THE ROLE OF NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHOHYDROLASE, A GENETICALLY VARIABLE ENZYME, IN INOSINE TRIPHOSPHATE METABOLISM IN HUMAN ERYTHROCYTES

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ABSTRACT

THE ROLE OF NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHOHYDROLASE, A GENETICALLY VARIABLE ENZYME, IN INOSINE TRIPHOSPHATE METABOLISM IN HUMAN ERYTHROCYTES

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Preliminary genetic information is presented concerning the inheritance of nucleoside triphosphate pyrophosphohydrolase (NTPH) activity found in human erythrocytes. Previous data had shown that NTPH activity varied over 100-fold in the red cells of a human population, but that the specific activity of any one individual was constant for a period of years. To confirm that the specific activity is indeed a genetic trait, NTPH analyses of the red cells of the members of a few selected families are presented. Secondly, to demonstrate that the variation in this trait is not a unique property of red cells, the specific activity in samples of human granulocytes, lymphocytes, or platelets obtained from selected individuals is shown to reflect the relative value of the specific activity of the erythrocytes of each of these people. Finally, to explore the molecular basis of the variation of NTPH specific activity, evidence is presented which shows that the variation cannot be attributed to differences in the K_m for its substrate ITP or the presence of intracellular effectors.

The relationship between NTPH activity and ITP in the red cell is examined by two approaches. First, the accumulation of [^{14}]ITP in

erythrocytes incubated in vitro with [^{14}C]hypoxanthine is shown to be inversely related to the NTPH activity determined in hemolysates of these erythrocytes. In fact the relationship between these two parameters follows the relationship of substrate concentration and enzyme activity predicted by Michaelis-Menten kinetics. Second, endogenous ITP, analyzed by high pressure liquid chromatography (HPLC), was detected in an extract of fresh whole blood from an individual with very low NTPH activity. This evidence suggests that NTPH limits the concentration of ITP allowed to exist in erythrocytes and that ITP is present at undetectable to very low concentrations in the red cells of the general population.

A methylene analog of ITP (IPCPOP) was chemically synthesized in order to inhibit NTPH activity in hemolysates and thus observe ITP synthesis in a cell-free system. IPCPOP was synthesized by two methods and characterized by HPLC, UV absorption, IR absorption and ^{13}C-NMR. IPCPOP is shown to be a competitive inhibitor for ITP with partially purified rabbit and human NTPH with K_i equal to 3.7 and 5.9 μM, respectively. An affinity resin was chemically synthesized by covalently coupling the γ phosphate of IPCPOP to Separose-4B by means of a six carbon spacer molecule. This resin is shown to be able to purify NTPH 200-fold from hemolysates.

ITP synthesis by fresh hemolysates was monitored by HPLC. Manipulation of the incubation conditions indicated that IDP and ITP synthesis were significantly enhanced by the presence of phosphoribosylpyrophosphate (PRPP) and inosine. Furthermore, the incorporation of [^{14}C]inosine and [^{3}H]hypoxanthine into IDP and ITP in these cell-free

systems was stimulated by the presence of unlabelled IMP and inosine, respectively. These *in vitro* experiments have been interpreted to indicate that an IMP kinase may not be involved in IDP synthesis but rather that a unique pyrophosphotransferase reaction may convert inosine directly to IDP. The stimulation of IDP/ITP synthesis with PRPP suggests that PRPP may be the pyrophosphoryl donor.

A proposed salvage role of IDP/ITP synthesis from inosine and PRPP coupled to NTPH degradation of ITP is discussed in terms of the possible implications of this mechanism in purine metabolism.

To my parents; my wife, Nan;

and my children, Philip and Renee

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Enzymes: ADase, adenosine deaminase; APRT, adenine phosphoribosyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; NTPH, nucleoside triphosphate pyrophosphohydrolase; PNP, purine nucleoside phosphorylase.

Reagents: APCP, α,β-methyleneadenosine 5'-diphosphate; APCPOP, α,β-methyleneadenosine 5'-triphosphate; CDI, 1,1'-carbonyldiimidazole; DCC, dicyclohexylearbodiimide; DMF, dimethylformamide; 2,3-DPG, 2,3-diphosphoglycerate; DTT, dithiothreitol; HAP, 6-amino-1-hexanol phosphate; HMPA, hexamethylphosphoremide; Hyp, hypoxanthine; IPCP, α,β-methyleneinosine 5'-diphosphate; IPCPOP, α,β-methyleneinosine 5'-triphosphate; PCA, perchloric acid; PRPP, 5-phosphoribosylpyrophosphate; R1P, α,D-ribose 1-phosphate; R5P, D-ribose 5-phosphate; TCA, trichloroacetic acid; TEAB, triethylammonium bicarbonate; TFA-HAP, N-triflouroacetyl-6-amino-1-hexanol phosphate.

Miscellaneous: ACD, acid citrate dextrose; BSS, buffered salt solution; CID, combined immunodeficiency; IR, infrared; Hb, hemoglobin; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; PRP, platelet rich plasma; UV, ultraviolet.

CHAPTER I

INTRODUCTION

Prior investigations in this laboratory of *in vitro* globin synthesis by rabbit reticulocyte lysates led to the observation that a GTP hydrolase activity was present in these lysates. This activity was purified 2000-fold and characterized as a nucleoside triphosphate pyrophosphohydrolase (NTPH) which catalyzes the general reaction, NTP + $H_2O \rightarrow NMP + PP_i$, where NTP and NMP refer to nucleoside triphosphate and monophosphate, respectively (1). The best substrates for NTPH were ITP, dITP and XTP. GTP was hydrolyzed at 10% the rate of ITP; and IDP, IMP and ATP were not hydrolyzed at all. The purified enzyme required at least 10 mM $MgCl_2$, a sulfhydryl reagent and a pH of 9.75 for optimal activity. The molecular weight of NTPH was estimated to be 37,000 daltons by sucrose density sedimentation.

A survey of NTPH in various tissues of the rabbit indicated that activity was present in the thirteen tissues examined and was highest in terms of activity per cell (determined by NTPH and DNA analyses) in brain, liver and kidney but lowest in erythrocytes (2). NTPH purified from rabbit liver exhibited the same characteristics as that previously purified from reticulocytes. No isozymes were detected by disc gel electrophoresis in NTPH preparations from rabbit liver or erythrocytes or preparations from human erythrocytes.

NTPH activity has also been detected in the red cells of twelve species including those of human. A survey of the specific activity (units/mg hemoglobin) in erythrocytes obtained from various human individuals indicated that the activity differed more than 100-fold from one individual to another yet was constant in any one individual over a period of years. A survey of 6000 subjects by Vanderheiden in 1969 gave evidence for the genetic transmission of an "inosine triphosphatase," the activity of which inversely correlated with the presence of ITP in red blood cells (3). He was inadvertently measuring a coupled reaction between NTPH and endogenous inorganic pyrophosphatase (4). These observations suggested that NTPH activity in the human erythrocyte may be an inherited trait.

Further work on NTPH was carried on by S. A. Fuller, a graduate student in genetics, and myself. The data presented here support the thesis that NTPH at a genetically defined activity determines the level of ITP that is allowed to exist in human erythrocytes; and furthermore, that this enzyme may complete a previously unrecognized salvage cycle in which inosine, a metabolic waste product, is converted to IMP, a metabolite which plays a central role in the biosynthesis and catabolism of AMP and GMP.

Preliminary observations were made in an attempt to determine the genetic or molecular basis for the remarkable enzyme variation from one individual to another. A population distribution study performed by S. A. Fuller and NTPH analyses of the members of two families indicate that the specific activity is an inherited trait but that its mode of inheritance may be rather complex. A mixing study using red cell lysates of selected individuals with greatly different NTPH activities

gave no evidence for intracellular activators or inhibitors. The K_m for ITP of NTPH of hemolysates prepared from red cells of selected individuals was independent of the specific activities of these individuals and thus variation of the K_m of NTPH does not account for the variation of NTPH specific activity in the red cell. Finally, the NTPH activity per cell found in granulocytes, lymphocytes, or platelets was directly correlated with the activity per cell found in erythrocytes. These studies show that the specific activity for NTPH of an individual's red cells is associated with NTPH specific activity in other blood cell types. These experiments support the conclusion that NTPH specific activity in all tissues is inherited in a defined manner and that variation of the K_m for ITP or the presence of intracellular factors are unrelated to the origin of the wide variation in NTPH levels found in the human population.

In a cooperative study with J. F. Henderson's laboratory at the University of Alberta (Edmonton, Alberta, Canada) it was possible to examine the relationship between NTPH activity of erythrocytes and the ability of erythrocytes incubated in vitro to accumulate [^{14}C]ITP from [^{14}C]hypoxanthine (5). The relationship between these two parameters closely fits a theoretical relationship predicted by employing Michaelis-Menten kinetics for the relationship of an enzyme activity with the concentration of its substrate. Furthermore, ITP could be detected in the perchloric acid extract of fresh blood of one individual, a person who had a very low level of NTPH activity. These experiments support Vanderheiden's inverse correlation between "ITPase" activity and concentrations of ITP in the cells (3).

A methylene analog of ITP (IPCPOP) was chemically synthesized to explore ITP metabolism in erythrocyte lysates. This compound exhibited competitive inhibition for ITP with either partially purified human or rabbit enzyme with K_i values of 3.7 and 5.9 μM, respectively. The γ phosphate of IPCPOP was attached to Sepharose by means of six carbon spacer molecule and the affinity column produced in this manner could be used to rapidly purify NTPH 200-fold from hemolysates by virtue of the affinity column's unique specificity for the enzyme.

Preliminary data indicated that the cell-free accumulation of IDP and ITP in a three hour incubation was greatly enhanced by inosine and PRPP as opposed to IMP and PRPP. The role of inosine, IMP, PRPP, and NTPH in IDP/ITP accumulation was studied by the addition of various inhibitors, radioactive precursors, or intermediary metabolites to the incubation media. The proposed scheme for the synthesis of ITP and the role of NTPH which is suggested by these data is presented in Figure 1.

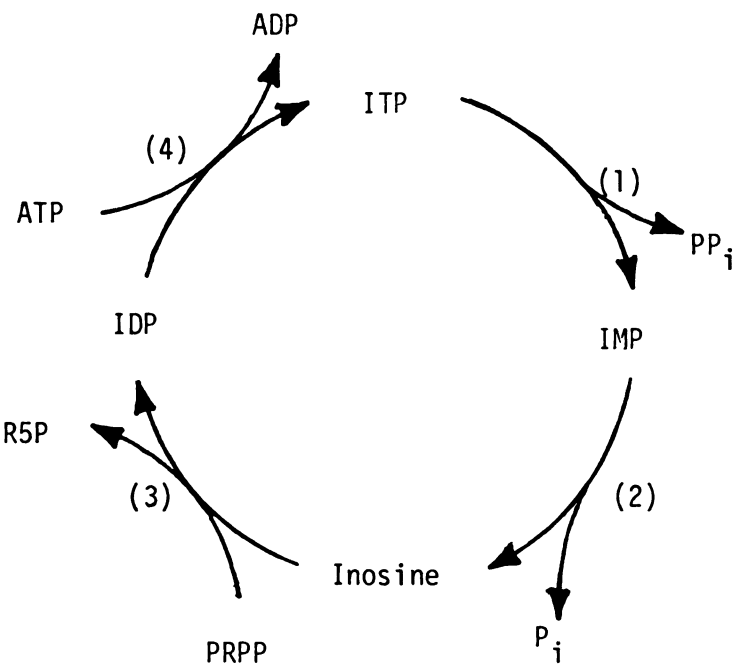

Figure 1. Proposed Role of NTPH in ITP Metabolism in the Red Cell

The enzymes involved in this cycle are the following: (1) NTPH, (2) 5'-nucleotidase, (3) proposed pyrophosphotransferase, (4) nucleoside diphosphokinase. PRPP and R5P refer to phosphoribosylpyrophosphate and D-ribose 5-phosphate.

CHAPTER II

LITERATURE REVIEW

A. Transport and Turnover of Preformed Purines by the Red Cells

Human erythrocytes lack three enzymes of purine metabolism usually found in other tissues, phosphoribosylpyrophosphate amidotransferase, adenylosuccinate synthetase and xanthine oxidase (6, 7, 8, 9). Therefore erythrocytes cannot synthesize purines de novo, AMP from IMP or oxidize hypoxanthine to xanthine or uric acid, and the red cells must depend on salvage and transport mechanisms to maintain their proper levels of nucleotides. Two reviews by Murray give a good background to these mechanisms by emphasizing the significance of the salvage pathways and the regulatory controls of nucleotide biosynthesis from preformed purines (10, 11).

The transport of purines across the red cell membrane does not seem to be a simple diffusion process. Although transportation of hypoxanthine, guanine and adenine has been shown to be very rapid (12, 13), Lassen has been able to distinguish a saturable from a nonsaturable transport system for one of the bases, hypoxanthine (13). Other investigators, deBruyn and Oei, have suggested that incorporation of purine bases by intact red cells may be regulated by salvage enzymes associated with the cell membrane (14). The transport of nucleosides across the plasma membrane is also complex; Oliver and Paterson have described the transport as mediated by a "nonconcentrative,

'facilitated diffusion' mechanism of broad specificity" (15). Furthermore, they have shown nucleoside transport to be independent of the base or sugar transport mechanisms (16). Agarwal and Parks suggest that the metabolic fate of adenosine may be determined at the membrane surface by adenosine deaminase (17). Nucleotides have not been shown to be transported across membranes, probably due to the highly charged phospate moieties on the ribose.

The transport processes described above not only supply the purine requirements of the erythrocytes but also allow the red cells to function as distributors of purines synthesized by the liver to other tissues not capable of de novo biosynthesis (18, 19). Prichard et al. have recently shown that rat or rabbit liver clears a perfusate of the hydroxylated purines, hypoxanthine, xanthine and uric acid, but releases adenosine into the hepatic circulation (20). They postulate therefore that adenosine is the transported form of the purines which supply the requirements of tissues lacking de novo biosynthesis.

Free purines or nucleosides normally are not found in the cell but exist in the form of nucleotides. Anion exchange chromatography of acid extracts of fresh erythrocytes indicates that adenine is present as AMP (13-60 μM), ADP (100-270 μM) or ADP (0.85-1.7 μM) while guanine exists as GDP (20 μM) or GTP (10-35 μM) and hypoxanthine as IMP (0-50 μM) and ITP (0-183 μM) (21, 22, 23, 24, 25, 26). An interesting experiment by Mager et al. illustrates the metabolic turnover of these nucleotide pools in vivo (27). First red cells were incubated in vitro to prelabel the nucleotide pools. Cells incubated with [^{14}C]adenine incorporated label into AMP (4%), ADP (14%) and ATP (82%). Cells incubated with [^{14}C]guanine incorporated label into IMP (10%), GDP (12%) and GTP (78%) while cells incubated with [^{14}C]hypoxanthine incorporated [^{14}C] label into GMP (27%) and IMP (73%).

Prelabelled cells were then injected into individuals, and the half-lives of the purine nucleotides were determined. Their results indicated that adenine had a half-life of 8-9 days, guanine, 5-7 hours and hypoxanthine, 1 hour. Bartlett has also illustrated the turnover of the nucleotide pools by in vitro labelling and subsequent analysis of the specific activities of these matebolites (23).

In vitro experiments with rabbit erythrocytes indicates that adenine is primarily used for adenine nucleotide synthesis while xanthine, guanine and guanosine are precursors of guanine nucleotides, and unlike human erythrocytes, hypoxanthine, inosine and adenosine can be converted to both guanine and adenine nucleotides (28). The only purines secreted from prelabelled cells are hypoxanthine, xanthine and xanthosine regardless of which [^{14}C] precursor is used (29). Combining the evidence derived from both rabbit and human erythrocytes and taking into account the enzyme deficiencies mentioned above, Figure 2 describes the interconversions of purines in the human red blood cell. As may be seen in the figure, purines synthesized de novo enter the scheme at the level of IMP (30). Thus biosynthesis and catabolism of purines is accomplished by controlled and defined pathways with IMP playing a key intermediary role.

B. Enzymes Involved in Red Cell Purine Metabolism

Several enzymes catalyzing purine conversions have been purified and characterized from human erythrocytes. A careful study of their kinetic parameters and susceptibility to regulation by other metabolites has added a great deal to the understanding of purine interconversions. This section will briefly list and describe some of these

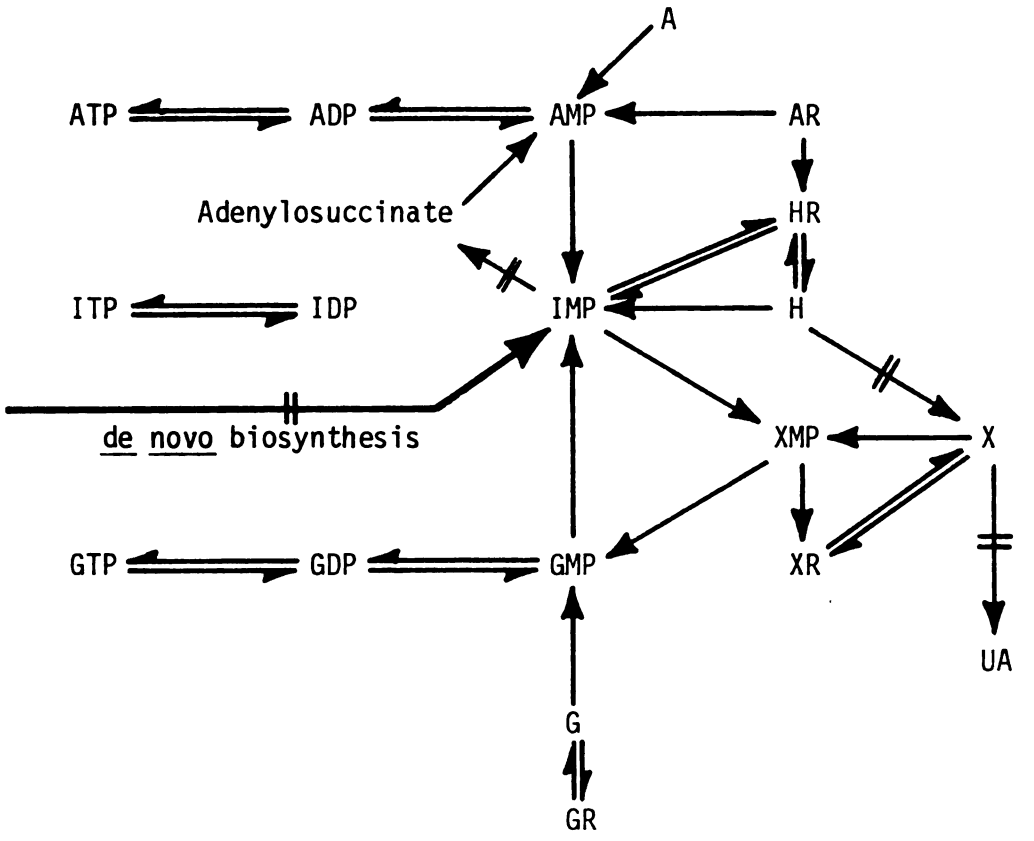

FIGURE 2. Interconversions of Purine Nucleotides in the Red Cell

The following abbreviations are used in this figure: A, AR, AMP, ADP and ATP refer to adenine, adenosine, adenosine mono-, di-, and triphosphate, respectively; H, HR, IMP, IDP and ITP refer to hypoxanthine, inosine, inosine mono-, di-, and triphosphate, respectively; UA, X, XR and XMP refer to uric acid, xanthine, xanthosine and xanthosine monophosphate, respectively; and G, GR, GMP, GDP and GTP refer to guanine, guanosine, guanosine mono-, di-, and triphosphate, respectively.

enzymes, note any significant properties, and describe their role in metabolism.

1. Purine Nucleoside Phosphorylase (PNP)

Purine Nucleoside Phosphorylase catalyzes the phosphorolysis of nucleosides: nucleoside + phosphate $\rightleftarrows$ base + ribose-1-phosphate. It has been purified from human erythrocytes and characterized in terms of

its physical and kinetic parameters (31). It shows specificity for hypoxanthine, xanthine and guanine and their nucleosides but will not react with adenosine. Zimmerman et al. have shown that the K_m for adenine is much higher than that for hypoxanthine (32). Equilibrium favors the formation of the nucleoside but the enzyme is thought to function only in the catabolic sense in vivo. Formycin B, an analog of inosine with a C-C glycosidic bond, is a good competitive inhibitor of purified PNP (K_i = 100 μM) or PNP found in intact cells or hemolysates (33).

2. Phosphoribosyltransferase (PRT)

Human erythrocytes have two enzymes which catalyze the general reaction: base + PRPP $\rightleftarrows$ nucleoside 5'-monophosphate + pyrophosphate (10, 11). Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is specific for hypoxanthine and guanine with little activity for xanthine while adenine phosphoribosyltransferase (APRT) is specific only for adenine. HGPRT has only recently been purified to homogeneity (34). Studies examining the properties and kinetics of red cell HGPRT have been hampered by the less than pure enzyme preparations used and by the fact that PRPP, one of its substrates, is rather unstable (35, 36). However, the evidence available indicates that HGPRT does not follow classical Michaelis-Menten kinetics but behaves as an allosteric enzyme (37). Red cell HGPRT is competitively inhibited by GMP and to a lesser extent by IMP for the binding of the substrate PRPP (38, 39). Developmental changes in both APRT and HGPRT activities have been noted (40). The significance of HGPRT as a salvage enzyme is illustrated by those individuals suffering Lesch-Nyhan syndrome which has been associated with a total deficiency of this enzyme (41).

3. PRPP Synthetase

PRPP synthetase catalyzes the following reaction: ATP + ribose 5 -phosphate $\rightleftarrows$ AMP + PRPP (42). Physiological concentrations of ADP, GDP and 2,3-DPG inhibit PRPP synthetase in hemolysates 200-fold but elevated phosphate concentrations can alleviate this inhibition (43). Nucleosides seem to inhibit production of PRPP at low phosphate concentrations by incorporating the phosphate that is present into nucleotides or α,D-ribose 1-phosphate (44). Many nucleotides including IDP and ITP can inhibit this enzyme (45). Recently, it has been observed that the functional PRPP synthetase is composed of 16 or 32 subunits and some of the effectors mentioned above have been shown to regulate the association or disassociation of the enzyme (46, 47). The concentration of PRPP, which is in part controlled by PRPP synthetase, may be critical for the salvage of purine bases or synthesis of nucleotides de novo in the cell (48).

4. Kinases

Kinases in the red cell which convert nucleosides to nucleoside monophosphate include adenosine kinase and inosine kinase (49). Adenosine kinase competes with adenosine deaminase to determine the metabolic fate of adenosine (50, 51, 52). Inosine kinase activity has been reported only in a crude hemolysate (53).

Kinases which convert nucleoside monophosphates to nucleoside diphosphates have been reported for GMP (guanylate kinase) and AMP (adenylate kinase) (49, 54, 55, 56). Some isozymes of guanylate kinase may use IMP 0.4 to 1% the rate of GMP (57), but this low rate is usually not considered physiologically significant.

Nucleoside diphosphokinases convert nucleoside diphosphates to nucleoside triphosphates in a very nonspecific manner (58). Since it is one of the most active purine metabolizing enzymes found in the red cell, Parks and Agarwal point out that one might expect equilibration of high energy phosphate bonds (59). However, this does not seem to be the case in red cells since the ATP/ADP ratio is not the same as the GTP/GDP ratio (60).

5. Deaminases

Two important enzymes which determine the metabolic fate of adenine derivatives are adenosine deaminase and AMP deaminase, enzymes which catalyze the reactions which convert adenosine to inosine and AMP to IMP, respectively (61). Adenosine deaminase from red cells has recently been purified and characterized (62). It exhibits product inhibition by inosine (K_i = 0.7 mM). AMP deaminase has been studied in hemolysates and may be regulated by cations, ATP and 2,3-DPG (63, 64).

Deamination of ADP has not been demonstrated in red cells although it may occur by some preparations of adenylate deaminase from skeletal muscles (11, 65).

Conversion of GMP to IMP is catalyzed by GMP reductase which uses NADPH as a cofactor to catalyze a reduction as well as a deamination (66). Unlike the biosynthetic pathway of GMP, XMP is not an intermediate in its catabolism. Guanine deaminase has not been observed in red cells.

6. Phosphatases

No specific 5'-nucleotidase has been described for the red cell (67). Furthermore very little alkaline phosphatase activity is present (68), but acid phosphatase and inorganic pyrophosphatase activities have been described (69, 70). Acid phosphatase has been widely studied because of the genetic variability of its isozymes. The role of this enzyme in metabolism has not been examined in detail but one would expect that it at least partially accounts for the hydrolysis of nucleotides to their nucleosides. Henderson, et al. measured an IMP dephosphorylase activity which may in fact be due to acid phosphatase (71). Inorganic pyrophosphatase probably causes pyrophosphate-producing reactions to be irreversible by hydrolyzing the pyrophosphate to inorganic phosphate.

7. Other Enzymes

IMP dehydrogenase and GMP synthetase have also been measured in red cells. Thus IMP may be converted to GMP through the intermediate XMP (71). Henderson has shown that this is not a very significant pathway for the utilization of exogenous hypoxanthine supplied to intact red cells (72). Adenylosuccinase activity, a reaction which converts adenylosuccinate to AMP, has been detected in erythrocyte lysates (9).

C. Physiological Properties of Purine Nucleosides

The vasodilating effect of adenosine and adenine nucleotides on the vascular system was suggested early in the study of the molecular physiology of the circulatory system (73).

Recently, Herlihy et al. have shown that adenosine is a smooth muscle relaxant, and they suggest that adenosine may alter the Ca^{++} permeability of the membrane or alter the membrane potential (74). Adenosine has been postulated as a regulator of skeletal muscle blood flow (75), cardiac blood flow (76, 77) and supply of blood to the brain (78).

Adenosine and adenine have also been shown to decrease the contractile force of the isolated rabbit heart (79). Whether this effect is due to the role of adenosine as a vasodilator is unclear.

Conflicting reports appear in the literature concerning the effect of inosine on myocardial hemodynamics. Both inosine and hypoxanthine have been reported to increase the contractile force of the isolated rabbit heart (79). Kypson and Hait have recently concluded that this positive inotropic effect on rabbit hearts was not mediated by catacholamines since a study of the metabolic changes following perfusion of the heart with inosine were not characteristic of a catacholamine-mediated effect (80). On the other hand, Gross et al. were not able to demonstrate any effect of inosine or hypoxanthine on the myocardial hemodynamics of an isolated supported dog heart preparation (81). Differences in the concentration of inosine used in their studies compared with those used in rabbit heart preparations may account for the physiological differences observed.

Small doses of inosine and IMP (.75-.85 μm/kg) caused a 16% increase in the blood pressure in the rat, while hypoxanthine or α,D-ribose 1-phosphate had no effect (82). Inosine did not change the heart rate and seemed to act independently of the autonomic nervous

system. Other investigators have not found inosine to be a vasoconstrictor (77).

Guanine and guanosine have also been observed to increase the contractile force of isolated rabbit hearts (79), but these metabolites have not received much further attention in terms of their effects on the vascular system.

In summary, it seems that both adenosine and inosine may have a role in the regulation of blood flow. It is interesting that these metabolites have opposite effects on the contraction force of the isolated heart. Since adenosine may readily be deaminated to inosine by adenosine deaminase (ADase), it may be that ADase has a role in the regulation of the heart hemodynamics. Certainly the mechanism of the action of these nucleosides on the vascular system will be a subject of future investigations.

D. Diseases Associated with Enzyme Defects in Purine Metabolism

Biochemical alterations in the enzymes involved in purine metabolism result in a wide range of clinical symptoms and may have very serious consequences. The etiologies of some forms of gout, the Lesch-Nylan syndrome, xanthinurea and severe combined immunodeficiency have been attributed to specific enzyme defects in purine metabolism. These and other diseases which may cause altered purine metabolism have been reviewed extensively and will be discussed briefly here (83-87).

1. Gout

One of the classical examples of a defect in purine metabolism is the clinical condition known as gout. In this disease the ultimate

degradation product of purine metabolism, uric acid, exceeds saturation in body fluids, a condition which often results in the deposition of monosodium urate crystals in and around the joints, causing severe inflammation. Although supersaturation of uric acid may be caused by the reduced ability of the kidney to excrete this metabolite, gout has also been associated with excess purine synthesis de novo. The increased production of uric acid has been correlated with the increased activity of PRPP synthetase which results in an increased intracellular concentration of PRPP (88, 89). Since the concentration of PRPP in the cell is in the range of the K_m for PRPP of PRPP amidotransferase, the first and probably rate-limiting enzyme of purine synthesis, Fox and Kelley have suggested that PRPP concentrations may determine the rate of purine synthesis de novo (90). Furthermore, PRPP has been shown to be an allosteric effector of PRPP amidotransferase and thus high concentrations of PRPP may stimulate this enzyme (91).

Another enzyme defect associated with increased uric acid production is an altered PRPP amidotransferase which is resistant to feedback inhibition by nucleotides (92, 93). As noted above, amidotransferase is probably the rate-limiting enzyme for de novo purine synthesis and is likely under stringent metabolic control in vivo.

Gout has also been associated with partial HGPRT deficiency (94). The reduced activity of this salvage enzyme has been correlated with increased intracellular concentrations of PRPP which in turn could affect purine de novo synthesis as described above (95). Increased PRPP concentrations in this case can be rationalized as a result of the reduced use of PRPP by HGPRT for the salvage of free bases. However, Graf et al. have reported that altered HGPRT molecules with

0-40% of normal activity are often associated with increased PRPP synthetase (96). They suggest that the HGPRT gene may have a regulatory role in the expression of the structural gene of PRPP synthetase. Thus in the case of partial HGPRT deficiency, elevated PRPP concentrations, and thus increased de novo purine synthesis, may be due in part to the elevated PRPP synthetase activity.

2. Lesch-Nyhan Syndrome

A second disease which has been attributed to a defect in purine metabolism is the Lesch-Nyhan syndrome. This x-linked disorder is characterized by self-mutilation, spasticity, growth and mental retardation and hyperuricemia. This syndrome is correlated with very low to undetectable activity of HGPRT (97) and appears to be the extreme form of partial HGPRT deficiency discussed above. It seems that the less HGPRT activity present in tissues, the more severe are the neurological disorders (98).

3. Xanthinurea

A third disease, xanthinurea, is associated with the lack of xanthine oxidase activity which normally oxidizes hypoxanthine to xanthine and xanthine to uric acid (99). The disease is recognized by the symptoms of hypouricemia and decreased excretion of uric acid. The accumulation and precipitation of xanthine appears to cause renal deposits and muscular disease.

4. Severe Combined Immunodeficiency

Severe combined immunodeficiency (CID) has sometimes been associated with either of two enzyme defects, adenosine deaminase (ADase) deficiency (100) or purine nucleoside phosphorylase (PNP) deficiency

(101). ADase deficiency in one case of CID has been shown to be a structural defect in the enzyme (102), and thus it is argued that this deficiency is the primary defect in the disease (103).

Purine production and metabolism have recently been studied in a patient with PNP deficiency associated with CID (104). The individual had severe hypouricemia and hypouricosuria and excreted inosine, guanosine, deoxyinosine, deoxyguanosine and uric acid 9-N riboside in the urine. Both inosine and PRPP concentrations were increased in the patient's erythrocytes. It was suggested that since inosine is an inhibitor of adenosine deaminase (62), the immunodeficiency symptoms associated with PNP deficiency may be caused by the effect of the elevated inosine concentrations on ADase activity.

Current investigations are being conducted to determine the mechanism of the toxicity caused by loss of ADase activity. Most recent suggestions have implied a role for deoxyadenosine as an inhibitor of lymphocyte activation (105) or the role of adenosine as a prostaglandin antagonist (106). But up to this time, the mechanism of the immunological dysfunction associated with PNP and ADase deficiencies remains undefined.

5. Other Diseases

A case study has been reported of the clinical symptoms associated with complete APRT deficiency (107). The patient exhibited hyperuricemia and had renal stones composed of 2,8-dihydroxyadenine. Glycogen-storage disease, type I, has also been associated with increased purine production (108). Kelley and Wyngaarden have suggested that de novo synthesis may be stimulated by the increased production of ribose 5 -phosphate and PRPP in the liver (85).

E. Blood Storage

The storage of blood for transfusion purposes has stimulated interest concerning the conditions which best preserve the cells' viability. Since Gabrio first noted that storage of blood with adenosine enhanced its post-transfusion viability, many investigators have given particular attention to the changes in the levels of phosphorylated carbohydrate intermediates during storage (109). Blood stored in an acid citrate dextrose solution (ACD) at 4°C metabolizes ATP to IMP and hypoxanthine and dramatically breaks down 2,3-DPG, an important effector of hemoglobin's affinity for oxygen (110, 111). Bartlett has shown that during a two week period, IMP increases from 30 μM to 129 μM, ATP decreases from 1 mM to 0.5 mM and 2,3-DPG decreases from 5 mM to 0.06 mM (112). Further studies have indicated that these metabolites may be regenerated by incubation media usually containing some combination of glucose, adenine, pyruvate, inosine and phosphate (113, 114, 115). It seems that inosine by phosphorolysis to ribose 1-phosphate, or glucose by phosphorylation to glucose 6-phosphate can each provide the cell with an energy source. High levels of phosphate increase PRPP synthetase activity (43) and thus increase the concentration of PRPP which can then salvage adenine by APRT to generate AMP. Pyruvate may be added to stimulate glyceraldehyde-3-phosphate dehydrogenase by reoxidizing the NADH or NADPH produced during synthesis of ATP or 2,3-DPG.

Under certain incubation conditions, ITP as well as ATP may accumulate in the cells. Four week old erythrocytes stored in ACD incubated five hours in Krebs-Ringer Phosphate plus 10 mM inosine yielded 0.07% ITP from inosine (116). Furthermore, Zachara has shown that incubation of out-dated blood for two hours in a medium containing either 10 mM

inosine or adenosine, 10 mM pyruvate and 50 mM phosphate could accumulate concentrations of ITP up to 1.4 mM, thus surpassing even the physiological levels of ATP (117-120)! Therefore the manipulation of red cells *in vitro* under artificial conditions is valuable in determining the pathways and controls of intermediary metabolites in the red cell as well as in developing better methods for the storage of blood cells.

F. ITP Metabolism

Up to this time I have deferred a discussion of ITP metabolism in the red cell in order to present a more coherent overview of this topic at this time. The synthesis and role of ITP in the red cell has not been clearly defined in the literature and remains the topic of this thesis. On the other hand, at least three investigators have examined the catabolism of ITP and have shown that the red cell has a specific pyrophosphohydrolase, NTPH which hydrolyzes ITP to IMP and pyrophosphate (1, 4, 121).

1. Accumulation of ITP in Erythrocytes

It has already been noted that stored red cells incubated under certain conditions can accumulate high levels of ITP. Other investigators have also studied this phenomenon in fresh erythrocytes. Agarwal *et al*. studied purine metabolism in erythrocytes of patients with adenosine deaminase deficiency and severe combined immunodeficiency (122). They noted that incubation of the erythrocytes of two patients previously treated with bone marrow or fetal liver transplantation resulted in the accumulation of ITP from exogenous inosine. IMP concentration did not change after one hour, while ITP accumulated linearly during the three hour incubation. This ITP accumulation was

decreased under the same conditions when one of the patients was reexamined ten and fifteen months later. Agarwal et al. observed much less accumulation and considerable variability in the accumulation of ITP in the erythrocytes of normal individuals and two other patients having adenosine deaminase deficiency.

Vanderheiden suggested that three groups of people may be distinguished by the ability of their red cells to incorporate [^{14}C]inosine into ITP (123). By his criteria, individuals were classified as accumulating high levels of [^{14}C]ITP if the [^{14}C]ITP/[^{14}C]IMP ratio of their red cells was 0.14 to 0.21 following one hour of incubation. Intermediate levels were defined as a ratio of 0.01 to 0.10 and low levels, 0.001 to 0.003. Vanderheiden later attributed the variation in [^{14}C]ITP accumulation to the genetic variation of an "ITPase" present in red cells (3) and was able to show synthesis of [^{14}C]ITP in a lysate prepared from the red cells of a person deficient in this "ITPase" (124).

Investigators in Henderson's laboratory studied the variation of ITP accumulation from [^{14}C]hypoxanthine in a control and mentally retarded population (72). They suggested that there were two classes of individuals, those whose red cells could accumulate 0 to 70 nmoles [^{14}C]ITP/10^{10} cells and those whose cells could accumulate 70 to 240 nmoles [^{14}C]ITP/10^{10} cells during two hours of incubation. The data show that 5% of the control population (80 individuals) compared to 15% of a mentally retarded population (100 individuals) could be classified in the high category.

In a collaborative effort between our laboratory and Henderson's laboratory we were able to establish that a substrate-enzyme

relationship defined by Michaelis-Menten kinetics exists between the accumulation of [^{14}C]ITP and NTPH activity in red cells obtained from various individuals (5). Our observations confirm the relationship between ITP accumulation and an "ITPase" which had been suggested by Vanderheiden (3).

Further studies by Henderson et al. indicated that various factors affect the synthesis of inosine nucleotides (125). First, they demonstrated a correlation between the intracellular levels of PRPP and the ability of the red cells to incorporate [^{14}C]hypoxanthine into inosine nucleotides. Second, they showed that storage of the cells in Krebs-Ringer medium containing 25 mM phosphate, pH 7.4, increased their ability to accumulate [^{14}C]ITP. Finally, Henderson et al. observed that the rate of [^{14}C]ITP accumulation increased dramatically with the time of incubation. Neither of these last two observations could be correlated with a loss of NTPH activity.

In the most recent report of ITP accumulation, Nelson et al. observed that cells incubated for 24 hours in the presence of 0.5 mM hypoxanthine or inosine, 25 mM glucose and 50 mM phosphate accumulated 0.4 mM ITP (126). However, some kinetic difference existed between the accumulation of ITP from hypoxanthine and the accumulation from inosine. In the presence of hypoxanthine, the intracellular level of IMP had stabilized at 12 hours but ITP accumulation was linear through 24 hours, while on the other hand, the presence of inosine caused an initial burst of synthesis of IMP and ITP followed by linear accumulation of each. These authors do not offer any explanation for the kinetic differences exhibited by these two precursors but suggest that

their results provide evidence that ITP is synthesized by an "IMP kinase" coupled to NDP kinase.

One can conclude from these studies that first, red cells have mechanisms which can synthesize ITP under the proper conditions and second, that this synthesis does not usually cause accumulation of ITP because of the presence of the degradative enzyme, NTPH.

2. Synthesis of ITP

The pathway of ITP synthesis has not, as yet, been defined. IMP kinase activity could not be detected in E. coli, pig kidney extracts or calf liver extracts (127-130). Guanylate kinase as mentioned before has very poor affinity for IMP (56, 57).

3. Endogenous ITP Concentrations

Early literature has scattered reports that ITP is a normal constituent of tissues. ITP has been reported in extracts of frog muscle (131), rabbit muscle (132), guinea pig liver and skeletal muscle (133) and rat liver mitochondria (134). Vanderheiden has presented evidence that ITP may normally be present in human red cells, an observation which will be discussed here in more detail.

In a study reported in 1964, high concentrations of ITP (153- 183 μM) were detected in the red cells of two siblings (26). Furthermore, in a survey of over 6000 people Vanderheiden found seven individuals who also had relatively high levels of ITP in their red cells (3); furthermore, this trait could be demonstrated in the families of two of these individuals. Vanderheiden suggested then that the elevated concentrations of ITP in the blood cells of some people as well as the ability of these same cells to accumulate large amounts of [^{14}C]ITP

during an incubation was attributable to a genetic deficiency in a degradative enzyme "ITPase." Later he evaluated the concentration of ITP in other "normal" individuals to be 0-16 μM with an average of 6.4 μM (25).

However the quality of the analyses reported by Vanderheiden may be challenged. The analysis of ITP concentrations in red cells involved either labelling the nucleotide pools including ITP by incubating the cells or lysates in vitro with [^{32}P]orthophosphate for 0.5 to 1 hour, or the analysis involved the addition of a "known" amount of [^{32}P] ITP to the neutralized trichloroacetic acid extracts to aid in the visualization of the area of the paper chromatogram containing ITP. Either method introduces an uncertainty factor. The first method has no control for changes in ITP concentration during the incubation, while the second increases the amount of background phosphate which must be subtracted to quantitate the actual endogenous ITP present in red cells. The neutralized trichloroacetic acid extracts containing the nucleotides were separated by high voltage paper electrophoresis followed by detection of the [^{32}P]labelled spots by x-ray film. The appropriate areas of the chromatogram such as that containing ITP were cut out, eluted with a solvent, and analyzed for total phosphate. However, the area containing ITP was consistently contaminated with sedoheptulose-1,7-diphosphate, and thus additional chromatography or analysis was required to distinguish these two compounds. In summary Vanderheiden's chromatographic system and quantitative analyses may not have given accurate estimates of endogenous ITP concentration in red cells because (a) manipulation of the red cells prior to extraction may have altered endogenous nucleotide concentrations, (b) the area of

the chromatogram containing ITP was seriously (50% or more) contaminated with background phosphate unrelated to endogenous ITP, (c) the trichloroacetic acid extracts contained no internal standard to estimate the efficiency of the extraction or the degradation of ITP during the procedure and (d) his data indicate considerable variation in the quantitative results.

4. Degradation of ITP

ITP may be substituted for ATP in reactions catalyzed by a wide variety of enzymes. For example, ITP can substitute for ATP as phosphoryl donor in many NMP and NDP kinase reactions (49, 58) and may be used by phosphofructokinase (135), phosphoenolpyruvate carboxykinase (136), succinate thiokinase (137), pyruvate kinase (138), and glucose 6-phosphatase (139). Yeast inorganic pyrophosphatase will also hydrolyze ITP in the presence of Zn^{++} (140) while rat liver alkaline pyrophosphatase will hydrolyze ITP even in the absence of Zn^{++} (141). ITP hydrolysis in red cells occurs by means of a pyrophosphohydrolase, NTPH, giving IMP and pyrophosphate (1, 4, 121). Since this dissertation deals with the role of this enzyme in inosine metabolism, the properties of NTPH will be described in some detail below.

NTPH was originally purified and characterized by Chern, MacDonald and Morris in 1969 (1). They achieved a 2000-fold purification from rabbit red cell lysates and demonstrated that the enzyme catalyzed a pyrophosphate hydrolysis, had a pH optimum of 9.75 and required at least 10 mM $MgCl_2$ and 1 mM sulfhydryl reagent. Monovalent cations as well as high substrate concentrations inhibited NTPH. The molecular weight as determined by sucrose density sedimentation was 37,000 daltons. ITP, dITP and XTP were by far the best substrates

while GTP was hydrolyzed at 10% the rate of ITP; and IDP, IMP, or ATP were not hydrolyzed. Methods for the purification of NTPH from rabbit liver and human red cells as well as rabbit red cells have recently been described by Morris (142).

In 1969 Hershko et al. reported a 100-fold purification of the same enzyme from rabbit red cells (121). The properties of that enzyme preparation were consistent with the results obtained in Morris' lab with the exception that Hershko et al. did not demonstrate a sulfhydryl requirement. They suggested that the physiological substrate of NTPH is GTP even though ITP was shown to be a better substrate for the enzyme.

NTPH analysis has usually been performed by a coupled enzyme assay which includes yeast pyrophosphatase to hydrolyze the pyrophosphate produced by NTPH followed by colorimetric analysis of the orthophosphate (1, 121, 4). However Vanderheiden has described a micro assay of NTPH by liquid scintillation which appears to be a valid but tedious method (143).

Two tissue distribution studies of NTPH can be found in the literature. Wang and Morris have shown that the activity per cell is highest in the brain, liver and kidney of the rabbit but lowest in its red cells (2). Moreover, NTPH was detected in all thirteen tissues examined suggesting that the enzyme plays a significant metabolic role in the body. NTPH was distinguished from nonspecific phosphatases in these studies by incubating control reactions with ATP instead of ITP. Purified liver NTPH had the same general properties as red cell NTPH, and enzymes from both tissues exhibited only one band on disc gel electrophoresis specific for the hydrolysis of ITP. Wang and Morris

suggest that the biological role of NTPH may be to prevent incorporation of ITP or XTP into RNA or dITP into DNA.

A study of ITP pyrophosphohydrolase and IDP phosphohydrolase activity in rat tissues has been reported by Vanderheiden (144). He also reported finding NTPH activity in all the tissues which he examined. The adrenal, brain, liver, lung, ovary and thymus tissues had the highest activity per mg protein. Vanderheiden attempted to distinguish the NTPH activity from nonspecific phosphatases by denaturing them by heat treatment at 57°C for five minutes. Although NTPH activity of red cells and liver has been shown to be comparatively stable to heat treatment (2, 121), this treatment may cause erroneous estimates of activity per mg tissue if the enzyme stability varies among tissues, if the enzyme is partially degraded, or if the kinetic parameters of the enzyme are altered by the high temperatures. One interesting observation by Vanderheiden is that the relatively low NTPH activity in the erythrocytes of one rat was also reflected in its other tissues. Vanderheiden suggested that NTPH plays a role in an ITP-IMP cycle which regulates the level of ATP.

Interest in the inheritance of NTPH activity and its genetic characteristics was stimulated by the correlation suggested by Vanderheiden that an "ITPase" activity seemed to be limiting the amount of ITP which normally exists in the red cell as well as limiting the ability of these cells to accumulate ITP from radioactive inosine (3). Vanderheiden suggested also that the "ITPase" specific activity is inherited as an autosomal codominant genetic trait. However, this proposal is not well established by the data presented in his paper.

Harris and Hopkinson have described an electrophoretic and staining system for the separation and detection of NTPH isozymes (145). They report that an intense zone was often associated with one or two minor more anodal zones. The intensities of these isozyme bands were dependent on the time of storage of the tissue extracts. They suggested that the change during storage was caused by oxidation of reduced sulfhydryl groups and could be prevented by the addition of 10 mM β-mercaptoethanol. Harris and Hopkinson could identify no electrophoretic variants in red cell lysates of a "large number" of Europeans, Blacks and Indians.

It is unclear whether the "isozymes" which are described above are indeed products of different NTPH genes. First Harris and Hopkinson made no attempt to determine whether the bands on their gels were actually caused by the pyrophosphohydrolyses of ITP by NTPH or whether these bands were caused by the release of phosphate by nonspecific phosphatases. Second, if the bands can be attributed to NTPH activity, the change in their intensity with different storage conditions indicates that these bands may be artifactual alterations of the same molecule.

Two recent reports suggest that NTPH is located on chromosome 20 in humans (146, 147). Using the technique of somatic cell hybridization these investigators found only 4 out of 24 hybrids which showed discordance between NTPH and adenosine deaminase, an enzyme which had been previously located on chromosome 20. Three of these discordant hybrids were derived from the same fusion experiment. However the possibility that NTPH is found on chromosomes 7 or 22 could not be excluded by the hybrids which were analyzed. As in the case of the

Harris and Hopkinson technique, the enzyme assay which these investigators used to identify NTPH in the cell hybrids may not have excluded the activity of nonspecific phosphatases.

CHAPTER III

REAGENTS, MATERIALS AND METHODS

A. Reagents

1. Commercial

The following chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri: dithiothreitol, bovine serum albumin, glutathione, β-mercaptoethanol, 1,1'-carbonyldiimidazole, α,D-ribose 1-phosphate dicyclohexylammonium salt, D-ribose 5-phosphate sodium salt, inosine, hypoxanthine, Wright's stain, ninhydrin, tri-n-octylamine, pyruvate, dextran (clinical grade, 240,000), dimethyldichlorosilane, 5-phosphoribosylpyrophosphate sodium salt, Dowex anion exchange resins, DEAE-Sephadex A-25-120 and Sepharose-4B.

The following reagents were acquired from P. L. Biochemicals, Milwaukee, Wisconsin: the ribonucleoside 5'-mono-, 5'-di- and 5'-triphosphate sodium salts, adenosine 5'-triphosphate potassium salt, deoxyribonucleoside 5'-triphosphate sodium salts, methylene diphosphonic acid, α,β-methyleneadenosine 5'-triphosphate sodium salt, α,β-methyleneadenosine 5'-diphosphate sodium salt, β,γ-methyleneadenosine 5'-triphosphate sodium salt.

Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, supplied the following: triethylamine, dicyclohexylcarbodiimide, 2',3'-isopropylideneadenosine, tri-n-butylamine, hexamethylphosphoramide, cyanogen bromide, naphthalene (scintillation grade), acetonitrile and iodomethane.

Miscellaneous reagents were obtained from the following sources. Hycel cyanmethemoglobin reagent (No. 116E) was obtained from Hycel Inc., Houston, Texas. 3,3'-dimethoxybenzidine was supplied by Eastman Organic Chemicals, Rochester, New York. Silver oxide, Norit-A and phosphorus pentoxide were obtained from Fisher Scientific Co., Pittsburgh, Pennsylvania. AG 1x8 (200-400 mesh) anion exchange resin and Bio-Gel P2 were obtained from Bio-Rad Laboratories, Richmond, California. Ficoll-paque was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey. Triton X-100, PPO (2,5-diphenyloxazole) and dimethyl-POPOP {1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]}-benzene were from Research Products, Inc., Elk Grove Village, Illinois. Kerosene (APCO #476) was obtained from Carrier-Stephens Co., Lansing, Michigan. Calcium hydride was purchased from K & K Laboratories, Inc., Jamaica, New York. Stock Geimsa stain was obtained from Sargent-Welch Scientific Company, Skokie, Illinois, and orcinol from Matheson, Coleman and Bell, Norwood, Ohio. Yeast inorganic pyrophosphatase (600-800 units/mg) was purchased from Nutritional Biochemicals, Cleveland, Ohio. [8-^{14}C]Inosine was obtained from Schwarz/Mann, Orangeburg, New York. [2-^{3}H]ATP and [G-^{3}H]hypoxanthine were purchased from Amersham/Searle, Arlington Heights, Illinois.

All other chemicals used were reagent grade.

2. Distillation of Solvents

Triethylamine was routinely distilled prior to use and collected over a temperature range, 88-89°C, at ambient pressure. Dimethylformamide was distilled and collected in a flask containing molecular seives over a temperature range, 151-154°C at ambient pressure. Hexamethylphosphoramide was also distilled at ambient pressure over a

range 234-239°C and collected on molecular seives. Tri-n-butylamine was twice distilled at reduced pressure over a range, 91-94°C. Pyridine was dried by refluxing it over calcium hydride for 4 hours and then distilling the pyridine over a temperature range, 115-116°C at ambient pressure, and the distillate was collected in a flask containing fresh calcium hydride. Water was distilled and passed over a mixed bed resin or redistilled.

3. Purification of ITP and PRPP

ITP was purified for some studies by a method similar to that used by Yount et al. for the purification of adenylyl imidodiphosphate (148). DEAE-Sephadex A-25-120 was allowed to swell overnight in 0.5 M ammonium bicarbonate. 'Fines' were removed twice by settling in the same buffer, and then the resin was suspended in 2 volumes of 0.3 M triethylamine bicarbonate (TEAB) at pH 7.5. A 2.5 x 40 cm. column was poured and washed overnight at ambient temperature with the TEAB buffer at a flow rate of 150 ml/hour. ITP (1.4 mmoles) was loaded in a total volume of 35 ml at the same ionic strength and pH as the initial buffer. The column was developed at ambient temperature with a 3 liter linear gradient from 0.3 M TEAB pH 7.5 to 0.8 M TEAB pH 7.5. The effluent was monitored at a wavelength of 254 nm with an ISCO UA5 absorbance monitor (Instrument Specialties Co., Lincoln, Nebraska) and was collected in 20 ml fractions. Fractions containing the largest peak of those resolved were pooled and concentrated with a flask evaporator on a water aspirator with the temperature set at 30°C. The concentrated syrup was dissolved in a small volume of water and concentrated again. This process was repeated twice. Finally, the syrup was dried to a glass with a high vacuum pump attached to the evaporator.

The ITP was converted to its sodium salt by the following method. A small amount of Dowex 50 (10-15 ml) was washed with 2 N HCl, rinsed with distilled water and placed in a cold scintered glass funnel. The triethylammonium salt of ITP was dissolved in 25 ml of ice cold water and passed through the Dowex within 15 seconds into a vacuum flask containing 4 equivalents of NaOH stirring in an ice bath. The Dowex was washed with two 20 ml aliquots of cold water which were combined with the ITP solution. This solution (pH 7-8) was lyophilized to the dry sodium salt of ITP and stored dessicated at -20°C.

PRPP was sometimes purified and converted to its magnesium salt as described by Flaks (149). A column (1.4 x 15 cm) of AG 1 x 8 (200-400 mesh) was prepared in the cold room at 4°C and washed with 500 ml 2N HCl and rinsed with distilled water. The resin was converted to its formate salt as described in the Bio-Rad catalog (Price List 2, March, 1974) and washed with cold water. PRPP (25 mg) dissolved in cold water was loaded on the column and was eluted with a 400 ml linear gradient from water to 1.5 M ammonium formate pH 5.0 at a flow rate of 164 ml/hour. Fractions containing 5 ml were collected and 0.1 ml aliquots of every third tube were analyzed by the orcinol determination for ribose (150, 151). Fractions containing the major peak were pooled and combined with 5 ml of 0.5 M $MgCl_2$ and 3 volumes of ethanol (-15°C) to precipitate the magnesium salt. The precipitate was stored overnight at -20°C and was harvested the next day by centrifugation at -15°C at 10,000 g for 10 minutes. The magnesium salt was washed twice with acetone and twice with ether and stored dessicated at -20°C.

4. Special Reagents

a. Triethylammonium bicarbonate buffers

A 1.0 M solution was prepared by diluting 140 ml distilled triethylamine with water to ca. 900 ml. This solution was titrated to pH 7.5 with carbon dioxide generated in the following manner. Concentrated HCl was dripped by means of a peristaltic pump into a vacuum flask containing a suspension of sodium bicarbonate stirring in water. The carbon dioxide generated was directed through a distilled water trap and bubbled into the triethylamine solution with the aid of a gas dispersion tube. The pH was monitored with test paper until it was ca. 8 and then it was monitored with a pH meter. The solution was adjusted to 1 liter and stored in a tightly stoppered container. When this stock solution was diluted or allowed to set for a long period of time, it was titrated again with carbon dioxide to the appropriate pH prior to use.

b. Synthesis of methyl-tri-n-octylammonium hydroxide

Methyl-tri-n-octylammonium hydroxide was synthesized by the method of Letters and Michelson (152). Tri-n-octylamine (8 ml) was mixed with 8 ml iodomethane in a 50 ml round bottom flask. A condensor was placed on the flask and the solution refluxed over low heat (heating mantle at 30 volts) for three hours. The flask and condensor were rinsed with 100% ethanol into a 250 ml flask, and the ethanol and excess iodomethane were removed by evaporating the solution with a flash evaporator with the vacuum drawn by a water aspirator and the temperature at 40°C. The methyl-tri-n-octylammonium iodide was converted to its hydroxide by adding 15 g silver oxide to a solution of 34 ml 100% ethanol, 3 ml of water and the syrup. This solution was stirred for

30 minutes and filtered through celite. The flask and celite were rinsed with ethanol to improve the recovery. The methyl-tri-n-octylammonium hydroxide was titrated with standardized hydrochloric acid with phenolphthalein as an indicator.

c. Preparation of [^{3}H]ITP from [^{3}H]ATP by deamination

Deamination of [^{3}H]ATP was performed with an aqueous solution of nitrous acid (153). About 100 μCi of [2-^{3}H]ATP (27 Ci/mmole) was diluted with water and lyophilized to dryness overnight. The residue was suspended in 0.35 ml of 2.86 N formic acid to which 40 mg of sodium nitrite dissolved in 0.15 ml water were added. This solution was stored at room temperature for one day followed by lyophilization to dryness overnight. DEAE-Sephadex A-25-120 was prepared as described under section III,A,3. A 0.8 x 60 cm column of the resin was prepared and washed with 0.4 M TEAB, pH 7.5, overnight. The sample was taken up in the TEAB buffer and loaded on the column. The column was washed with another 20 ml of the initial buffer followed by elution with a 200 ml linear gradient from 0.4 M to 0.7 M TEAB, pH 7.5, at a flow rate of 9 ml/hour. Fractions containing 2.5 ml were collected, and 5 μl aliquots of every third tube were analyzed by liquid scintillation. The major peak was pooled and concentrated to dryness with a flash evaporator at 30°C with a water aspirator vacuum. The residue was suspended in a small volume of water and concentrated again three times. Finally, the residue was suspended in 0.5 ml of 100% ethanol plus 0.5 ml 0.1 M ammonium hydroxide and stored at -20°C. Radiochemical purity was monitored by HPLC.

d. Preparation of IPCPOP from APCPOP by deamination

Commercial APCPOP was deaminated to obtain a small amount of IPCPOP which could be used as an independent standard in the HPLC analysis of IPCPOP synthesized by another method. The deamination procedure is similar to that used in the deamination of [^{3}H]ATP described above. APCPOP (6.5 μmoles) was dissolved in 1.0 ml 2 N formic acid to which was added 0.4 ml of a sodium nitrite solution (800 mg sodium nitrite in 3 ml water). The reaction was allowed to stand at room temperature for 3 hours and then was neutralized with sodium hydroxide. HPLC analysis demonstrated that the reaction was 85-90% complete. One volume of cold absolute ethanol and 91 μl of 25% $BaCl_2$ were added to the reaction mix to precipitate the barium salt of IPCPOP. The precipitate was centrifuged at 500 g for 2 minutes at 4°C in a clinical centrifuge. The pellet was washed once with cold absolute ethanol and dissolved in cold 0.01 N HCl. Sodium sulfate was added to precipitate the barium sulfate which was then removed at 500 g for 5 minutes at 4°C. The supernatant was neutralized with sodium hydroxide, lyophilized and stored dessicated.

B. Materials

1. Commercial

Vacutainers and needles were obtained from Becton-Dickinson, Rutherford, New Jersey. Heparinized microhematocrit capillaries and 0.45 micron filters were purchased from Arthur H. Thomas Co., Philadelphia, Pennsylvania. Molecular seives (3A°, 8-12 mesh, grade 564) were obtained from Fisher Scientific Company, Pittsburgh, Pennsylvania. PM 10 dialysis membranes were purchased from Amicon, Lexington, Massachusetts. Whatman paper 40 and N-triflouroacetyl-6-amino-1-hexanol

phosphate were gifts of Dr. R. Barker, Department of Biochemistry, Michigan State University. Other Whatman filter papers, AS Pellionex SAX and a prepacked Partisil-10 SAX column were obtained from Whatman, Inc., Clifton, New Jersey. Silicon septums for the Perkins-Elmer 1250 HPLC were purchased from Anspec, Ann Arbor, Michigan.

2. Special Preparation of Glassware

Glassware to be silanized was cleaned in an equal volume of ethanol and 2 N KOH. It was rinsed, dried and dipped in 1% dimethyldichlorosilane in carbon tetrachloride for 5 minutes. The glassware was placed in a 150° oven overnight, rinsed with distilled water and dried.

3. Biological

Units of outdated whole blood and random samples for a Caucasian population survey were obtained from the American Red Cross, Lansing, Michigan. Blood from the Od family and Biochemistry personnel was drawn by venipuncture at different times of the day with the aid of vacutainers containing heparin or EDTA. Blood from the Gr family was obtained through Hurley Hospital in Flint, Michigan. Blood samples used in the collaborative study with Dr. J. F. Henderson's laboratory were shipped as packed cells in sealed ampules on dry ice. Blood samples of patients with muscular dystrophy were obtained by Dr. C. Suelter, Department of Biochemistry, Michigan State University.

Partially purified rabbit NTPH (247,000 units/mg) was a gift of S. A. Fuller. Human NTPH was partially purified from out-dated blood by the method of Morris (142). Final specific activity was 3500 units/mg.

C. Methods

1. Analytical

a. NTPH, phosphate, hemoglobin and protein analyses

NTPH catalyzes the general reaction: $NTP + H_2O \rightarrow NMP + PP_i$. According to the method of Chern et al., the pyrophosphate produced during the incubation of NTPH with ITP may be hydrolyzed by yeast inorganic pyrophosphatase (1). The inorganic phosphate thus formed may then be analyzed colorimetrically by the method of Rathbun and Betlach using K_2HPO_4 as a reference standard (154).

Unless otherwise noted, the assay mixtures contained 50 mM β alanine buffer (pH 9.5), 10 mM $MgCl_2$, 1 or 2 mM DTT, 1 unit of yeast inorganic pyrophosphatase, 0.5 mM ITP and the NTPH-containing solution. The use of 2 mM DTT enhanced the reproducibility of the determinations especially when a great number of assays were conducted simultaneously. Control mixtures, one lacking enzyme and another lacking ITP, were incubated concurrently to account for the trace phosphate contamination in both the ITP and NTPH-containing solutions. 0.5 mM ATP was sometimes substituted for ITP to test for the presence of nonspecific phosphatases. All analyses were conducted in triplicate. Following the incubation of these mixes for 20 minutes at 37°C, the reactions were terminated by the addition of 2.2 ml cold 7.27% trichloroacetic acid. The precipitated protein was removed by centrifugation (1000 g for 5 minutes at 4°C) and the supernatants were decanted into phosphate-free test tubes for inorganic phosphate determination. Phosphate analysis was accomplished by adjusting the pH to ca. 4.5 with 1.88 ml 3 M acetate buffer (1:1 mixture of 3.0 M sodium acetate and 3.0 N acetic acid) to which 1/10 part 37% formaldehyde (10 ml formaldehyde for every 90 ml

acetate buffer) had been added to diminish the interference of sulfhydryl reagents in the color development step. Then 0.2 ml 2% ammonium molybdate followed immediately by 0.4 ml 6.75 mM stannous chloride were added to each tube. After 15 minutes the absorbance at 700 nm of each of the solutions was determined with a Gilford 300 micro-sample spectrophotometer.

A unit of NTPH activity is defined as that amount of enzyme which hydrolyzes one nmole of ITP at 37°C in the standard 20 minutes incubation. The specific activity may be expressed as units per mg protein, units per mg hemoglobin, or units per cell.

The hemoglobin concentration in hemolysates was determined by the method of Austin and Drabkin (155). The determinations were facilitated by the use of Hycel cyanmethemoglobin reagent. The absorbance at 540 nm of a 1 mg/ml cyanmethemoglobin solution is 0.718.

Other protein analyses were carried out by the method of Lowry et al. with bovine serum albumin as the reference standard (156).

b. Staining and counting procedures for blood cells

Blood cells were counted at an appropriate dilution with isotonic saline or buffer by means of a counting chamber (improved Neubaur, Scientific Apparatus) and a Zeiss Microscope at 500-fold amplification (Zeiss standard W6 research microscope). If platelets were to be counted, the chamber was placed in a moist atmosphere for 15 minutes to allow the platelets to settle to the bottom of the chamber. Platelets could easily be distinguished from other blood cells by their size. A total of at least 500 cells were counted in two chambers for each sample.

Smears of white cells were prepared by placing a concentrated drop on one end of the slide and tipping the slide to allow the cells to disperse. After the smears air dried, they were stained for 3 minutes with a filtered solution of Wright's stain (diluted with an equal volume of water) followed by a 10 minute treatment with filtered Geimsa stain (diluted 1:20 with water) (157). The slides were washed thoroughly with distilled water and allowed to air dry. The cells were identified by their morphology (158) and counted under oil immersion at 1280-fold amplification. At least 300 cells were counted on each slide. Lymphocytes and monocytes were often difficult to distinguish.

c. Paper chromatography and visualization of spots

Descending paper chromatography of nucleoside derivatives was performed on the full length of Whatman 1 with the solvent, isobutyric acid-1.0 N ammonia-0.1 M EDTA, 100:60:1.6 (v./v.) for 17 hours, and the chromatogram was dried in a hood (159). Ascending paper chromatography was performed on 23 cm Whatman 40 with isobutyrate-conc. ammonium hydroxide-water, 66:1:33, as solvent I; isopropanol-conc. ammonium hydroxide-water, 6:3:1, as solvent II and isopropanol-conc. ammonium hydroxide-water, 8:1:1, as solvent III. These chromatograms were developed for ca. 5 hours.

The UV-absorbing compounds could be observed as dark spots under short wave UV irradiation on the dried chromatograms. The chromatograms could be further developed with a molybdate spray for phosphates (160), a ninhydrin test for amines (161), the Pauly test for imidazoles (162) and a periodate-benzidine spray for the detection of vic-hydroxyls (163).

Phosphate was detected by spraying the chromatograms with Hanes-Isherwood reagent prepared in the following manner: 25 ml 4% ammonium molybdate (w/v) were added to 5 ml 60% perchloric acid (w/w), 10 ml 1.0 N HCl and 60 ml water. The chromatograms were dried in the warm, glassware drying oven and developed under short wave UV light.

A buffered ninhydrin solution was prepared containing 0.5 g ninhydrin, 80 ml ethanol, 20 ml glacial acetic acid and 1 ml collidine. The chromatogram was sprayed and the blue color developed in a warm dark place.

The Pauly test was used by spraying the chromatogram with an equal mixture of 1% sulfanilic acid in 1.0 N HCl and 5% sodium nitrite. After the chromatograms had air dried, they were sprayed with 15% sodium carbonate. An orange or yellow color indicated the presence of imidazole.

Vic-hydroxyls were detected by spraying the chromatograms with 0.5% sodium periodate followed 2 minutes later with a benzidine spray prepared by dissolving 0.5 g 3,3'-dimethoxybenzidine in 20 ml g acetic acid and 80 ml absolute ethanol. Vic-hydroxyls appeared as a white spot on a blue background.

d. UV, IR and ^{13}C-NMR spectrometry

UV spectra were obtained with a Cary Recording Spectrophotometer (Model 15). Routine UV absorbance measurements were obtained with a Gilford 2400S Spectrophotometer or a Beckman DU equipped with a Gilford absorbance indicator.

IR spectra were obtained with a Perkin-Elmer 167 Grating Infrared Spectrophotometer. KBr pellets containing the sample were prepared by

standard techniques after 1 mg of the sample and 80 mg KBr were mixed thoroughly and dried over phosphorous pentoxide.

Proton decoupled ^{13}C-NMR spectra were obtained using a Bruker WP-60 spectrometer, equipped for Fourier transform operation, with 10-mm tubes and a capillary containing D_2O to provide lock. All chemical shifts are given in parts per million relative to an external tetramethylsilane standard. Resolution was 0.733 hertz per point. Samples were prepared by passing them through a small bed of Chelex in the sodium form to remove any divalent metal ions prior to analysis. Each sample was adjusted to a final concentration of 50 mg/ml water at neutral pH and analyzed at 294°K for ca. 16 hours.

e. High pressure liquid chromatography

Nucleotides have been separated by HPLC by means of pellicular anion exchange resins (164), microparticulate anion exchange resins (165) and microparticulate reversed-phase resins (166). The best system in terms of reproducibility and resolution is the microparticulate strong anion exchange resin.

All analyses described here were performed at ambient temperature with a Perkin-Elmer 1250 HPLC equipped with a prepacked Partisil-10 SAX column and UV detector. The buffer reservoir was maintained at 65-70°C to degas the buffer. The pump was routinely operated at 35% capacity giving a flow rate of 55 ml per hour with 600 PSI pressure.

All buffers were prepared with double distilled water, stored in glass containers and filtered through a 0.45 micron filter prior to use. Appropriate solvent systems for a particular separation were determined empirically, but the following guidelines were used. Nucleoside 5'-monophosphates could be separated from each other with 50 mM

KH_2PO_4, pH 3.4; nucleoside 5'-diphosphates could be resolved with 0.2 M KH_2PO_4, pH 3.4, and nucleoside 5'-triphosphates required ca. 0.5 M KH_2PO_4, pH 3.4. This pH was critical for the separation of inosine, adenosine and guanosine derivatives.

Variable amounts of samples up to 10 μliters were injected on the column through a septum injection port by means of a 10 μliter Hamilton syringe. However reproducible quantitative analysis was best achieved by delivering a constant volume of 10 μliters for all samples and standards. Quantitative analysis was performed by standardizing the peak height of a particular nucleotide with known concentrations. The purity of standards or other samples was determined by estimating the peak areas of the mono-, di-, and triphosphate peaks. Standards were analyzed within a few days of the samples in order to minimize small variations in the calibration curve. When appropriate, the detector effluent was collected by means of a fraction collector set in the time mode for 0.5 or 1.0 minutes depending on the resolution desired. To synchronize the collector with the UV recorder, it was necessary to allow a 15 second delay to account for the length of tubing between the detector and collector. Radioactive fractions were counted as described below.

f. Liquid scintillation analysis

All radioactive analyses were conducted using a flourophor solution (Formula B) containing 100 g naphthalene in 400 ml xylenes, 4 g PPO, 50 mg dimethyl-POPOP, 200 mg kerosene and 400 ml Triton X100. For routine analysis 0.4 ml water and 5 ml Formula B were added to each vial containing the radioactive sample. To radioactive fractions of the HPLC effluent containing 0.2 M or more KH_2PO_4, 0.8 ml water and 10

ml Formula B were added to each vial to obtain a clear solution. Vials were counted by means of a Nuclear Chicago Unilux or Beckman LS-230 liquid scintillation counter at efficiencies of ca. 20% and 50% for ^{3}H and ^{14}C nuclides respectively.

g. Computer analysis of data

Lines were fitted to data points by the use of either of two computer programs stored in the CDC 6500 available through a teletype intercom system at Michigan State University. Kinetic data plotted in Lineweaver-Burk graphs were analyzed by the weighted method of Wilkinson (167). Correlation data were analyzed by a linear least squares program.

2. Synthetic Procedures

a. Synthesis of IPCP

The procedure for the synthesis of IPCP involves the condensation of methylene diphosphonic acid with 2', 3'-isopropylideneadenosine by the method of Myers et al. (159). The isopropylidene blocking group was then removed and the product, APCP, was purified by DEAE-Sephadex chromatography. APCP was then deaminated with a solution of nitrous acid to form IPCP (153) which was again purified from the reagents by column chromatography.

All glassware was dried prior to use by heating in a 150°C oven and cooling in a dry box (Lab Con Company). 2.8 g (16 mmoles) methylene diphosphonic acid, 200 ml dry pyridine and 28 ml double distilled tri-*n*-butylamine were mixed in a 500 ml double necked round bottom flask fitted with a drying tube and thermometer. The mixture was stirred and heated to 60°C until a clear solution was obtained. The flask was

cooled to room temperature and 1.24 g (4 mmoles) 2',3'-isopropylideneadenosine and 13 g dicyclohexylcarbodiimide were added. The solution was again heated to 60°C with stirring. After 14 hours the reaction mixture appeared yellow and contained a white precipitate (N,N'-dicyclohexylurea). The slurry was evaporated at 35°C with a flash evaporator on a water aspirator. The gummy solid was taken up in 50-60 ml water and filtered through Whatman 3 paper. The flask and precipitate were rinsed with four 20 ml portions of water which were combined with the other filtrate. The filtrate was filtered again through Whatman 2 and the flask and residue rinsed 3 times with water. This final clear filtrate was extracted with five 100 ml portions of ether. The aqueous layer, a clear yellow liquid, was evaporated at 35°C as before. The syrup was dissolved in water and reconcentrated 3 times with a high vacuum pump attached to the flash evaporator. The final syrup was taken up in 30 ml water, frozen and lyophilized to a yellow solid.

The isopropylidene blocking group was removed by dissolving the product in 10% acetic acid and refluxing this solution for 1 hour. A small aliquot was spotted on filter paper and sprayed for the detection of vic-hydroxyls (see section III, C, 1b). The solution was allowed to cool and was then concentrated with the flash evaporator and vacuum pump as described above. The final syrup was dissolved in 30 ml water and the total yield estimated by the absorbance at 260 nm. The pH was adjusted to 8 with triethylamine, and the solution was diluted to 200-300 ml to dissolve the precipitate that formed. A slight amount of oil remained that could not be dissolved.

A 3.4 x 67 cm DEAE-Sephadex A-25-120 column was prepared by swelling and removal of 'fines' in 0.5 M ammonium bicarbonate. The

resin was then allowed to equilibrate with degassed water and poured into the column. The column was washed with water, loaded with sample and washed with another liter of water at 210 ml/hour. The column was eluted with a 6 liter linear gradient from water to 0.8 M TEAB, pH 7.5 at a flow rate of 216 ml/hour. The effluent was monitored at 254 mn, with the ISCO UA5 UV monitor, and 20 ml fractions were collected. The major peak was further resolved by appropriate dilutions of aliquots and analysis of the absorbance at 260 nm with a Beckman DU. The conductivity of every tenth fraction was tested with a conductivity meter (Radiometer Copenhagen).

The major peak containing APCP was characterized by HPLC retention time (R_T), descending paper chromatography (R_{ADP}) and the stain for the presence of vic-hydroxyls. APCP was concentrated with the flash evaporator at 35°C. The final residue was taken up in water and concentrated at 35°C with the high vacuum pump.

APCP was dissolved in 400 ml 2 N formic acid in a 1 liter round bottom flask. 32 g sodium nitrite dissolved in 120 ml water were added dropwise, and the solution was stirred for 3 hours at room temperature. Progress of the deamination was monitored by HPLC. At the end of three hours the reaction mix was transferred to a 1.5 liter beaker and neutralized with saturated sodium hydroxide. One volume of ethanol (ca. 700 ml) and 24 ml 25% $BaCl_2$ were added to the reaction mix. The barium IPCP precipitate was collected by centrifugation at 400 g for 5 minutes at 4°C and was washed with absolute ethanol. The barium salt was dissolved in 2.5 l of 0.01 N HCl and 16 g sodium sulfate were added. The barium sulfate precipitate was removed by centrifugation as above and the supernatant solution was neutralized with 1.0 N sodium hydroxide. The supernatant solution was concentrated to

about 200 ml by the flash evaporator and filtered through a 0.45 micron filter to remove the remaining barium sulfate. Recovery was estimated by its absorbance at 248.5 nm.

The large DEAE-Sephadex column used for the purification of APCP was washed with water and loaded with IPCP. The column was eluted with 1 liter of water followed by 2 liters 0.6 M TEAB, pH 7.5 at 150 ml/hour. The effluent was monitored and collected as before. The fractions containing the major peak, IPCP, were combined and concentrated as before and characterized by UV absorption and HPLC. A neutralized concentrated solution of IPCP was stable at -20°C indefinitely.

b. Synthesis of IPCPOP

The chemical synthesis of IPCPOP involved the imidazolide-activated condensation of IPCP and orthophosphate by the method of Barker et al. (168). Briefly, the first step involved conversion of IPCP to its methyl-tri-n-octylammonium form followed by synthesis of the IPCP-imidazolide by reaction of IPCP with 1,1'-carbodiimidazole (CDI) under anhydrous conditions. The second step involved reaction of the IPCP-imidazolide with orthophosphate under anhydrous conditions to yield IPCPOP.

IPCP (0.5 mmoles) was passed through a small column of Dowex 50 (H^+). The acidic eluent was combined with 1 mmole methyl-tri-n-octylammonium hydroxide. Ethanol was added to give a clear solution which was then evaporated with the flash evaporator attached to the water aspirator and temperature at 35°C. The syrup was dissolved in dry dimethylformamide (DMF) and concentrated three times by means of the flash evaporator attached to the high vacuum pump. The syrup was redissolved in ca. 5 ml dry DMF.

CDI (2.5 mmoles, 205 mg, m.p. > 105°C) was dissolved in dry DMF and concentrated in a 50 ml round bottom flask containing a stir bar. The CDI was redissolved in 5 ml dry DMF, and the IPCP-containing solution was added in three aliquots over a 15 minute period with constant stirring. The solution was stirred for 30 minutes more after which 2.5 mmoles (45 μliters) water were added. The solution was stirred for a final 30 minutes in the presence of this water to hydrolyze the remaining CDI and prevent the formation of branched structures in subsequent condensations with phosphate. A small aliquot of the reaction mixture was analyzed for the formation of the IPCP-imidazolide by ascending paper chromatography in solvent I. The solution was concentrated twice with dry DMF as before and resuspended in 26 ml DMF.

Phosphoric acid (2.5 mmoles) was added to an equivalent amount of methyl-tri-n-octylammonium hydroxide. This phosphate syrup was concentrated and resuspended in dry DMF three times. The phosphate solution was finally resuspended in 10 ml of dry DMF, and the IPCP-imidazolide was added to the phosphate with a pasteur pipet. The reaction mix was dried twice more with DMF and finally suspended in ca. 20 ml dry DMF, and the mix was stored over phosphorus pentoxide for four days.

To convert the IPCPOP to the ammonium salt, the reaction mix was concentrated to a syrup and dissolved in 50 ml ether followed by the addition of 50 mmoles (3.15 g) ammonium formate in 50 ml water. The aqueous phase containing the IPCPOP ammonium salt was collected with the aid of a separatory funnel and evaporated with the flash evaporator at 30°C. The solid was dissolved in water and reconcentrated to remove the ammonium formate.

A 2.8 x 25 cm DEAE-Sephadex column was prepared as before (see section III, A, 3) and washed with 0.4 M TEAB, pH 7.5. IPCPOP was resuspended in water and adjusted to the same pH and ionic strength as the TEAB buffer and loaded on the column in about 300 ml solution. The column was then washed with 400 ml 0.4 M buffer followed by a 3 liter linear gradient from 0.4 M to 0.8 M TEAB, pH 7.5. The effluent was monitored for UV absorption and conductivity. Fractions containing the major peak, IPCPOP, were combined, concentrated by the flash evaporator at 30°C, and the IPCPOP was converted to its sodium salt in a method similar to that already described for ITP (see section III, A, 3). The IPCPOP sodium salt was characterized by its UV, IR, and ^{13}C-NMR absorption spectra, and the purity of IPCPOP was determined by HPLC and the UV-absorption of a weighed sample.

c. Synthesis of Sepharose-4B covalently attached to IPCPOP

Preparation of an affinity resin coupling IPCPOP through the γ-phosphate to a six carbon spacer molecule covalently attached to Sepharose-4B was accomplished by the method of Barker et al. (168, 169). The N-triflouroacetyl-o-phosphoryl 6-amino-1-hexanol imidazolide was prepared and reacted with IPCP to form γ-(N-triflouroacetyl-6-aminohexyl)-IPCPOP which was then purified by column chromatography. After the triflouroacetyl blocking group had been removed, the resulting γ-(6-aminohexyl)-IPCPOP was reacted with cyanogen bromide-activated Sepharose-4B (170).

N-triflouroacetyl-6-amino-1-hexanol phosphate (TFA-HAP) was purified by dissolving it in water and passing it through a 2.2 x 10 cm Dowex 50 x 8 (20-50 mesh, H^+) column. The acidic effluent was collected and concentrated by flash evaporation at 35°C. The compound was

estimated to be pure by ascending paper chromatography in solvent II. TFA-HAP was dried twice with dry DMF using the flash evaporator at 40°C attached to a high vacuum pump. 234 mg (~0.75 mmoles) of the syrup were dissolved in DMF, dried with DMF as above and resuspended in 2 ml dry DMF.

CDI (3 mmoles, 487 mg) was dissolved and stirred in 5 ml dry DMF to which the TFA-HAP prepared above was added in two aliquots 10 minutes apart. After 30 minutes 3 mmoles (54 µliters) water were added, and the solution stirred for 20 minutes more. The formation of the imidazolide was demonstrated by ascending paper chromatography in solvent III. The compound was concentrated, dried twice with DMF as before and redissolved in ca. 3 ml distilled hexamethylphosphoramide (HMPA).

IPCP (0.5 mmoles) was converted to its methyl-tri-n-octylammonium form and dried with DMF as described (see section III, C, 2b). The IPCP was then dissolved in ca. 3 ml HMPA and combined with the TFA-HAP imidazolide. The reaction mixture was placed in vacuo over phosphorus pentoxide for 3 days.

A 1.3 x 45 cm Dowex 1x2 (200-400 mesh, Cl^-) was prepared in 50% ethanol. The HMPA containing the product was diluted 4-fold with 50% ethanol and loaded on the column. The column was washed with 250 ml 50% ethanol followed by a 1 liter linear gradient from 0.01 N HCl to 0.01 N HCl containing 1.0 M LiCl. The effluent was monitored with an ISCO UA5 UV monitor at 254 nm. and collected in 15 ml fractions. The tubes containing the major peak were combined and the solution was neutralized with LiOH and concentrated to ca. 30 ml.

To remove the TFA blocking group, the solution was adjusted to ca. pH 14 with LiOH and stirred for 2-3 days. HPLC was used to monitor the

course of the reaction, and the final product, γ-(6-aminohexyl)-IPCPOP was ninhydrin positive.

A 3.4 x 40 cm Bio-Gel P2 column (200-400 mesh) was prepared and washed with 50 mM TEAB, pH 7.5 at a flow rate of 60 ml/hour. Thirty ml of the γ-(6-aminohexyl)-IPCPOP solution was carefully layered on the column and eluted with the 50 mM TEAB buffer. The effluent was monitored for UV-absorbing material and conductivity. The desalted fractions containing the UV peak were concentrated and dissolved in water to a final concentration of 10 mM γ-(6-aminohexyl)-IPCPOP and cooled to 4°C.

'Fines' were removed from Sepharose-4B, and 10 ml of the resin were combined with 20 ml 2.5 M potassium phosphate, pH 12.0. The Sepharose-4B was cooled to 3°C and stirred while cyanogen bromide (1 g/ml acetonitrile) was rapidly dispersed into the solution with a syringe. The reaction was allowed to proceed exactly 8 minutes, after which the mix was filtered rapidly through a scintered glass funnel. The resin was washed immediately with 200 ml cold water (within 1.5 minutes total), and the cake was added to the cold γ-(6-aminohexyl)-IPCPOP and adjusted to pH 9.5 with concentrated NaOH. The Sepharose-4B was stirred overnight, after which it was stored in the reaction mix without stirring for two days. The UV absorbance of the supernatant was determined to calculate by difference the amount of γ-(6-aminohexyl)-IPCPOP coupled to the resin.

3. General Procedures

a. Preparation of hemolysates

For routine analysis of NTPH whole blood was centrifuged at 4000 *g* for 5 minutes at 4°C to sediment the cells. The plasma and buffy

coat were removed by aspiration, and the red cells washed once or twice with 3-5 volumes 0.9% saline and collected by centrifugation. The washings were discarded and the packed cells lysed with 9 volumes cold 1 or 2 mM DTT. The debris was removed by centrifugation at 30,000 g for 10-15 minutes and the top half of the supernatant was used for NTPH and hemoglobin analyses. In some special cases the lysate was prepared more concentrated or dilute than that described here.

For analysis of the cell-free synthesis of ITP, the procedure was modified in that the packed cells were lysed with an equal volume of cold water for 10 minutes. In some experiments, lysates prepared in this manner were given one to three treatments of Norit A (10 mg/ml) for 5 minutes to remove endogenous nucleotides. The charcoal was removed by centrifugation at 30,000 g for 10 minutes at 4°C.

b. Blood cell separations and sonication procedures

Cell separations methods have been reviewed by various authors (171-174). A combination of a few of these methods was used to separate blood components for the comparison of NTPH activity in platelets, granulocytes, lymphocytes and erythrocytes.

Only carefully cleaned silanized glassware (see III, B, 2) or plastic materials were used in these experiments. Twenty ml of blood was drawn fresh daily by venipuncture using EDTA-containing vacutainers. Two ml of 3.8% sodium citrate, pH 7.4, was mixed with 10 ml of whole blood, and platelet rich plasma (PRP) was obtained by centrifugation of the blood at 740 g_{av} in a swinging bucket rotor (IEC Model HN centrifuge) for 5 minutes at room temperature. The PRP was removed with a pipet and the remaining cells were washed twice with a buffered salt solution (BSS) containing .01% glucose, 5 μM $CaCl_2$, 98 μM $MgCl_2$, 0.54

mM KCl, 1 mM sodium EDTA, 5 mM β-mercaptoethanol, 14.5 mM Tris and 126 mM NaCl at pH 7.4. The cells were centrifuged as before, and the two washings were combined with the PRP to obtain the platelet fraction of the blood. After the number of platelets was determined in this solution (see III, C, 1b), the platelets were cooled to 4°C for 2 hours and then centrifuged at 35,000 g for 15 minutes. The supernatant was carefully removed by aspiration; and the pellet was stored at 4°C until it could be sonicated.

White cells were separated from erythrocytes by dextran sedimentation (172). Following the removal of the platelets, the blood cells were adjusted to 18 ml with BSS and mixed with 3.6 ml 6% dextran prepared in BSS. With the tube placed in a vertical position, the red cells were allowed to settle for 45 minutes at room temperature leaving a leukocyte-rich supernatant. The white cells were collected by centrifuging this supernatant at 740 g_{av} for 5 minutes at room temperature and resuspended gently with the aid of a pasteur pipet in 5 ml fresh BSS. An aliquot of erythrocytes from the bottom of the tube was washed three times with BSS. The cells were collected each time by centrifugation at 740 g_{av} for 5 minutes at room temperature. An appropriate dilution of the cells were counted and the remainder were centrifuged as above, the supernatant was removed and the red cells were placed at 4°C.

The lymphocytes were separated from the granulocytes by the Isopaque-Ficoll method of Boyum (174). Two ml of Ficoll-paque were placed in each of two silanized glass ignition tubes. Each of these was carefully layered with 2.5 ml of the suspended white cells and centrifuged at 400 g_{av} for 30 minutes at room temperature in a swinging

bucket rotor. The interphase between the BSS and Ficoll-paque was collected with a pasteur pipet and washed three times with at least 3 volumes BSS. The cells were collected by centrifugation at 740 g_{av} each time for 5 minutes at room temperature. These cells found at the interphase were characterized by a staining technique (see III, C, 1b) and suspended in 1 ml BSS for counting. The cells, predominately lymphocytes and monocytes, were again centrifuged as above, the supernatant was carefully removed and the pellet placed at 4°C prior to sonication.

The red and white cells collected at the bottom of the Ficoll-paque were washed three times with 0.87% NH_4Cl to lyse the remaining erythrocytes. Each time the cells were collected by centrifugation at 740 g_{av} for 5 minutes at room temperature. The last pellet was characterized by a staining procedure (see III, C, 1b) and suspended in 2.0 ml BSS for counting. The cells, predominately granulocytes, were centrifuged as before, the supernatant was removed and the pellet was placed at 4°C.

The various fractions were sonicated to insure that all the cells were lysed completely. The erythrocyte pellet was suspended in ca. 9 volumes cold 2 mM DTT. The granulocyte and lymphocyte pellets were each suspended in 1 ml cold 2 mM DTT and the platelet pellet was suspended in 2 or 4 ml cold 2 mM DTT. These four suspensions were sonicated at 4°C with two 15 second bursts delivered with a microprobe powered by a Biosonik sonicator at 50% capacity (Model BIO III, Bronwill Scientific). The debris was removed by centrifugation at 30,000 g for 20 minutes at 4°C. The volumes of the granulocyte, lymphocyte and platelet debris were considered negligible but the volume of the erythrocyte lysate was measured since considerable debris was present.

All NTPH analyses were performed on the same day that the blood was drawn. Both 0.5 mM ITP and 0.5 mM ATP were used in all NTPH analyses to determine the enzyme activity specific for the hydrolysis of ITP. NTPH and protein analyses have been described previously (see III, C, 1a).

c. Henderson's methods for the accumulation of [^{14}C]ITP in intact erythrocytes

Henderson's procedures for the analysis of [^{14}C]ITP in intact erythrocytes have been described in detail (5, 72). Briefly his procedure involves the following methods. Erythrocytes, 2% suspensions in modified Fisher's medium, were incubated at 37°C for 2 hours in the presence of 100 μM [^{14}C]hypoxanthine. Neutralized perchloric acid extracts were prepared and separated by one-dimensional, polyethylenimine-cellulose, thin-layer chromatography. Areas containing IMP, IDP and ITP were cut out and the radioactivity analyzed. [^{14}C]ITP accumulation was reported to be linear for two hours with negligible radioactivity accumulating in IDP.

d. Analysis of nucleotides in blood or lysates

Various procedures for the extraction of free nucleotides from cells have been reviewed by Mandel (175). P. R. Brown has compared some of these techniques for use with HPLC (176-178). Perchloric acid extraction followed by neutralization with potassium hyroxide was used successfully to determine the ITP concentration in fresh blood. Trichloroacetic acid extraction followed by neutralization with Tris was most convenient for the determination of the cell-free synthesis of ITP in hemolysates. Neutralized extracts containing nucleotides are stable for months at -20°C (176, 179).

Twenty ml of blood were drawn by venipuncture into a heparinized vacutainer. The hematocrit was determined by filling a small hematocrit capillary, plugging its end with a seal, centrifuging it in an IEC MB centrifuge for 5 minutes and measuring the percentage of packed cells to total volume. Fifteen ml of whole blood measured in a marked silanized tube were mixed with 50 µliters of a [^{3}H]ITP internal standard (see III, A, 4c) and combined with 30 ml 0.6 N perchloric acid in a 50 ml centrifuge tube. The precipitated protein was stirred vigorously at 4°C for 5 minutes and removed by centrifugation at 35,000 g for 10 minutes at 4°C. The supernatant was filtered through Whatman 1 paper and neutralized with ca. 9 ml 10% KOH. The potassium perchlorate salt was removed by centrifugation at 20,000 g for 10 minutes at 4°C. The pH was adjusted to ca. 7.5 and the extract was lyophilized to dryness. The solid was taken up in 10 ml cold water, centrifuged and lyophilized a second time. If necessary this process was repeated to obtain a precipitation free solution with a total volume of ca. 1 ml. The exact volume was noted and 10 µliter aliquots were analyzed by HPLC, and the effluent of the HPLC in the ITP region was monitored for radioactivity (see III, C, 1e). The neutralized extract was stored at -20°C with very little decomposition in a month.

Reaction mixtures for synthesis of ITP in cell-free hemolysates were extracted with two volumes ice cold 12% tricholoacetic acid (TCA); usually 0.1 ml of the reaction mix was added to 0.2 ml 12% TCA. The precipitate was removed by centrifugation at 20,000 g for 5 minutes at 4°C. A 100 µliter aliquot of the supernatant was neutralized with 7 mg Tris and stored at -20°C until it was analyzed by HPLC (see III, C, 1e).

e. Determination of K_m and K_i for NTPH

Methods for the determination of enzyme kinetic constants have been reviewed by Segel (180). For K_m or K_i determinations, either lysates or partially purified enzymes were used at a concentration which gave a maximum NTPH activity of 10 to 20 units. The method of Lee and Wilson was used to compensate for the significant utilization of substrate at low substrate concentrations (181). Thus the average substrate concentrations were determined by averaging each initial and final concentration ($\bar{S}$), and the values so obtained were substituted for the initial substrate concentrations in the determination of the K_m by Lineweaver-Burk plots. The K_m for a set of $\bar{S}$ and "initial" velocity pairs was determined by a computer program using the method of Wilkinson (167, see also III, C, 1g) and plotted in the form, $1/\bar{S}$ vs. $\frac{1}{v_i}$. Similarly Lineweaver-Burk plots were obtained at four different inhibitor concentrations. The apparent K_m values from each of these analyses were plotted against the inhibitor concentration of each, and the K_i was determined as the negative of the intercept on the inhibitor axis. This method of analysis was valid since the inhibition was shown to be competitive.

f. Affinity chromatography of NTPH

The techniques for the practical application of affinity chromatography have been reviewed by Lowe and Dean (182). The following procedure provided the best combination of conditions tested for the purification of NTPH by means of the IPCPOP-Sepharose affinity resin (see III, C, 2c). All procedures were conducted at 4°C. The NTPH-containing solution (15 ml of a 1:4 lysate) was dialyzed against 1500 ml containing 50 mM Tris-Cl, pH 7.4, 1 mM $MgCl_2$ and 1 mM GSH. A 0.9 x

6.5 cm affinity column was prepared with this buffer, and the sample was loaded at ca. 60 ml/hour. Following elution of the major peak of protein, an additional 40 ml buffer were passed through the column. The NTPH was eluted by allowing the resin to equilibrate with 1.5 ml buffer containing 10 mM $MgCl_2$, 10 mM ITP, 1 mM GSH and 50 mM Tris-Cl, pH 7.4, for 30 minutes followed by washing the column with ca. 40 ml of the initial buffer.

The protein solutions were concentrated and separated from ITP by pressure dialysis using an Amicon Model 52 apparatus with a 43 mm PM 10 ultrafiltration membrane. The concentrated solution was resuspended in initial buffer and reconcentrated two additional times to remove the ITP. The NTPH and protein were assayed by procedures described before (see III, C, 1a).

g. Cell-free synthesis of ITP

Hemolysates were prepared as described (see III, C, 3a). Incubations were carried out in 10 x 75 mm culture tubes and included 0.2 ml of the lysate, 50 mM sodium phosphate buffer, pH 7.0, and other substances indicated. When Na_4 PRPP was substituted for Mg_2PRPP, an additional 5 mM $MgCl_2$ was added to the mixes. The reactions were carried out for 3 hours with aliquots removed and extracted (see III, C, 3d) at indicated time points. HPLC analysis of the nucleotides in these extracts has previously been described (see III, C, 1e).

CHAPTER IV

RESULTS

A. Genetic Variability of NTPH

1. Population Distribution

The population study presented in Figure 3 was performed by S. A. Fuller, a graduate student in genetics. A random survey of NTPH specific activity in the erythrocytes of a Caucasian population (262 individuals) in the mid-Michigan area was conducted over a two month period. At least two different populations of individuals are readily apparent from this distribution. The specific activity of the low NTPH group, 18% of the population, ranges from undetectable levels to 27.5 nmoles ITP cleaved/mg hemoglobin. The high NTPH group, 82% of the population, includes specific activities that range from 27.5 to 125 nmoles ITP cleaved/mg hemoglobin. Other subgroups are suggested by these data, but whether or not they are indeed distinct classes must await confirming evidence by an independent method of analysis.

These results are comparable with the overall characteristics of the population survey for erythrocyte "ITPase" conducted by Vanderheiden (3). His population data was also resolved into two classes with the lower, 19% of the population, ranging from undetectable to 180 μmoles P_i released/hour/g hemoglobin and the upper, 81% of the population, ranging from 180 to 720 μmoles P_i released/hour/g hemoglobin. Since Vanderheiden also presented some evidence that the "ITPase" was

Figure 3. A Random Survey of NTPH Specific Activity in the Red Cells of a Caucasian Population

The NTPH activity of a red cell lysate of each individual was analyzed in triplicate under standard assay conditions with ITP as the substrate. A unit of NTPH activity is defined as that amount of the enzyme which hydrolyzes 1 nmole ITP in 20 minutes at 37°C under standard assay conditions.

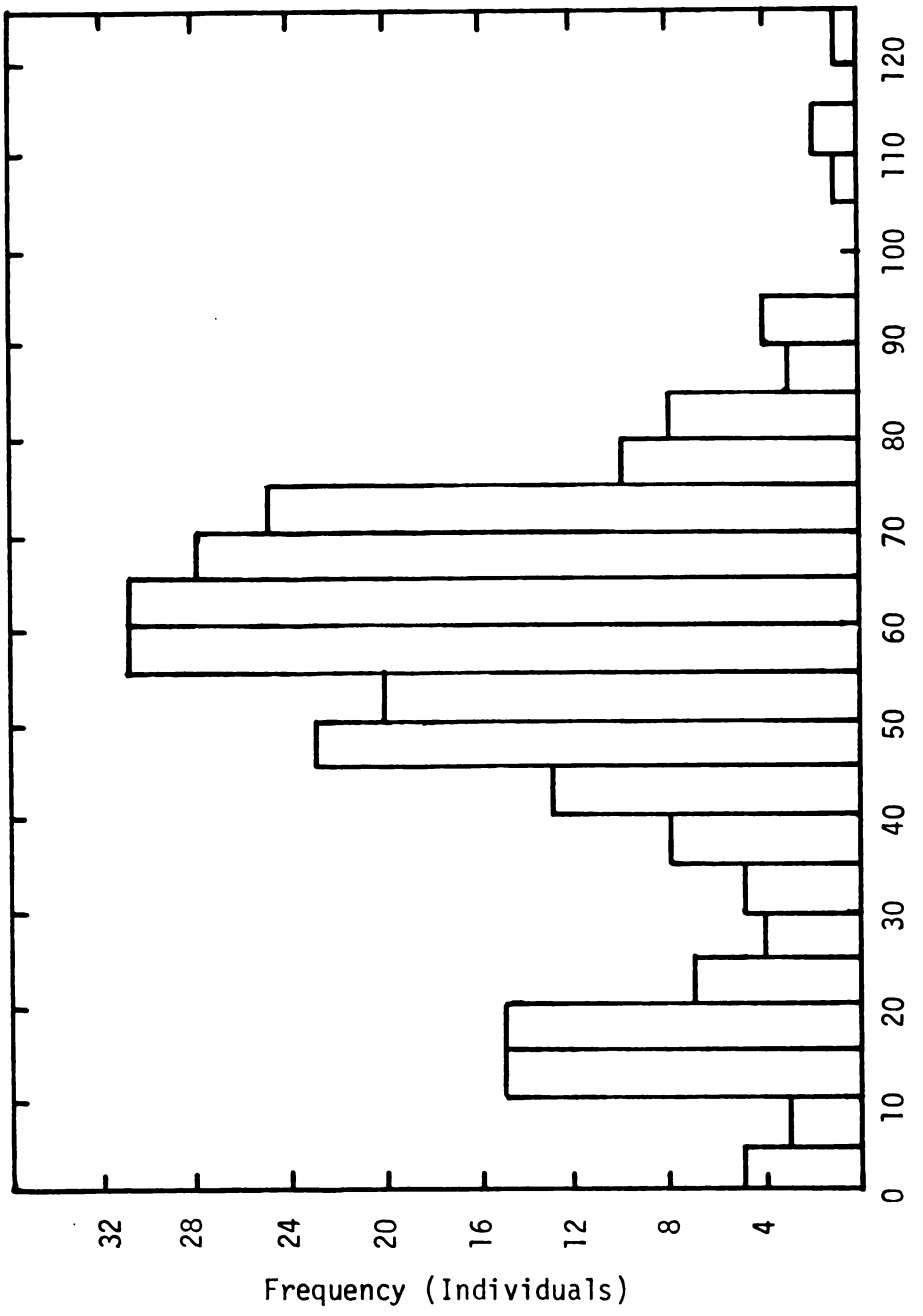

Figure 3. A Random Survey of NTPH Specific Activity in the Red Cells of a Caucasian Population

inherited as a codominant trait in human erythrocytes, we further pursued the analysis of NTPH in related individuals.

2. Inheritance of NTPH in Two Families

Two interesting family studies were conducted early in the investigation of the inherited variability of NTPH in red cells (Figure 4). The first is a family (Od) with two parents who have relatively high specific activities. The children in this family also have high NTPH levels. The second family study (Gr) demonstrates parents who have very low to undetectable levels of NTPH. Four of the progeny also have very low levels of NTPH while the other has a comparatively high NTPH level. However, this latter person was receiving blood transfusions at the time of the analyses; thus the relatively high NTPH level determined for red cells drawn from him may not be characteristic of red cells produced by his own marrow. To verify the NTPH level of the members of the Gr family, the analyses were repeated by S. A. Fuller; these results are also shown in Figure 4. Blood from more families is currently being collected to define more clearly the inheritance of the red cell NTPH specific activity.

3. Mixing Study with Hemolysates of Red Cells of Selected Individuals

To determine whether intracellular inhibitors or activators of NTPH are present in the red cell, equal volumes of different hemolysates were mixed to observe if the NTPH activities were additive. As can be seen in Table 1, three of the four experiments gave additive results, while the other experiment indicated a significant difference between the observed and expected result with the observed 26% less than the expected. Since experiment 3 was essentially repeated in experiment 4

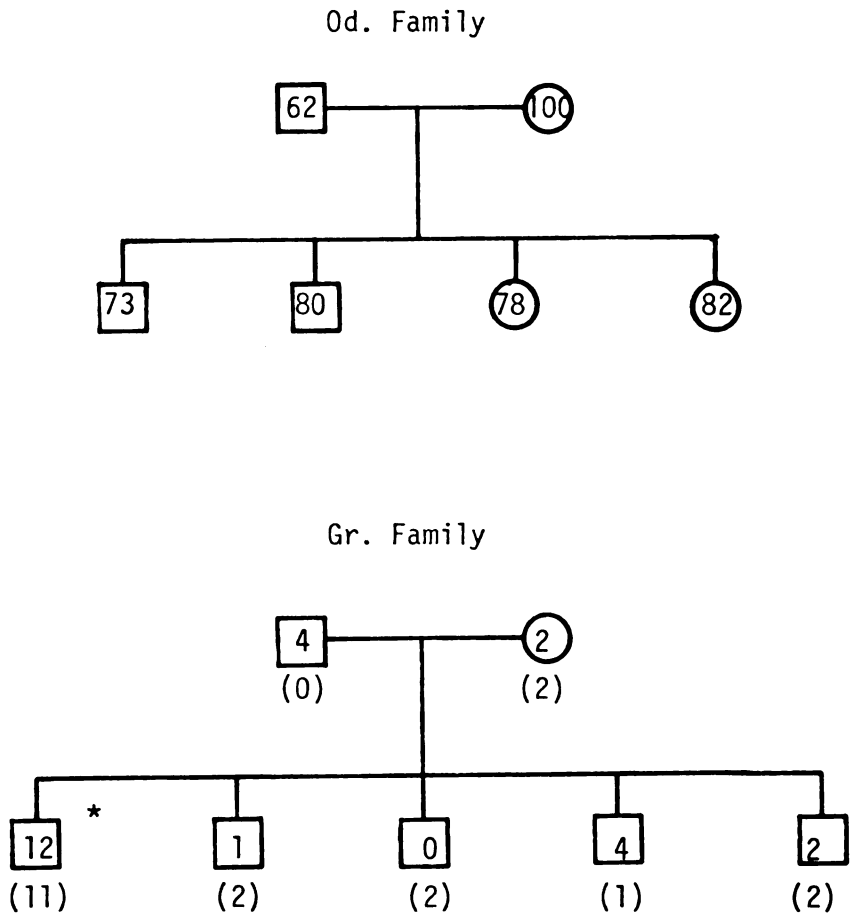

Figure 4. NTPH Specific Activity in the Red Cells of Members of Two Families.

The numerical values represent the NTPH activity expressed as nmoles ITP cleaved/mg hemoglobin under standard conditions. The parenthetical values are the results of an independent NTPH analysis.

*This individual was receiving periodic blood transfusions at the time his blood was drawn for this NTPH analysis.

Table 1. Study of NTPH Activity in Mixed Hemolysates*

Experiment	Lysate Source	NTPH Activity Expected	NTPH Activity Observed	Student t Test
1	VLV + MG	298	335	N.S.
2	SF + MG	484	354	N.S.
3	AJM + MG	777	575	$.05 > p' > .01$
4	JOD + MG	942	873	N.S.

* Hemolysates (1:10) of blood of five individuals were prepared, analyzed for NTPH activity separately and analyzed in equal volume mixtures as indicated in the table. Each analysis was done in triplicate and all experiments were performed in one day. The expected and observed activities are expressed in units/ml lysate. NTPH specific activities (nmoles ITP cleaved/mg hemoglobin) of the subjects studied were as follows: VLV, 13; SF, 31; AJM, 69; JOD, 64 and MG, 6.

with negative results, the search for intracellular effectors of NTPH by this method was not continued.

Neither were effectors detected by studies in which partially purified NTPH was mixed with lysates of blood cells of selected individuals (5).

4. The Michaelis Constant for ITP of NTPH in Hemolysates of Selected Individuals

A study was undertaken to determine if the K_m of NTPH was the same in NTPH-containing lysates of red cells of selected individuals with greatly different NTPH specific activities. The data presented in Table 2 indicates that no correlation can be observed between the NTPH specific activities and the respective K_m values among these individuals. The variability of the K_m that is reflected in the high standard deviations is probably due to the technical difficulty of getting good kinetic data at low enzyme activity in hemolysates.

Table 2. The Michaelis Constant (K_m) of NTPH of Human Red Cell Lysates from Selected Individuals*

NTPH Specific Activity (Units/mg Hemoglobin)	$K_m \pm$ S.D. ($\times 10^5$ M)
12	3.1 ± 0.63
17	1.5 ± 0.80
18	2.4 ± 0.26
28	2.5 ± 0.33
36	4.4 ± 0.49
46	1.2 ± 0.42
49	2.5 ± 0.26
70	3.7 ± 0.32
84	2.5 ± 0.52
106	5.1 ± 0.56
124	2.0 ± 0.34

* The NTPH specific activity for the red cells of each individual was determined in triplicate under the standard assay conditions. The initial velocity of NTPH in lysates was determined for 12 to 14 ITP concentrations over the range, 10-500 μM. K_m values were determined by using average substrate concentrations and a computer program of the method of Wilkinson (see Methods).

5. A Comparative Study of NTPH Activity Found in Erythrocytes, Granulocytes, Lymphocytes and Platelets

Since NTPH activity had previously been found in all tissues examined of the rabbit and rat (2,144), it was of special interest to determine whether the genetic variability expressed in human red cells was reflected in other human tissues. Thus procedures were developed to separate blood cells into defined populations and to analyze each type for NTPH activity.

The complete characterization of cell populations and NTPH analyses of erythrocytes, granulocytes, lymphocytes and platelets from selected individuals are listed in Tables 3, 4, 5 and 6, respectively. The mean cell hemoglobin concentration in the 19 erythrocyte analyses was 2.90 ± 0.071 (S.E.) mg hemoglobin/10^8 cells, and the mean red cell protein concentration as determined by the Lowry method in 17 of these samples was 3.87 ± 0.082 (S.E.). These results indicate good internal consistency in the methodology over the two month period that these analyses were performed. One can see that individuals were selected to represent an NTPH range of 3 to 97 nmoles ITP cleaved/mg hemoglobin. The units of activity/10^7 cells were adjusted for nonspecific ITP hydrolysis (0-27%) measured by substituting ATP for ITP in the reaction mix.

The granulocyte populations were composed of 92-99% granulocytes and 1-8% lymphocytes and contained an average of 5.10 ± 0.35 (S.E., N=17) mg protein/10^8 cells. The units/10^7 cells were adjusted for activity due to platelet contamination (0-11%) and for nonspecific hydrolysis of ITP (0-39%).

The lymphocyte populations were composed of 84-95% lymphocytes, 2-14% monocytes and 1-12% granulocytes. The cells had an average 6.5 ± 0.37 (S.E., N=16) mg protein/10^8 cells. The units/10^7 cells were adjusted for activity due to platelet contamination (0-5%) and for nonspecific hydrolysis of ITP (0-29%).

Platelet preparations were contaminated with activity, 2-10%, and protein, 1-8%, due to large cells. The average amount of protein per cell was calculated as 0.174 ± 0.0078 (S.E., N=17) mg protein/10^8 cell.

Table 3. Characterization and NTPH Analyses of Populations of Erythrocytes

Subject	NTPH Specific Activity				Protein/Cell	
	Units[a] / mg Hb	ATP Units[b] / 10^7 Cells	Net Units[c] / 10^7 Cells	Net Units[c] / mg Protein	mg Hb / 10^8 Cells	mg Protein / 10^8 Cells
VLV	14	0.34	3.7	9.8	2.94	3.77
AJM	76	0.71	20.6	53	2.80	3.90
DL	92	0	19.8	71	2.16	2.77
CV	33	0.18	11.1	25	3.39	4.46
SF	32	0.02	8.2	24	2.60	3.40
VLV	16	0	4.2	12	2.69	3.48
AJM	64	0.43	16.3	45	2.60	3.60
SF	28	0.08	7.3	20	2.68	3.70
DL	97	0.86	21.0	70	2.25	3.01
CV	35	0	11.2	25	3.19	4.46
SF	36	0.10	8.6	-	2.38	-
VLV	15	0.08	5.2	-	3.61	-
BH	18	0.37	3.5	11	2.10	3.06
CV	53	0	15.3	36	2.88	4.24
SF	43	0.16	13.0	30	3.07	4.32
DY	3	0.26	0.71	1.8	3.03	3.92
SF	44	0.25	12.3	32	2.86	3.84
CV	37	0.02	10.6	30	2.89	3.55
AJM	76	0	21.2	59	2.80	3.59

Activity was determined in triplicate for each sample under standard NTPH assay conditions and was calculated as (a) nmoles ITP hydrolyzed, (b) nmoles ATP hydrolyzed and (c) nmoles ITP hydrolyzed - nmoles ATP hydrolyzed. Protein was determined in triplicate for each sample by the cyanmethemoglobin or Lowry methods (see Methods). Aliquots of cells to be analyzed were diluted and counted with the aid of a counting chamber. The individuals are listed in the chronological order that their cells were analyzed.

Table 4. Characterization and NTPH Analyses of Populations of Granulocytes

Subject	NTPH Specific Activity			Characterization			
	ATP Units[a] / 10^7 Cells	Net Units[b] / 10^7 Cells	Net Units[b] / mg Protein	mg Protein / 10^8 Cells	Gran. %	Lym. %	Plat.c %
VLV	0	56	162	3.45	92	8	11
AJM	14	97	257	3.79	95	5	2
DL	5	163	580	2.80	97	3	2
CV	0	51	88	5.76	97	3	4
SF	0	101	160	6.32	95	5	1
VLV	0	60	138	4.33	-	-	2
AJM	13	112	364	3.10	98	2	2
SF	0	150	211	7.10	98	2	0
DL	0	180	236	7.61	99	1	1
CV	2	132	210	6.28	99	1	0
SF	0	101	-	-	98	2	-
VLV	0	46	-	-	97	3	-
BH	11	65	126	5.15	97	3	2
CV	0	117	181	6.47	99	1	0
SF	15	128	192	6.70	95	5	1
DY	11	17	41	4.19	99	1	0
SF	27	139	280	4.96	97	3	0
DV	12	87	176	4.96	99	1	1
AJM	6	201	543	3.69	99	1	0

Activity was determined in triplicate for each sample under standard NTPH assay conditions and was calculated as (a) nmoles ATP hydrolyzed and (b) nmoles ITP hydrolyzed - nmoles ATP hydrolyzed. Protein was determined in triplicate by the Lowry method (156). Aliquots of cells to be analyzed were diluted and counted with the aid of a counting chamber and a representative aliquot of each population was stained with Wright and Geimsa stains. The types of cells were classified by their morphology (see Methods for further details). The platelet contamination (c) is expressed as the percent of the total NTPH activity which was contributed by the platelets. The individuals are listed in the chronological order that their cells were analyzed and correspond to the individuals listed in Table 3.

Table 5. Characterization and NTPH Analyses of Populations of Lymphocytes

Activity was determined in triplicate for each sample under standard NTPH assay conditions and was calculated as (a) nmoles ATP hydrolyzed and (b) nmoles ITP hydrolyzed-nmoles ATP hydrolyzed. Protein was determined in triplicate by the Lowry method (156). Aliquots of cells to be analyzed were diluted and counted with the aid of a counting chamber and a representative aliquot of each population was stained with Wright and Geimsa stains. The types of cells were classified by their morphology (see Methods for further details). The platelet contamination (c) is expressed as the percent of the total NTPH activity which was contributed by the platelets. The individuals are listed in the chronological order that their cells were analyzed and correspond to the individuals listed in Table 3.

Table 5. Characterization and NTPH Analyses of Populations of Lymphocytes

Subject	NTPH Specific Activity			Characterization				
	ATP Units[a] / 10^7 Cells	Net Units[b] / 10^7 Cells	Net Units[b] / mg Protein	mg Protein / 10^8 Cells	Lym. %	Mono. %	Gran. %	Plat.c %
VLV	29	100	179	5.58	84	14	2	5
AJM	59	327	788	4.14	95	4	2	0.5
DL	11	389	767	5.07	90	9	1	1
CV	0	167	224	7.46	84	11	5	1
SF	20	234	285	8.22	90	7	3	0
VLV	0	204	240	8.49	-	-	-	2
AJM	45	394	786	5.00	91	5	4	0.5
SF	-	-	-	-	-	-	-	-
DL	27	363	593	6.11	90	6	4	1
CV	37	292	321	9.10	84	4	12	1
SF	7	316	-	-	90	8	2	-
VLV	14	136	-	-	90	5	5	-
BH	20	217	328	6.60	78	10	12	4
CV	24	407	499	8.15	93	3	4	0.5
SF	32	370	482	7.68	96	2	2	0.5
DY	37	91	148	6.16	95	3	2	1
SF	82	267	458	5.83	89	5	6	2
CV	33	337	525	6.42	93	5	2	1
AJM	36	462	985	4.68	90	5	5	0

Table 6. Characterization and NTPH Analyses of Populations of Platelets

Activity was determined in triplicate for each sample under standard NTPH assay conditions and was calculated as (a) nmoles ATP hydrolyzed and (b) nmoles ITP hydrolyzed-nmoles ATP hydrolyzed. Protein was determined in triplicate by the Lowry method (156). Representative aliquots of cells to be analyzed were diluted and counted with the aid of a counting chamber. The large cell contamination (c) is expressed as either the percent activity or percent protein contributed by these cells. The individuals are listed in the chronological order that their cells were analyzed and correspond to the individuals listed in Table 3.

Table 6. Characterization and NTPH Analyses of Populations of Platelets

Subject	NTPH Specific Activity			Characterization		
	ATP Units[a] / 10^7 Cells	Net Units[b] / 10^7 Cells	Net Units[b] / mg Protein	mg Protein / 10^8 Cells	Large Cell[c] Activity (%)	Large Cell[c] Protein (%)
VLV	1.11	2.5	128	0.20	2	1
AJM	0.64	5.4	456	0.12	2	1
DL	0.88	7.2	398	0.18	5	3
CV	0.59	4.1	254	0.16	2	3
SF	0.93	4.0	229	0.18	10	8
VLV	0.83	3.2	138	0.23	6	4
AJM	0.99	6.0	398	0.15	5	3
SF	0.52	3.9	252	0.15	10	7
DL	1.00	6.2	294	0.21	6	4
CV	0.79	4.2	250	0.17	3	2
SF	-	-	-	-	-	-
VLV	-	-	-	-	-	-
BH	0.96	4.0	173	0.23	2	1
CV	0.54	4.7	309	0.15	7	4
SF	1.22	5.6	282	0.20	8	4
DY	0.74	1.1	68	0.17	8	4
SF	1.22	5.1	295	0.17	8	5
CV	0.77	4.2	269	0.16	3	2
AJM	0.68	6.0	465	0.13	4	2

The units/10^7 cells were adjusted for nonspecific hydrolysis of ITP (9-40%).

The variation of NTPH activity in human red cells is correlated with the activity in human granulocytes, lymphocytes and platelets in Figures 5, 6 and 7, respectively. In each case there is a significant positive correlation ($p<.01$) of activities indicating that the variation is reflected in each of these cell types. It should also be noted that the activity per cell and the range of the variation in each type is quite different. In the individuals tested in this study the erythrocytes ranged from 0.7 to 21 units/10^7 cells, the granulocytes from 17 to 201 units/10^7 cells, the lymphocytes from 91 to 462 units/10^7 cells and the platelets from 1.1 to 7.2 units/10^7 cells.

These data provide evidence that the inherited variability of NTPH activity of the red cell demonstrated by the population survey and family studies in Figures 3 and 4 is also expressed in other blood cell types.

6. NTPH Activity in the Red Cells of 13 Patients with Muscular Dystrophy

Blood from 13 patients with various forms of muscular dystrophy became available through the laboratory of Dr. C. Suelter, Michigan State University. The NTPH activities of these people, listed in Table 7, exhibited little deviation from the activity found in the general population (Figure 3).

B. Relationship Between NTPH and the Accumulation of [^{14}C]ITP in Intact Erythrocytes

It came to our attention that Dr. J. F. Henderson of the University of Alberta was interested in the basis for the variation of

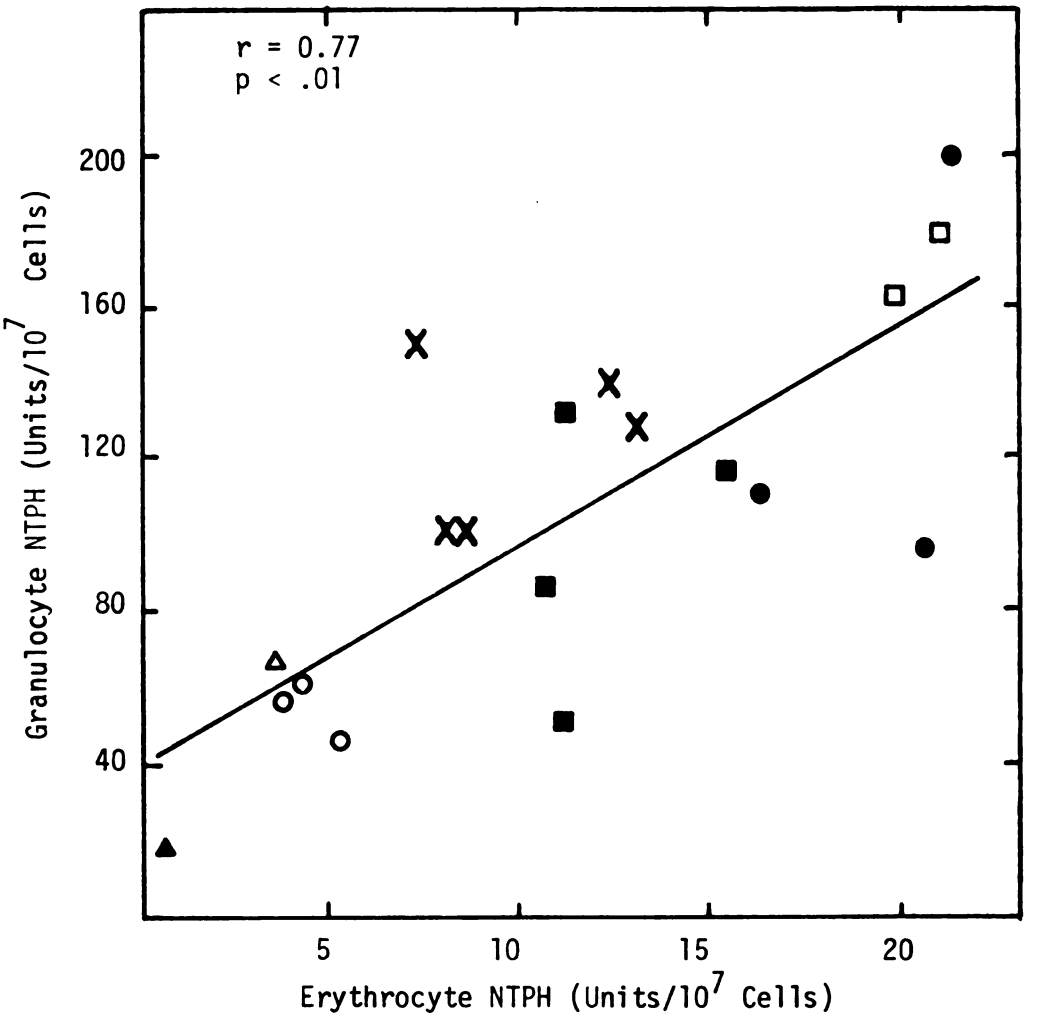

Figure 5. Correlation of NTPH Activities in Granulocytes and Erythrocytes

The NTPH analyses for each data point were conducted in triplicate on a single day, and the activity for each cell type was adjusted for nonspecific hydrolysis of ITP and activity due to platelet contamination (see Methods). The seven individuals involved in the study are represented by the following symbols: DY (▲), VLV (o), BH (△), SF (x), CV (■), AJM (●) and DL (□).

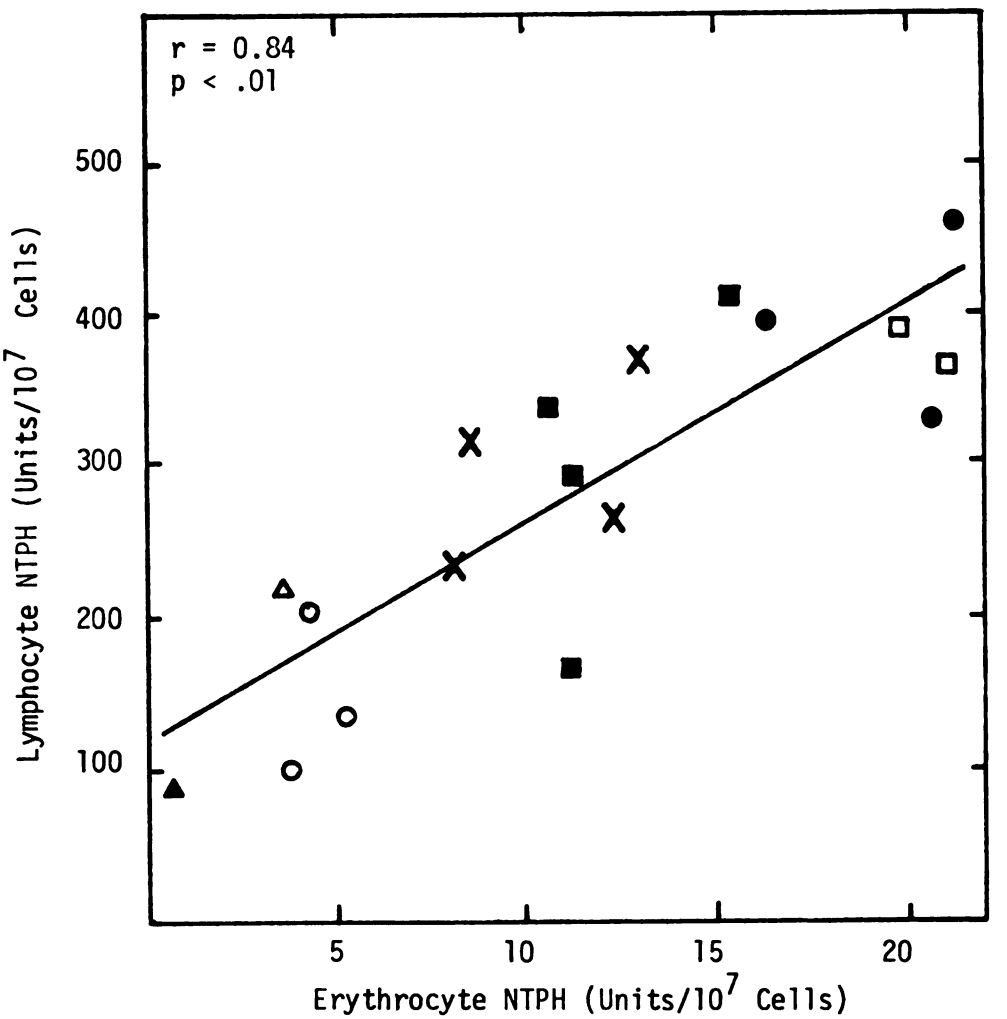

Figure 6. Correlation of NTPH Activities in Lymphocytes and Erythrocytes

The NTPH analyses for each data point were conducted in triplicate on a single day, and the activity for each cell type was adjusted for nonspecific hydrolysis of ITP and activity due to platelet contamination (see Methods). The seven individuals involved in the study are represented by the following symbols: DY (▲), VLV (o), BH (Δ), SF (x), CV (■), AJM (●) and DL (□).

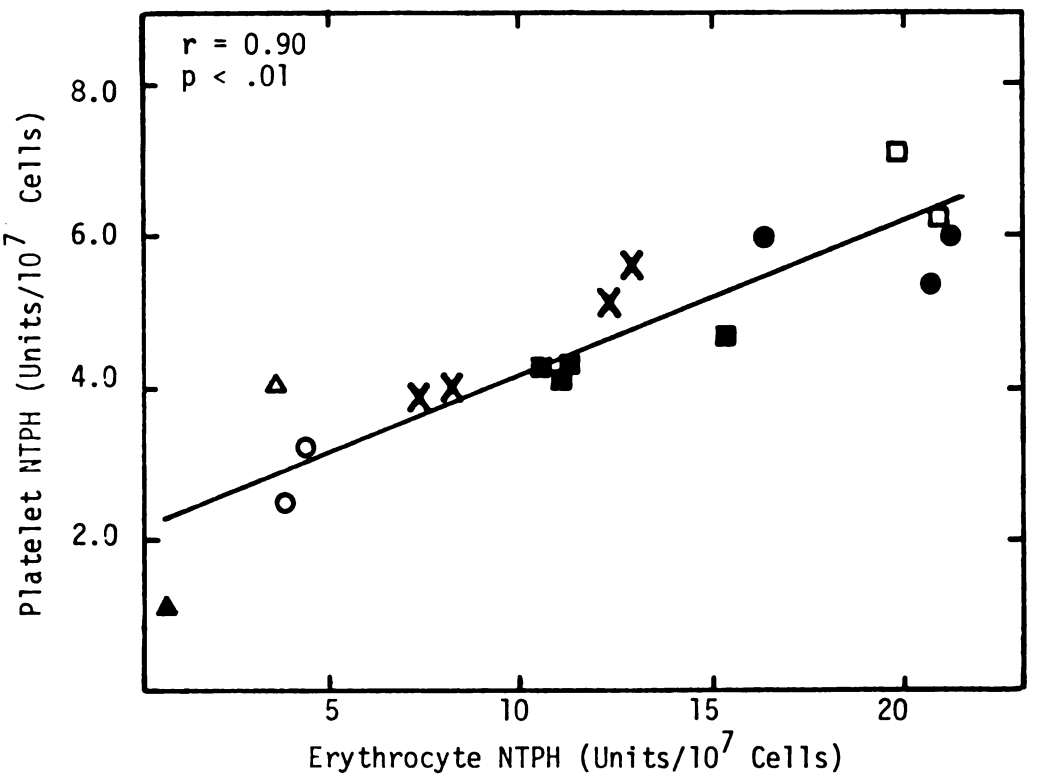

Figure 7. Correlation of NTPH Activities in Platelets and Erythrocytes

The NTPH analyses for each data point were conducted in triplicate on a single day, and the activity for each cell type was adjusted for non-specific hydrolysis of ITP (see Methods). The seven individuals involved in the study are represented by the following symbols: DY (▲), VLV (o), BH (Δ), SF (x), CV (■), AJM (●) and DL (□).

Table 7. NTPH Activity in the Red Cells of Patients with Muscular Dystrophy*

Patient	Sex	Age (years)	Diagnosis	NTPH Specific Activity (Units/mg Hemoglobin)
1	F	19	limb girdle	47
2	M	10	Duchenne	56
3	M	13	Duchenne	65
4	M	15	Duchenne	67
5	M	17	Duchenne	64
6	M	18	Duchenne	65
7	M	15	Duchenne	72
8	M	17	Duchenne	71
9	M	18	Duchenne	64
10	M	18	Duchenne	76
11	F	37	Myotonia	60
12	M	21	Duchenne	53
13	M	19	Duchenne	37

*Blood was obtained from these patients by Dr. C. Suelter, Department of Biochemistry, Michigan State University. The NTPH activity in the washed red cells of each individual was determined in triplicate under standard assay conditions.

[^{14}C]ITP accumulation from [^{14}C]hypoxanthine in intact erythrocytes from various individuals (72). We undertook a cooperative study with his laboratory in which the red cells of 93 individuals were analyzed for [^{14}C]ITP accumulation and NTPH specific activity. Since some of the blood was stored at 4°C and -20°C prior to NTPH analysis, the stability of NTPH was tested under these conditions.

Storage of blood at 4°C for 5 weeks had little effect on the activity of NTPH (Figure 8); however, storage under these conditions did increase the endogenous phosphate level in the red cells and hence the absorbance values obtained in the no substrate controls of the NTPH

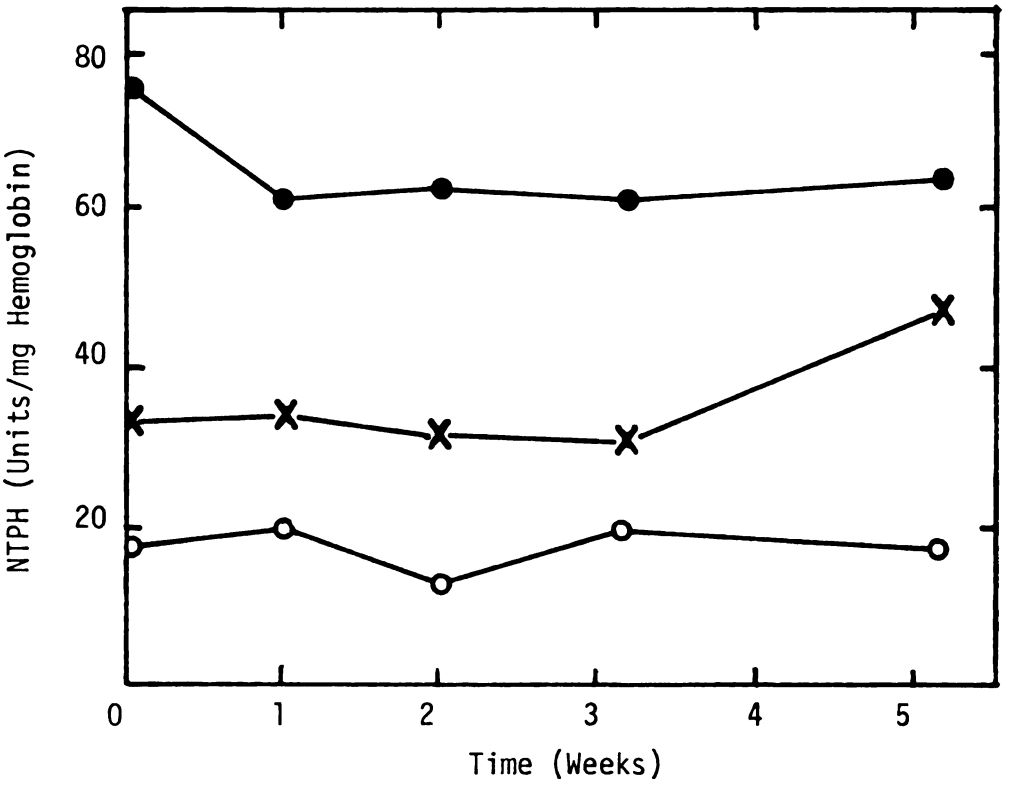

Figure 8. NTPH Activity in Red Cells Stored in Heparin for 5 Weeks

Three samples of whole blood were stored in heparinized vacutainers for 5 weeks. At each time point indicated, aliquots from each sample were analyzed in triplicate for NTPH activity under standard conditions. Blood from the following individuals was used in this study: AJM (●), WGC (X) and VLV (O).

assay. Other evidence indicated that NTPH was stable for months during the storage of frozen packed red cells.

Figure 9 indicates that [^{14}C]ITP accumulation and NTPH specific activity in blood cells of selected individuals may be described by an inverse hyperbolic relationship. A theoretical curve is fitted to the data which describes a substrate-enzyme relationship between % [^{14}C]ITP accumulation and NTPH, predicted by assuming no variation in the synthesis of ITP among individuals, assuming steady state conditions and assuming Michaelis-Menten kinetics (see Appendix A).

This relationship corroborates and expands the data of Vanderheiden that suggest that [^{14}C]ITP accumulation from [^{14}C]inosine is limited by an "ITPase" (3). Whereas Vanderheiden suggested three classes of individuals that accumulated different amounts of [^{14}C]ITP, Figure 9 indicates that a continuous spectrum of individuals are scattered along a hyperbolic relationship between % [^{14}C]ITP accumulation and NTPH specific activity.

C. Analysis of Endogenous Levels of ITP in Whole Blood

Since the theoretical consideration of the data in Figure 9 suggests that ITP may be a normal constituent of red cells (see Appendix A) and since Vanderheiden had previously reported the presence of ITP in fresh extracts of human erythrocytes, experiments were designed to quantitate the ITP extracted from blood of selected individuals. Fresh blood without removal of serum or leukocytes was used in these experiments to minimize any change in the pool size of endogenous nucleotides.

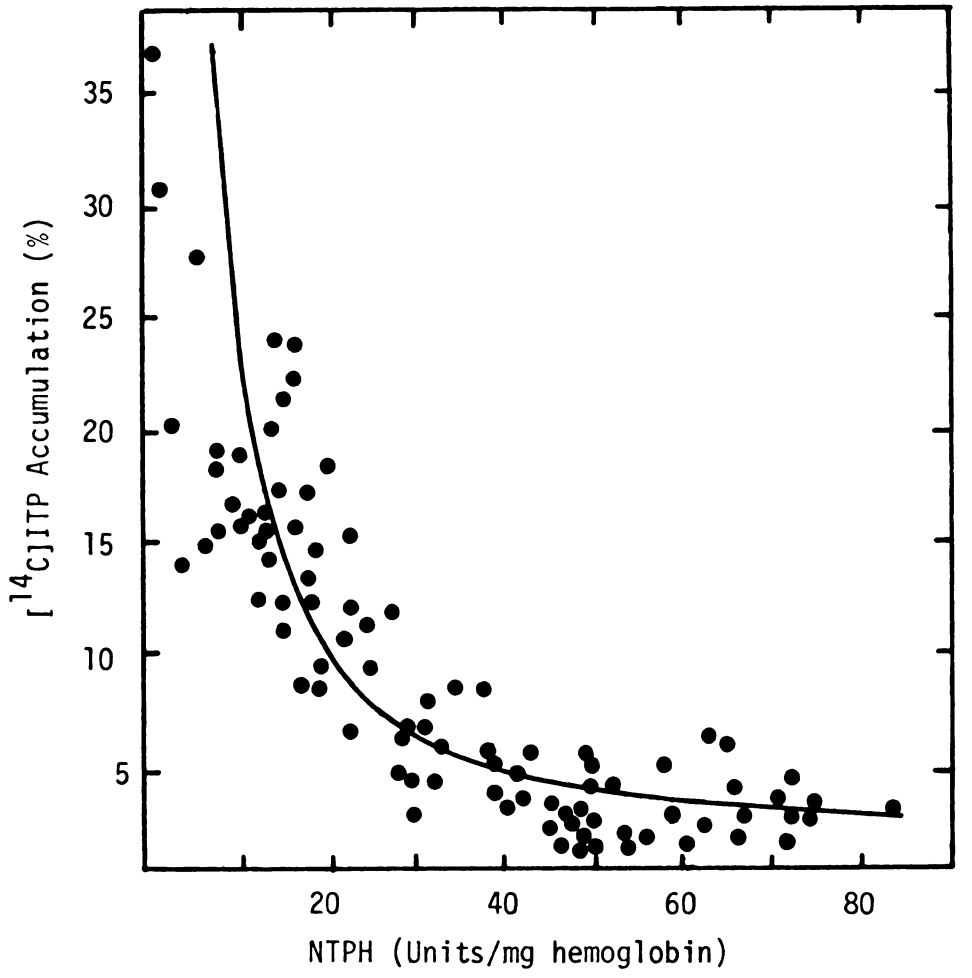

Figure 9. The Relationship Between [^{14}C]ITP Accumulation in Intact Red Cells and NTPH Specific Activity

[^{14}C]ITP accumulation in red cells of selected individuals was analyzed by Dr. J. F. Henderson's laboratory at the University of Alberta and expressed as a percent defined as ([^{14}C]ITP/[^{14}C]ITP + [^{14}C]IMP) x 100. NTPH activity was determined in triplicate for each sample under standard assay conditions.

1. Preparation of the Internal Standard, [^{3}H]ITP

To monitor the recovery of ITP by acid extraction of blood, [^{3}H]ITP of high specific activity was prepared by deamination of [2-^{3}H]ATP (27 Ci/mmole). DEAE-Sephadex purification of the [^{3}H]ITP is shown in Figure 10. The major peak was pooled, concentrated and analyzed by HPLC (Figure 11). The standard was 90% pure with major contaminants, [^{3}H]ATP (5%) and [^{3}H]nucleoside mono- and diphosphates (5%). Reanalysis of this standard following storage in ethanol and ammonium hydroxide at -20°C indicated less than 1% loss of the radiochemical purity of [^{3}H]ITP. HPLC analysis of this standard on the most sensitive UV scale failed to detect any UV-absorbing material in the ITP region of the chromatogram (Figure 12). This analysis also served to quantitate the [^{3}H]ITP in an aliquot of the standard solution of [^{3}H]ITP.

2. Resolution of Nucleotides by HPLC

In order to demonstrate the resolution of nucleoside 5'-triphosphates and to identify various peaks in the HPLC chromatograms of biological extracts, the retention times were determined for various standards (Table 8). XTP is the nucleotide least resolved from ITP.

3. Analysis of Endogenous ITP Levels in the Blood

The blood of three individuals with different NTPH specific activities was analyzed for the presence of endogenous ITP pools by HPLC. Figure 13 is a chromatogram of an extract of blood of an individual with very low NTPH activity, 3 nmoles ITP cleaved/mg hemoglobin. One can observe the presence of a small UV-absorbing peak at a retention time coinciding with the internal [^{3}H]ITP. This HPLC analysis was repeated four times on the same extract of this individual with similar results.

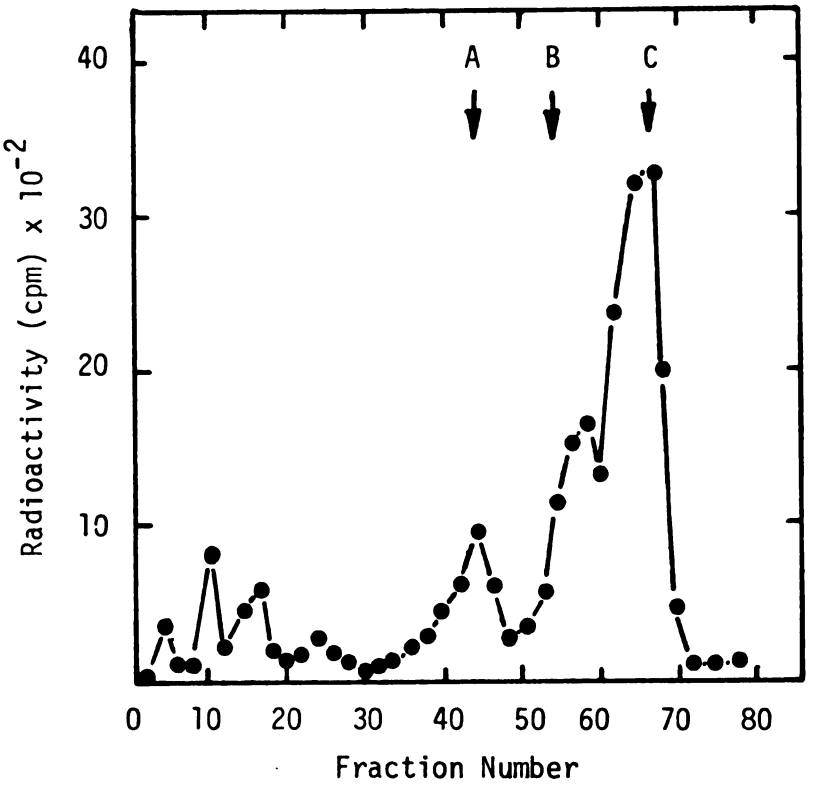

Figure 10. DEAE-Sephadex Purification of [^{3}H]ITP

[^{3}H]ITP was purified following deamination of [^{3}H]ATP by DEAE-Sephadex chromatography with a 200 ml linear gradient from 0.4 M to 0.7 M TEAB, pH 7.5, at a flow rate of 9 ml/hour. Fractions (2.5 ml each) were collected and aliquots were analyzed by liquid scintillation. Peak fractions were characterized by HPLC as described in the Methods as (A) a mixture of [^{3}H]ADP and [^{3}H]IDP and (B and C) a mixture of [^{3}H]ATP and [^{3}H]ITP.

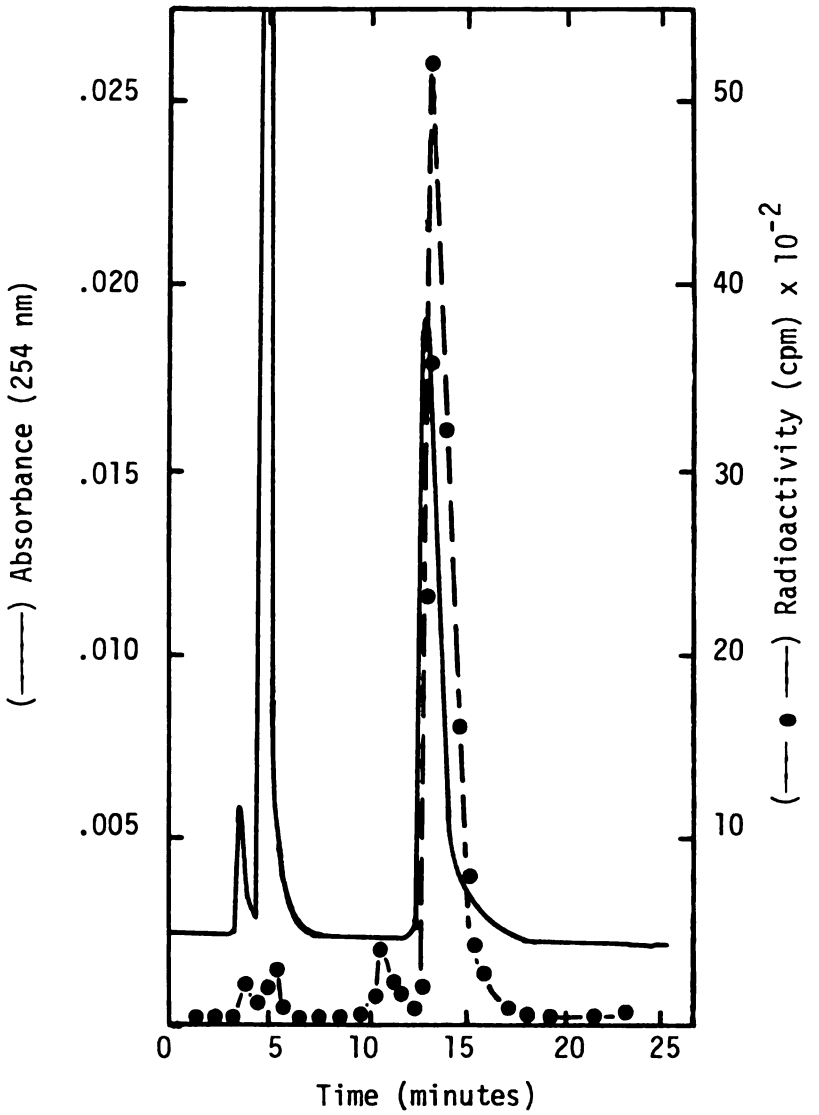

Figure 11. Radiochemical Purity of the Internal Standard, [^{3}H]ITP

The nucleotides were separated on a Perkin-Elmer 1250 HPLC equipped with a Partisil-10 SAX column in a buffer, 0.6 M KH_2PO_4, pH 3.4, at a flow rate of 55 ml/hour. The [^{3}H]ITP was injected ^{2}simultaneously with a standard ITP solution. Fractions (0.5 minute) were collected and analyzed by liquid scintillation.

Figure 12. UV-absorbing Impurities in the Internal Standard, [^{3}H]ITP

A 10 µliter aliquot of a 1:10 dilution of the internal standard solution of [^{3}H]ITP was analyzed on a Perkin-Elmer 1250 HPLC with a Partisil-10 SAX column in a buffer, 0.4 M KH_2PO_4, pH 3.4, at a flow rate of 42 ml/hour. Fractions (0.5 minute) were collected in the ITP region and analyzed by liquid scintillation.

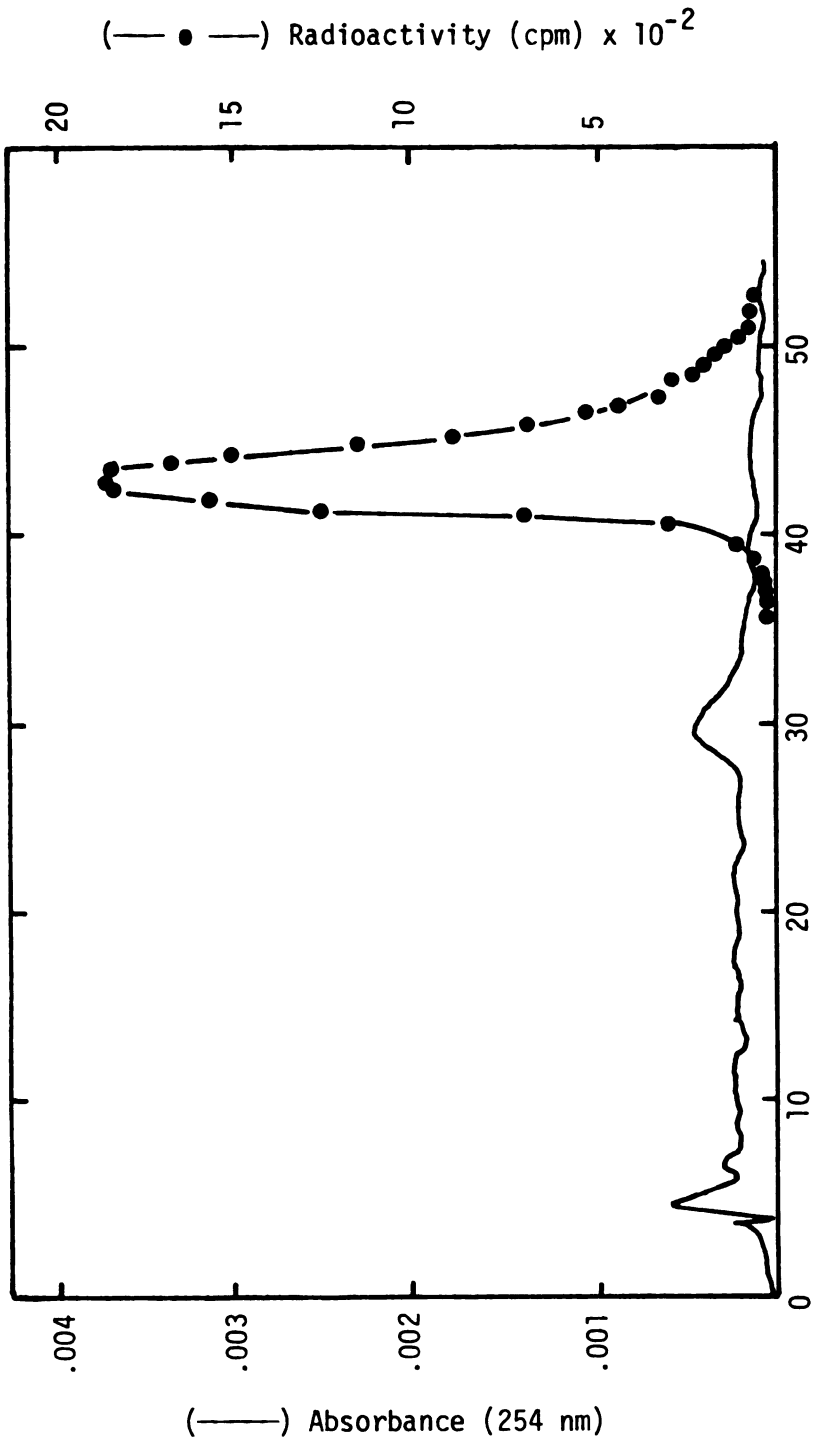

Figure 12. UV-absorbing Impurities in the Internal Standard, [^{3}H]ITP

Figure 13. HPLC Analysis of Endogenous Nucleotides in a PCA Extract of DY Blood

A 10 μliter aliquot of a PCA extract of DY blood (NTPH specific activity: 3 nmoles ITP hydrolyzed/mg hemoglobin) was analyzed on a Perkin-Elmer 1250 HPLC with a Partisil-10 SAX column in a buffer, 0.4 M KH_2PO_4, pH 3.4, at a flow rate of 42 ml/hour. Fractions (0.5 minute) were collected in the ITP region and radioactivity was detected by liquid scintillation. The internal standard, [^{3}H]ITP, indicated a 43% recovery of ITP in the extraction and analysis procedures.

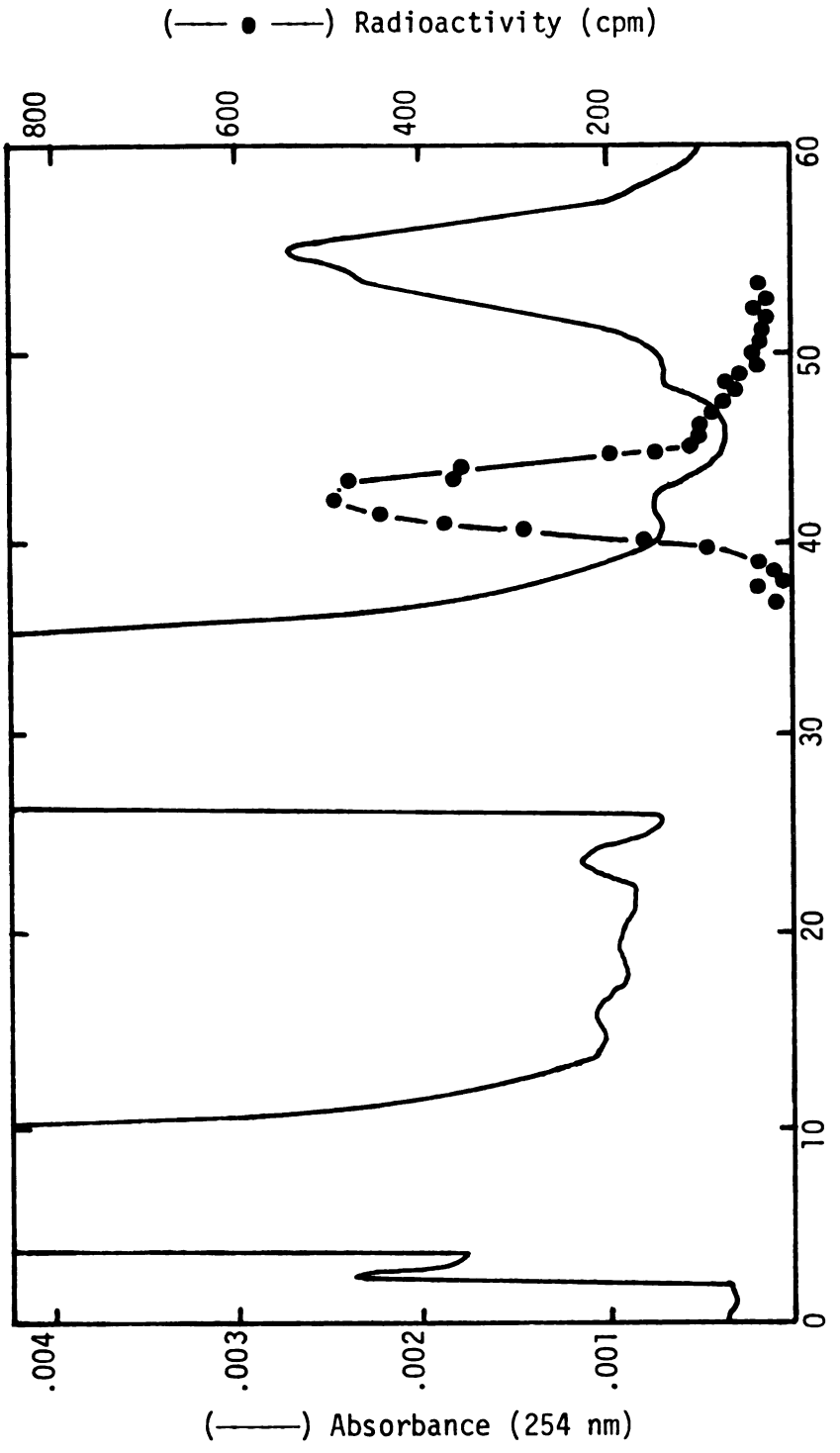

Figure 13. HPLC Analysis of Endogenous Nucleotides in a PCA Extract of DY Blood

Table 8. Retention Times for Standard Nucleotides by HPLC Analysis*

Standard	R_T (minutes)	Standard	R_T (minutes)
CTP	19.5	ITP	43
dCTP	20.5	dITP	52.5
UTP	24	GTP	55
ATP	29	dGTP	61
dATP	36		
XTP	39		

* HPLC was performed with a Partisil-10 SAX column in a buffer, 0.4 M KH_2PO_4, pH 3.4, at a flow rate of 42 ml/hour. Retention times (R_T) were measured from the time of injection to the time at the apex of the peak.

If this peak is quantitated by comparison of its peak height with that of known amounts of ITP and by adjusting the value obtained by the percentage recovered as determined by the internal standard, one can calculate that 5 ± 0.8 nmoles/ml packed cells (Mean ± S.D. of 4 HPLC analyses of the same extract) ITP is present in the cells of this individual. On the other hand, no endogenous ITP was detectable in the blood of two individuals with higher NTPH activities, 15 and 35 nmoles ITP cleaved/mg hemoglobin, Figures 14 and 15, respectively.

These results raise questions concerning the prevalence of ITP as a normal constituent of blood cells as reported by Vanderheiden since he claimed ITP to be present at concentrations on the order of 6.5 μM in the general population (25). It does seem however that, as Vanderheiden suggested, endogenous ITP levels in red cells can be correlated with the decreased activity of the degradative enzyme for ITP.

Figure 14. HPLC Analysis of Endogenous Nucleotides in a PCA Extract of VLV Blood

A 10 μliter aliquot of a PCA extract of VLV blood (NTPH specific activity: 15 nmoles ITP hydrolyzed/mg hemoglobin) was analyzed on a Perkin-Elmer 1250 HPLC with a Partisil-10 SAX column in a buffer, 0.4 M KH_2PO_4, pH 3.4, at a flow rate of 42 ml/hour. Fractions (0.5 minute) were collected in the ITP region and radioactivity was detected by liquid scintillation. The internal standard, [^{3}H]ITP, indicated a 42% recovery of ITP in the extraction and analysis procedures.

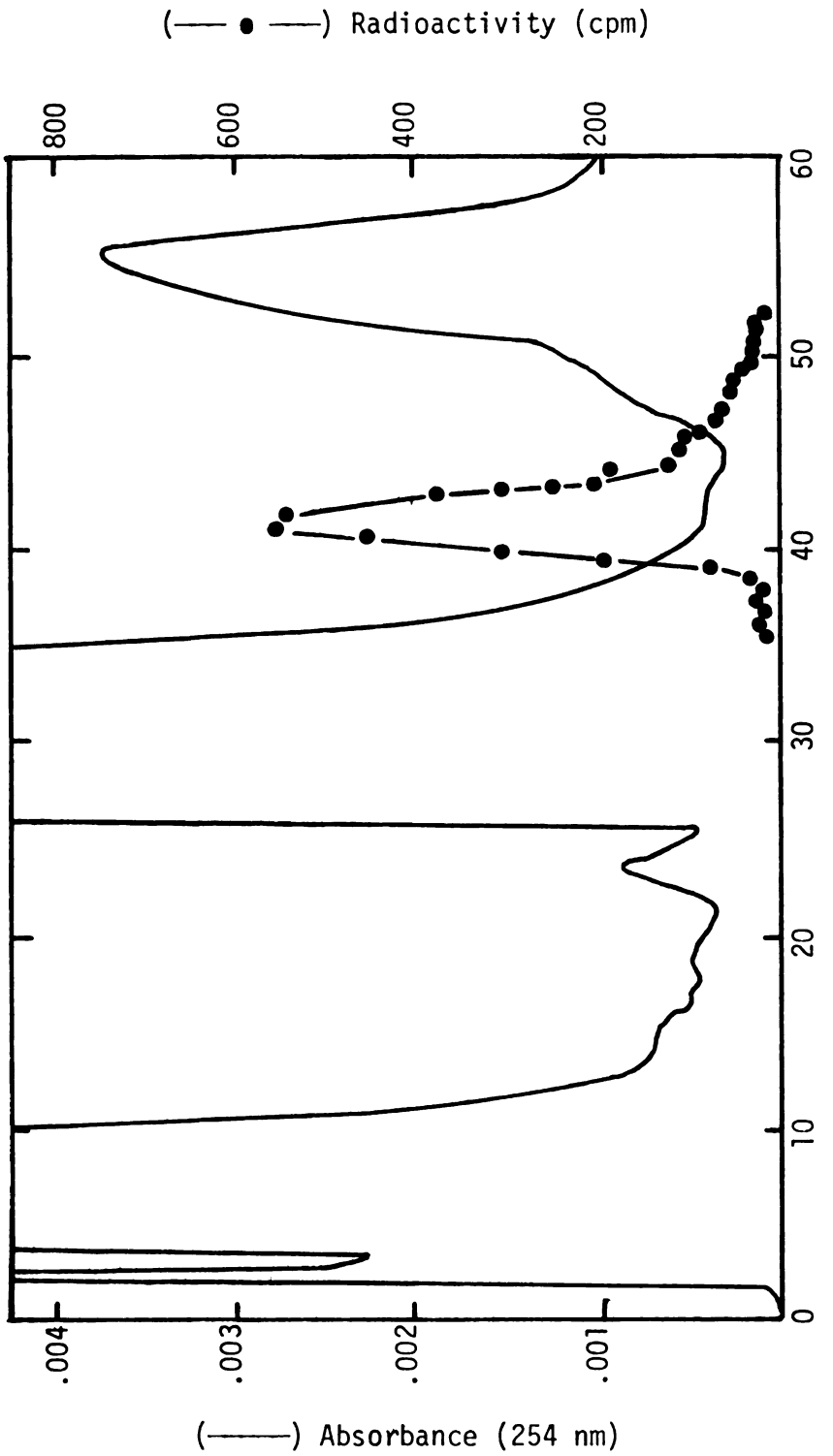

Figure 14. HPLC Analysis of Endogenous Nucleotides in a PCA Extract of VLV Blood

Figure 15. HPLC Analysis of Endogenous Nucleotides in a PCA Extract of SF Blood

A 10 μliter aliquot of a PCA extract of SF blood (NTPH specific activity: 35 nmoles ITP hydrolyzed/mg hemoglobin) was analyzed on a Perkin-Elmer 1250 HPLC with a Partisil-10 SAX column in a buffer, 0.4 M KH_2PO_4, pH 3.4, at a flow rate of 42 ml/hour. Fractions (0.5 minute) were collected in the ITP region and radioactivity was detected by liquid scintillation. The internal standard, [^{3}H]ITP, indicated a 36% recovery of ITP in the extraction and analysis procedures.

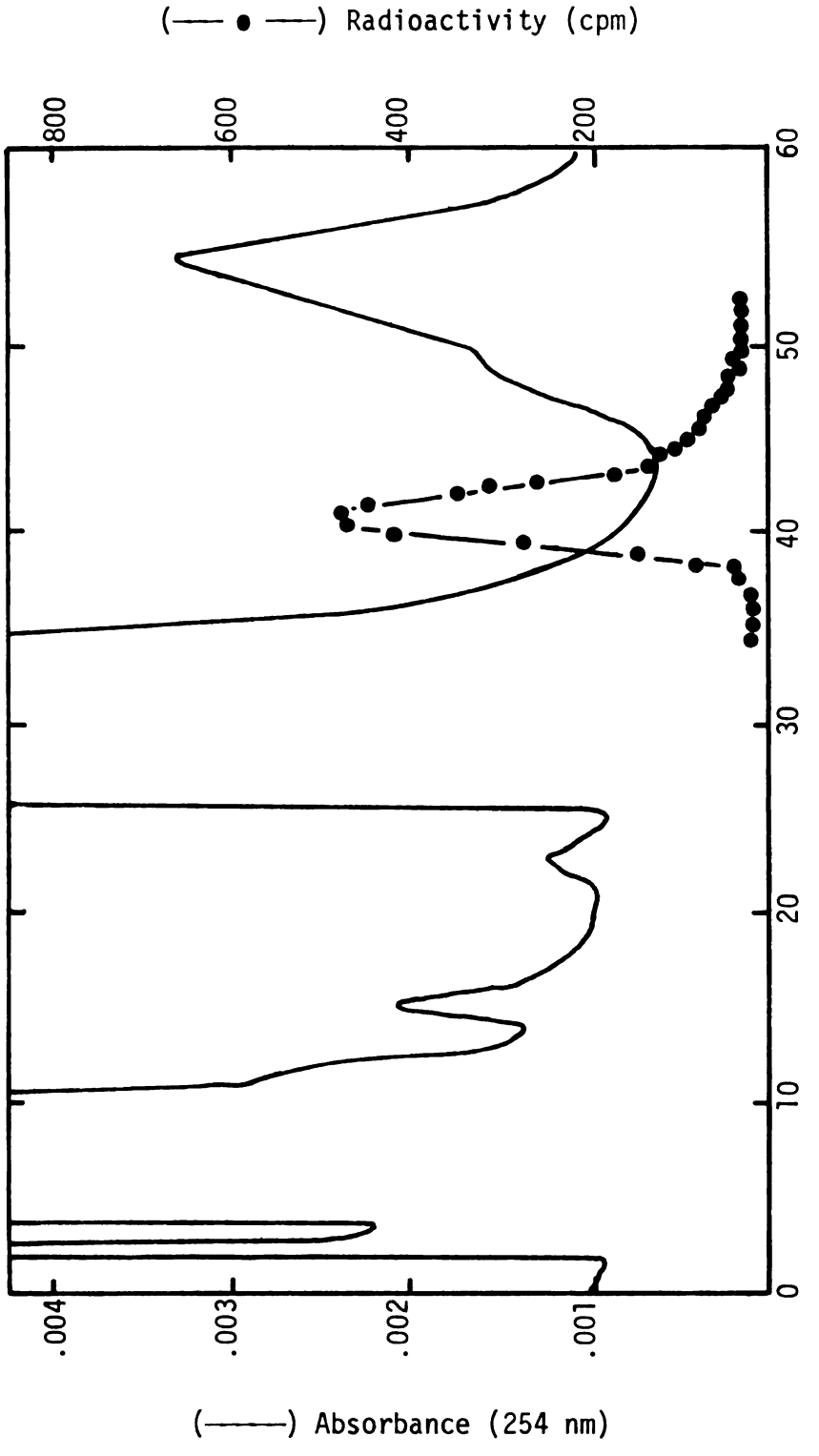

Figure 15. HPLC Analysis of Endogenous Nucleotides in a PCA Extract of SF Blood

D. Synthesis of Methylene Analogs of ITP

Modification of the triphosphate chain of ATP has received considerable attention since it is the site of many enzyme catalyzed reactions. The methylene derivatives of ATP are resistant to hydrolysis and may often be substituted for ATP as a substrate, inhibitor or effector in an enzyme-catalyzed reaction (183). With this in mind, we devised procedures to prepare α,β-methylene-ITP with the hope of using it as an inhibitor of NTPH. Furthermore this analog was attached through its γ-phosphate to Sepharose-4B to yield an affinity resin specific for NTPH but resistant to hydrolysis by NTPH.

1. Synthesis of ITP

a. Synthesis of APCP

2',3'-isopropylideneadenosine was condensed with methylene diphosphonic acid in the presence of DCC by the established procedure of Myers et al. (159). The isopropylidene blocking group was then removed by refluxing the compound 1 hour in 10% acetic acid. Since preliminary results indicated that the Dowex purification described in the paper of Myers et al. may lead to acid hydrolysis of the N-glycosidic bond, APCP was purified by DEAE-Sephadex chromatography at pH 7.5 (see Figure 16). A contaminant APCPA is eluted prior to APCP. The yield of the pooled concentrated product was 56% of the starting material.

APCP prepared in this manner was characterized by comparison of its retention time to commercial APCP on HPLC, by comparison of its mobility (relative to ADP) by paper chromatography and by detection of the presence of vic-hydroxyls by the method of Viscontini et al. (163). A positive result in the latter test ascertained the removal of the isopropylidene blocking group. The results are presented in Table 9.

Figure 16. Purification of APCP by DEAE-Sephadex Column Chromatography

A column (3.4 x 67 cm) of DEAE-Sephadex was loaded with sample and eluted with water followed by a 6 liter linear gradient from water to 0.8 M TEAB, pH 7.5. Fractions, 20 ml each, were collected and analyzed for absorbance and conductivity.

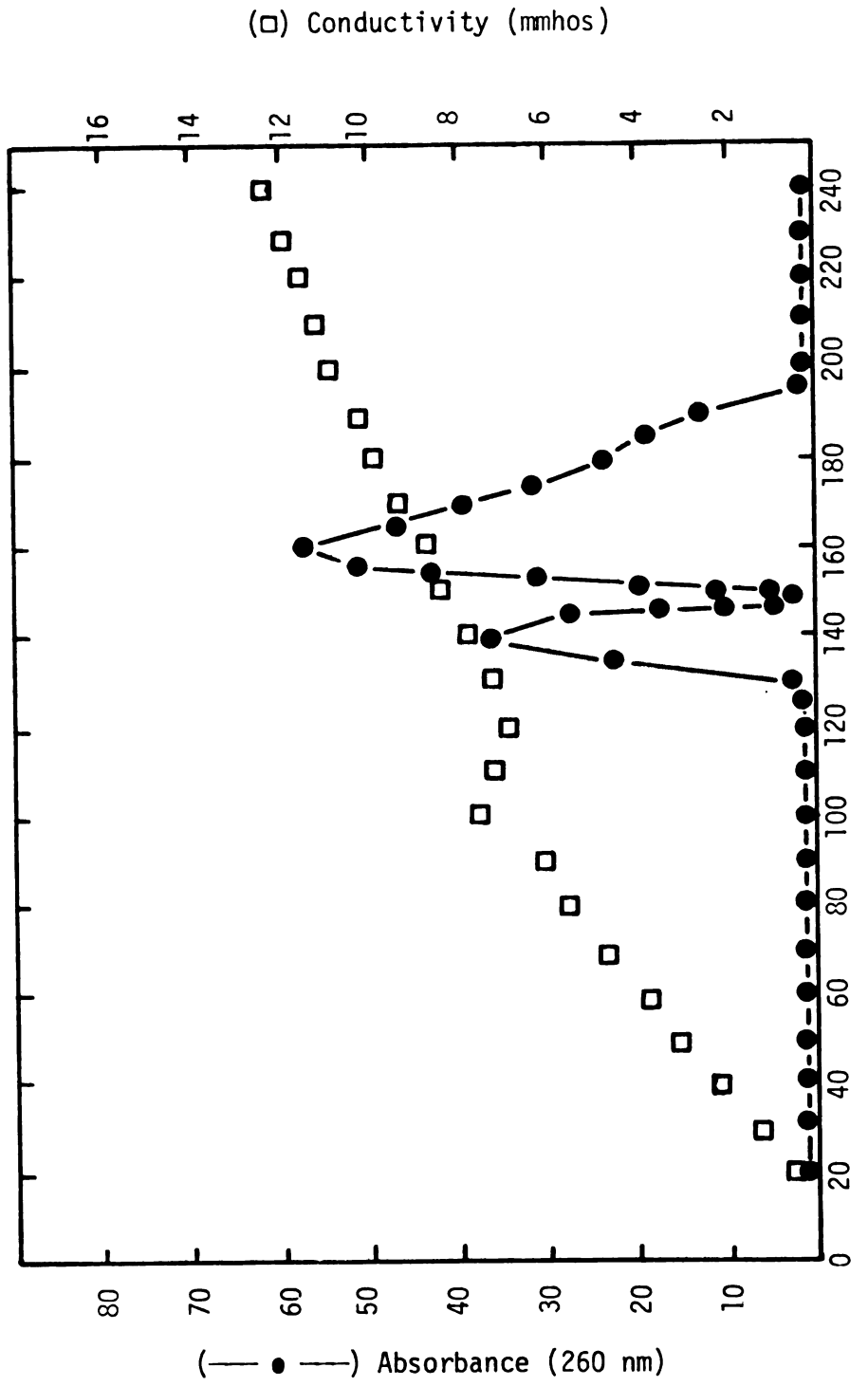

Figure 16. Purification of APCP by DEAE-Sephadex Column Chromatography

Table 9. Identification and Characterization of Synthesized APCP

Method	Synthesized APCP	Control
HPLC (R_T)*	7.5 minutes	7.5 minutes
Paper Chromatography (R_{ADP})**	1.14	1.15
vic-hydroxyls ***	yes	no

* HPLC analyses were performed on a Perkin-Elmer 1250 HPLC with a Partisil-10 SAX column using a phosphate buffer, 0.25 M KH_2PO_4, pH 3.4. Commercial APCP was used as the control.

** Descending paper chromatography was performed on Whatman 1 in the solvent, isobutyric acid - 1.0 N ammonia - 0.1 M disodium EDTA, 100:60:1.6 (v./v.). The control is the published R_{ADP} for APCP in this system (159).

*** Vic-hydroxyls were detected by the periodate-benzidine spray of Viscontini et al. (163). The control was 2',3'-isopropylideneadenosine.

b. Deamination of APCP

APCP was deaminated with an aqueous solution of nitrous acid (153). Deamination was monitored by HPLC and was complete in 3 hours (Figure 17). The product was purified by DEAE-Sephadex column chromatography as described in the Methods and stored at -20°C until further use.

2. Synthesis of IPCPOP

a. Preparation of the IPCP-imidazolide and its reaction with orthophosphate

The chemical synthesis of IPCPOP involved the imidazolide activation of IPCP followed by its reaction with orthophosphate by the method of Barker et al. (168). The methyl-tri-n-octylammonium salt form of IPCP was prepared and reacted with an anhydrous solution of 1,1'-carbodiimidazole. Formation of the imidazolide of IPCP was verified by paper chromatography (Figure 18). Formation of the imidazolide

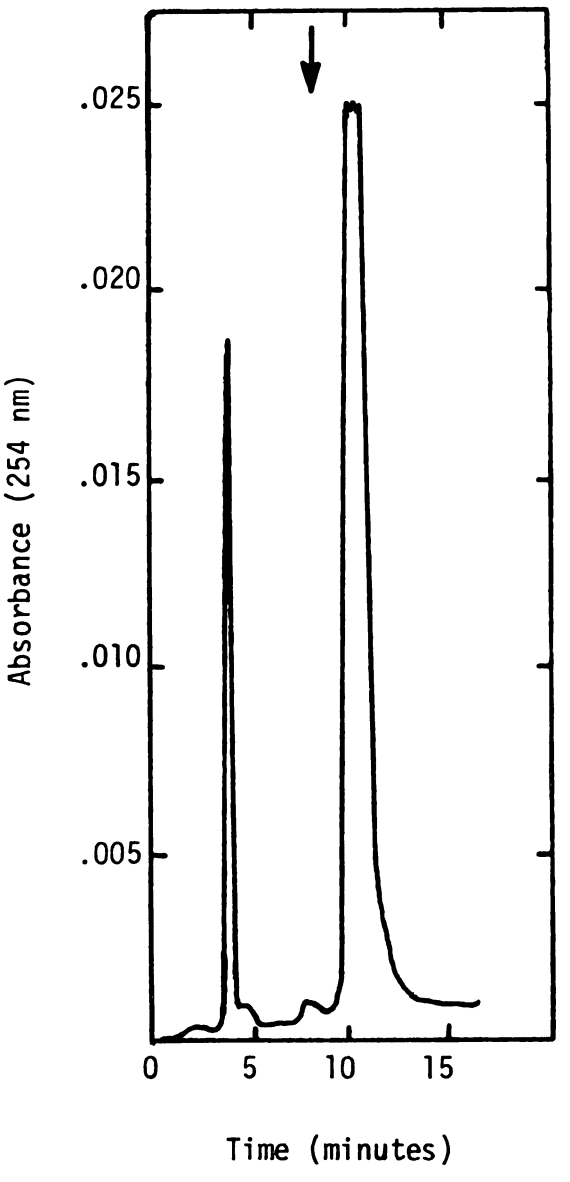

Figure 17. HPLC Analysis of Deaminated APCP

The analysis was performed on a Perkin-Elmer 1250 HPLC with a Partisil-10 SAX column using a phosphate buffer, 0.25 M KH_2PO_4, pH 3.4. The arrow indicates the retention time of standard APCP.

increased the R_f of IPCP from 0.24 to 0.36 in this system, however the IPCP-imidazole did not react with the Pauly test. The major imidazole-positive area at R_f=.60 is the imidazole released during the reaction.

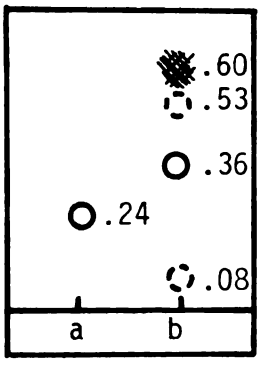

Figure 18. Characterization of the IPCP-imidazolide by Paper Chromatography

Paper chromatography of IPCP (a) and the IPCP-imidazolide (b) was performed with Whatman 40 in solvent I. The circled areas indicate UV-absorbing material while the cross-hatched area is imidazole-positive. The numerical values are the R_f at the center of each spot. The impurities at R_f=.53 and R_f=.08 were estimated by their intensity to be ca. 25% and 10%, respectively.

b. Purification and characterization of IPCPOP

The IPCP-imidazolide was reacted with orthophosphate and the product purified by DEAE-Sephadex chromtography (Figure 19). The major peak, which was pooled and concentrated, represented a 56% yield from the starting material, IPCP.

1) UV Absorption

The product was characterized by a comparison of its UV absorption spectral characteristics with those of ITP (Table 10). Using the extinction coefficient of ITP, 12.2 mM at 248.5 nm, the purity of IPCPOP by weight was estimated as 78 ± 3% (S.D.). The contaminant was probably sodium bicarbonate (see ^{13}C-NMR Absorption below.

2) HPLC

HPLC analysis of IPCPOP indicated that it was 96% pure by UV

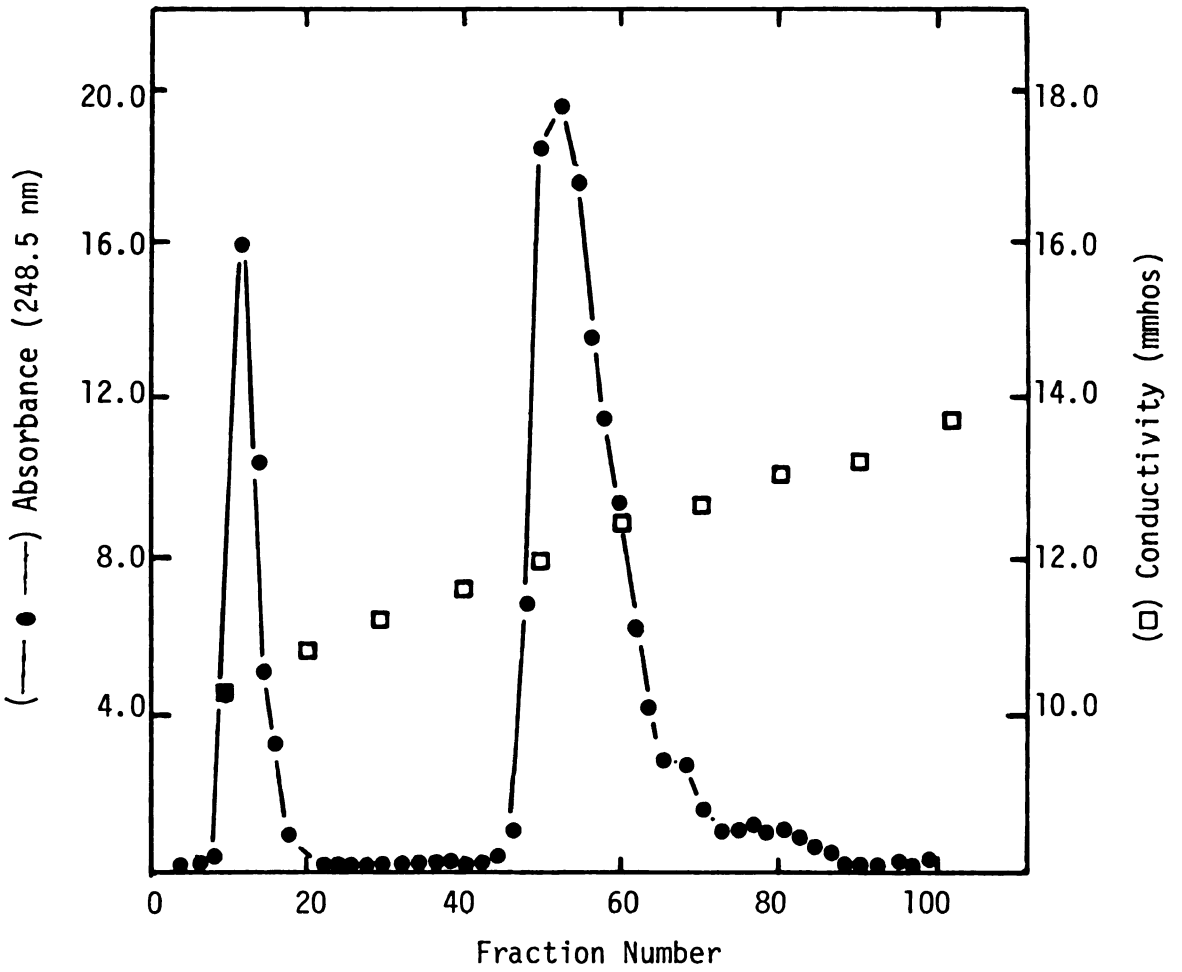

Figure 19. DEAE-Sephadex Purification of IPCPOP

A column (2.8 x 25 cm) of DEAE-Sephadex was loaded with the sample and eluted with a 3 l linear gradient from 0.4 to 0.8 M TEAB, pH 7.5. Fractions, 15 ml each, were collected and monitored for absorbance and conductivity. Only one-half of the gradient was necessary to elute the major peak.

Table 10. Comparison of the UV Absorption Spectral Characteristics of IPCPOP and ITP*

	pH 2			pH 11		
	λ_{max}	λ_{min}	A_{250}/A_{260}	λ_{max}	λ_{min}	A_{250}/A_{260}
ITP	249	222	1.49	254	225	1.08
IPCPOP	249	222	1.51	254	225	1.06

* Absorption spectra were obtained by a Cary Recording Spectrophotometer (Model 15) with the appropriate acid or basic solution used as the blank. λ refers to wavelength.

absorption. The retention time for the synthesized IPCPOP was exactly the same as that of IPCPOP prepared by deamination of commerical APCPOP (Figure 20).

3) IR Absorption

Figure 21 is a comparison of the IR absorption spectrum of IPCPOP with that of ITP. The most obvious feature is that the P=O stretch (184) at a wavenumber of 1240 cm^{-1} for ITP is shifted to lower enery, wavenumber of 1220 cm^{-1}, in the IPCPOP spectrum. This might be expected since the methylene is an effective insulator for electron sharing among the P-O bonds. Less distinct differences between ITP and IPCPOP also appear in the region of the P-O-P stretch (900 cm^{-1}) and the region of P-OH and P-O-C stretching (1000 cm^{-1}).

4) ^{13}C-NMR Absorption

Figure 22 is a comparison of the proton-decoupled ^{13}C-NMR spectra of IPCPOP and ITP. If one compares the spectrum in Figure 22A with a published spectrum of IMP (185), one can see that the peak corresponding to C-5 of the purine ring (124.6 ppm) is missing in the ITP spectrum. However, when the pulse and recycle times were changed as in

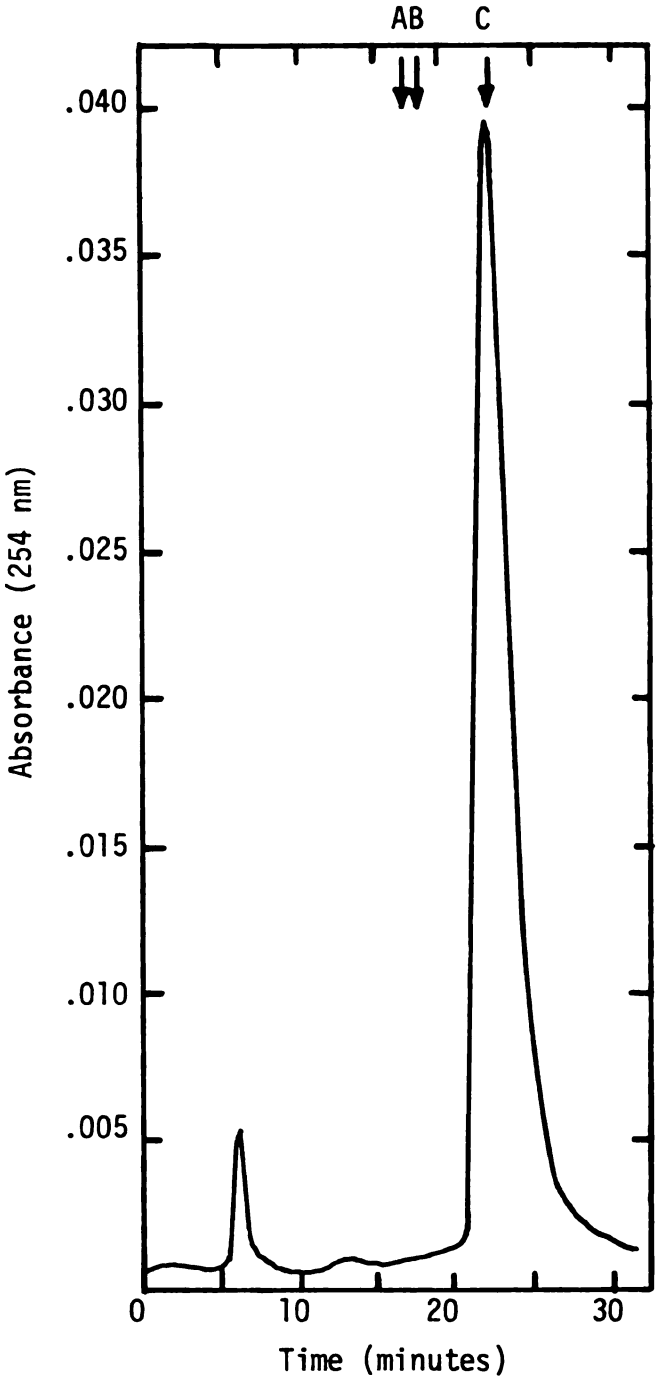

Figure 20. HPLC Analysis of Synthesized IPCPOP

The analysis was performed on a Perkin-Elmer 1250 HPLC with a Partisil-10 SAX column using a phosphate buffer, 0.6 M KH_2PO_4, pH 3.4. The arrows indicate the retention times of (A) ITP, (B) APCPOP and (C) deaminated APCPOP.

Figure 21. IR Absorption Spectra of ITP and IPCPOP

KBr pellets, each containing a mg of ITP or IPCPOP and 80 mg KBr were prepared as described in the methods and analyzed by a Perkin-Elmer 167 Grating Infrared Spectrophotometer. The absorption spectrum of ITP is represented by the solid line and the spectrum of IPCPOP is represented by the dashed line.

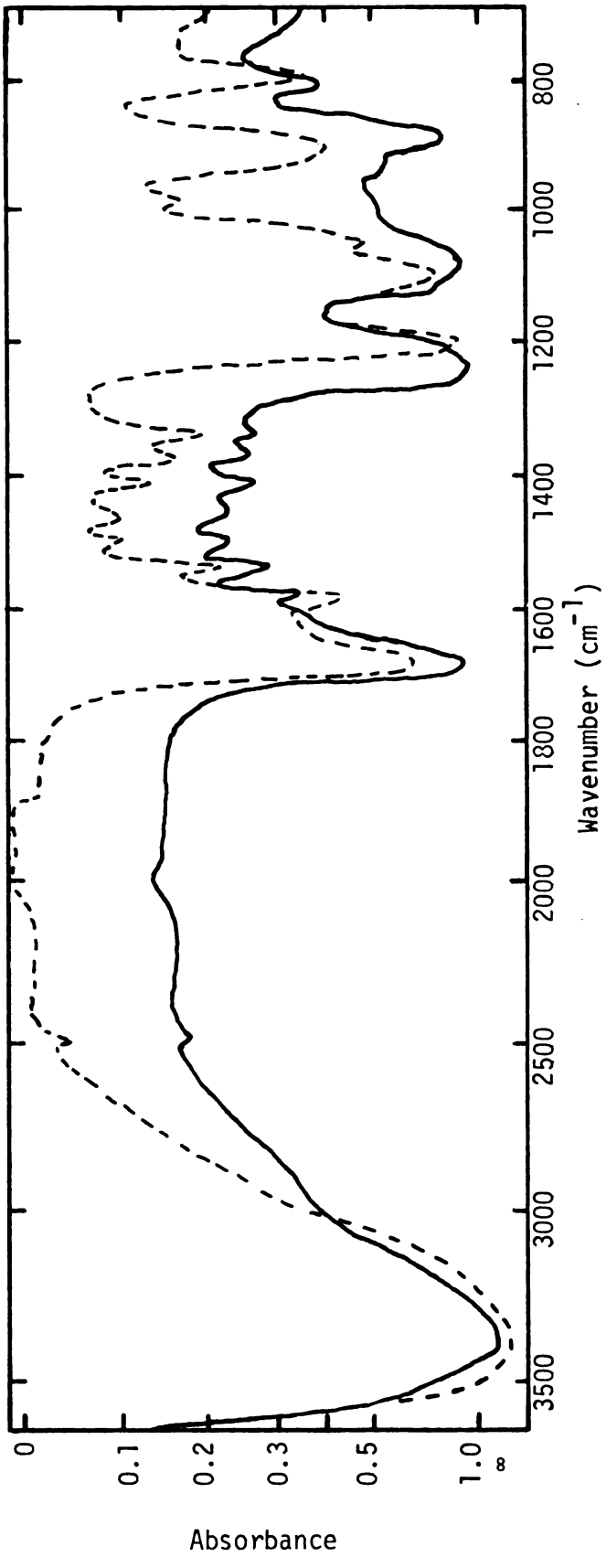

Figure 21. IR Absorption Spectra of ITP and IPCPOP

Figure 22. The 15.08-MHz, Decoupled ^{13}C-NMR Spectra of (A) ITP, (B) IPCPOP, and (C) the Expanded Methylene Region of IPCPOP

These ^{13}C-NMR spectra were obtained as described in the Methods. The spectrum for A was accumulated with 15, 368 scans having a 16 μ second pulse with no recycle time and was analyzed with the line broadening set at 1.00 Hz. The spectrum for B was accumulated with 14, 512 scans having a 12 μ second pulse with a 3 second recycle time and was analyzed with the line broadening set at 1.00 Hz. Spectrum C was accumulated under the same conditions as B but analyzed with the line broadening set at 4 Hz.

The arrow indicates an impurity in the IPCPOP sodium salt. Chemical shifts are expressed in parts per million downfield from tetramethylsilane.

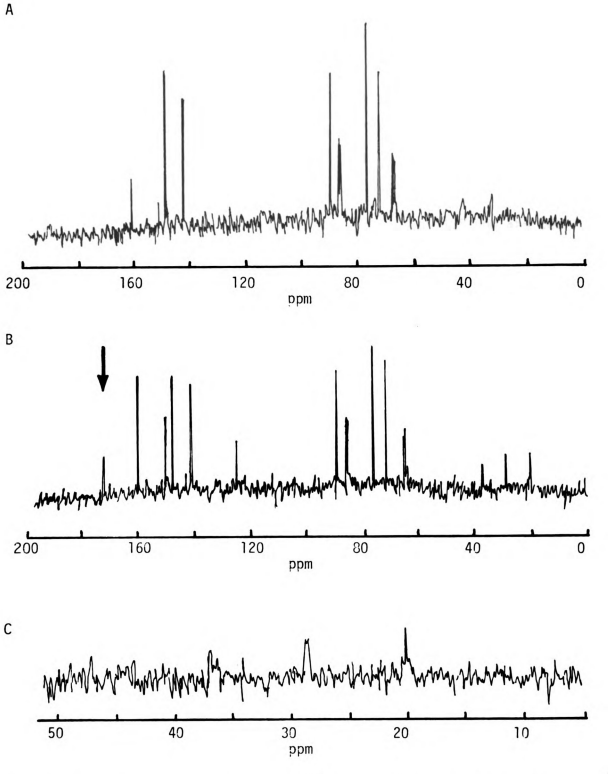

Figure 22. The 15.08-MHz, Decoupled ^{13}C-NMR Spectra of (A) ITP, (B) IPCPOP, and (C) the Expanded Methylene Region of IPCPOP.

the spectrum for IPCPOP (Figure 22B), the C-5 peak is clearly visible. Thus the missing peak in the ITP spectrum may be explained by the experimental conditions during data collection. The IPCPOP spectrum also shows an impurity at 172 ppm which is very likely sodium bicarbonate since the bicarbonate carbon appears in the region of 162 ppm and was present during the purification of IPCPOP.

The following features distinguish the IPCPOP spectrum from the ITP spectrum. The C-5' doublet is shifted upfield by 1.5 ppm and the coupling constant, $^{2}J_{P-C}$, is decreased from 6.6 in the ITP spectrum to 5.2 Hz in the IPCPOP spectrum. The methylene itself appears upfield at 28.8 ppm as a triplet in Figure 22B which is partially resolved into a quartet in Figure 22C ($^{1}J_{P-C}$ = 124-131 Hz). Thus the coupling constants for the two P-C bonds are almost identical.

The chemical shifts and coupling constants for the carbons of ITP and IPCPOP are listed in Table 11. Peak assignments were made by comparison of these data with the spectrum for IMP published by Kotowycz et al. (185).

3. Synthesis of IPCPOP Covalently Attached to Separose-4B

An affinity resin was prepared by coupling the γ-phosphate of IPCPOP by means of a 6-carbon spacer molecule to Sepharose-4B. This was accomplished by the method of Barker et al. (168, 169).

N-triflouracetyl-6-aminohexane-1-phosphate (TFA-HAP) was purified by cation exchange chromatography and analyzed by paper chromatography (Figure 23).

The imidazole of TFA-HAP was prepared by reacting TFA-HAP with 1,1'-carbodiimidazole and the product was characterized by paper

Table 11. The 15.08 MHz, Decoupled ^{13}C-NMR Chemical Shifts and Coupling Constants of ITP and IPCPOP in Aqueous Solution*

Carbon	ITP		IPCPOP	
	Chemical Shift (ppm)	Coupling Constant (Hz)	Chemical Shift (ppm)	Coupling Constant (Hz)
Impurity			172.2	
C-6	159.7		159.8	
C-4	149.8		149.7	
C-2	147.4		147.5	
C-8	140.8		140.9	
C-5	N.D.		124.6	
C-1'	88.4		88.5	
C-4'	85.1	$^{3}J_{P-C}$=8.8	85.0	$^{3}J_{P-C}$=8.1
C-2'	75.7		75.6	
C-3'	71.3		71.2	
C-5'	66.1	$^{2}J_{P-C}$=6.6	64.6	$^{2}J_{P-C}$=5.2
Methylene	-		28.8	$^{1}J_{P-C}$=124-131

* The peak assignments were made from the ^{13}C-NMR IMP spectrum published by Kotowycz et al. (185). The chemical shifts are expressed in parts per million downfield from the resonance of tetramethylsilane. The impurity in the IPCPOP is likely sodium bicarbonate (see Text).

chromatography in solvent III as shown in Figure 24 below. However, the free imidazole and product were not separated by this system.

The methyl-tri-n-octylammonium form of IPCP was reacted with TFA-HAP-imidazolide under anhydrous conditions. HPLC indicated the appearance of a product with a retention time greater than that of IPCP. This product was purified by Dowex 1 column chromatography as shown in Figure 25 and characterized by HPLC, Figure 26A. The impurity in the product coincided with the R_T of IPCP but may also be γ-(6-aminohexyl)-IPCPOP as shown in Figure 26B. Figure 26 further demonstrates that 83% of the TFA-blocking group was hydrolyzed during a 3 day treatment with

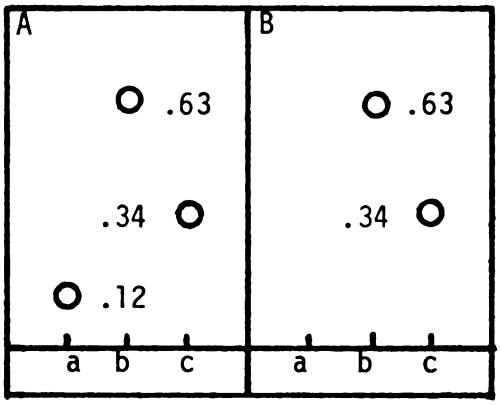

Figure 23. Characterization of N-triflouracetyl-6-aminohexane-1-phosphate by Paper Chromatography

Paper chromatography of (a) P_i, (b) TFA-HAP and (c) HAP was carried out in solvent II. Chromatogram A was developed with an ammonium molybdate spray for the detection of phosphate, and chromatogram B was developed by spraying with 1.0 N NaOH and drying to hydrolyze the triflouracetyl blocking group followed by spraying with ninhydrin for the detection of primary amines. The numerical values indicate the R_f at the center of each spot.

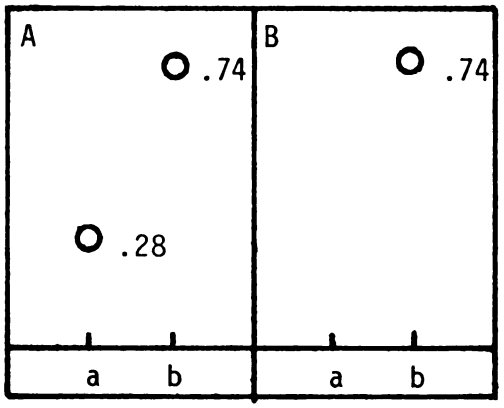

Figure 24. Characterization of the Imidazolide of N-triflouracetyl-6-aminohexane-1-phosphate by Paper Chromatography

Paper chromatography of (a) TFA-HAP and (b) TFA-HAP-Imidazolide was performed in solvent III. Chromatogram A was developed by spraying with 1.0 N NaOH and drying followed by spraying with ninhydrin. Chromatogram B was developed by the use of an imidazole-specific spray. The numerical values indicate the R_f at the center of each spot.

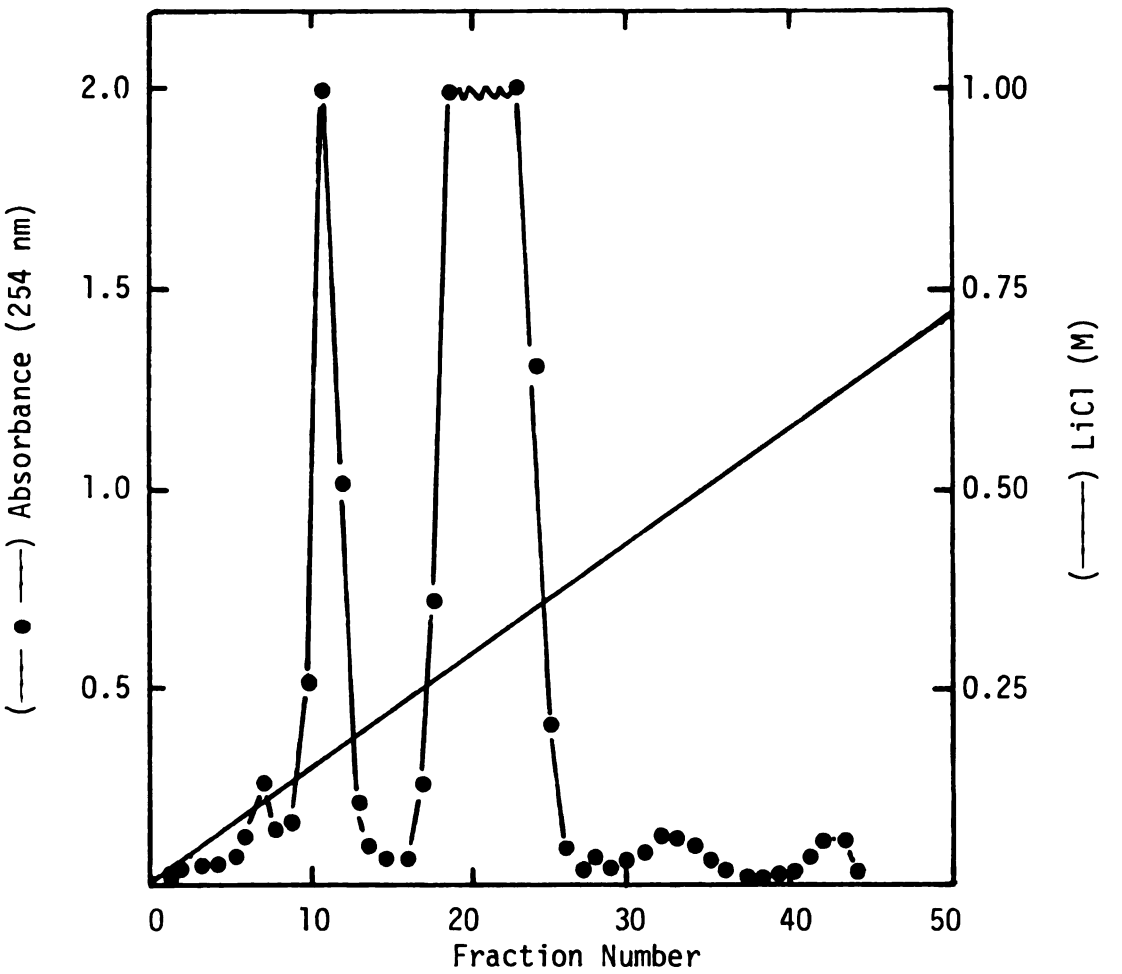

Figure 25. Purification of γ-(N-triflouracetyl-6-aminohexyl)-IPCPOP by Dowex Column Chromatography

A column (1.3 x 45 cm) containing Dowex 1 x 2 (200-400 mesh, Cl form) was loaded with the sample and eluted with 250 ml ethanol followed by a 1 liter linear gradient from 0.01 N HCl to 0.01 N HCl containing 1.0 M LiCl. Fractions, 15 ml each, were collected and monitored at 254 nm.

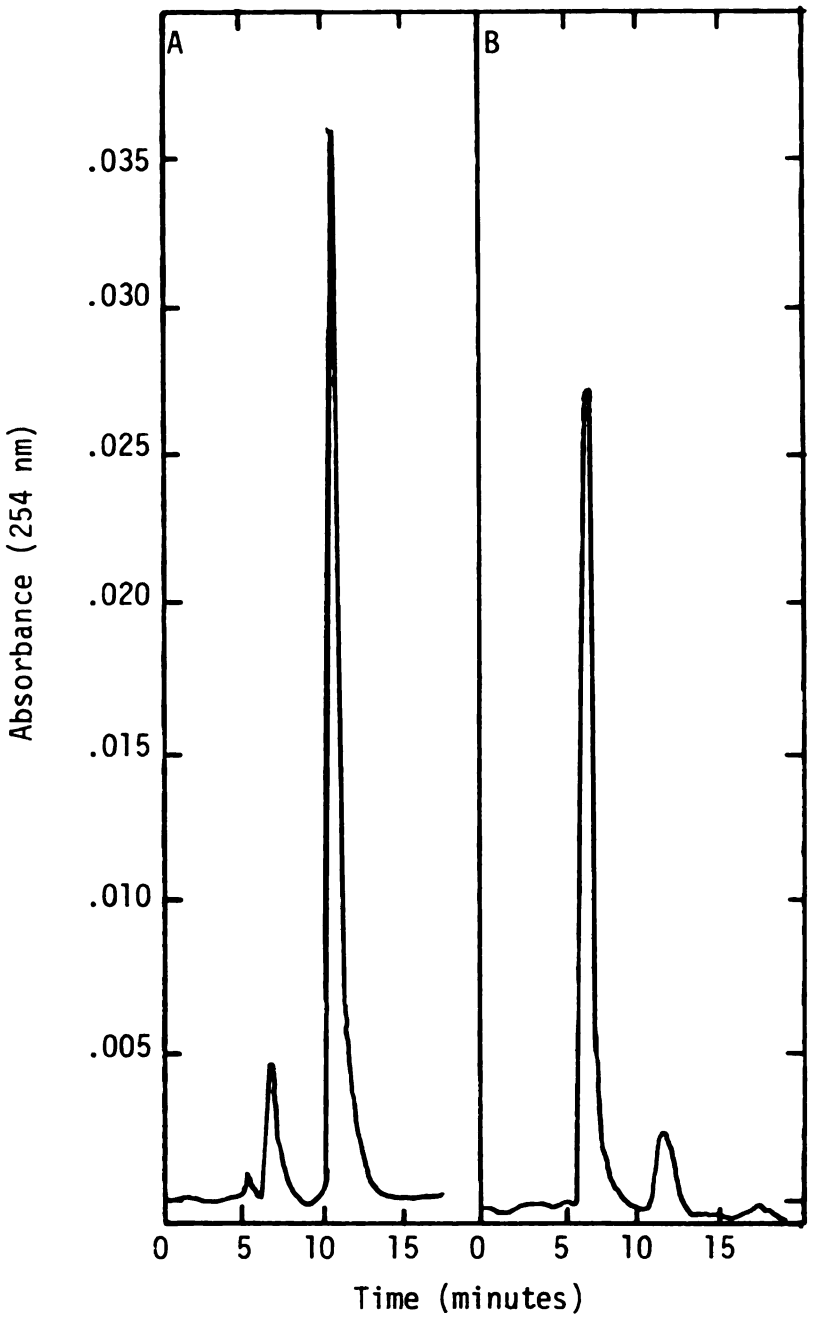

Figure 26. HPLC Analysis of (A) γ-(N-triflouracetyl-6-aminohexyl)-IPCPOP and (B) γ-(6-aminohexyl)-IPCPOP

Analyses were performed on a Perkin-Elmer 1250 HPLC with a Partisil-10 SAX column using a phosphate buffer, 0.75 M KH_2PO_4, pH 3.4. Chromatogram B is the analysis of a solution of γ-(N-triflouracetyl-6-aminohexyl)-IPCPOP treated 3 days with 1.0 N LiOH.

1.0 N LiOH. Finally the ninhydrin-positive γ-(6-aminohexyl)-IPCPOP was desalted with Bio-Gel P2 column chromatography (Figure 27). About 24% of the IPCP starting material was recovered as γ-(6-aminohexyl)-IPCPOP. A sketch of this product is shown in Figure 28 below. This ligand was then coupled through its primary amine to 10 ml CNBr-activated Sepharose-4B. Final analysis (see III, C, 2c) indicated a concentration of 7.3 μmoles ligand/ml Sepharose.

E. Application of α,β-Methylene-ITP Analogs to the Study of NTPH

1. Inhibition of NTPH with IPCPOP

To be sure that the coupling enzyme, yeast pyrophosphatase, was not being affected by IPCPOP, a range of concentrations of IPCPOP and PP_i were treated under the standard NTPH assay conditions at a reduced concentration of yeast pyrophosphatase (0.01 unit/ml reaction mixture). The IPCPOP/PP_i ratio was varied from 0 to 7.5 with no effect on the activity of the pyrophosphatase.

Figure 29 clearly shows the competitive inhibition of NTPH with IPCPOP. The V_{max} remains the same while the apparent K_m for ITP increases with increasing concentrations of the inhibitor. A replot of the apparent K_m, determined by the method described in Figure 29, versus the concentration of IPCPOP is shown in Figure 30. The K_i was calculated from the x-intercept of the replot as 5.9 μM for partially purified human NTPH and 3.7 μM for partially purified rabbit NTPH. Kinetic analysis of an NTPH containing lysate also indicated competitive inhibition by IPCPOP, but the data did not fit a straight line in the replot of apparent K_m versus IPCPOP concentration (Figure 30). This nonlinearity could not be accounted for by the hydrolysis of IPCPOP during the experiment, and no alternative explanation has been tested.

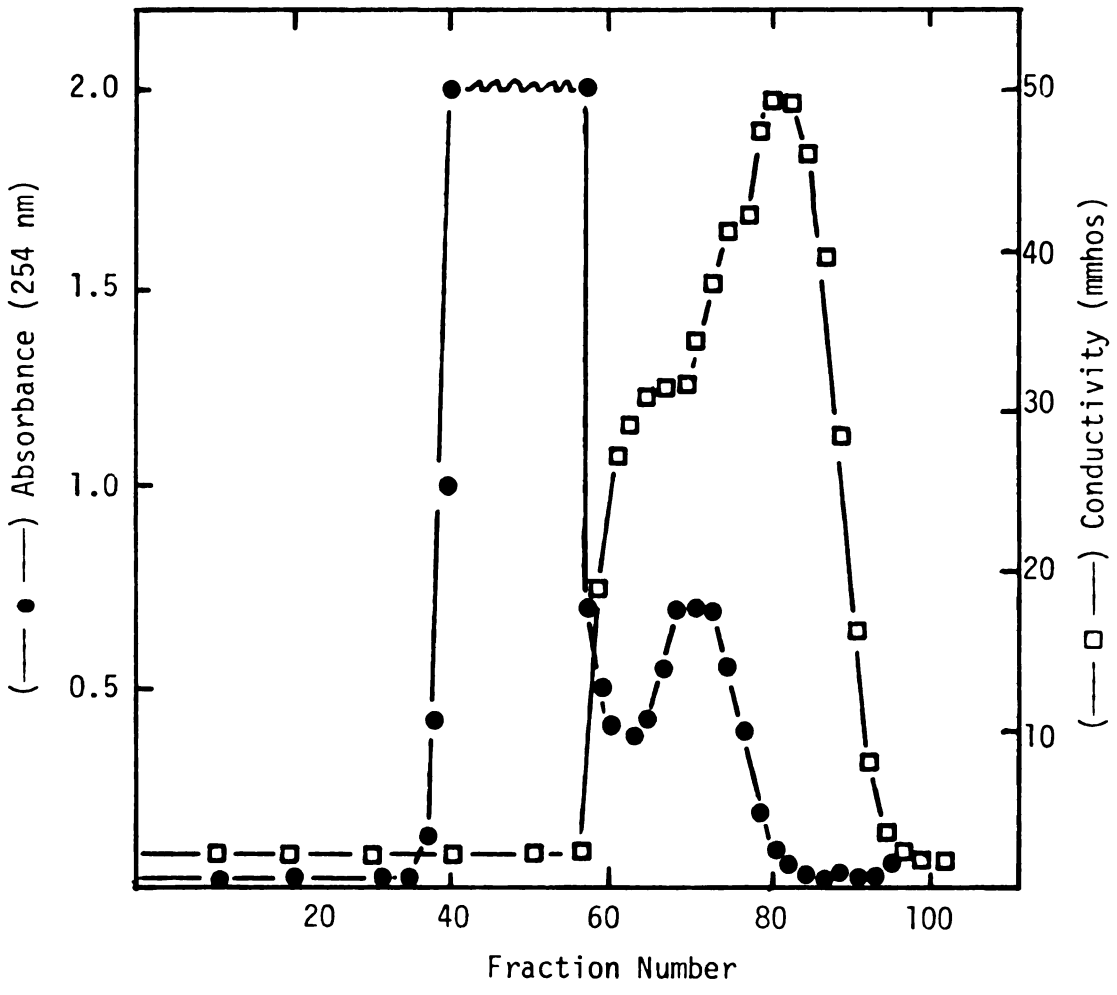

Figure 27. Bio-Gel P2 Column Chromatography of γ-(6-aminohexyl)-IPCPOP

A column (3.4 x 40 cm) containing Bio-Gel P2 (200-400 mesh) was loaded with the sample and eluted with 50 mM TEAB, pH 7.5. Fractions, 5 ml each, were collected and monitored for absorbance and conductivity.

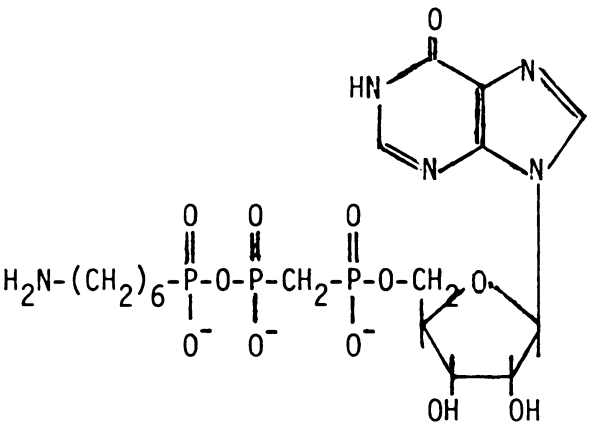

Figure 28. Structure of γ-(6-aminohexyl)-IPCPOP

Purified ITP (96% by HPLC analysis) was used in these studies to lower the phosphate background in the NTPH assay controls which lacked enzyme and thus increase the reproducibility of the enzyme assay.

2. Affinity Chromatography of NTPH

Since affinity chromatography of an enzyme may be affected by temperature, concentration of the ligand, pH, ionic strength of the buffer, concentration of the protein as well as other factors (182), the effect of these variables on the purification of NTPH from hemolysates was tested extensively by S. A. Fuller and the best results are presented here. Experiment 1 in Table 12 shows the purification of NTPH from a lysate of red cells. Greater than 76% of the activity loaded on the column was recovered in the effluent. About 53% of the activity was recovered following an ITP elution and indicated a 200-fold purification of NTPH from the initial lysate.

Experiment 2 in Table 12 shows the results of the affinity chromatography of a partially purified preparation of NTPH from human red cells. Thus 123% of the activity loaded on the column was recovered in

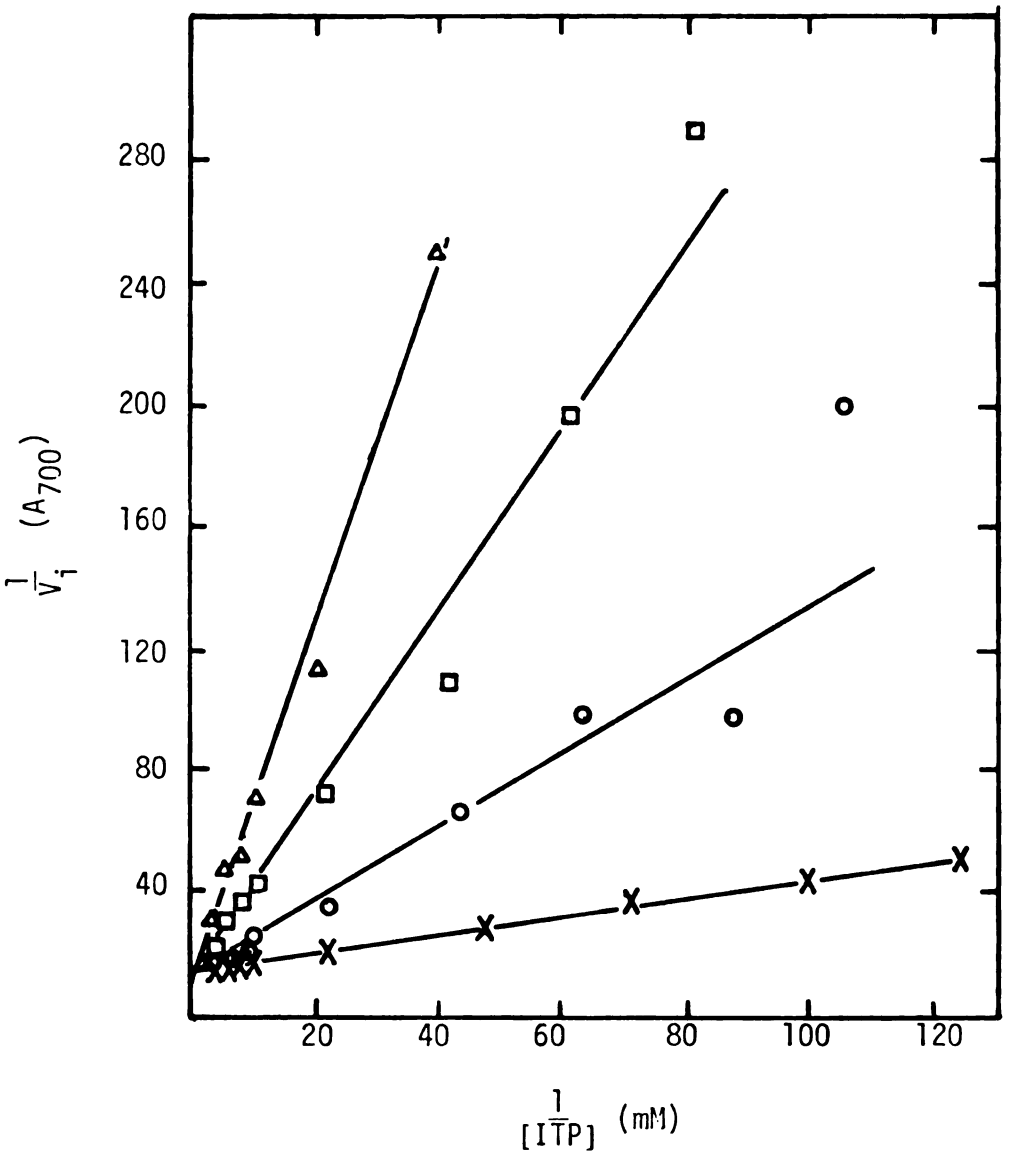

Figure 29. Competitive Inhibition of Partially Purified Human NTPH with IPCPOP

NTPH (specific activity = 3500 units/mg protein) was determined under standard conditions at various concentrations of purified ITP (96%). The velocity is expressed as the net absorbance at 700 nm in the Rathbun and Betlach assay for phosphate (see Methods). Substrate concentrations were recalculated by the method of Lee and Wilson (181) and lines were fit to the data by a computer program using the method of Wilkinson (167). Each point plotted here represents an average of two determinations. The following IPCPOP concentrations were used: 0 mM (x), 0.025 mM (o), 0.075 mM (□) and 0.15 mM (Δ).

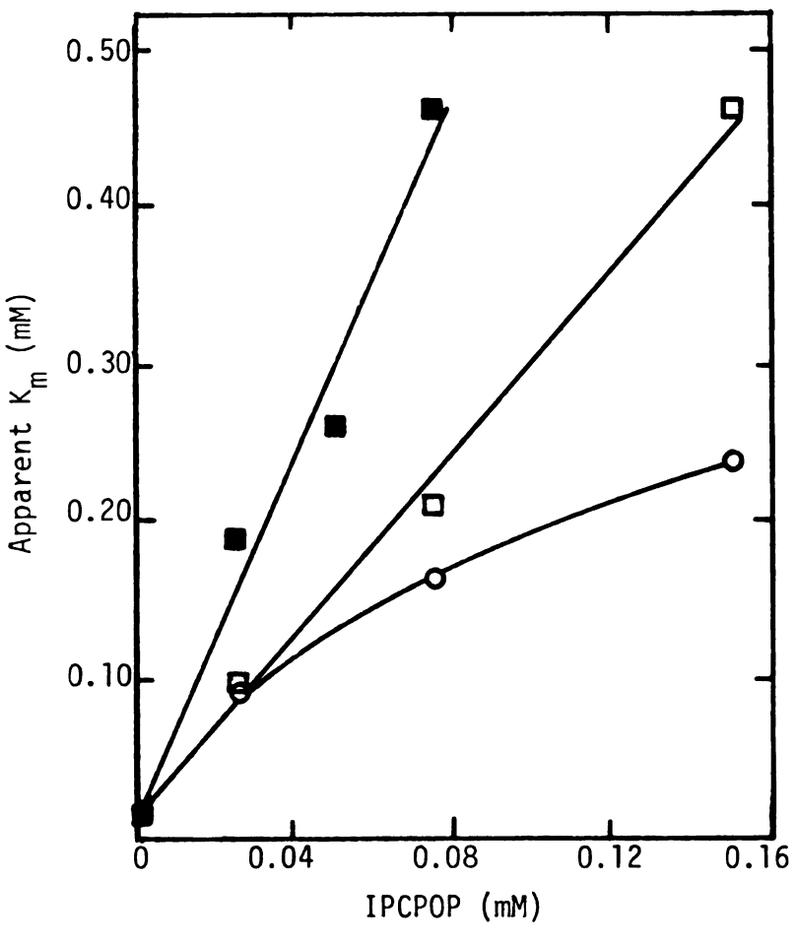

Figure 30. Effect of IPCPOP on the Apparent K_m of NTPH

The apparent K_m was calculated at each IPCPOP concentration as described in Figure 29. The two straight lines were fit to the data by the method of least squares. Legend: (■) partially purified rabbit NTPH, specific activity = 247,000 units/mg, (□) partially purified human NTPH, specific activity = 3500 units/mg, (o) NTPH-containing lysate, specific activity = 15 units/mg.

Table 12. Purification of NTPH by Affinity Chromatography*

	Recovery (%)	Specific Activity (Units/mg)	Purification
Experiment 1			
NTPH-containing lysate	100	30	
Buffer elution	21		
Buffer + ITP elution	55	6080	200
Experiment 2			
Partially purified NTPH	100	1140	
Buffer elution	17		
Buffer + ITP elution	106	4990	4.4

* Affinity chromatography was performed with a 0.9 x 6.5 cm column containing IPCPOP-Sepharose-4B at 4°C. The column was eluted with buffer at pH 7.4 containing 1.0 mM $MgCl_2$ followed by a buffer containing ITP and 10 mM $MgCl_2$ as described in the Methods. NTPH-containing lysate was prepared by a 1:4 hypotonic lysis of red cells, and partially purified NTPH was prepared by the method of Morris (142). The protein was determined by the method of Lowry et al. (156).

the effluent with 106% appearing during the ITP elution at a greater than 4-fold purification.

F. Kinetic Differences Between Human and Rabbit NTPH

Table 13 is a comparison of the kinetic constants of human and rabbit NTPH. The K_m for ITP, K_m for GTP and K_i for IPCPOP was in each case less for the rabbit enzyme than the human. The Lineweaver-Burk plot for the determination of the K_m for GTP in Figure 31 indicates that GTP at high concentrations inhibits the rabbit enzyme in agreement with results reported earlier (153). Furthermore, high ITP concentrations have also been reported to inhibit the rabbit NTPH (1). In contrast to these results neither GTP (up to 1.0 mM, Figure 31) or ITP (up to 2 mM, data not shown) was observed to inhibit the human NTPH.

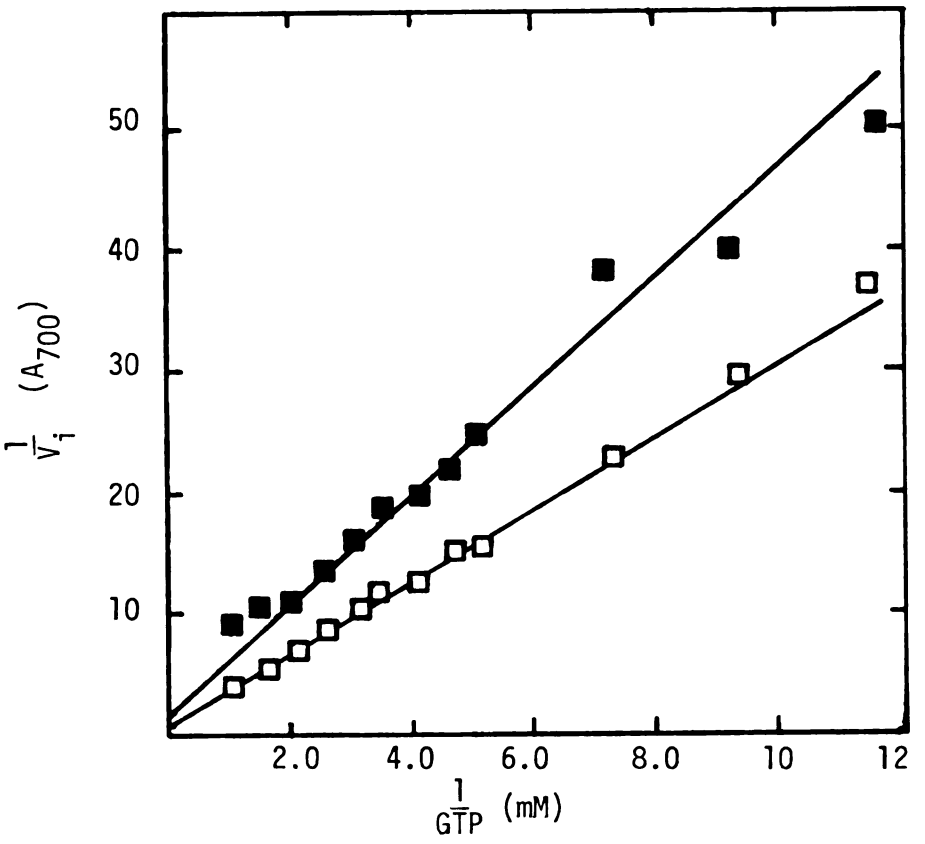

Figure 31. Lineweaver-Burk Plot for the Determination of the K_m for GTP of Rabbit and Human NTPH

NTPH was determined under standard conditions with varying concentrations of GTP. The velocity is expressed as the net absorbance at 700 nm in the Rathbun and Betlach assay for phosphate (see Methods). Lines were fit by a computer program using the method of Wilkinson (167). Legend: (■) partially purified rabbit NTPH (specific activity = 247,000 units/mg), (□) partially purified human NTPH (specific activity = 3500 units/mg).

Table 13. Kinetic Constants for Human and Rabbit NTPH*

	Human NTPH	Rabbit NTPH
K_m for ITP	23.5 ± 2.2 μM	16.0 ± 1.0 μM
K_m for GTP	3.82 ± 0.46 mM	2.74 ± 0.71 mM
K_i for IPCPOP	5.9 μM	3.7 μM

* Human and rabbit NTPH were partially purified by the method of Morris (142). The specific activities of human and rabbit NTPH were 3500 units/mg and 247,000 units/mg, respectively. The K_m for ITP and GTP was determined as described in Figure 29 and 31, respectively, and the K_i was determined as described in Figure 30. The standard deviation is given for the K_m values.

Whether these kinetic differences indicate a molecular difference in the NTPH from these two sources is not known.

G. Cell-free Synthesis of ITP

Zachara reported that red cells incubated in the IPP media (10 mM inosine:10 mM pyruvate:50 mM phosphate) of Duhm, Deuticke and Gerlach (186) accumulated 98 μmoles ITP per 100 ml cells during a 2 hour period at 37°C (119). Although Zachara used Dowex 1-χ8 to analyze the ITP accumulation in these cells, studies in our laboratory indicated that nucleotides including ITP could readily be separated and quantitated by HPLC (see Figures 32 and 33). Suspension of intact red cells (NTPH activity = 15 nmoles ITP hydrolyzed/mg hemoglobin) in the IPP media at 37°C for 2 hours followed by analysis of the nucleotides by HPLC indicated an accumulation of 64 μmoles ITP/100 ml red cells, a result similar to that reported by Zachara. Experiments were then designed to analyze cell-free synthesis of ITP.

Figure 32. HPLC Analysis of Hypoxanthine, Inosine, IMP, GMP, IDP, ITP, and ATP

TCA extracts of lysates were analyzed on a Perkin-Elmer 1250 HPLC with a Partisil-10 SAX column eluted with (A) 0.05 M KH_2PO_4, pH 3.4, (B) 0.2 M KH_2PO_4, pH 3.4, and (C) 0.5 M KH_2PO_4, pH 3.4 at a flow rate of 55 ml/hour. The retention time of standards are marked as follows: (a) inosine, (b) hypoxanthine, (c) IMP, (d) GMP, (e) ADP, (f) IDP, (g) GDP, (h) ATP, (i) ITP and (j) GTP.

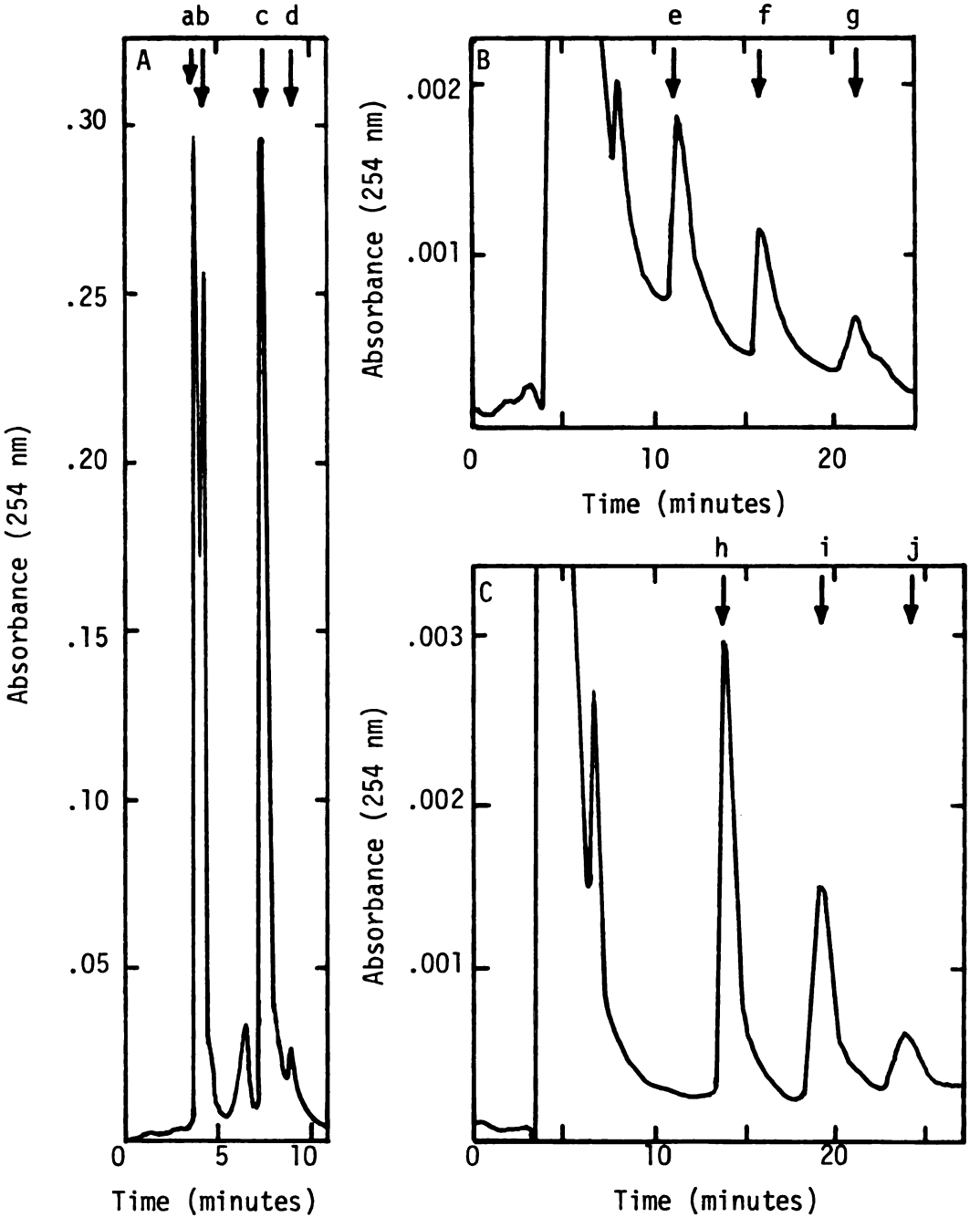

Figure 32. HPLC Analysis of Hypoxanthine, Inosine, IMP, GMP, IDP, ITP, and ATP

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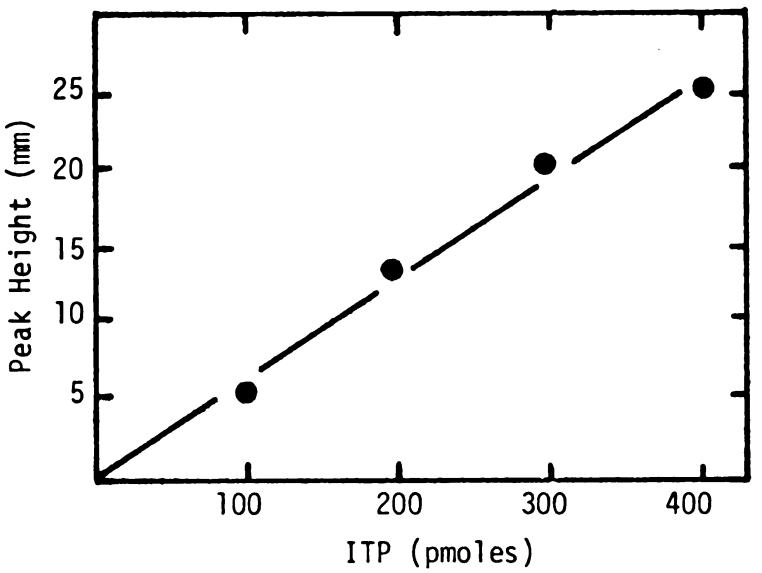


Figure 33. Standard Curve for the Quantitation of ITP by HPLC

HPLC analysis of ITP was performed as described in Figure 32C. The peak height of ITP is plotted against the amount of ITP in the 10 µliters injected on the column. ITP concentrations were determined by the UV absorption of ITP at 248.5 nm (extinction coefficient = 12.2 mM).

1. Stimulation of Cell-free Synthesis with PRPP

In agreement with Hershko et al. (29), we were not able to find an IMP kinase activity in hemolysates by the substitution of IMP for GMP in the guanylate kinase assay (187). Furthermore, in an attempt to observe ITP synthesis in hemolysates either by sonicating the cells in the IPP medium described above or lysing the cells with water followed by addition of the components of the IPP medium, we could detect very little ITP accumulation by HPLC even if the hemolysates were prepared from blood of individuals with relatively low NTPH activity and IPCPOP was included during the incubation to inhibit the remaining NTPH. Thus a search was initiated to find a metabolite or set of conditions which might enhance the synthesis of ITP. The results in Table 14 indicate that the combination of PRPP and inosine gave a 4-fold stimulation of the synthesis of IDP and ITP. On the other hand, this stimulation was

Table 14. Stimulation of Cell-free ITP Synthesis with PRPP

All incubations in these experiments were performed at 37°C for 3 hours. The reaction mixtures (0.3 ml each) included 0.2 ml of a lysate (packed cells-water, 1:1) of VLV red cells (NTPH activity, 15 nmoles ITP hydrolyzed/mg hemoglobin), 50 mM sodium phosphate buffer, pH 7.0, and the metabolites listed. R1P and R5P refer to α,D-ribose 1-phosphate and D-ribose 5-phosphate, respectively. Mg_2 PRPP was purified as described in the Methods. TCA extracts of the mixtures were neutralized and analyzed by a Perkin-Elmer 1250 HPLC equipped with a Partisil-10 SAX column (see Figure 32). Nucleotides were quantitated by peak height (see Figure 33).

Experiment 1

Incubation Mixture					Nucleotides Analyzed by HPLC			
Metabolites (mM)					(nmoles/ml mix)			
Ino	IMP	IDP	Mg_2PRPP	Glucose	ATP	IDP	ITP	IDP+ITP
0	0	0	0	5	175	0	0	0
5	0	0	0	5	151	9	24	33
5	0	0	5	5	119	69	76	145
0	5	0	0	5	131	25	19	44
0	5	0	5	5	163	13	19	32
0	0	5	0	5	96	-	874	-

Experiment 2

Incubation Mixture				Nucleotides Analyzed by HPLC			
Metabolites (mM)				(nmoles/ml mix)			
Ino	Mg_2PRPP	R1P	PP_i	ATP	IDP	ITP	IDP+ITP
0	5	5	0	119	-	0	-
5	5	5	0	103	21	59	80
5	0	5	0	95	9	0	9
5	0	0	5	131	-	0	-

Experiment 3

Incubation Mixture						Nucleotides Analyzed	
Metabolites (mM)						(nmoles/ml mix)	
Ino	Mg_2PRPP	Glucose	Mg_2Cl_2	R5P	ATP	ATP	ITP
0	0	5	0	0	0	235	0
0	5	5	0	0	0	262	0
5	0	5	0	0	0	168	17
5	5	5	0	0	0	176	70
5	5	5	0	0	0	172	58
5	0	5	5	0	0	163	21
5	0	5	0	0	2	434	12
5	0	5	0	5	2	319	12

not observed when any of the metabolites, α,D-ribose 1-phosphate, PP_i, $MgCl_2$, D-ribose 5-phosphate or ATP was substituted for PRPP. Furthermore, the stimulation of the synthesis of IDP/ITP by PRPP was independent of the presence of glucose.

2. Correlation of IDP/ITP Synthesis with the Presence of Inosine and Hypoxanthine

The results in Table 14 further indicate that inosine cannot be substituted by IMP. Thus inosine and PRPP gave 4-fold more synthesis of IDP/ITP than the same concentrations of IMP and PRPP. This suggests that the biosynthesis of IDP/ITP may not occur through IMP as an intermediate. Table 15, Experiments 1 and 2, presents additional evidence that cell-free IDP/ITP synthesis is best correlated with elevated concentration of hypoxanthine and inosine rather than the concentrations of IMP in the reaction mix. It may also be noted from this table that inhibition of HGPRT with 5 mM IMP, 0.5 mM GMP, or 5 mM GMP (39) eliminates the requirement for PRPP, perhaps by increasing the availability of endogenous PRPP or the PRPP synthesized from inosine (43). IMP, and perhaps GMP, may also inhibit NTPH (1) and thus enhance the accumulation of IDP/ITP.

3. Incorporation of [^{14}C]inosine and [^{3}H]hypoxanthine into IDP/ITP

In contrast to the accumulation of IDP/ITP from high concentrations of unlabeled inosine, accumulation of [^{14}C]ITP from [^{14}C]inosine not only requires PRPP but also requires the additional presence of IMP (Table 16, Experiment 1). It seems likely that at low concentrations, [^{14}C]inosine in the presence of PRPP is rapidly incorporated into IMP through the combined action of purine nucleoside phosphorylase and HGPRT. A high concentration of IMP would then slow this incorporation

Table 15. Correlation of Cell-free IDP/ITP Synthesis with Elevated Concentrations of Hypoxanthine and Inosine

The VLV lysate used in Experiment 1 and the DY lysate used in Experiment 2 (NTPH activities of 15 and 3 nmoles ITP hydrolyzed/mg hemoglobin, respectively) were treated with 10 mg charcoal/ml lysate for 5 minutes and the charcoal was removed by centrifugation. All reaction mixtures included 5 mM $MgCl_2$ and 50 mM sodium phosphate, pH 7.0. R5P refers to D-ribose 5-phosphate. Metabolite analyses were performed as described in Table 14.

Experiment 1

Incubation Mixture				Metabolites Analyzed by HPLC						
Time	Metabolites (mM)			(μmoles/ml mix)			(nmoles/ml mix)			
(hrs.)	Ino	IMP	Na_4PRPP	Hyp	Ino	IMP	ATP	IDP	ITP	IDP+ITP
3	5	0	0	3.1	.029	1.6	110	9	19	28
3	0	5	0	0.25	.010	6.5	81	0	0	0
3	0	0	5	.009	.010	0.48	192	0	0	0
3	5	5	0	5.9	.086	3.0	156	27	118	145
3	5	0	5	1.2	.057	7.0	150	25	57	82
3	0	5	5	0.8	.048	7.2	132	15	43	58
0	5	5	5	3.2	6.23	0.05	90	-	0	-
3	5	5	5	4.4	.238	7.7	122	19	99	118

Experiment 2

Incubation Mixture						Metabolites Analyzed by HPLC							
Time	Metabolites (mM)					(μmoles/ml mix)				(nmoles/ml mix)			
(hrs.)	Ino	IMP	GMP	R5P	Na_4PRPP	Hyp	Ino	IMP	GMP	ATP	IDP	ITP	IDP+ITP
3	5	0	0	0	0	3.5	.057	1.2	-	90	19	57	76
3	0	5	0	0	0	0.31	.009	6.4	-	111	0	19	19
3	0	0	0	0	5	.009	.009	0.43	-	35	0	0	0
0	5	5	0	0	0	1.9	3.1	6.8	-	63	0	0	0
3	5	5	0	0	0	3.5	.076	7.6	-	177	49	133	182
3	5	0	0	0	5	1.1	.038	6.1	-	192	37	142	179
3	0	5	0	0	5	.073	.009	7.2	-	78	9	38	47
3	5	5	0	0	5	1.6	.048	11.7	-	189	43	13	176
3	0	5	0	5	0	.093	.019	7.3	-	132	15	24	39
3	0	5	0	.5	0	.009	.009	6.7	-	108	9	19	28
3	5	0	5	0	0	3.1	.038	1.8	7.3	165	37	90	127
3	5	0	.5	0	0	3.5	.048	1.8	1.3	144	55	123	178

Table 16. Cell-free Incorporation of [^{14}C]Inosine and [^{3}H]Hypoxanthine into IDP/ITP

Each reaction mixture in Experiments 1 and 2 included DY lysate (NTPH activity, 3 nmoles ITP hydrolyzed/mg hemoglobin), 5 mM $MgCl_2$, and 50 mM sodium phosphate, pH 7.0. The specific activity of the [^{14}C]inosine used was 52 mCi/mmole.

Each reaction mixture in Experiments 3 and 4 included VLV lysate (NTPH activity, 15 nmoles ITP hydrolyzed/mg hemoglobin) and 50 mM sodium phosphate, pH 7.0. The Mg_2PRPP was purified as described in the Methods. RIP refers to α,D-ribose 1-phosphate. The [^{3}H]hypoxanthine used in these experiments had a specific activity of 1.8 Ci/mmole.

All incubations were carried out for 3 hours at 37°C and the metabolites were analyzed as described in Table 14. Radioactivity in each region was collected and analyzed as described in the Methods.

Experiment 1

Incubation Mixture			Metabolites Analyzed by HPLC		
Metabolites (mM)			(cpm)		
[^{14}C]Ino	Na_4PRPP	IMP	Hyp+Ino	IMP	ITP
0.13	5	0	1317	39,727	20
0.13	5	5	1337	41,700	216

Experiment 2

Incubation Mixture					Metabolites Analyzed by HPLC		
Time (hrs.)	Metabolites (mM)				(cpm)		
	[^{14}C]Ino	Ino	IMP	Na_4PRPP	Hyp+Ino	IMP	ITP
0.5	0.13	5	5	5	27,635	6,909	N.D.
1.5	0.13	5	5	5	13,506	21,107	31
3.0	0.13	5	5	5	3,801	32,033	194

Experiment 3

Incubation Mixture				Metabolites Analyzed by HPLC		
Metabolites (mM)				(cpm)		
Ino	Mg_2PRPP	Glucose	[^{3}H]Hyp	Hyp+Ino	IMP	ITP
5	5	5	.033	23,119	51,415	959
0	5	5	.033	3,979	80,353	35

Experiment 4

Incubation Mixture				Metabolites Analyzed by HPLC		
Metabolites (mM)				(cpm)		
Ino	Mg_2PRPP	R1P	[^{3}H]Hyp	Hyp+Ino+IMP	IDP	ITP
5	5	5	.033	78,643	212	767
5	0	5	.033	80,114	68	169
0	5	5	.033	74,559	-	0
0	0	5	.033	71,852	-	0

of radioactivity into IMP by its mass action effect on the HGPRT catalyzed reaction as well as the actual inhibition of HGPRT (39) and thus conserve [^{14}C]inosine for synthesis of IDP/ITP.

Further evidence that the concentration of inosine during the incubation may be directly correlated with the incorporation of [^{14}C]inosine into ITP is presented in Table 16, Experiment 2. Unlabelled inosine in this experiment was increased by 5 mM over that in Experiment 1. Comparison of the results of these two experiments indicates that 5 mM [^{14}C]inosine at a specific activity of 1.3 mCi/mmole was incorporated to the same extent as 0.13 mM inosine at a specific activity of 52 mCi/mmole. It may be observed that following 3 hours of incubation, the radioactivity in the hypoxanthine and inosine region of the chromatogram was 3.4-fold higher in the case with high initial inosine concentration (Experiment 2) than in the case with low initial inosine concentration (Experiment 1). Thus it seems that the incorporation of [^{14}C]inosine into IDP/ITP as well as the incorporation of unlabelled inosine into IDP/ITP may be correlated with conditions which prevent or slow the inosine incorporation into IMP.

Table 16, Experiments 3 and 4, demonstrates that at low concentrations [^{3}H]hypoxanthine is readily incorporated into IMP but not IDP or ITP. However a high concentration of unlabelled inosine with the same amount of [^{3}H]hypoxanthine yielded more than 1% incorporation of the label into IDP/ITP. In this case it seems that inosine itself may have been labelled by the [^{3}H]hypoxanthine by means of the reversable purine nucleoside phosphorylase reaction (188) and the high concentration of inosine prevented all the radioactivity from being incorporated into IMP during the 3 hour incubation (see Experiment 3). α,D-ribose

1-phosphate (dicyclohexylammonium salt) alone could not stimulate the incorporation of [^{3}H]hypoxanthine into ITP (see Experiment 4), but this may simply be due to the low concentration of hypoxanthine in this experiment.

4. Effect of NTPH Activity on IDP/ITP Accumulation

As might be expected, the activity of NTPH in the lysates affected the rate of ITP accumulation in the three hour incubations. As demonstrated in Table 17, the highest accumulation of ITP was obtained with the lysates with lowest NTPH activity.

Table 17. Effect of NTPH Activity on the Cell-free Synthesis of IDP/ITP*

Incubation Mixture						Nucleotides Analyzed		
Lysate		Metabolites (mM)				(nmoles/ml mix)		
Subject	NTPH ($\frac{\text{Units}}{\text{mg Hb}}$)	Ino	IMP	GMP	Na_4PRPP	IDP	ITP	IDP+ITP
DY	3	5	5	0	0	49	133	182
DY	3	5	5	0	5	43	133	176
DY	3	5	0	0.5	0	55	123	178
VLV	15	5	5	0	0	27	118	145
AJM	65	5	0	0.5	5	15	33	48

* All reaction mixes included lysates, which had been treated once with charcoal (10 mg/ml lysate); 5 mM $MgCl_2$ and 50 mM sodium phosphate, pH 7.0. The 3 hour incubation procedures for the lysate of the red cells of each subject were performed on different days. The nucleotide analyses were performed as described in Table 14.

5. Other Conditions Affecting Cell-free IDP/ITP Synthesis

To uncouple IDP from ITP synthesis by removing ATP and to test whether IDP synthesis was ATP dependent, the endogenous nucleotides were removed by treatment of the lysates with charcoal. As can be seen

in Table 18, extensive charcoal treatment reduced both the ATP concentration and capability of these lysates to synthesize IDP/ITP. Experiment 2 in Table 18 indicates that very little IDP/ITP synthesis was recovered even when ATP or PRPP was added back to these lysates. Thus the experiments so far have not demonstrated an uncoupling of IDP from ITP synthesis or shown that IDP synthesis is completely independent of ATP concentrations.

Sonification of crude hemolysates seemed to decrease endogenous levels of ATP, perhaps by release of a phosphatase associated with the membrane, however these lysates were no longer capable of cell-free synthesis of IDP or ITP.

When stored for months at -20°C, the washed packed red cells from one individual (DY) retained the ability to accumulate ITP under cell-free conditions.

Table 18. Effect of Extensive Charcoal Treatment of Hemolysates on Cell-free IDP/ITP Synthesis

Experiment 1

Incubation Mixture			Metabolites Analyzed by HPLC						
Lysate Charcoal Treatment	Metabolites (mM) Ino	IMP	(μmoles/ml mix) Hyp	Ino	IMP	(nmoles/ml mix) ATP	IDP	ITP	IDP+ITP
1 X	5	5	3.5	.076	7.6	177	49	133	182
2 X	5	5	3.8	.048	6.1	18	0	17	17
3 X	5	5	-	-	-	30	9	24	33

Experiment 2

Incubation Mixture					Metabolites Analyzed by HPLC						
Lysate Charcoal Treatment	Metabolites (mM) Ino	GMP	Na_4PRPP	ATP	(μmoles/ml mix) Hyp	Ino	IMP	(nmoles/ml mix) ATP	IDP	ITP	IDP+ITP
2 X	5	0.5	0	0	-	-	-	-	0	-	-
2 X	5	0.5	0	1	-	-	-	-	5	-	-
2 X	5	0.5	5	0	1.01	.057	6.6	-	8	-	-
2 X	5	0.5	5	1	1.03	.076	6.7	123	6	10	16

The reaction mixtures in Experiment 1 contain DY lysate (NTPH activity, 3 nmoles ITP hydrolyzed/mg hemoglobin) and the mixes in Experiment 2 contain VLV lysate (NTPH activity, 15 nmoles ITP hydrolyzed/mg hemoglobin). Prior to the 3 hr incubation these lysates were treated with charcoal, 10 mg/ml lysate, for 5 min and the charcoal was removed by centrifugation. This process was repeated where indicated. All mixtures also include 5 mM $MgCl_2$ and 50 mM sodium phosphate, pH 7.0. Metabolite analyses were performed as described in Table 14.

CHAPTER V

DISCUSSION

A. The Genetic Variability of NTPH

At the time of the purification and characterization of NTPH from rabbit reticulocytes by Chern, MacDonald and Morris (1), it was observed that the NTPH activity in human red cells varied more than 100-fold from one individual to another, yet the specific activity was constant in the red cells of any one individual over a period of years. Concurrent with these observations Vanderheiden published a population distribution and 15 family studies of an "ITPase" activity in the red cells, data which he interpreted as evidence for the codominant transmission of this activity (3). Vanderheiden was inadvertantly measuring a coupled reaction between NTPH and endogenous red cell pyrophosphatase and thus either variation in NTPH or red cell pyrophosphatase activity could have accounted for his observed distribution of "ITPase" activity in the human population. To ascertain whether this distribution reflected the variation of NTPH activity and to establish a framework for the study of the inheritance of NTPH as well as for a study of the metabolic implications of this activity, S. A. Fuller conducted a population survey of the specific activity of NTPH in red cells. The survey clearly demonstrates the variation of NTPH activity among individuals and furthermore suggests that individuals may be classified into at least two groups, those with low or high activity based on the

specific activity of their red cells (Figure 3). This distribution corresponds well with the population survey presented by Vanderheiden indicating that the variation of the "ITPase" which he observed was primarily due to variation of NTPH. Vanderheiden however suggested yet a third classification of individuals, those with very low activity. Whether this third classification is indeed a distinct group which represents a unique genotype cannot be established by evidence presented by Vanderheiden or supported by data presented in this dissertation. However as discussed below, the metabolic consequence of very low red cell NTPH activity (0-3 nmoles ITP hydrolyzed/mg hemoglobin) may be distinct from the metabolic consequence of low activity (3-27 nmoles ITP cleaved/mg hemoglobin). Thus it seems that the specific activity of NTPH in the red cells is an inherited trait by virtue of the similarity of the two population distributions described here and the fact that NTPH activity remains unchanged in any individual over a period of years.

Other data presented in this dissertation also support this conclusion. First, the two family studies, particularly the Gr family (Figure 4), indicate that the NTPH activity of the progeny reflect the activity of their parents. S. A. Fuller is currently expanding these studies to include other families with the hope of defining the pattern of inheritance. Second, the results of the survey of NTPH activity in granulocytes, lymphocytes and platelets emphasize that the variation of NTPH activity is not limited to the erythroid cells but is reflected in other tissues as well. Thus any explanation of the molecular basis of this diversity cannot be limited to red cell development or metabolism.

Consistent with the results of the tissue survey of the rabbit (2), the NTPH activity per cell was lowest in the erythrocyte (see Figure 5-7). Although the NTPH per platelet was similar to the red cell activity, the platelet activity probably reflects the NTPH activity of a small portion of the cytoplasm of a megakaryocyte, the platelet-producing cell.

Possible explanations for the molecular basis of the NTPH variation may be classified into two groups. First, the actual amount of NTPH protein may vary due to alterations in the synthesis or degradation of the enzyme in the cells or second, variation may be caused by NTPH molecules with altered kinetic parameters for the substrate ITP which may be caused by the presence of isozymes or intracellular effectors in different proportions or combinations in the cells of different individuals. A number of these possibilities has been tested. Previous data from this laboratory as well as the data offered by Harris and Hopkinson suggest that the variation cannot be attributed to electrophoretic isozymes. With NTPH from rabbit liver, rabbit red cells and human red cells, Wang and Morris could observe only one band on disc gel electrophoresis specific for the hydrolysis of ITP (2). Although Harris and Hopkinson report that by their method NTPH migrates as 2 or 3 electrophoretic bands, they could not observe qualitative differences of NTPH electrophoretic mobility while screening a "large number of red cell lysates" (145, see also Literature Review). Table 2 indicates that the K_m for ITP is the same for NTPH in lysates of selected individuals which exhibit a wide range of NTPH specific activities. The mixing study in Table 1 and data reported earlier (5) give no indication of intracellular inhibitors or activators. Current

studies in this laboratory are designed to eliminate or test other possible molecular causes of this variation.

B. The Metabolic Consequences of the Genetic Variation of NTPH

A number of hypotheses have been offered over the past ten years concerning the physiological significance of NTPH. Hershko, et al. suggested that NTPH may be involved in the control of the intracellular concentration of GTP, a substrate of NTPH hydrolyzed at 10% the rate of ITP (121); Wang and Morris suggested that NTPH is necessary to prevent incorporation of ITP into RNA or dITP into DNA (2) and Vanderheiden postulated that NTPH is one of the enzymes of an ITP-IMP cycle which controls intracellular concentrations of ATP (144). To support the assumption inherent in the last two hypotheses that ITP is the physiological substrate of NTPH, it is important to establish that the substrate-enzyme relationship exists between ITP and NTPH. Vanderheiden had suggested as early as 1965 that the presence of ITP in the red cells of some individuals could be attributed to the lack of an "ITPase" and furthermore that the variation in the accumulation of [^{14}C]ITP from [^{14}C]inosine by intact cells could be attributed to variation in this same "ITPase" (26, 3). The data in Figure 9 as discussed in Appendix A support the conclusion that the rate of [^{14}C]ITP accumulation by intact red cells in the presence of [^{14}C]hypoxanthine is related to the NTPH specific activity of these cells in a manner predicted by Michaelis-Menten kinetics. Furthermore direct analysis of nucleotides by HPLC indicated that a UV-absorbing peak coinciding with ITP was present in extracts of blood which had a very low red cell NTPH activity, 3 nmoles ITP cleaved/mg hemoglobin (see Figure 13). On the other hand concentrations of GTP (Table 8 and

Figures 13-15) were quite similar for blood of individuals with a 10-fold range of red cell NTPH activity. One can conclude therefore that NTPH activity has a physiologically significant enzyme-substrate relationship with ITP in the red cell both in artificial incubation media and *in vivo*.

Since ca. 1% of the population may have undetectable NTPH activity in their red cells, the question may be asked whether other tissues of these individuals have undetectable activity as well and if so, whether the ITP concentration is unusually high in these tissues. Although we were not able to analyze tissues other than the red cells of individuals with undetectable NTPH activity, it is interesting that the one individual with very low red cell NTPH has greater than 5-fold more granulocyte NTPH activity and greater than 30-fold more lymphocyte NTPH activities compared to the red cell level of that enzyme (Tables 3-5). Thus tissue differences with regard to NTPH activity may prevent endogenous accumulation of ITP in cells other than the red cell, a cell which is not capable of DNA or RNA synthesis. On the other hand tissues may differ in the ability to synthesize ITP, a question which has not yet been studied.

C. The Physiological Role of ITP and NTPH

If we proceed with the hypothesis that NTPH is present to prevent incorporation of ITP or dITP into RNA or DNA, we may ask why ITP is synthesized at all. Since the red cell and perhaps other tissues clearly can synthesize ITP, it is possible that either ITP or intermediates of its synthesis or an ITP-IMP cycle may have some physiological role. Since the biosynthesis of ITP has not been defined or studied

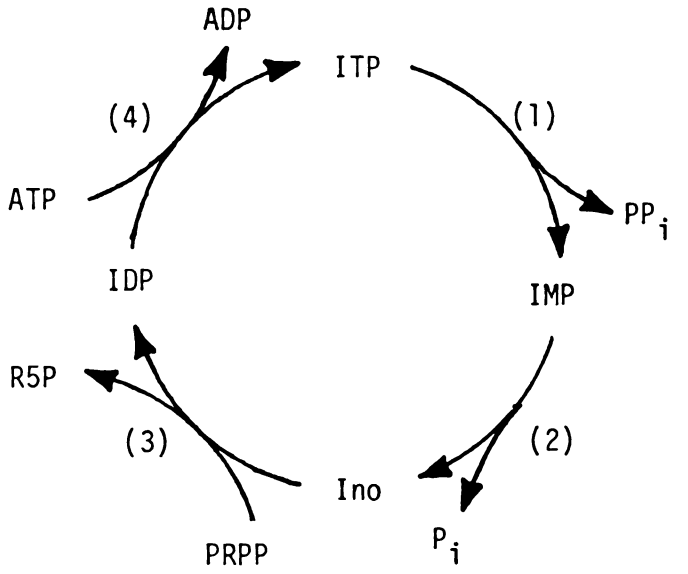
in red cell lysates, we worked to establish a cell-free system which had the capability to accumulate ITP.

The substrate-enzyme relationship between ITP and NTPH in intact cells suggested that to observe any ITP synthesis it would be necessary to use the red cells of an individual with very low NTPH activity or find an inhibitor which is highly specific for NTPH in order to prevent degradation of ITP as soon as it was synthesized. Since the availability of individuals with very low NTPH activity is limited, we found it more practical to prepare an inhibitor. The work of Yount (183) with methylene analogs of ATP suggested that substitution of the oxygen with a methylene in the α,β-position of ITP might yield a very specific competitive inhibitor of NTPH.

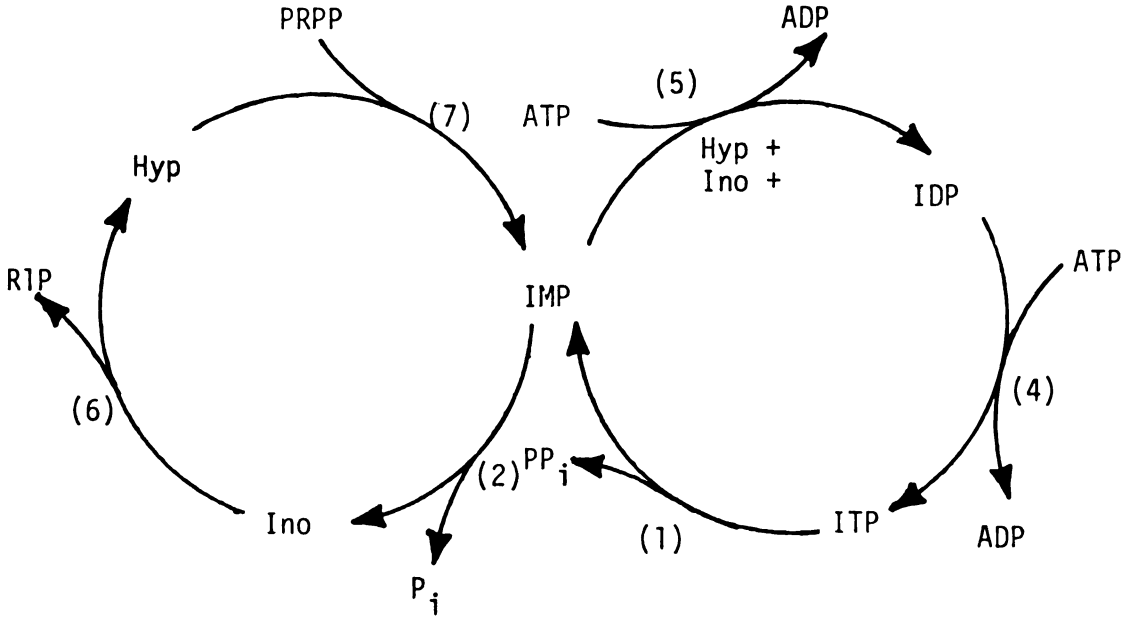
IPCPOP was successfully prepared by two methods, deamination of commercial APCPOP and synthesis from adenosine and methylene diphosphonic acid followed by deamination and phosphorylation of the resulting APCP. IPCPOP was a very good competitive inhibitor of partially purified human NTPH (K_i = 5.9 μM) and NTPH present in lysates (Figure 29). IPCPOP was also coupled to Sepharose-4B to yield an affinity resin which was used to purify NTPH from hemolysates or partially purified preparation (Table 12). Preliminary cell-free experiments designed to observe accumulation of ITP by lysates incubated with the inosine, pyruvate and phosphate media used by Zachara (119) were unsuccessful even when lysate with low NTPH activity, 15 nmoles ITP cleaved/mg hemoglobin, was used in combination with the IPCPOP inhibitor. These studies indicated that the cell-free accumulation of ITP was not limited by NTPH alone but probably required a cofactor or set of conditions not being supplied by the incubation conditions.

Many of the obvious cofactors such as nucleoside triphosphates or conditions such as variation of the pH did not stimulate ITP synthesis. But the observation that PRPP enhanced IDP and ITP synthesis in the presence of inosine but not in the presence of IMP (Table 14) suggested that synthesis of ITP may involve a unique reaction in which the pyrophosphoryl moiety of PRPP is transferred to the 5'-ribose position of inosine to yield IDP (Model I of Figure 34) as opposed to synthesis of IDP by phosphorylation of IMP (Model II of Figure 34). Evidence for this pyrophosphotransferase reaction is discussed below.

The direct involvement of PRPP in IDP/ITP synthesis was deduced from the following observations. First, the inosine-phosphate media used in these studies is conducive for the synthesis of PRPP in that inosine can be metabolized to R5P and combine with ATP to form PRPP, a reaction catalyzed by PRPP synthetase in the presence of high phosphate (43). But the production of PRPP by this pathway sacrifices inosine which also appears to be necessary for the synthesis of IDP/ITP. Thus the addition of exogenous PRPP stimulates IDP/ITP synthesis, perhaps because in this case neither inosine or PRPP is rate-limiting. Second, to confirm that the stimulation by PRPP was not caused by one of its degradation products such as PP_i, R5P or R1P, these metabolites were substituted for PRPP in the cell-free system but none could stimulate the synthesis of IDP/ITP. Third, since R5P can potentially yield ATP in this system by entering glycolysis at the level of glyceraldehyde-3-phosphate, exogenous ATP was tested and not found to be an effector of IDP/ITP synthesis. However the possibility that the endogenous ATP provided by the lysates was sufficient to effect the IDP/ITP synthesis cannot be excluded by data described here. Fourth, conditions



MODEL I



MODEL II

Figure 34. Two Models for the Role of NTPH in the Metabolism of ITP

The enzymes involved in these cycles are the following: (1) NTPH, (2) 5'-nucleotidase, (3) proposed pyrophosphotransferase, (4) NDP Kinase, (5) hypothetical IMP Kinase, (6) PNP, (7) HGPRT.

which prevented the utilization of PRPP in other reactions also stimulated IDP/ITP synthesis. Thus 0.5 mM GMP or 5 mM IMP, known competitive inhibitors for PRPP of HGPRT (K_i = .014 and .14 mM, respectively, 39), could stimulate synthesis even if exogenous PRPP was not provided.

The direct involvement of inosine in the synthesis of IDP/ITP was deduced from the following data. First, inosine and PRPP yield 4-fold more IDP/ITP synthesis than either IMP alone or IMP and PRPP. Second, elevated concentrations of inosine and hypoxanthine as detected by HPLC following 3 hours of incubation were consistently associated with increased synthesis of IDP/ITP during this incubation period. Third, conditions which prevented the utilization of hypoxanthine by HGPRT also stimulated IDP/ITP synthesis. Thus either 0.5 mM GMP or 5 mM IMP, inhibitors of HGPRT as noted above, not only spares PRPP but also spares hypoxanthine and thus prevents complete degradation of inosine during the three hour incubation (Table 15).

Table 14 shows clearly that IDP is readily converted to ITP in the cell-free system. Attempts to uncouple IDP synthesis from ITP synthesis by removal of endogenous ATP by extensive charcoal treatment of the lysates were not successful since the charcoal treatment also altered the ability of these lysates to synthesize IDP (Table 18). Synthesis of IDP or ITP could not be recovered even when ATP was added back to the system.

From these data alone and the observations that NTPH activity affects the accumulation of ITP in the cell, one can develop a coherent model for the direct involvement of both inosine and PRPP as substrates in a reaction synthesizing IDP/ITP with subsequent hydrolysis to IMP (Model I), however one may argue that these correlations could also

support Model II in which inosine or hypoxanthine stimulates an "IMP kinase" which transfers a phosphate from ATP to IMP to yield IDP. It may be argued that even in the situation with maximum stimulation of IDP/ITP synthesis from inosine in the presence of 0.5 mM GMP (Table 15), enough IMP may be produced during the 3 hour incubation such that the concentration of IMP is not limiting the rate of the "IMP kinase." But one must then assume that the synthesis of IDP/ITP is regulated very closely by hypoxanthine or inosine since the combination of ATP, R5P and IMP gave no stimulation of IDP/ITP synthesis (Table 15).

In an attempt to resolve which model represents synthesis of IDP/ITP in this cell-free system, experiments were designed with radioactive precursors to determine the conditions necessary for maximum incorporation of labelled inosine or hypoxanthine into IDP/ITP. First, it was observed that [^{3}H]hypoxanthine in the presence of PRPP is only incorporated into IMP, but [^{3}H]hypoxanthine in the presence of both PRPP and inosine is incorporated into IDP/ITP as well. Although these data might be rationalized by Model II, the incorporation of [^{3}H]hypoxanthine stimulated by inosine may be explained by an exchange reaction between inosine and [^{3}H]hypoxanthine under the conditions of the experiment. This explanation receives some support by the fact that in the presence of inosine, only 69% of the labelled hypoxanthine was incorporated into IMP while in the absence of inosine 95% of the label was incorporated into IMP. Second, it is particularly significant that IMP stimulated the incorporation of [^{14}C]inosine into IDP/ITP. In the presence or absence of added IMP most of the label from [^{14}C]inosine appeared in the IMP region of the chromatogram following 3 hours of incubation. However, the stimulation of the incorporation of label

into IDP/ITP by unlabelled IMP is easily rationalized by Model I in which the large pool of IMP would act to inhibit HGPRT and thus slow incorporation of [^{14}C]inosine into IMP and allow the [^{14}C]inosine to be incorporated into IDP/ITP. An unlikely alternate explanation would be that the small amount of added [^{14}C]inosine in these experiments both labels a huge IMP pool and yet the inosine or hypoxanthine remains at a concentration which is capable of stimulating the incorporation of radioactivity from this IMP pool into IDP/ITP.

Obviously the situation is complicated in these red cell lysates by reactions which mask the effect of exogenous inosine and PRPP. Inosine can be metabolized by purine nucleoside phosphorylase to hypoxanthine and R1P. The R1P in turn may be converted by phosphoribomutase (191) to R5P which may be used to synthesize PRPP by means of PRPP synthetase and ATP or enter glycolysis at the level of glyceraldehyde 3-phosphate and stimulate production of ATP. On the other hand, hypoxanthine could be salvaged by PRPP to form IMP which may then recycle to inosine and phosphate. The cell-free system would be greatly simplified by an inhibitor of purine nucleoside phosphorylase (PNP). p-Chloromercuribenzoate does inhibit PNP (192) but also destroys the capability of these lysates to accumulate IDP/ITP (data not shown). Formycin B, a C-glycoside analog of inosine, is a potent inhibitor of PNP (33) but was not tested since there is a high probability that it would inhibit all inosine reactions. The most productive approach to the problem may be to purify the IDP/ITP synthetic activity from the lysates until it is free of PNP activity and then reexamine the dependance of the activity on inosine and PRPP. One should be able to characterize the products of the reaction if the

purified preparation is also free of NDP Kinase and phosphoribomutase. Use of [^{32}P]labelled ATP or PRPP should establish which of these is the pyrophosphoryl donor.

A model for ITP synthesis involving pyrophosphoryl group transfer from PRPP to inosine is consistent with several observations in the literature concerning the kinetics of incorporation of radioactive hypoxanthine or inosine into IMP and ITP by intact red cells. First, Henderson et al. proposed that the nonlinear incorporation of [^{14}C]hypoxanthine into ITP with time (125) was due to the increasing intracellular concentration of IMP which in turn increased the activity of an "IMP Kinase" by normal Michaelis-Menten kinetics. An alternative explanation for nonlinear accumulation of [^{14}C]ITP with time suggested by Model I and supported by the data discussed above may be that the increasing concentration of IMP tends to inhibit HGPRT which in turn increases the intracellular concentration of hypoxanthine, inosine and PRPP. By Model I the higher the intracellular concentrations of these precursors, the greater the stimulation of the synthesis of IDP/ITP. Second, Nelson et al. observed that in intact red cells in the presence of 0.5 mM hypoxanthine, 25 mM glucose and 50 mM phosphate the intracellular IMP pool did not reach steady state concentration until after 12 hours of incubation but the ITP concentration increased linearly for the whole 24 hours of the experiment (126). Incubation of cells in hypoxanthine may be expected to rapidly increase the intracellular concentration of hypoxanthine by virtue of its high rate of transport as well as increase the intracellular concentration of inosine by the reversability of purine nucleoside phosphorylase. Thus hypoxanthine pools may be gradually incorporated into IMP until IMP inhibits HGPRT

while the elevated inosine concentration immediately stimulates linear synthesis of IDP/ITP as suggested by Model I. Nelson et al. have also shown that incubation of red cells in the presence of 0.5 mM inosine, 25 mM glucose and 50 mM phosphate yielded an initial burst of accumulation of IMP and ITP followed by linear accumulation of each (126). Once again these results are readily rationalized by Model I in which one would expect rapid synthesis of ITP and IMP (by the action of NTPH) in the presence of high concentrations of inosine until the inosine is depleted by purine nucleoside phosphorylase, at which time synthesis of IDP/ITP would proceed at a slower rate.

The reaction proposed between inosine and PRPP in Model I is thermodynamically favorable. Switzer has determined the free energy of the PRPP synthetase reaction as -2.0 ± 0.5 kcal/mole (42). He further suggests that if one uses -11 kcal/mole for the free energy of hydrolysis of ATP as calculated by Alberty (189), one can calculate the free energy of hydrolysis of the P-glycosidic bond of PRPP to be ca. -9 kcal/mole at pH 7.5. On the other hand, the free energy of the phosphate hydrolysis of glucose 6-phosphate at pH 7.0 is reported to be -3.3 kcal/mole (190). Thus if one assumes that hydrolysis of the P-O-C bond of IDP is on the same order as hydrolysis of the P-O-C bond of glucose 6-phosphate, the reaction between PRPP and inosine to yield IDP would be expected to have a negative free energy on the order of 5 to 6 kcal/mole.

Model II would seemingly provide a futile cycle for IMP and ITP with the net result of a loss each cycle of two high energy pyrophosphate bonds and a decrease in the adenylate energy charge. It make little sense physiologically that the cell reduce the energy charge

during the situation of elevated concentrations of purine catabolic waste products inosine and hypoxanthine, particularly the latter which is already being salvaged into nucleoside monophosphates. On the other hand, Model I is much more attractive since it represents the salvage of the metabolic waste product inosine to a useful metabolite IMP which has central importance in the biosynthesis of AMP and GMP (see Figure 2).

If Model I is indeed the mechanism by which IDP/ITP is synthesized, it raises several interesting predictions and questions. First, red cells of patients with purine nucleoside phosphorylase deficiency associated with severe combined immunodeficiency have been found to contain both elevated concentrations of inosine and PRPP (104). Model I predicts that these same cells would have increased concentrations of ITP particularly if these red cells had low NTPH activity. One may ask whether other tissues in humans with this disease might also have elevated inosine and ITP concentrations. For example, if ITP does exist even at very low levels in the lymphocytes might it not interfere with transcription of RNA and might this be the primary lesion associated with the immunodeficiency symptoms of this disease? Second, the question arises whether IDP/ITP synthetic activity of red cells is the same in all individuals or if the variation expressed in the ITP degradative enzyme NTPH is reflected or compensated in some way by the synthetic enzymes for ITP. Third, one may question the substrate specificity of the inosine salvage pathway. Can guanosine be substituted for inosine in this reaction? Is there another enzyme specific for adenosine? Finally, one may ask how important physiologically is the

salvage of purine ribosides in relation to the salvage of purine bases in the over-all salvage of purines.

The possible significance of a unique salvage pathway suggested by data in this dissertation emphasizes the importance and need for the purification of the enzyme or enzymes involved in the biosynthetic pathway of ITP and identification of the substrates and products involved in the reaction.

CHAPTER VI

SUMMARY

1. NTPH is shown to be a genetically variable enzyme by (a) a random survey of the specific activity in the red cells of a human population, (b) a survey of the activity in the members of two families, (c) analysis of NTPH specific activity and demonstration of its variability in granulocytes, lymphocytes and platelets.

2. The variation of NTPH specific activity of red cells is independent of the K_m for its substrate ITP. Intracellular effectors of NTPH activity were not detected.

3. NTPH specific activity is inversely related to the accumulation of [^{14}C]ITP in erythrocytes incubated <u>in vitro</u> with [^{14}C]hypoxanthine in a manner predicted by Michaelis-Menten kinetics for an enzyme and its substrate. Endogenous ITP was detected in an extract of blood of an individual with very low NTPH activity. This evidence is interpreted to indicate that NTPH limits the concentration of ITP allowed to exist in erythrocytes.

4. A methylene analog of ITP (IPCPOP) is a good competitive inhibitor for ITP with a K_i = 5.9 μM for partially purified human NTPH. Attachment of IPCPOP to Sepharose-4B by means of a six carbon spacer molecule provides an affinity resin for the purification of NTPH.

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5. A cell-free system was established to study the biosynthesis of ITP in red cell hemolysates. Manipulation of the incubation conditions indicated that IDP may be synthesized by a unique pyrophosphotransferase which transfers the pyrophosphate of PRPP to the 5'-carbon of inosine.

6. NTPH is proposed to complete a metabolic cycle in which inosine is salvaged by PRPP to form IDP which is converted to ITP by NDP Kinase. The ITP thus formed is hydrolyzed by NTPH to prevent interference of ITP in transcription of RNA and to produce IMP, a metabolite essential in the de novo biosynthesis of GMP and AMP.

APPENDIX A

Theoretical Relationship Between Endogenous ITP in Human Red Cells and NTPH Specific Activity

APPENDIX A

THEORETICAL RELATIONSHIP BETWEEN ENDOGENOUS ITP IN HUMAN RED CELLS AND NTPH SPECIFIC ACTIVITY

By the Michaelis-Menten equation one can relate substrate concentration to the initial velocity of a reaction as given in Equation [1], where V_i is the initial velocity of an enzyme reaction, V_{max} is the maximum velocity, [S] is the substrate concentration and K_m is the Michaelis constant.

$$[1] \quad V_i = V_{max} \quad [S]/[S] + K_m$$

Furthermore, in the case of an irreversible enzyme such as NTPH (153), the velocity of the enzyme reaction at a steady state substrate concentration could be substituted for the initial velocity in Equation [1].

If, for the purposes of developing a theoretical relationship between the specific activities of NTPH and %[^{14}C]ITP accumulation in the red cells of two individuals, one assumes (a) that the rate of ITP synthesis is constant among the subjects studied and equal to the rate of ITP degradation, (b) that the K_m value for ITP is a constant among the subjects studied, and (c) that the %[^{14}C]ITP accumulation observed in these analyses is proportional to the steady-state ITP concentration in the human red cells, then one may substitute the specific activity value of NTPH for V_{max} and relate percentage of [^{14}C]ITP accumulation to [S] by using a proportionality constant in the following equation:

$$[2] \quad V_{max_1} [S]_1/([S]_1 + K_m) = V_{max_2}[S]_2 /([S]_2 + K_m).$$

NTPH is assayed under conditions of optimal activity (1) and hence closely approximates V_{max}. The K_m for ITP is independent of the specific activity of NTPH in lysates of red cells from selected individuals with markedly different NTPH levels (Table 2). The proportionality constant relating the steady state ITP concentrations with the observed %[^{14}C]ITP accumulation was calculated as 5.1×10^{-7} M by use of an average K_m value of 2.8×10^{-5} M (from Table 2) and the reference points 2.5% [^{14}C]ITP, specific activity 70, and 14% [^{14}C]ITP, specific activity 15. A best-fit theoretical curve relating [^{14}C]ITP accumulation and NTPH activity was then prepared as shown in Figure 9. The entire range of experimental values correlate rather closely with the relationship defined by this mathematical treatment.

If one recalculates the theoretical relationship in terms of the steady state level of ITP predicted by the proportionality constant above, then one can define the expected steady state levels of ITP in the red cells of individuals with different NTPH specific activities as shown in Figure 35. From this curve one would predict ITP levels to be greater than 5 μM for any individual with an NTPH specific activity less than 20 units/mg. Moreover individuals with very low NTPH activity may be expected to have 50 μM or more ITP present in their red cells if no other mechanism limits the physiological level.

In constrast to these predictions the HPLC analysis of endogenous ITP in the blood of an individual with an NTPH activity of 3 units/mg hemoglobin indicated that only 5 μM ITP was detectable (Figure 13). No ITP could be detected in the blood of two individuals with NTPH activities, 15 and 30 units/mg hemoglobin (Figures 14 and 15). Special care

was taken in these experiments to minimize the amount of time between venipuncture and extraction of nucleotides (ca. 5 minutes). A [^{3}H]ITP internal standard was added as the cells were extracted to monitor recovery throughout the extraction and analytical procedures. It may be however that the ITP present in intracellular pools did not completely mix with the added standard during the extraction, in which case the HPLC analysis may be an underestimate of the endogenous pool sizes.

An alternative explanation for the discrepancy between the theoretical prediction of endogenous ITP levels and the HPLC analysis may be that the theoretical curve is based on a faulty assumption. In the mathematical treatment, it is assumed that Henderson's method for labelling the ITP pools does not significantly alter the pool size. This may not necessarily be true since Henderson et al. have shown in one case that prior to incubation of red cells with [^{14}C]hypoxanthine no ITP could be detected by HPLC whereas after incubation, ITP was observed (72).

Thus the analysis of the endogenous concentration of ITP in red cells is a problem which is unresolved, but it does seem that NTPH activity has a role in determining the intracellular steady state concentration.

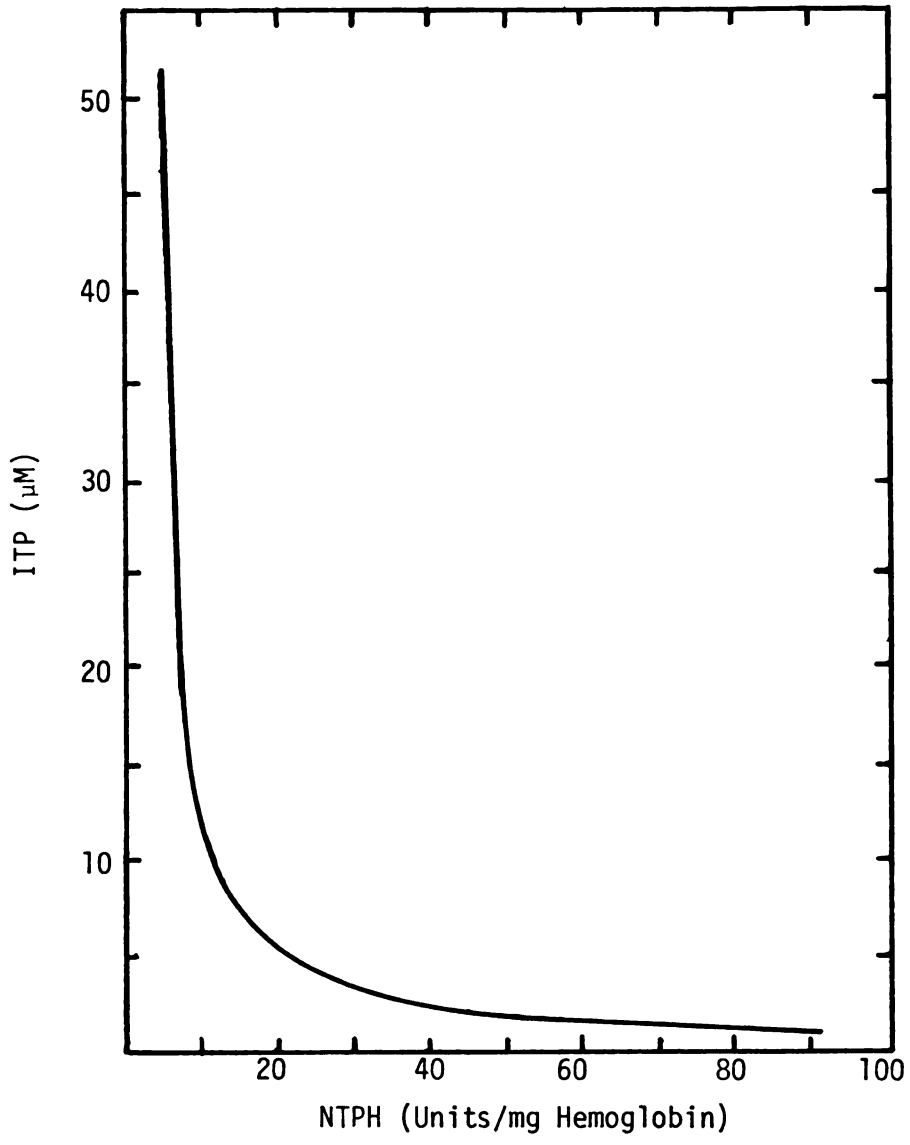

Figure 35. Theoretical Relationship Between Endogenous ITP in Human Red Cells and NTPH Specific Activity

This figure is the result of the mathematical treatment described in Appendix A on the data presented in Figure 9.

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