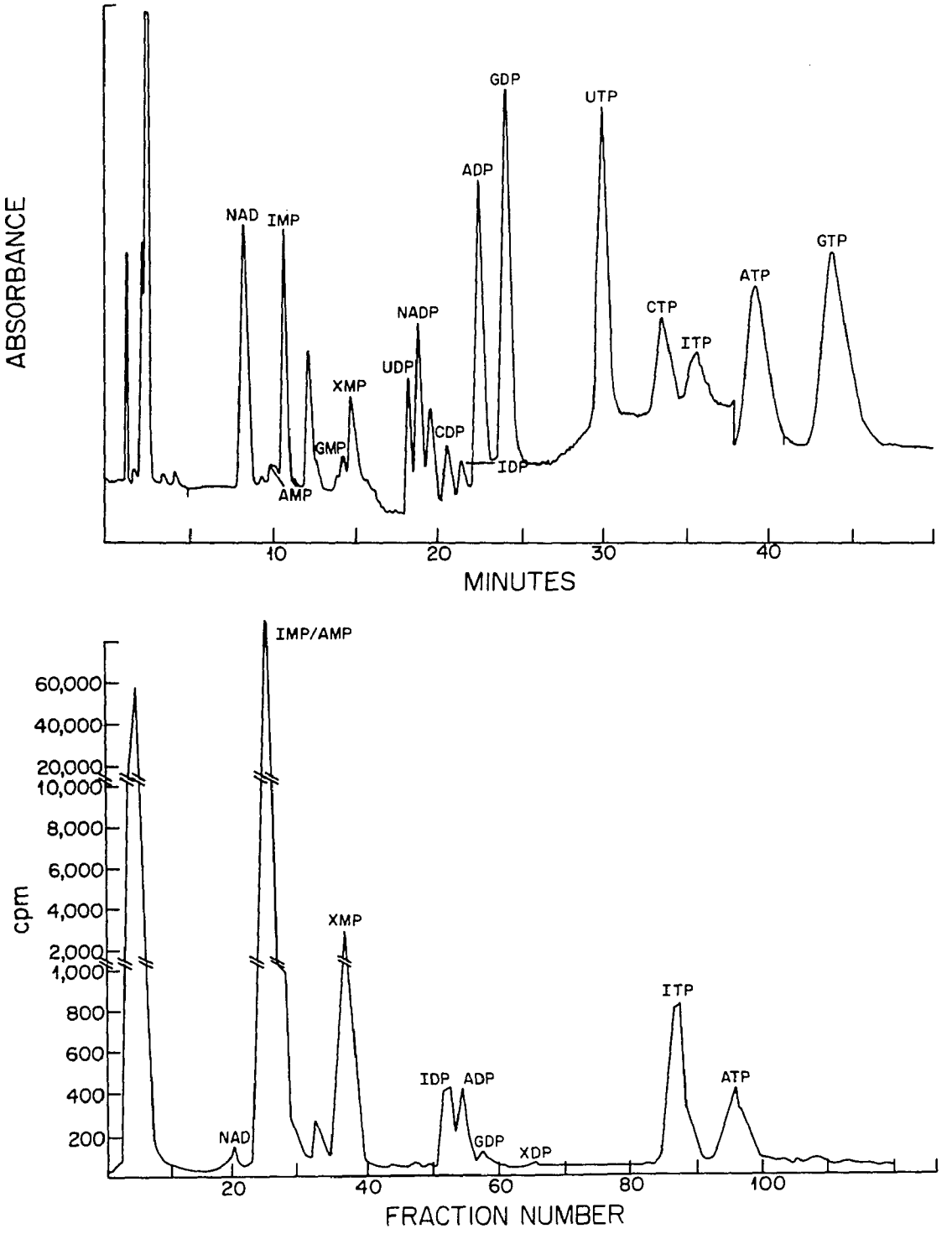
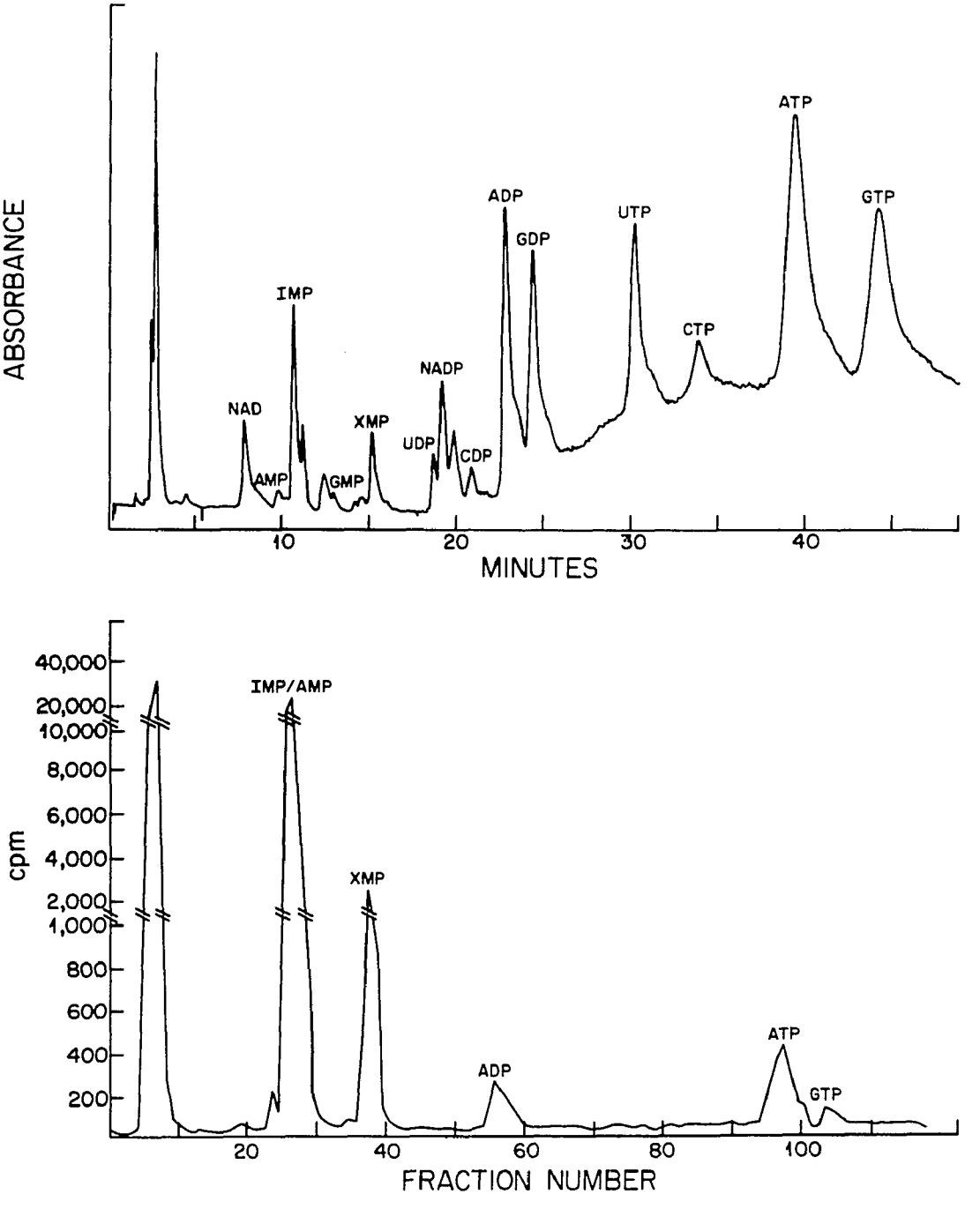

ABSORBANCE
NAD
IMP
AMP
GMP
XMP
UDP
NADP
CDP
ADP
IDP
GDP
UTP
CTP
ITP
ATP
GTP
10
20
30
40
MINUTES
cpm
60,000
40,000
20,000
10,000
8,000
6,000
4,000
2,000
1,000
800
600
400
200
IMP/AMP
NAD
XMP
IDP
ADP
GDP
XDP
ITP
ATP
20
40
60
80
100
FRACTION NUMBER

Figure 30

Free Nucleotide Content and Radioactivity
Present in a ITPase Normal Lymphoid Line
Incubated with 8-^{14}C Inosine for Two Hours
Analyzed by HPLC
Absorbance at 248 nanometers
Full scale .08 to XMP
.01 to CTP
.02 ATP and CTP
Note full scale change during analysis


ABSORBANCE
NAD
AMP
IMP
GMP
XMP
NADP
UDP
CDP
ADP
GDP
UTP
CTP
ATP
GTP
10
20
30
40
MINUTES
cpm
40,000
20,000
10,000
8,000
6,000
4,000
2,000
1,000
800
600
400
200
IMP/AMP
XMP
ADP
ATP
GTP
20
40
60
80
100
FRACTION NUMBER

Cells were resuspended in fresh complete media at a density of 2 x 10^5 cells per milliliter and allowed to grow for seven days. Cells were counted and harvested at daily intervals. The cells entered log phase on day 1 and were in stationary phase on day three. Nucleotide content was found to increase during log growth and decrease in stationary phase (Figure 31), confirming the observation of Brenton et al. (163). Incubation with labeled inosine on day 3 did not result in ITP synthesis in either the normal or the variant. Incubation with hypoxanthine on day 3 also did not result in ITP synthesis. In later experiments cells in late stationary phase also did not accumulate ITP.

The next parameter investigated was the composition of the culture medium. IMP has several alternative pathways available. Conversion of IMP to AMP is dependent on the presence of aspartate as a cofactor. Conversion of IMP to GMP is dependent on glutamine as a cofactor. Limitations in the availability of either of these cofactors would result in blockage of an alternate pathway. Inosine incorporation studies demonstrated that, when glutamine was absent and aspartate was present in the media, ITP was synthesized (0.011 nanomole/10^6 cells/two hours). In contrast, cells that were grown in media which either contained both aspartate and glutamine or glutamine without aspartate for twenty four hours did not accumulate ITP when incubated with inosine (Table 14).

Figure 31

Free Nucleotide Content at Various Times of Subculture in a ITPase Variant and a ITPase Normal Lymphoid Line

Analysis by HPLC

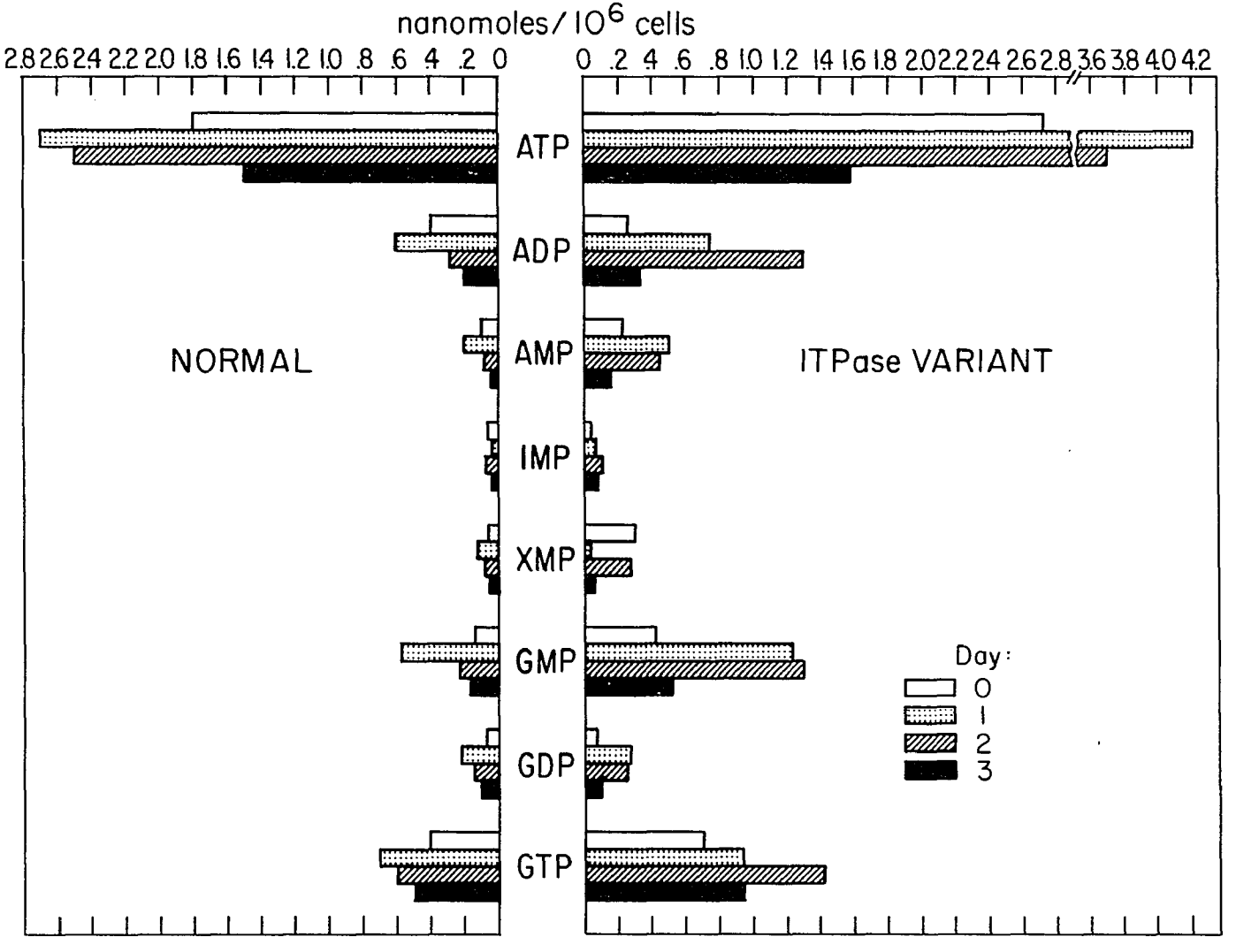

nanomoles/ 10^6 cells
2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 .8 .6 .4 .2 0
0 .2 .4 .6 .8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.6 3.8 4.0 4.2
ATP
ADP
AMP
IMP
XMP
GMP
GDP
GTP
NORMAL
ITPase VARIANT
Day:
0
1
2
3

Table 14

ITP nanomoles/10^6 cells

	Random		+Glut. +Asp.		+Glut. -Asp.		-Glut. +Asp.		-Glut. -Asp.	
	Normal	Variant	Normal	Variant	Normal	Variant	Normal	Variant	Normal	Variant
0 time	0	0	0	0.001	0	0	0.001	0.005	0	0.011
gluc 0	0	0.072	0	0.002	0	0.001	0	0.011	0	0.010
1' ino	0.001	0.038	0	0.001	0	0	0.001	0.003	0.002	0.005
2' ino	0	0.068	0	0	0	0	0	0.001	0	0.010
				IDP						
0 time	0.005	0.169	0	0.002	0.001	0.001	0.001	0.001	0.010	0
gluc 0	0	0.004	0	0	0	0	0	0.001	0	0
1' ino	0	0.006	0	0	0.001	0	0	0.002	0	0.002
2' ino	0	0.014	0	0	0	0	0	0.002	0	0.030
				IMP						
0 time	0.267	0.526	0.127	0.137	0.137	0.131	--	--	0.034	0.023
gluc 0	1.120	1.163	0.142	0.204	0.851	0.429	0.106	0.125	0.104	0.549
1' ino	1.013	2.338	0.859	0.884	0.893	0.652	0.073	0.883	0.070	1.970
2' ino	1.220	2.350	0.460	0.910	0.787	--	0.211	1.380	0.058	1.970

In addition to alterations in ITP content, other alterations were observed in cells grown in glutamine free media when incubated with exogenous inosine. The ratio of ATP/GTP dropped significantly in the variant line from that observed in the normal control under the same conditions (normal 4.65 vs. variant 1.05). The drop in this ratio was due to a drop in the content of ATP in the variant line, not due to increased GTP (Tables 15 and 16). AMP was present in greater amounts in the variant line (0.979 in the variant line, 0.047 in the normal line for one hour incubation with inosine) (Table 17). Total adenine nucleotides were not different in the normal and variant lines. In addition, two times as much IMP was present under conditions of ITP synthesis.

These alterations were observed under all conditions where ITP accumulation was noted. Thus, Tables 14 and 22 demonstrate that the ratio of ATP/GTP is significantly different under conditions of ITP synthesis in the ITPase variant line and normals. The ratio of ATP/GTP is not significantly different in the variant line from normal lines when no ITP is accumulated.

Many of the differential effects observed in glutamine free media were also present in glutamine free aspartate free media. However, the ATP/GTP ratio drop was not as evident in cells grown in the media that was missing aspartate and glutamine as those grown in glutamine free, aspartate positive media.

Table 15

ATP nanomoles/10^6 cells

	Random		+Glut. + Asp.		+Glut. -Asp.		-Glut. +Asp.		-Glut. -Asp.	
	Normal	Variant	Normal	Variant	Normal	Variant	Normal	Variant	Normal	Variant
0 time	2.608	3.290	3.935	5.220	3.003	1.478	1.210	2.055	0.230	0.739
gluc 0	0.892	0.959	2.518	1.149	3.334	1.030	1.266	1.080	1.171	1.711
1' ino	1.140	1.510	2.590	1.053	0.237	0.870	1.452	0.670	1.000	0.690
2' ino	0.905	1.297	0.894	1.053	1.137	0.625	1.312	0.783	1.192	0.783
					ADP					
0 time	0.861	0.680	0.661	1.078	0.508	0.262	0.252	0.244	0.054	0.092
gluc 0	0.362	0.223	0.129	0.136	0.203	0.181	0.116	0.115	0.085	0.260
1' ino	0.191	0.240	--	0.137	1.610	0.461	0.066	0.186	0.051	0.380
2' ino	0.245	0.259	0.307	0.301	1.194	0.353	0.147	0.595	0.054	0.235
					AMP					
0 time	0.090	0.234	0.099	0.215	0.143	0.122	0.219	0.087	0.046	0.047
gluc 0	0.167	0.154	0.031	0.019	--	0.108	0.041	0.074	0.039	0.233
1' ino	0.074	0.110	--	0.474	0.556	0.445	0.046	0.144	0.051	0.342
2' ino	0.128	0.124	0.176	0.672	1.855	0.384	0.047	0.929	0.019	0.873

Table 16

GTP nanomoles/10^6 cells

	Random		+Glut. +Asp.		+Glut. -Asp.		-Glut. +Asp.		-Glut. -Asp.	
	Normal	Variant	Normal	Variant	Normal	Variant	Normal	Variant	Normal	Variant
0 time	0.588	1.210	0.763	0.962	0.756	0.318	0.391	0.257	--	0.627
gluc 0	0.701	1.081	0.298	0.394	--	0.288	0.164	0.237	0.081	0.801
1' ino	1.182	1.350	0.527	1.183	0.382	0.320	0.184	0.595	0.097	0.352
2' ino	0.706	1.345	0.503	1.114	0.610	0.557	0.740	0.969	0.685	0.787
					GDP					
0 time	0.154	0.158	0.040	0.043	0.119	0.077	0.063	0.054	0.022	0.114
gluc 0	0.320	0.263	0.181	0.267	0.109	0.088	0.054	0.069	0.031	0.035
1' ino	0.194	0.150	0.576	0.166	0.031	0.196	0.032	0.144	0.054	0.168
2' ino	0.209	0.184	0.220	0.387	0.538	0.580	0.075	0.134	0.016	0.265
					AMP					
0 time	0.252	0.971	0.167	0.206	0.152	0.143	0.231	0.073	0.024	0.049
gluc 0	0.075	--	0.082	0.085	0.219	0.091	0.062	0.054	0.052	0.034
1' ino	0.247	0.306	0.434	0.132	0.275	0.150	0.014	0.081	--	0.118
2' ino	0.071	0.326	0.432	0.274	0.413	0.333	0.050	0.125	0.020	0.138

Table 17

Ratio of ATP/GTP Content of Lymphoid Lines Under Conditions of ITP Synthesis

	mutant	normal
1' inosine	1.07 ± 0.17	2.16 ± 0.53
2' inosine	1.02 ± 0.15	3.82 ± 0.93

Ratio of ATP/GTP Content of Lymphoid Lines Under Conditions of No ITP Accumulation

1' inosine	2.03 ± 1.01	2.55 ± 0.81
2' inosine	2.69 ± 1.32	2.08 ± 0.91

The only differential effect observed between normal and variant cells grown in aspartate free glutamine positive was a slightly lower amount of ATP in the variant at zero time.

Thus, the factor that seems to be the most important in the synthesis of ITP is the absence of glutamine in the media.

Levels of Other Nucleotides in ITPase Variant and Normal Lymphoid Cells Under All Conditions of Growth and Incubation

Table 18 indicates the average nucleotide content found in extracts for normal lymphoid cells and the ITPase variant under all conditions investigated. No significant difference other than ITP levels existed in the mean level of any nucleotides present in cell culture. In most of these experiments cells were in stationary phase at which time levels of free nucleotides were lower than when cells are in log phase (see page 167). Table 19 indicated the mean levels of free nucleotides found in the ITPase variant and normal lymphoid lines which had been incubated in a glucose phosphate buffer for twenty-five minutes (glucose zero). Again, no significant difference was observed. Table 20 indicates the mean values of various nucleotides incubated for one hour with inosine. The only significant difference observed was the level of IMP. IMP was increased above normal in the variant at one hour of inosine incubation. The difference was greater at two hours, with the ITPase variant possessing two times as much IMP (Table 21).

Table 18

Levels of Free Nucleotides

Zero Time

	Normal		Variant	
	Mean	s.e.m.	Mean	s.e.m.
NAD*	0.428	0.07	0.595	0.13
AMP**	0.207	0.09	0.187	0.03
IMP**	0.099	0.02	0.074	0.02
GMP**	0.210	0.05	0.258	0.04
m_3*	0.150	0.02	0.317	0.05
XMP**	0.093	0.02	0.134	0.05
UDP*	0.025	0.01	0.016	0.00
NADP*	0.030	0.00	0.049	0.01
CDP*	0.030	0.00	0.037	0.01
D_1*	0.010	0.00	0.022	0.00
IDP**	0.001	0.00	0.009	0.01
ADP**	0.378	0.07	0.432	0.07
GDP**	0.092	0.01	0.176	0.08
XDP**	0.001	0.00	0.009	0.01
UTP*	0.190	0.02	0.260	0.05
CTP*	0.078	0.01	0.092	0.01
ITP**	0.003	0.00	0.008	0.01
ATP**	1.902	0.32	2.646	0.37
GTP**	0.510	0.07	0.587	0.07

*expressed as $cm^2 \times 10^3/10^6$ cells

**expressed as nanomoles/10^6 cells

Table 19

Levels of Free Nucleotides

Glucose Zero

	Normal		Variant	
	Mean	s.e.m.	Mean	s.e.m.
NAD*	0.315	0.14	0.393	0.09
AMP**	0.051	0.02	0.086	0.02
IMP**	0.450	0.25	0.448	0.12
GMP**	0.084	0.02	0.147	0.03
m_3*	0.211	0.09	0.115	0.02
XMP**	0.246	0.16	0.191	0.09
UDP*	0.063	0.02	0.023	0.00
NADP*	0.039	0.01	0.049	0.01
CDP*	0.046	0.01	0.048	0.01
D1*	0.025	0.01	0.019	0.00
IDP**	0.001	0.00	0.001	0.00
ADP**	0.189	0.05	0.148	0.02
GDP**	0.108	0.04	0.075	0.01
XDP**	0.001	0.00	0.026	0.01
UTP*	0.089	0.03	0.138	0.02
CTP*	0.048	0.01	0.064	0.01
ITP**	0.007	0.00	0.015	0.01
ATP**	0.976	0.42	1.032	0.01
GTP**	0.444	0.14	0.506	0.11

*expressed as $cm^2 \times 10^3/10^6$ cells

**expressed as nanomoles/10^6 cells

Table 20

Levels of Free Nucleotides

1' Glucose Incubation

	Normal		Variant	
	-Inosine	+Inosine	-Inosine	+Inosine
NAD*	0.101	0.329	0.202	0.664
AMP**	0.083	0.151	0.122	0.261
IMP**	0.116	0.525	0.131	1.082
m_3*	0.110	0.130	0.294	0.114
GMP**	0.058	0.157	0.143	0.149
m_2*	0.024	0.116	0.168	0.089
XMP**	0.078	0.123	0.041	0.140
UDP*	0.011	0.020	0.016	0.026
NADP*	0.015	0.029	0.020	0.052
CDP*	0.013	0.033	0.024	0.043
D_1*	0.007	0.011	0.013	0.026
IDP**	0.004	0.001	0.001	0.002
ADP**	0.135	0.325	0.262	0.258
GDP**	0.072	0.151	0.088	0.193
XDP**	0.001	0.001	0.005	0.003
UTP*	0.055	0.148	0.115	0.234
CTP*	0.025	0.063	0.028	0.095
ITP**	0.003	0.010	0.001	0.010
ATP**	0.445	0.971	0.739	0.827
GTP**	0.179	0.428	0.361	0.546

*Expressed as $cm^2 \times 10^3/10^6$ cells

**Expressed as nanomoles/10^6 cells

Table 21

Levels of Free Nucleotides

2' Incubation

	Normal		Variant	
	-Inosine	+Inosine	-Inosine	+Inosine
NAD*	0.081	0.447	0.445	0.929
AMP**	0.067	0.290	0.067	0.469
IMP**	0.058	0.573	0.041	1.161
m_3*	0.098	0.168	0.011	0.094
GMP**	0.081	0.121	0.073	0.296
m_2*	0.036	0.108	0.008	0.096
XMP**	0.032	0.095	0.119	0.147
UDP*	0.008	0.013	0.008	0.035
NADP*	0.019	0.037	0.028	0.099
CDP*	0.008	0.023	0.065	0.069
D1*	0.005	0.013	0.014	0.034
IDP**	0.000	0.002	0.001	0.003
ADP**	0.207	0.286	0.244	0.334
GDP**	0.068	0.165	0.069	0.267
XDP**	0.001	0.001	0.001	0.001
UTP*	0.085	0.098	0.104	0.199
CTP*	0.036	0.047	0.075	0.083
ITP**	0.001	0.003	0.001	0.014
ATP**	0.518	0.614	0.768	0.848
GTP**	0.335	0.402	0.257	0.747

*Expressed as $cm^2 \times 10^3/10^6$ cells

**Expressed as nanomoles/10^6 cells

Table 22

Mean Ratio of Purine Compounds Under Various Incubation Conditions

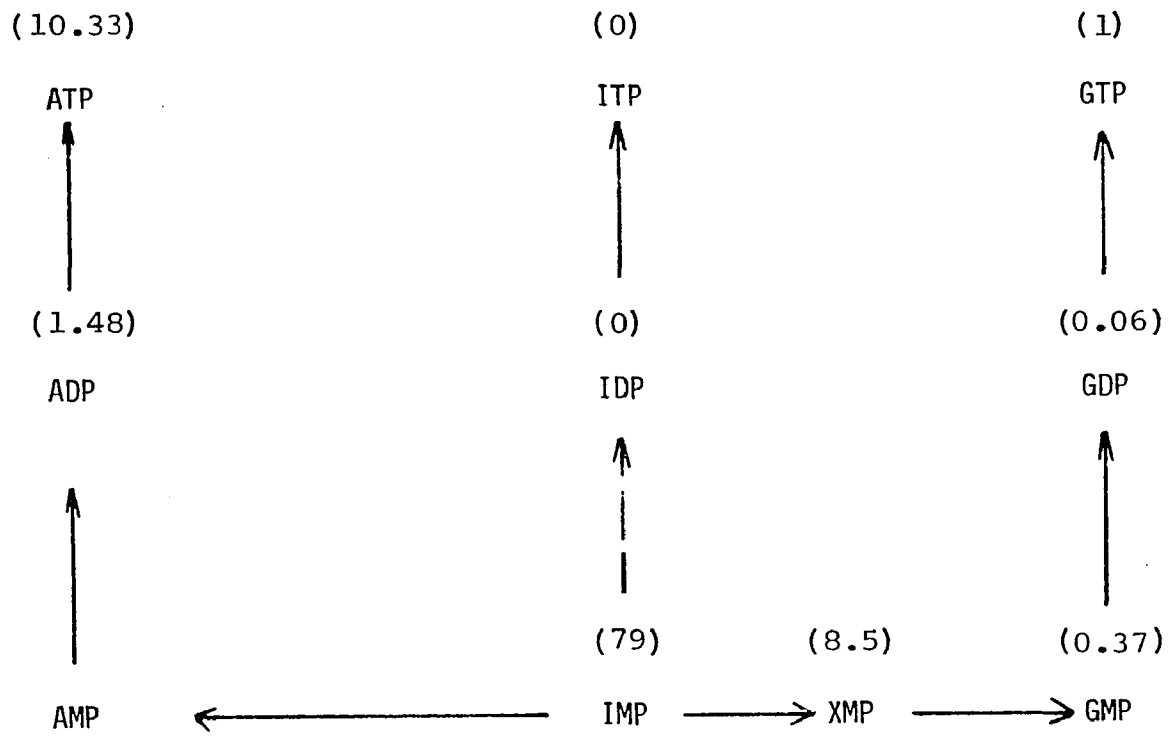
	Zero		gluc. zero		1' inosine		2' inosine	
	Normal	Variant	Normal	Variant	Normal	Variant	Normal	Variant
Total A	2.49	3.26	1.22	1.26	1.45	1.34	1.19	1.66
Total G	0.82	1.02	0.64	0.73	0.74	0.89	0.67	1.31
Total Hx	0.11	0.09	0.46	0.48	0.54	1.09	0.58	1.18
ATP/GTP	3.73	5.79	1.97	2.41	2.39	2.03	2.11	1.55
ATP/ADP	5.03	7.63	4.88	7.63	4.87	4.24	2.99	4.34
IMP/ATP	0.06	0.03	0.39	0.54	0.60	1.37	0.93	1.37
IMP/AMP	0.48	0.40	9.00	5.21	3.48	4.15	1.98	2.48
IMP/GMP	0.48	0.29	5.36	3.05	3.34	9.49	4.74	5.44
IMP/XMP	1.06	0.55	1.83	2.35	4.27	7.73	6.03	7.90

Several different parameters of nucleotide metabolism have been monitored. In addition to determining free nucleotide content, under various incubation conditions the pattern of synthesis and the specific activity of the nucleotide compounds were followed. The latter two were accomplished by incubation of the lymphoid lines with a radio-labeled precursor. Theoretically, the changes in nucleotide levels may have been due secondarily to the release of RlP by metabolism of inosine and subsequent stimulation of the HMP shunt. Cells treated under identical conditions but with no inosine added were also monitored.

Response of Lymphoid Lines to Incubation with 8-^{14}C Inosine

On incubation of lymphoid lines with inosine, various purine nucleotides were synthesized. Approximately two to five percent of the exogenous inosine was incorporated into the cell. Of this fifty percent was present in nucleotides with the remainder present in nucleosides. Figures 32-36 indicate the pattern of synthesis of nucleotides in lymphoid cells incubated for two hours with inosine under conditions of ITP accumulation and also under conditions where there was a lack of ITP accumulation. As demonstrated in these figures, a higher percentage of total nucleotides synthesized was IMP under conditions of ITP synthesis. Few guanine nucleotides were synthesized under conditions of ITP accumulation. In addition, there was reduced ATP synthesis during ITP synthesis

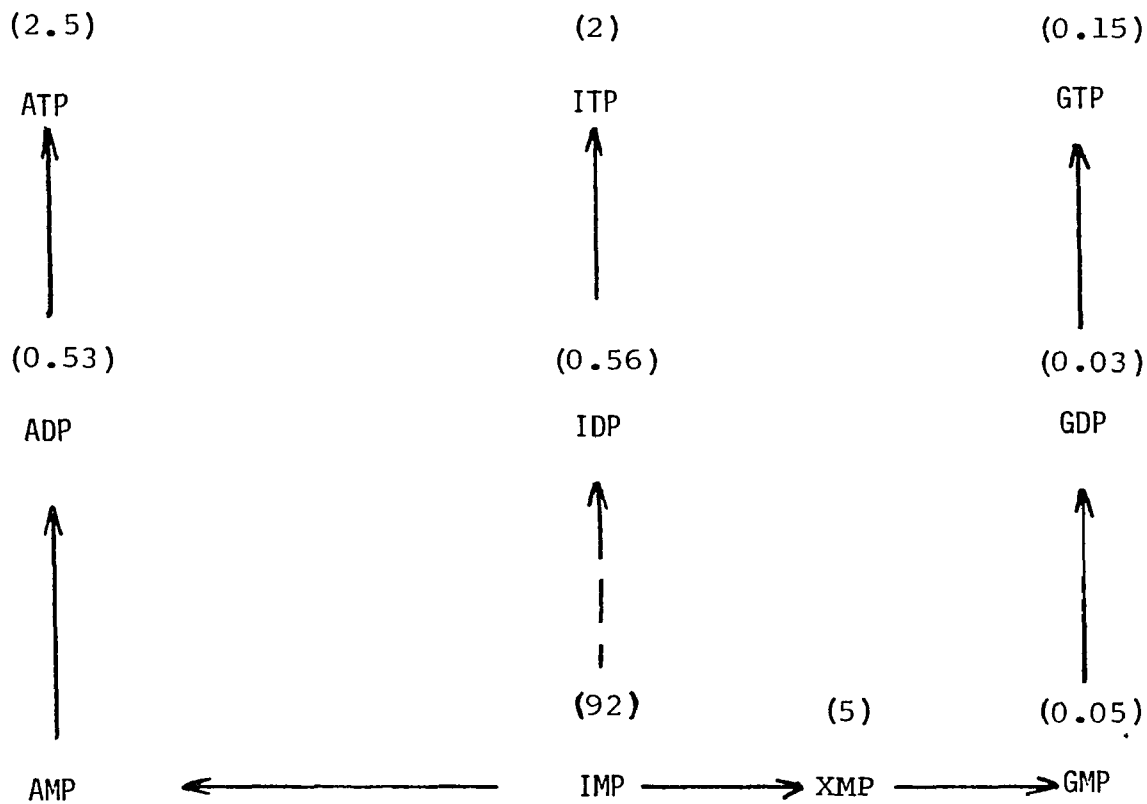
Figure 32 Distribution of Radioactive Inosine Incorporated at Two Hours by Normal Lymphoid Lines Under Conditions of ITP Synthesis



Numbers in parentheses are percent of total nucleotides incorporated.

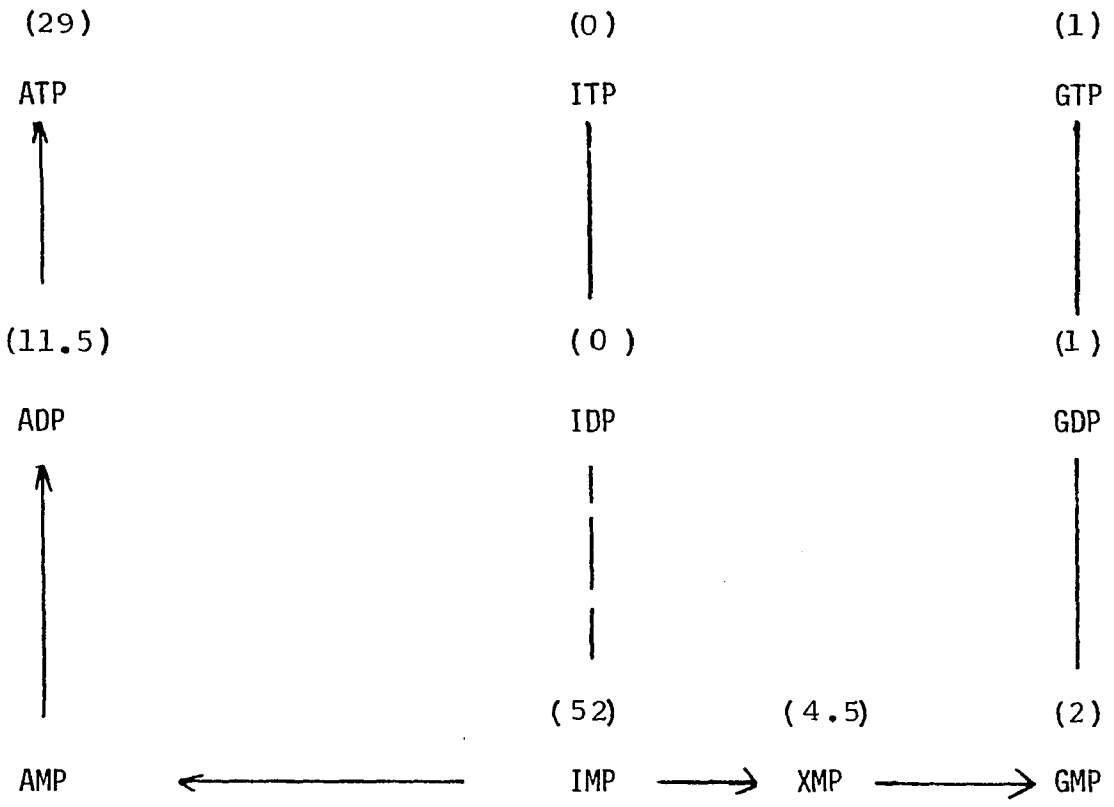
Figure 33

Distribution of Radioactive Inosine Incorporated at Two Hours by ITPase Variant Under Conditions of ITP Synthesis



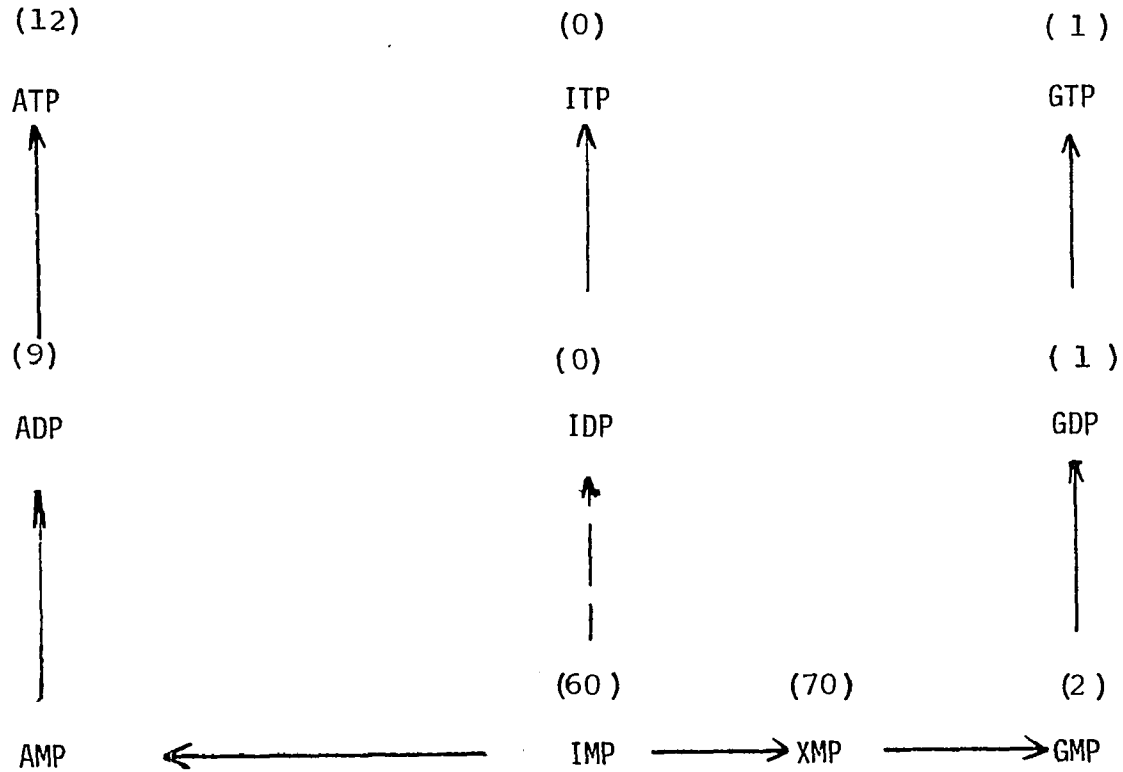
Numbers in parentheses are percent of total nucleotides incorporated.

Figure 34 Distribution of Radioactive Inosine Incorporated at Two Hours by Normal Lymphoid Lines Under Conditions of No ITP Synthesis



Numbers in parentheses are percent of total nucleotides incorporated.

Figure 35 Distribution of Radioactive Inosine Incorporated at Two Hours by ITPase Variant Under Conditions of No ITP Synthesis



Numbers in parentheses are percent of total nucleotides incorporated.

in the variant. At two hours the normal line was also found to synthesize less ATP (under conditions where ITP accumulation was found at one hour). The variant line, however, exhibited a lower level of ATP synthesis.

On several occasions low levels of radioactivity were found in the region of XTP in the variant line. Both the normal lines and the variant line seem to have the ability to accumulate radio-labeled XDP. This extends the observation of Nelson et al. that normal human erythrocytes are capable of synthesizing XDP and XTP. Conditions of XDP synthesis were not defined.

Specific Activity of Nucleotides in Lymphoid Lines Incubated with 8-^{14}C Inosine

On incubation of lymphoid lines with inosine, Table 23 indicates the specific activity of lymphoid cells incubated for one hour with 50 uM inosine. Table 24 indicates the pattern obtained if lymphoid cells were incubated for two hours with inosine. A striking feature is the high specific activity of XMP.

Two alternative modes of ITP synthesis have been proposed based on the relative specific activity of ITP and IDP. In one ITP has a higher specific activity than IDP, the other IDP has a higher specific activity.

This data does not permit distinction of two alternative modes of synthesis of ITP, the pyrophosphorylation of IMP

Table 23

One Hour Inosine Incubation

Specific Activity--CPM/pmole

Normal

	IMP/AMP	IDP	ITP	ADP	ATP	XMP	GMP	GDP	GTP	XDP
1	5.5	--	7.43	0.12	0.21	16.7	--	--	--	--
2a	29.8	--	8.35	0.43	0.59	7.73	--	--	0.02	--
2b	9.8	--	7.43	0.88	1.01	38.40	--	--	0.01	--
4	2.1	--	--	2.68	2.33	0.91	1.40	1.46	0.27	--
5	18.34	--	--	0.55	1.00	5.03	--	0.32	0.04	
6	13.59	--	4.17	1.10	1.14	6.67	--	--	0.34	--
8	18.97	--	--	1.05	1.35	9.67	0.42	0.30	--	--
9	17.82	--	--	1.18	1.52	6.65	0.20	0.44	--	--
10	20.81	--	--	2.23	2.36	10.62	0.20	0.52	--	--
11	4.82	--	--	0.64	0.75	5.03	--	0.13	0.11	--

Table 23 (Continued)

	Variant									
	IMP/AMP	IDP	ITP	ADP	ATP	XMP	GMP	GDP	GTP	XDP
1	5.34	2.27	4.64	0.04	0.13	6.99	--	--	0.01	--
2	14.70	17.30	9.9	0.30	0.36	2.7	--	--	0.02	--
3	8.46	--	--	2.27	5.7	11.0	0.28	0.32	--	--
4	8.73	--	--	2.64	2.45	12.9	--	0.35	0.71	--
5	21.52	--	--	0.89	1.13	5.15	--	--	0.06	--
6	16.34	--	--	0.45	0.48	6.45	--	0.27	0.08	8.71
7	3.78	--	--	5.81	2.27	20.35	0.29	0.20	0.11	--
8	25.08	--	--	3.21	6.80	18.04	--	0.23	0.70	--
9	18.61	--	--	1.41	1.43	8.65	0.16	0.15	0.14	--
10	30.28	--	--	1.73	1.98	15.34	0.33	0.09	0.13	--
11	1.19	--	--	1.96	2.08	9.65	0.24	0.54	0.50	--
12	12.68	--	--	0.36	0.36	2.89	0.29	0.11	0.04	7.51

Table 24

Two Hour Inosine Incubation

Specific Activity--CPM/pmole

	IMP/AMP	IDP	ITP	ADP	ATP	XMP	GMP	GDP	GTP	XDP
					Normal					
1	5.42	--	--	0.36	0.42	4.48	--	0.10	0.24	--
2a	6.44	--	--	1.65	1.60	23.32	0.07	--	0.15	--
2b	24.93	--	--	0.32	1.06	4.83	0.06	--	0.12	--
4	1.94	--	--	3.19	2.49	1.46	0.31	0.45	0.24	--
5	17.21	--	--	0.53	2.55	11.10	--	0.38	0.16	0.88
*6	15.69	--	4.17	1.39	1.63	10.48	1.32	1.18	1.56	--
7	13.72	--	--	2.38	0.06	10.02	0.20	0.14	0.15	--
8	6.42	--	--	1.74	1.43	13.75	0.20	0.22	0.20	--
*9	13.55	--	--	2.31	2.54	10.34	0.37	0.51	0.45	--
**10	34.85	--	--	1.40	3.39	9.77	0.59	0.25	0.14	--
***11	--	--	--	--	--	--	--	--	--	--
***12	8.07	--	--	0.66	1.48	2.53	0.38	0.38	0.51	8.50

Table 24 (Continued)

Variant	IMP/AMP	IDP	ITP	ADP	ATP	XMP	GMP	GDP	GTP	XDP
1	8.92	2.26	4.17	0.45	0.49	10.81	--	--	0.01	0.17
2a	15.0	16.50	12.60	0.08	0.84	2.90	--	--	0.02	--
3	8.1	--	--	3.35	2.86	10.6	0.42	0.67	0.51	--
4	7.7	--	--	2.99	3.17	2.99	0.26	0.18	0.14	--
5	30.7	--	--	2.06	1.80	8.19	--	0.44	0.07	--
*6	14.10	--	4.69	0.89	1.80	10.78	0.27	0.31	0.11	--
7	17.60	--	--	1.95	4.60	17.92	0.57	0.22	0.23	--
8	13.29	--	--	2.73	2.55	10.80	0.14	0.61	0.46	10.17
9	14.12	--	--	1.13	1.22	15.95	0.18	0.11	0.06	--
**10	29.78	--	4.00	1.14	1.14	13.88	0.20	0.20	0.08	--
***11	13.33	--	0.93	5.76	0.72	5.76	0.38	0.30	0.11	9.17
***12	5.27	--	--	1.63	1.99	10.85	0.37	--	0.89	10.00

*grown in glu free media, homocystinuric line

**grown in glu free asp free media

***hypoxanthine substituted instead of inosine

to ITP or the stepwise phosphorylation of IMP to IDP to ITP. However, in all situations in which radio-labeled IDP was present radio-labeled ITP was also present. The approximate ratio of the amount of ITP and IDP was three to one. Thus, it is apparent that IDP is involved in either the degradation or synthesis of ITP in intact lymphoid cells.

Small amounts of XDP with high specific activity were found on several occasions. The ability of lymphoid lines to synthesize XDP has not been previously reported to my knowledge.

Correlation of the Amount of ITP with Other Variables

Computer assisted analysis indicated that the amount of ITP present was strongly positively correlated with the ratio of IMP/AMP (0.91) at all time points in the ITPase variant line. In addition, the amount of ITP correlated with the amount of IDP present at all times (0.96). In samples with radioactivity there was a strong negative correlation (-0.93) of ITP with the specific activity of GTP (all at a p value of 0.0001). In the normal lines the amount of ITP present was negatively correlated with specific activity of GTP (-0.92) but no significant correlation was obtained for the ratio of IMP/AMP. In all instances of ITP synthesis there was over 1 nanogram/10^6 cells of IMP. However, there were many instances of over 1 nanogram IMP/10^6 cells in which no ITP accumulated.

Correlation of the Synthesis and Accumulation of ITP

The amount of radioactivity present in ITP correlated positively with the amount of radioactivity present in IDP (0.96).

Correlation of the Specific Activity of ITP With Other Parameters

The specific activity of ITP correlated inversely with the specific activity of GTP in both normals and the variant.

Correlation of the Amount, Synthesis, and Specific Activity of IDP

The amount of IDP present positively correlated only with the amount of ITP present (0.98). The amount of radio-labeled IDP positively correlated (0.99) with the ratio of IMP to AMP and the IMP/ATP ratio at both one hour and two hour time points in the variant.

Correlation of the Amount, Synthesis, and Specific Activity of IMP With Other Variables

The amount of IMP present following glucose incubation (glucose 0) correlated with the total amount of guanine nucleotides present. The amount of IMP did not correlate with the cpm present in the nucleosides.

In response to exogenous inosine (50 uM), the ITPase variant accumulates two times the level of inosine nucleotides that normal lymphoid lines do. This is true for both one hour and two hour time points. No significant difference was observed in the mean levels of total adenine nucleotides, total guanine nucleotides or the ratio of mean levels of IMP/AMP, ATP/ADP, ATP/GTP, IMP/GMP, and IMP/XMP in normal

and ITPase variant cells. However, the ratio of IMP/ATP was significantly increased in response to exogenous inosine in the ITPase variant at both one hour and two hour incubation times.

Computer assisted analysis indicated that the mean value of ITPase variant cells was not significantly different than the mean value of normal ITPase with respect to levels of free nucleotides, the ratio of ATP/ADP/AMP, GTP/GDP, GTP/GDP/GMP, ATP/ADP, ITP/IDP, UTP/CTP, UTP/ATP, ATP/CTP, UTP/CTP, ADP/GDP, AMP/GMP, ATP/XMP, GMP/XMP in all conditions tested. The variant was not significantly different from normal lines in the amount of free nucleotides synthesized or the ratio of the amount of radioactivity found in ATP/ADP, GTP/GDP, GTP/GDP/GMP, GTP + GDP + GMP, GTP + GDP, ADP/GDP, IMP/XMP, XMP/GTP, and the ratio of total cpm in nucleotides to total cpm in nucleosides.

The ratio of the specific activity in ATP/ADP, ITP/ATP, ITP/GTP, IMP/XMP, XMP/ATP, and XMP/GTP was not significantly different in the variant from that found in normal lymphoid lines.

In summary, ITP accumulation was increased in a line variant for ITPase activity if the cells were grown for twenty-four hours in glutamine free media. The amount of ITP accumulating on incubation with exogenous inosine was

strongly negatively correlated with the synthesis of GTP (-0.99). These cells either synthesize GTP or ITP but not both to any considerable extent. The observation that ITP accumulation occurs in glutamine free media agrees well with the negative correlation of ITP accumulation with the specific activity of GTP. Both of these factors indicate reduced GTP synthesis. In addition, the variant cell line seems unable to maintain a normal level of ATP if the cells are grown in glutamine free media for twenty-four hours prior to incubation with exogenous inosine. However, glutamine limitation may be one of many predisposing factors to ITP accumulation since the level of ITP accumulation never reached that observed in the first experiment.

Under certain conditions lymphoid lines with normal ITPase activity are able to accumulate small amounts of ITP when incubated with inosine for one hour. However, these cells metabolize all of the ITP present and do not accumulate ITP if incubated with inosine for two hours.

XDP was also present in several lymphoid extracts. The conditions of XDP synthesis were not defined.

All parameters investigated indicate that ITP accumulation occurs if GTP synthesis is limited. The smaller content of ATP under these conditions may be a combination of reduced synthesis and increased degradation. The reduced level of ATP cannot be accounted for stoichiometrically by the amount of ITP accumulated.

Discussion

Enzymatic Studies

Development of an HPLC assay offers several advantages. The assay measures the direct conversion of ITP to IMP. Both compounds are easily visualized as widely separated U.V. absorbing peaks after anion exchange chromatography by HPLC. In addition, unexpected U.V. absorbing peaks would also be visible. In contrast, measurement of ITPase activity by phosphate determination relies on a linked enzyme system ($ITP \longrightarrow IMP + PP_i \longrightarrow 2P_i$) and determination of a nonspecific end product, inorganic phosphate.

The HPLC assay is not limited by high concentrations of phosphate as might be present in cell extracts or buffers. Phosphate contamination of laboratory vessels, as often happens from use of detergent, presents no problem if ITPase activity is analyzed by HPLC.

The sensitivity of the HPLC assay is in the range of picamoles. As little as 5 picamoles of IMP can be detected. However, due to contamination of commercially available ITP with IDP and IMP, approximately 25 picamoles of IMP would be needed to be easily visible. The lower limit that has been used in these studies is detection of 70 picamoles of IMP. The phosphate determining assay is sensitive in the micromole range.

Development of an HPLC assay also has the advantage of

reducing the number of cells necessary for each assay. With the miniaturization of the assay, as few as 10^3 cells can be assayed. ITPase activity as determined by phosphate production requires a minimum of 5×10^6 cells since the sensitivity is in the micromole range. Although the HPLC assay can be performed on 10^3 cells, the minimum number of cells assayed was 2×10^4 in the data presented here. Healthy lymphoid cell cultures usually grow at a density of approximately 4×10^5 cells/ml. Hence the HPLC assay of ITPase in theory requires less than one milliliter of a cell culture for analysis.

The HPLC assay is highly reproducible. As shown in Table 5, the standard error of the mean was 109.4 umoles IMP in six determinations of an individual lymphoid line. The standard error of the mean for six determinations of the same lymphoid line assayed by phosphate production was 372.3 umoles PP_i/hr/gm protein. Hence there was over three times as much variation from the mean in the phosphate assay as in the HPLC assay.

Both assays depend on a two-step system. In the HPLC assay ITP is converted to IMP + PP_i, then the reaction products ITP and IMP are separated by chromatography. In the phosphate detection assay, ITP is also converted to IMP + PP_i and the PP_i split to $2P_i$ in the same reaction mixture.

The second step is a phosphate determination. Data presented in Table 5 show that the reaction mixture of HPLC assay is stable to freezing for a minimum of 4 days before analysis on HPLC. There is a slight increase in the amount of IMP detected on day eight which may be due to the nonenzymatic degradation of ITP. ITP is known to be unstable to storage for prolonged periods. In contrast, if the reaction mixture is frozen before phosphate determination, it is impossible to quantitate the amount of phosphate present, as all samples read with an optical density of over two. Thus, assay of ITPase by HPLC offers the convenience of storing the reaction mixture before final analysis is performed.

As shown in Figure 6 and Figure 7, assay of ITPase by phosphate production shows a fall-off of activity above 500 ug protein per milliliter reaction mixture in preparations from lymphoid lines and a fall-off of activity above 100 ug of hemoglobin in human erythrocyte preparations. While the basis for this observation is unclear, this finding has been reported in the literature by Holmes et al. (127) and Vanderheiden (125) in human hemolysates. This observation would be consistent with inhibition of enzyme activity by a high concentration of inorganic phosphate. An alternative explanation might be another enzyme present in the hemolysate or lysate at low concentrations that utilized inorganic phosphate. This would explain less

phosphate present at higher concentration of enzyme preparation, as you could postulate the need for either a critical concentration of P_i (produced by the combined action of ITPase and PPase) or a critical amount of the theorized enzyme before phosphate utilizing activity became apparent.

The HPLC assay of ITPase was linear in all ranges tested from 10 ug protein to 110 ug protein in human hemolysates and from 5 to 75 ug in lymphoid lysates.

Comparison of linearity curves with protein of the two assay methods reveals a fall-off of activity above 100 ug of protein per milliliter reaction mixture of human hemolysates assayed by phosphate detection versus no fall-off of activity at 110 ug of protein per milliliter reaction mixture of human hemolysates assayed by HPLC. This would indicate that in human hemolysates fall-off of ITPase activity as measured by the production of phosphate was not due to decreased activity of ITPase. Evidence that the amount of phosphate present in the reaction mixture did not effect the activity of ITPase was found in data presented that showed the reaction was linear for 20 minutes. Two times as much phosphate would be present at a 20 minute time point as at a 10 minute time point in the reaction mixture. The level of ITPase activity would not be expected to be linear for 20 minutes if the amount of phosphate present influenced the activity of the enzyme. Assay of ITPase by HPLC revealed that the reaction

was linear for twenty minutes in extracts prepared from both lymphoid lines and human erythrocytes.

Comparison of the mean value of activities obtained by HPLC and phosphate production assay are in close agreement. The mean value of 10,188 $\pm$ 2928 umoles IMP/hr/gram protein by HPLC assay and 15,687 $\pm$ 750 umoles PP_i/hr/gram protein agree fairly well. The difference can be explained by the small sample size of both samples (n = 4 for phosphate production and n = 12 for HPLC).

Assay of the same line, a variant in ITPase activity, revealed a mean value 2,289 $\pm$ 268 umoles IMP/hr/gram protein (n = 6) by HPLC assay and a mean of 3,114 $\pm$ 912 umoles PP_i/ hr/gram protein (n = 6) by phosphate production assay. The mean of the HPLC assay was within one standard deviation of the mean of the phosphate determining assay. However, the mean of the phosphate determination assay was outside one standard deviation of the mean of the HPLC assay. This discrepancy was probably due to the larger variation present in the phosphate assay. As mentioned previously, the standard error of the mean, in six determinations of the level of ITPase activity in this line, was 372 umoles PP_i/hr/mg protein in the phosphate assay, while this value was 105 for the HPLC assay.

Since investigation of a line variant in ITPase activity

was in progress (approximately 25% of normal activity by phosphate assay, HPLC assay and starch gel electrophoresis), it was desirable to distinguish the products of the ITPase reaction. It was conceivable that the normal ITPase activity might cleave ITP to IMP + PP_i (as reported in the literature), while the variant might cleave ITP to IDP + P_i. First attempts were made to distinguish the two activities on the basis of pyrophosphate versus phosphate production. However, it was found that there was an endogenous pyrophosphatase activity that was active under the assay conditions utilized at pH 9.2, 8.5, and 8.0 (Table 2) in both lymphoid cells and erythrocytes. Previous studies reported that ITPase was a pyrophosphatase based on studies with either partially purified enzyme or γB^{32}PP-ITP.

Further investigation of the endogenous pyrophosphatase revealed that the pyrophosphatase was active at pH 9.2 and was not a limiting factor in detection of ITPase activity at various protein concentrations. Heating the hemolysate at 55°C for 5 minutes did not substantially reduce pyrophosphatase activity contrary to the report of Hershko et al. (129).

Assay by HPLC provided the necessary method for determination of products of ITPase assay. As shown in Figure 17, commercial preparations of ITP were not completely free of

contaminating IDP; hence IDP was a consistent feature of chromatograms. Although the amount of IDP present was fairly constant and not dependent on protein concentration, it was possible that IDP was being produced and quickly degraded to IMP by an active IDPase. Substrate specificity studies by HPLC and phosphate assay (Table 4) showed that IDP was utilized as a substrate at about 4% the rate of ITP utilization. Additional proof of IMP production was obtained by utilizing ^{14}C ITP as a substrate in an ITPase assay of lymphoid cells by HPLC. The data presented in Table 8 and Figure 17 show that both a normal line and the line variant in ITPase activity converted ITP to IMP if lysates centrifuged at 27,000 x g were used as an ITPase source. In addition, IMP is not cleaved to hypoxanthine or inosine under these assay conditions. This data also indicate that, if a crude lysate is used as the enzyme source, approximately two times the amount of ITP degradation takes place, and IDP is formed from ITP as well as IMP. The line variant in ITPase activity (27,000 x g) had approximately the same amount of activity in this particulate-bound ITPase. Data presented in Table 4 shows that in crude lysates ATP is cleaved as effectively as ITP, while ATP is not cleaved at all in preparations centrifuged at 27,000 x g. These studies have provided evidence that in 27,000 x g supernatant lysates

of lymphoid lines ITP is cleaved directly to IMP in both a normal lymphoid line and the ITPase variant. In addition, evidence is presented that indicates the HPLC assay can distinguish quite effectively between the nonspecific particulate-bound ITPase and the ITP pyrophosphohydrolase present in the cytosol. The variant ITPase line was found to have 25% of the pyrophosphohydrolase activity of normal cell lines and 100% of the activity of nonspecific ITP degradation.

These data further indicate that the 25% residual activity of the line variant in ITPase activity was not due to a particulate-bound ITPase, since the particulate-bound ITPase and the cytosol ITPase appear to have a different mechanism of action.

Heat inactivation studies at 50°C show that the ITPase variant exhibited a mild heat instability. Studies at 56°C confirm this finding.

Loss of activity of ITPase seems to plateau in both the variant and normal lymphoid line incubated at both 50°C and 56°C. The activity plateaus at 70% of initial activity in normal lymphoid lysates heated at 50°C and 48% of initial activity if heated at 56°C, while the values for the ITPase variant are 48% and 24% of initial activity respectively.

While there is no other evidence for isozymes of ITPase, it was possible that the 25% residual activity in the variant

ITPase line was due to an isozyme.

Therefore, an investigation of ITPase activity on starch gel electrophoresis was initiated. The ITPase from the variant line exhibited the same electrophoretic mobility as ITPase from a normal line, with a diminished intensity of the phosphate band, indicative of diminished ITPase activity.

While starch gel electrophoresis is not usually considered a quantitative method, serial dilutions of a lysate allow an estimate of the amount of enzyme activity present in comparison to other samples. Dilutions of the enzyme preparation indicated that the variant enzyme had approximately 25% of normal activity on starch gel electrophoresis.

There are several reports in the literature of variant human enzymes in which enzyme activity is normal or near normal with one substrate, while diminished with another. One such report is of a variant of hexosaminidase A whose activity is 50% of normal with an artificial substrate, 4 methyl-umbelliferyl but totally lacking in enzyme activity if the natural substrate G_{M2} is substituted instead of 4 methyl-umbelliferyl (164). Another such report is of a variant HGPRT with 10% of normal activity with hypoxanthine as substrate, 0.5% of normal activity with guanine as a substrate (77). Thus, it was of interest to establish the amount of enzyme activity with various substrates in the ITPase variant. Starch gel electrophoresis was chosen because it provided a partial purification of the enzyme while allowing

an estimate of enzyme activity. The variant ITPase line was shown to have the same reduced level of ITPase activity with ITP and XTP or dITP as substrate.

These data strongly suggest that the residual ITPase activity present in the variant line was not due to the presence of another isozyme of ITPase, although it is possible but not probable, that the isozyme would have the same electrophoretic mobility.

As mentioned earlier, ITPase activity has been found to exhibit a continuous spectrum of activity in human erythrocytes (31,136). However, based on the data presented (ITPase activity beneath two standard deviations from the mean, mild instability to heat, and reduced activity towards all known substrates of ITPase), in addition to no reported ITPase activity in red cells of the same individual (135), there is an extremely strong suggestion of a partial ITPase deficient lymphoid line which is distinct from the ITPase present in the normal population.

There are no reports in the literature of a partial ITPase deficiency (indeed, the boundary of levels of heterozygote-homozygote activity have not been established in red cells), but the model does exist. There is a report of a partial adenosine deaminase deficient individual. The enzyme in the lymphoid line established from this patient

seems to have some remarkably similar characteristics to the partial ITPase deficient. The ADA enzyme exhibits 25% of normal activity in lymphoid cells, 1-2% in erythrocytes and is postulated to be a heat stability mutant (115). In addition, an HGPRT variant has been reported that has 14% of normal activity in leukocyte lysates, no HGPRT activity in hemolysates, and 100% of normal activity if intact cells as assayed (^{14}C inosine conversion to ^{14}C IMP in whole cells). The enzyme was reported to be thermolabile in comparison to normal enzyme.

All enzymes reported to be partially deficient in the purine pathway have been involved in base-nucleoside metabolism. Adenosine deaminase, nucleoside phosphorylase, 5' nucleotidase, hypoxanthine guanine phosphoribosyl transferase, and adenine phosphoribosyl transferase all involve a base or nucleoside as a substrate or product. A report of partial deficiency of adenylate kinase is based on total deficiency of the isozyme, adenylate kinase 1. This is the first report of a partially deficient enzyme involved solely in nucleotide metabolism.

In summary, a lymphoid line has been found to have 25% of normal ITPase activity by phosphate assay, HPLC assay, and starch gel electrophoresis. The line has been found to be mildly unstable to heat. An HPLC assay has been developed for ITPase that is sensitive, accurate, and

reproducible. The assay has shown that the product of ITPase is IMP in both normal lymphoid cells and the ITPase variant, while a nonspecific particulate-bound triphosphatase will degrade ITP to IDP as well as IMP

Metabolic Studies

Purine interconversion and synthesis is achieved by a complex set of reactions with regulatory controls at many points. The balance between de novo synthesis and salvage is finely controlled. The conversion of IMP, a central metabolite in both de novo synthesis and salvage, to AMP and GMP is under several sets of controls. The conversion of IMP to AMP is modulated by two enzymes, adenylsuccinate synthetase, and adenylsuccinate lyase. AMP and GDP inhibit adenylsuccinate synthetase. In addition, GTP is a required cofactor in the interconversion of IMP to AMP. The conversion of IMP to GMP again requires two enzymes, IMP dehydrogenase and GMP synthetase. IMP dehydrogenase is inhibited by XMP and IDP as well as GTP. Glutamine is required for conversion of XMP to GMP. The effect of ITP as a regulatory compound on these reactions is unknown.

The use of HPLC equipment and radio-labeled precursors has enabled the monitoring of several parameters of purine metabolism. The cellular content of free nucleotides under several different conditions has been measured. In addition, the level of synthesis of a compound can be monitored by following the path of percent of total radioactivity incorporated into each compound. The calculation of specific activity, based on the level of radioactivity and the amount

of a compound present, is indicative of the pool size present. Each reaction involves a dilution of the radioactive precursor. Hence, in theory, a compound with a higher specific activity would have been synthesized prior to a compound with lower specific activity. The flow of the pathway can thus be monitored.

Lymphocytes and erythrocytes have very different alternatives available to them in terms of purine biosynthesis. Erythrocytes lack the capacity of de novo purine synthesis (165). ITP accumulation in intact tissues other than erythrocytes has not been previously demonstrated. The accumulation of ITP in a cell which has the capacity to synthesize purines de novo and to salvage purines is of particular interest. In cells possessing both the de novo and salvage pathways accumulation and synthesis of a purine compound is representative of a balance of the two pathways and thus is indicative of cellular function when alternative pathways are available. While enzyme defects are sometimes manifest primarily in erythrocytes, the more severe defects in purine metabolism have tended to be expressed as dysfunction of the immune system and neurologic dysfunction. The tissues involved in these defects are primarily lymphocytes and central nervous system tissue. Other tissues such as kidneys and joints have been secondarily involved due to an inability

to metabolize the products of purine metabolism.

Erythrocytes

Metabolic studies in human erythrocytes indicate that intact erythrocytes increase the levels of IMP and ATP present when incubated with 50 uM inosine in agreement with Blaire et al. (143) and Dean et al. (166). Inosine incorporation experiments demonstrated that the major nucleotide formed from inosine is IMP. Four percent of the radioactivity was found in ATP. Red cells are reported to be missing the enzyme adenylsuccinate synthetase (165) and lack the capacity to convert IMP to AMP. The low level conversion of IMP to AMP may have been due to contamination of the erythrocytes with leukocytes which possess the enzyme adenylosuccinate synthetase or to contamination of the inosine with adenosine. Nelson et al. have reported no radio-label in ATP or GTP when human erythrocytes are incubated with 8-^{14}C hypoxanthine if the hypoxanthine was purified. Approximately 2-3 percent of the nucleotides synthesized from radio-labeled inosine in erythrocytes have been reported to be adenine nucleotides by various investigators (143,145). The ability of exogenous inosine to increase the level of ATP or prevent degradation of ATP has been noted (166). The increase in ATP may be secondary to incubation with inosine as little ATP is synthesized from inosine. Under the stated incubation conditions, no ITP accumulated in erythrocytes from three normal individuals.

ITP accumulation in intact lymphoid cells has not been previously demonstrated, presumably because the lymphoid cell has high enough activity of ITPase to metabolize all of the ITP synthesized or lacks the capacity to synthesize ITP.

Discovery of an individual whose lymphoid cells in culture possessed reduced activity of ITPase presented a unique opportunity to investigate the ability of lymphoid cells to synthesize ITP and the parameters affecting ITP accumulation. This particular cell line exhibited 20% residual activity of ITPase. It was uncertain if the level of residual activity in this partial deficient was sufficient to prevent any metabolic abnormalities under the conditions chosen to ascertain these metabolic abnormalities.

Partial deficients have been reported to exhibit a variety of metabolic defects. These vary from no metabolic abnormalities detectable, metabolic abnormalities present in vitro but not in vivo, to metabolic abnormalities demonstrated both in vivo and in vitro. The metabolic abnormalities in vitro will depend on the tissue chosen to assay the metabolic defect and the method of ascertainment.

The ability of lymphoid cells in culture to synthesize ITP was demonstrated by the detection of ITP in the lymphoid line with reduced ITPase activity. Furthermore, initial studies indicated that, when these cells were incubated with ^{14}C inosine in a culture media containing 10 mM glucose,

50 mM PO_4, 50 uM inosine, and NaCl, these cells actively synthesized ITP. The conditions of incubation were chosen to ensure that these cells were actively synthesizing purine nucleotides. The glucose was present to help maintain energy charge and levels of R5P and the high phosphate concentration to increase PRPP levels intracellularly. Increase of intracellular PRPP would theoretically increase the amount of IMP synthesized when the cells were presented with exogenous inosine.

The first two incorporation experiments demonstrated that ITPase normal lymphoid cells had the capacity to synthesize ITP. The accumulation of ITP was found to occur if these cells were incubated with inosine for one hour. By two hours there was no accumulation of ITP. This could have been due to reduced synthesis of ITP at two hours or an increased ability to degrade ITP, since accumulation is the balance between synthesis and degradation.

In addition, the first two experiments indicated that the ability of the lymphoid line with reduced ITPase activity to accumulate ITP was variable. In the first experiment, 0.144 nanomoles/10^6 cells were present in cell culture and on glucose incubation. The second experiment demonstrated no ITP present at zero time or on glucose incubation, but ITP was synthesized when the cells were presented with exogenous inosine. In this experiment IDP

was present (0.104 nanomoles/10^6 cells) in ITPase variant cells growing in culture. Under conditions where the normal cell accumulated ITP, the variant line accumulated approximately 100 fold more ITP. Further investigation centered on conditions of cell culture that were permissive to ITP accumulation. As previously mentioned, ITPase from the variant line showed mild instability. It was possible that the line possessed enough ITPase activity to degrade all of the ITP synthesized while actively growing (in log phase) but at stationary phase lacked enough residual activity to degrade all the ITP synthesized. Investigation of time of subculture revealed no ITP was present at any time from zero to three days and zero to seven days after resuspension in fresh media at a density of 2 x 10^5 cells/ml. The cells were harvested at daily intervals. Incubation with labeled inosine on day 3 and day 7 resulted in no ITP being detected. Previous studies had been conducted with cultures that had been maintained by a 1 to 1 dilution with fresh media.

Synthesis of ITP from IMP is just one of several alternative pathways available to IMP. IMP can be converted to AMP, GMP (via XMP), or inosine. Alterations in the levels of AMP, GMP, or their metabolites may have an effect on the levels of ITP accumulation. Conversely, accumulation of ITP might alter levels of AMP, GMP, and their metabolites. To investigate the possibility of alterations of other metabolites

the full nucleotide profiles of cells obtained by HPLC were analyzed. Analysis revealed that the mean levels of various nucleotides listed in Tables 10 to 13 were not significantly different in the lymphoid line variant in ITPase activity and normal lines grown in complete culture conditions and 25 minute glucose incubation. However, on incubation with exogenous inosine for one and two hours the variant line possessed significantly more IMP (1.082 nanomoles/10^6 cells vs. 0.538 nanomoles/10^6 cells). The accumulation of ITP was not found to be correlated with the level of IMP present, in agreement with the work of Fraser et al. in erythrocytes (31). The ratio of IMP/ATP was found to be increased under these conditions as a result of increased IMP. The mean values obtained for levels of free nucleotides present in lymphoid cells cultured in complete media is in agreement with those reported in the literature by Brenton et al., although all values are slightly lower. The mean value of ATP (1.9 nanomoles/10^6) is lower than that reported by Brenton (4.23) et al. This is probably due to the stage of cell growth at which the cells were analyzed. Brenton et al. reported on the nucleotide levels of cells in log phase, while the majority of determinations presented here were from cells in stationary phase. As shown by the growth curve presented in Figure 31, the levels of nucleotides vary with the stage of cell growth. Total content of nucleotides is lower in stationary phase than log phase.

Cells analyzed in log phase revealed values of 4.3 in the normal and 5.5 in the variant respectively for ATP.

Composition of the culture medium was found to effect the synthesis of ITP. The presence or absence of glutamine, a cofactor in the conversion of IMP to GMP, was found to effect ITP accumulation. ITP was synthesized in cells that had been cultured in glutamine free media for twenty-four hours. Glutamine limitation of cultured human cells has been demonstrated to decrease the level of PRPP (167). In addition, glutamine is a required cofactor in purine biosynthesis de novo. Glutamine is also a cofactor for the conversion of XMP to GMP. Incubation conditions were chosen to increase the availability of PRPP and minimize de novo synthesis. Of the above mentioned factors, limitation of the conversion of XMP to GMP seems the most likely effect glutamine had on these cells, as reduced synthesis of guanine nucleotide was observed.

Although ITP was synthesized and accumulated in the absence of glutamine, the amount of ITP accumulated never reached the levels observed in the first two experiments. This indicated that other factors modified the accumulation of ITP. The presence or absence of aspartate did not seem to effect ITP accumulation.

The accumulation of ITP was found to be strongly

correlated with the ratio of IMP/AMP /correlation coefficient of 0.91 (p = 0.0001)/ and the amount of IDP present (0.96) in the variant ITPase line at all times. In addition, there was a strong negative correlation (-0.93) with specific activity of GTP in the variant. The inverse correlation with specific activity of GTP and the accumulation of ITP in cells grown in glutamine free media indicate that ITP accumulates when guanine nucleotides are not being synthesized. In normal cell lines, the accumulation of ITP was also correlated inversely (-0.95) with the specific activity of GTP. No significant correlation was obtained in the IMP/AMP ratio with the accumulation of ITP in normal cell lines. In both cases ITP accumulated when over one nanogram of IMP was present in response to exogenous inosine. However, in many nucleotide profiles over one nanogram IMP/10^6 cells were present and no ITP accumulated. The synthesis of ITP in the presence of large amounts of IMP could not be explained merely by a variation in the level of activity of HGPRT (synthesis of IMP) as accumulation of ITP did not correlate with the synthesis of IMP (cpm present) or the amount present. The incorporation experiments would indicate that a minimum level of IMP is necessary before ITP synthesis occurs. The finding of a large amount of IDP (0.169 nanomoles/10^6 cells) present in ITPase variant cells grown in culture media with a relatively

high level of IMP (0.569 nanomoles/10^6) supports this. The level of IMP present was higher than that found at any other time in cells growing in complete culture media. It would appear that a high IMP content is a necessary, but not sufficient, condition of ITP synthesis. The enzyme(s) responsible for synthesizing ITP from IMP may have a high Km for IMP. The observation that the level of ATP declined under conditions of ITP synthesis in the variant line was found to be a characteristic of the variant line. Data presented in Table 17 indicate that the ratio of ATP/GTP is significantly different than normal cell lines under conditions of ITP synthesis at both 1 hour and 2 hour incubation time points. Furthermore, the ratio of ATP/GTP is significantly different in the ITPase variant line when ITP is accumulated than when it is not. The reduction of ATP was not stoichiometric to the accumulation of ITP. There was no significant difference in the normal line or between the normal line and the variant line when no ITP accumulated.

All of these observations seem to indicate that ITP accumulates to a greater extent in the ITPase variant than the normal line. Conditions of synthesis and accumulation include reduced synthesis of guanine nucleotides, depletion of ATP pools, and an increased IMP/AMP ratio.

While the conditions utilized to demonstrate ITP accumulation are not physiologic, it is possible that in other

tissue types ITP accumulation does occur under physiologic conditions. The variant ITPase enzyme exhibits twenty percent activity in lymphoid lines and no reported activity in erythrocytes. The level of residual activity that this variant enzyme would possess in other tissues such as brain is uncertain. ITP has recently been reported to inhibit glutamic acid decarboxylase, an active enzyme in human brains.

Accumulation of greater amounts of ITP in the ITPase variant than normals is of great interest. Data presented previously demonstrated that the ITPase variant has an active particulate bound ITPase capable of catalyzing the degradation of ITP at the rate of 6.25 umoles/hr/10^6 cells. The accumulation of ITP in these cells could be due to several possibilities. One possibility is that the cells synthesize ITP in amounts greater than 6.25 umoles/hr/10^6 cells. The second possibility is that the synthesis of ITP occurs in a region where the particulate bound ITPase does not have access to ITP. A third possibility is the inactivity of the particulate bound ITPase in intact cells under incubation conditions. Compartmentalization of nucleotides has been reported in Novikoff tumor cells incubated with adenosine (168). ATP was reported to be compartmentalized into cytosolic and mitochondrial components in isolated rat liver cells (169). As suggested by Rappaport and Zamecnik (168), the compartmentalization need not be a physical barrier but may be a

localization of the nucleotide in the cytosol so that it is unavailable to the general cytosolic pool. Compartmentalization of adenine nucleotides in erythrocytes was postulated by Dean and Perret. Incorporation studies with ^{14}C adenine and ^{14}C adenosine resulted in a higher specific activity of ADP than ATP. ADP and ATP are rapidly equilibrated in the cytosol. In contrast, Beutler et al. (170) recently theorized a lack of ATP pools in erythrocytes based on ^{32}P labeling of ATP formed by phosphoglycerate kinase. Data from other species have indicated ITP is synthesized in association with membranes. Cain et al. reported ITP synthesis in frog muscle. Recently Plank et al. (171) have reported an ATP-ITP phosphate exchange in dog cardiac sarcoplasmic reticulum. While the level of ATP was noted to decrease in human lymphoid lines, the decrease in ATP was not stoichiometric to the increase in ITP as would be expected if a simple exchange mechanism was operating.

Data presented here did not allow the distinction of several alternative modes of ITP synthesis. This data did not clearly indicate which nucleotide, ITP or IDP, was synthesized first. In one experiment ITP had a higher specific activity indicating pyrophosphorylation, while in another experiment IDP had a higher specific activity, indicating stepwise phosphorylation of IMP to IDP to ITP. It is possible that lymphoid cells are capable of synthesizing ITP by both pyrophosphorylation

and stepwise phosphylation of IMP. Which of the alternative pathways are operative may depend on differences in culture conditions which are not as yet defined. The data from cell lysates has clearly indicated that in lymphoid cells ITP can be degraded to IMP (cytosolic ITPase) or IDP (particulate bound ITPase). The possibility exists that, if ITP is synthesized both by pyrophosphorylation of IMP and phosphorylation of IDP studies were not conducted in an in vitro system capable of distinguishing between the two.

ITP has been reported infrequently as a normal cellular substituent and few studies have included ITP as a possible inhibitor of enzyme activity. The data presented here indicate that reduced amounts of ATP and GTP synthesis, along with increased levels of IMP is not inconsistent with the hypothesis that ITP is a regulator and can serve as an inhibitor of GMP synthetase (XMP $\rightarrow$ GMP), and/or one of the enzymes involved in AMP synthesis. To postulate a regulatory role of ITP in purine metabolism is an exciting possibility particularly in light of the reported ability of ITP to inhibit L-glutamic acid decarboxylase, an enzyme whose reduced activity is known to be associated with schizophrenia. Vanderheiden has reported ITP to be synthesized by pyrophosphorylation of IMP in whole blood but by phosphorylation of IDP in lymphocyte lysates (141).

Guanylate kinase has been postulated to catalyze the phosphorylation of IMP to IDP (151). The data presented indicate that less radio-labeled GDP and GTP accumulates under conditions of IDP and ITP accumulation. These data can be interpreted in several ways. If GMP and IMP were competitive inhibitors of guanylate kinase, one would expect less GDP synthesized under high IMP conditions and vice versa. However, IDP synthesis was not correlated with the level of synthesis of GMP. Another alternative is that GDP and IDP are synthesized by two different enzymes which are not maximally active under the same conditions. Degradation of IDP and GDP are believed to be catalyzed by the same enzyme, nucleoside diphosphokinase.

All parameters investigated indicated that under conditions of ITP accumulation there was a reduced level of guanine nucleotide synthesis. This was evident from the percentage of total nucleotides synthesized, the specific activity of guanine nucleotides, and the accumulation of ITP under conditions of glutamine limitation. In addition, the variant line showed a marked degradation of ATP under conditions of ITP synthesis. The ratio of IMP/AMP was elevated in the variant line due to increased content of IMP.

Synthesis and Accumulation of Other Purine Bases

The presence of XMP with a high specific activity was a consistent and surprising feature of all lymphoid lines

incubated with inosine. Radio-labeled XMP has been reported to accumulate if lymphoid cells were incubated with inosine under conditions of glutamine limitation (22). In contrast, the data presented demonstrate the synthesis of XMP under conditions where glutamine is not limiting, as well as conditions of glutamine limitation. Small amounts of XDP were also observed in lymphoid preparations (0.001 nanomoles/ 10^6 cells). The presence or absence of XDP bore no correlation to the accumulation of ITP. The synthesis of XDP as monitored by accumulation of radioactivity has no obvious relationship to the presence or absence of ITP. XDP has not been previously reported to be present in lymphoid lines. In addition, a small amount of XTP was synthesized. This amount was detectable only in radioactivity and not in U.V. absorbing material. The low specific activity found in ATP and ADP are indicative of the large pool sizes present. ATP had the greatest proportion of radioactivity found in nucleotides except for IMP.

Conclusion

A high pressure liquid chromatography assay was developed for ITPase. Use of this assay has detected an ITPase variant lymphoid line with 25% residual activity. The variant enzyme was found to be mildly heat labile in comparison to normal if ITPase lysates were incubated at 50°C and 56°C for up to one hour. The enzyme was found to be a pyrophosphatase in both normal and the variant line. A sedimentable phosphatase was found to hydrolyze ITP. This enzyme had normal activity in the ITPase variant.

Metabolic studies demonstrated that intact normal lymphocytes have the ability to synthesize ITP. Maximal ITP accumulation was 100 times greater in the variant than in normal lines.

Lack of glutamine was found to be a contributing factor to ITP accumulation. The accumulation of ITP was found to be inversely correlated (-0.98, $p = 0.0001$) with the specific activity found in GTP, positively correlated with the ratio of IMP/AMP, and positively correlated with the amount of IDP present (0.96, $p = 0.0001$). In addition, a decreased content of ATP was found in the variant line under conditions of ITP synthesis. The decrease in ATP could not be accounted for stoichiometrically with the increase in levels of ITP present. In normal lines ITP accumulation was inversely

correlated with the specific activity of GTP (-0.92, $p = 0.0001$). This data suggests that ITP may serve a regulatory role in the conversion of IMP to adenine and guanine nucleotides.

Lymphoid lines were also found to have the ability to synthesize XDP and XTP. The conditions of XTP and XDP synthesis were not determined.

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