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Biochemical Profiling of Gout Patients

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BIOCHEMICAL PROFILING OF GOUT PATIENTS

by

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ABSTRACT

BIOCHEMICAL PROFILING OF GOUT PATIENTS

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The purpose of the research is to investigate the biochemical cause of gout in 82 patients. The gouty patients had been diagnosed with gout according to the criterion of the American Rheumatism Association. This gout patient population consisted of 58 men and 24 women with gout whose ages ranged from 11 to 84 years of age with a mean age of 44 years. Key purine metabolism enzyme activity levels and purine metabolite concentrations in the patients' plasma and urine were compared to the mean values of a healthy control group. The control group consisted of 33 males and 25 female who did not have primary or secondary hyperuricemia expressed for the past two generations, and did not have any immediate family members suffering from gouty diseases. Their ages ranged from 8 to 75 years with a mean age of 36. Based on the results of the purine metabolism enzyme activity levels, the patients are classified as one of eight groups.

The plasma urate was determined by the uricase-peroxidase coupling reaction. Plasma hypoxanthine and xanthine concentrations were determined by HPLC. Erythrocyte red blood cell phosphoribosylpyrophosphate (PRPP) synthetase, hypoxanthine-guanine phosphoribosyltransferase (HPRT), and adenine

phosphoribosyltransferase (APRT) activities were determined by using a combination of enzymatic and high performance liquid chromatography (HPLC) methods.

The study found that the mean levels of xanthine, uric acid, hypoxanthine, and erythrocyte PRPP synthetase levels for gouty patients were significantly higher than normal healthy controls. While no differences in biochemical parameters were found between male and female gout patients, there was a significant difference between the male and female control patient's biochemical parameters. The gout patients were further classified based on their biochemical profile of enzyme function.abnormalities, with 13% of the population found to have multiple abnormalities. These patients were in the first seven biochemical profiles. 48.9% of the remaining patients were classified as Group 8, which is classified with other possible factors, such as renal clearance issues. The involvement of renal clearance of urate should be further investigated.

ACKNOWLEDGMENTS

I would like to thank my committee, Dr. Yuan, Dr. Elliott, and Dr. Williams, for their time, guidance, and contributions to this thesis. I would especially like to thank them for the speed and patience. I would like to extend a special thank you to Dr. Yuan for endless patience and guidance. He has become a true friend and mentor over the last couple of years.

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INTRODUCTION

PURPOSE. The purpose of this study is to determine the underlying cause of gout in 82 patients. To do this, the patients' enzyme activity levels and purine metabolite concentration were compared to the mean of a control group's parameters. The control group consisted of 58 healthy subjects who did not have primary or secondary hyperuricemia within three generations. There are three main purines of interest: hypoxanthine, xanthine, and uric acid. An overproduction of uric acid has been linked to gout. Xanthine oxidase converts hypoxanthine and xanthine to uric acid. These three enzymes were examined to see if there was a significantly different concentration of these purines in gout patients versus the control group.

To determine if an enzyme abnormality was causing the patients gout, enzyme assays were preformed for hypoxanthine-guanine phosphoribosyltransferase (HPRT) , adenine phosphoribosyltransferase (APRT), and α-5-phospho-D-ribosyl-1-pyrophosphate synthetase (PRPP synthetase). These enzymes have been linked to the overproduction of uric acid, which can ultimately lead to hyperuricemia. The activity of each of these enzymes was taken to determine if a deficiency of HPRT or APRT or a superactivity of PRPP synthetase was behind the patients' gout. The activities of the enzymes were compared to the normal group to see if there was a significant difference. A combination of HPRT, APRT, and PRPP synthetase abnormalities can also be the cause of a patient's gout. If an enzyme abnormality such as an overactive or deficient enzyme was not found, then another factor is considered the culprit.

This thesis is modeled after the format in *Biochemistry.*

BACKGROUND. Gout is a type of arthritis that which is caused by the crystallization of urates within the joints (*1*). This condition has multiple causes, with no single enzyme defect solely responsible for the condition. The main requisite for gout is the patient must be hyperuricemic, which is an elevated plasma urate level above the saturation value (*2*). Gout has the distinctive features of acute arthritis, chronic tophaceous arthritis, and the presence of tophi in soft tissues.

There are many possible reasons why a patient may develop gout. A deficiency in certain key enzymes can lead to the development of gout by causing hyperuricemia. Environmental factors such as chronic alcoholism and obesity can make a patient more prone to developing hyperuricemia. Males are more likely to develop gout, and they are more likely to develop gout at a younger age. While average age for the first attack for males is thirty, women often do not see their first attack until after menopause(*1*).

The two types of gout are primary and secondary. Primary does not have a primary disease causing the gout, while secondary does (*2*). Primary gout accounts for 90% of all patients with the remaining 10% being secondary gout. Primary gout can be caused by the overproduction of uric acid with normal excretion, the overproduction of uric acid with increased excretion, and normal uric acid production with under excretion. Primary gout can also be caused by an incomplete deficiency in an enzyme, an overactive enzyme, a decreased renal clearance, or a combination of the three. Patients who have an overproduction of urates with an increased urinary excretion are at a greater risk for urate nephrolithiasis, the formation of urate kidney stones, while other types of primary gout are not as susceptible.

The overproduction of uric acid causes secondary gout. This type of gout is associated with the excessive breakdown of cells, which in turn causes an increased turnover in nucleic acid. An increased breakdown of purines causes the overproduction of uric acid. Secondary gout has been associated with myeloproliferative disorders, leukemias, chronic hemolytic anemias, and multiple myeloma. Any type of chronic renal disease that reduces glomerular filtration and tubular function may lead to hyperuricemia, which may ultimately lead to gout. In addition, reduced excretion of uric acid by the kidney may cause secondary gout. Drugs such as low-dose aspirin, pyrazinamide, nicotinic acid, and ethanol have been shown to induce hyperuricemia (*1*).

There are five different stages to gout. These are hyperuricemia, acute gouty arthritis, intercritical gout, recurrent gouty arthritis, and chronic gout. The first stage of gout is hyperuricemia, which is when a patient has an elevated plasma urate level. This stage is asymptomatic (*2*). Hyperuricemia is defined as having a plasma urate level above 7 mg/dL. This level was assigned because this is above the saturation value for urates in normal blood plasma pH and temperature, which is 6.5 mg/dL. While 2 to 18% of the entire population is thought to be hyperuricemic, only 0.13 to 0.37% exhibit gout. Clearly being hyperuricemic does not mean a patient will develop gout (*1*).

The second stage is acute gouty arthritis is the first gout attack where the formation of monosodium urates in the joint fluid and the synovial membrane is the culprit causing the gout attack. This causes the joint affected to become swollen, hot and tender. The age of the first onset depends on the degree and length of

hyperuricemia (*2*). Usually only one to two joints are affected at the same time, but in rare instances, multiple joints can be affected. For 90% of all gout patients, the great toe is the first joint that is affected. The reason why the big toe is the first joint affected is believed to be caused by the big toe's lower temperature as compared to other joints. Gout can affect other joints such as the instep, ankle, knee, and wrist. While these are the more common joints affected, any joint in the body can be affected by gout (*1*).

Uric acid is the main culprit behind gout attacks. At body fluid's pH of 7.4, uric acid is present mainly as monosodium urate. While it is known that the deposition of monosodium urate crystals in synovial fluids is due to the lack of solubility, the exact mechanism has not yet been determined. The formation in mainly avascular tissues suggests that the characteristics of the tissue might be affecting the solubility. Avascular areas rely on anaerobic glycolysis of glucose, which produces lactic acid. It is believed that the decreased solubility could be due to the lower pH found in synovial fluids. It has also been suggested that the inflammatory response to the crystal formation might cause other crystals to be produced (*1*).

The cause of some of the tenderness in the joint is the inflammatory response that occurs after the first deposition of crystals around the joint. The inflammation seems to be mediated by the physical shape and size of the monosodium urate crystals, rather than the chemical nature. The inflammatory response is caused by polymorphonuclear leukocytes and macrophages. While the exact method has not been completely determined, one of the possible contributors is the Hageman factor being activated. This leads to the production of inflammatory mediators such as

kinins. Another possible contributor is that the breaking down of monosodium urate crystals releases lysosomal products and toxic free radicals. This can cause an increased inflammatory reaction. The phagocytosis of the monosodium urate crystals by the monocytes and macrophages leads to the release of interleukin 1 (IL-1), (*1,3*).

IL-1 stimulates and activates collagenases, which are derived from synoviocytes, chondrocytes, and other cells. IL-1 also activates and stimulates the chodrocytes with release of PGE_2, collegenase, and other lytic enzymes. It inhibits the synthesis of proteoglycans by chondrocytes, and it acts as a chemo-attractant for neutrophils and lymphocytes (*1*).

Gout attacks are usually self-limiting in nature. Two theories have been proposed for why this occurs. This first theory is that the urate crystals have increased solubility due to the increase of temperature caused by the inflammatory response. As the inflammatory response increases, so does the vascular supply to the afflicted area, which increases the temperature. The second theory is that apo-B-lipoproteins bind to the surface of the urate crystals and modulate the inflammatory response (*1,3*).

The third stage of gout is the intercritical gout. During this period, the patient experiences none of the symptoms associated with gout. This period normally lasts from a couple of months to a couple years, with the occasional patient never developing gout again.

During the fourth stage, which is the recurrent gouty arthritis stage, the frequency and severity of acute gout attacks increases. Previously unaffected joints may become affected, and the severity of the attack may increase. During this stage is

possible to have more than one joint affected during an attack. The frequency of the gout attacks increases.

The final stage is the chronic gouty arthritis stage. This stage follows years of multiple recurrences of acute arthritis. Some characteristics of this stage are the formation of tophi in soft tissues and joint damage, which can be documented by radiographic evidence (*1*). Permanent damage and deformity can also occur because of erosion of the joint, and joint motion may have a mild to severe limitation. Renal dysfunction may also occur with many patients developing urate nephropathy and uric acid nephrolitihiasis (*2*). This progression to this stage can be suppressed by treatment. Due to medical advances in treatment options, fewer than 10 to 15% of patients developing this stage.

One characteristic of chronic gouty arthritis is the development of tophi. Tophi are crystalline or amorphous masses of urates that are surrounded by an intense inflammatory reaction. They are commonly seen in the helix and the antihelix of the ear, and tophi can be observed in the pyramids and medulla in the kidney. While tophi can be found in other body parts including the skin of the fingertips, the aorta and the nasal cartilage, they are never found in the nervous system (*1*).

Kidney complications have also been associated with gout patients. A diminished excretion level for hyperuricemic adults on a purine-free diet is set at excreting less than 250 to 300 mg of uric acid in a day. Eighty percent of patients exhibit a decrease in the renal excretion of urates (*4*). The mechanism for the renal clearance of urate is filtration at the glomerulus, reabsorption of the filtered urate, secretion, and finally postsecretory reabsorption in the proximal tubule (*5, 6*). The

final clearance of urate may amount to only 6-10% of creatine clearance. First, the kidney filters the urate at the glomerulus, and then the urate can be reabsorbed in the proximal tubule by an organic anion exchanger. URAT 1, the molecular target for urocisuric agents, was recently identified (*7*) through human genome database (SLC22A12) as the primary system for urate reabsorption. URAT 1 is thought to be expressed at the apical brush border of the proximal nephron, and it is thought to be responsible for the renal proximal tubule reabsorbing filtered urate. Urate is transferred from the lumen to the proximal tubular cell in exchange for anions. A secondary sodium dependent urate reabsorption by the proximal tubule was also reported (*8*). While the complete regulation of urate clearance is not well understood, several transporter systems are thought to regulate renal clearance (*9-13*).

Three possible diseases that may result from the hyperuricemia are acute uric acid nephropathy, nephrolithiasis, and chronic urate nephropathy. Acute uric acid nephropathy is an acute obstructive renal disease that is caused by free uric acid crystals intratubular deposition in the acid milieu of the collecting ducts. This is most commonly seen in patients with secondary gout. Uric acid nephrolithiasis is caused by stones in the collecting system. Chronic urate nephropathy is the deposition of urates in the interstitial tissue of the medulla (*1*). Proteinuria and hypertension are also associated with 20 to 40 percent of gouty patients (*2*).

DE NOVO PATHWAY. While gout can be caused by several different mechanisms, each one of them has to do with the bioprocessing of purines. Purines can be synthesized *de novo*, or they can be salvaged. Purines can be produced by three different means. The first one is through *de novo* synthesis. The second way is

through reconstruction from purine bases by the addition of a ribose phosphate moiety, and the final way is thru the phosphorylation of nucleosides. The salvaging of purines saves energy, as compared to the *de novo* synthesis of purines. There are several key control points that helps to maintain the levels of purines in the body (*1*). The three main enzymes involved in purine metabolism are hypoxanthine-guanine phosphoribosyltransferase (HPRT) , adenine phosphoribosyltransferase (APRT), and α-5-phospho-D-ribosyl-1-pyrophosphate synthetase (PRPP synthetase). While other enzymes are present in the synthesis and regulation of purines, HPRT, APRT, and PRPP synthetase A diagram showing the de novo pathway can is shown in Figure 1. These points will be further examined in the paragraphs below.

Gout is related to the *de novo* pathway for purine synthesis. The main requisite for gout is being hyperuricemic. To be hyperuricemic, a patient must have a concentration of uric acid that is greater than blood saturation level. Hyperuricemia is generally defined as having uric acid concentrations above 7 mg/dL. The production of uric acid is regulated through various enzymes that control purine biosynthesis. Therefore, to evaluate the possible causes of a patient's gout, the regulation of key enzymes in the *de novo* pathway and concentrations of purines in the body must be considered.

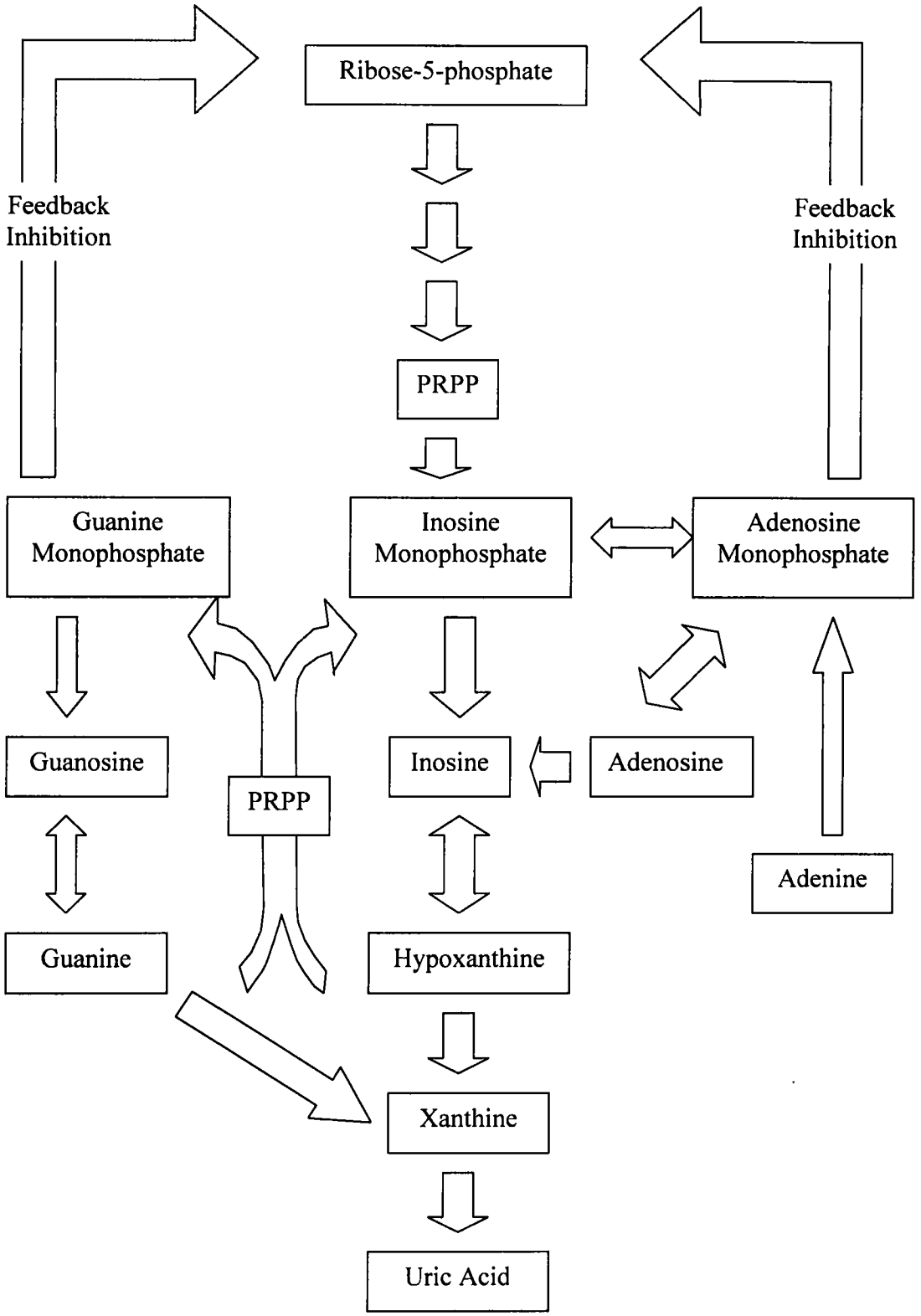

FIGURE 1: *De Novo* pathway. This shows the synthesis and salvaging of purines.

As seen in figure 1, PRPP is an important precursor to the entire system. PRPP is synthesized from D-ribose-5-phosphate and an ATP with PRPP synthetase (a.k.a. ribose-5-phosphate pyrophosphokinase or PRPP kinase) as the catalyst. To produce PRPP from D-ribose-5-phosphate, the terminal pyrophophate groups of ATP are transferred to the first position of the ribose-5-phosphate. This reaction is activated by inorganic phosphate (P_i). Mg^{+2} is a cofactor and an activator. Inhibitors of this reaction are ADP and glycerate-2, 3-biphosphate. AMP and GDP are also noncompetitive inhibitors. When energy stores are low, the inhibitors will stop the reaction (*2, 14, 15*). The reaction for the synthesis of PRPP is shown in Figure 2.

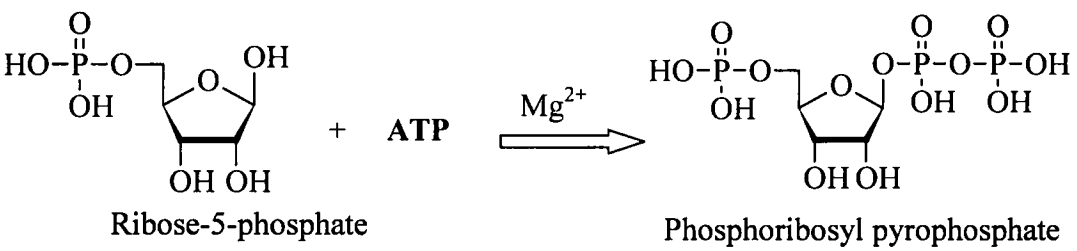

FIGURE 2: PRPP synthetase converts ribose-5-phosphate to PRPP. This reaction requires one ATP, and Mg^{2+} is a cofactor.

Once PRPP is formed, it can then be converted into inosine monophosphate through a series of ten reaction steps. These are illustrated in Figure 3 below. The first five steps involve the formation of the five sided ring in the inosine monophosphate. The first step is the formation of phosphoribosylamine. This step commits the PRPP to the biosynthetic pathway and uses glutamine phosphoribosylpyrophosphate amidotransferase as the catalyst. This step uses a

glutamine, and the step is done in the presence of Mg^{2+}. The N-1 amine group is added to the 5-phospho-α-D-ribosyl-1-pyrophosphate, and the ribose is converted to the β conformation. The second step is the formation of the amide bond between the amino group of the phosphoribosylamine and the carboxyl group of the glycine. ATP is necessary for the energy needed in this reaction, and it is hydrolyzed to ADP and P_i. This involves a synthase catalyst. The first two steps involve the addition of atoms 1, 2, 3, and 4, while the remaining steps will add on atoms one at a time. In step 3, a formyl group is transferred from 10-formyl- tetrahydrofolate. This adds carbon 5 and is catalyzed by formyltransferase. Step 4 involves the transfer of an amino group form a glutamine to phosphoribosylformyl- glycinamide. This step introduces the nitrogen 6, and requires a molecule of ATP. Step five requires one ATP, and it is the closure of the imidazole ring. This step also requires Mg^{2+} and K^+, and is catalyzed by a synthase.

The remaining steps involve the formation of the six membered ring. In step six, the C-7 of the purine ring is added by bicarbonate in the presence of a carboxylase. This proceeds through CO_2 fixation. Biotin serves as a cofactor for this step. Step seven involves the use of an ATP to get aspartic acid to form an amide with the 4-carboxyl group. This accounts for N-8. The cleavage of fumarate by adenylosuccinate is step 8. 10-Formyltetrahydrofolate donates a formyl group to the N-6 amine in step 9. The reaction is catalyzed by formyltransferase. Finally, water is eliminated and the six-membered ring closes using IMP cyclohydrolase. This produces insosine-5'-monophosphate. The entire conversion of PRPP to IMP requires six ATP (2, *15*).

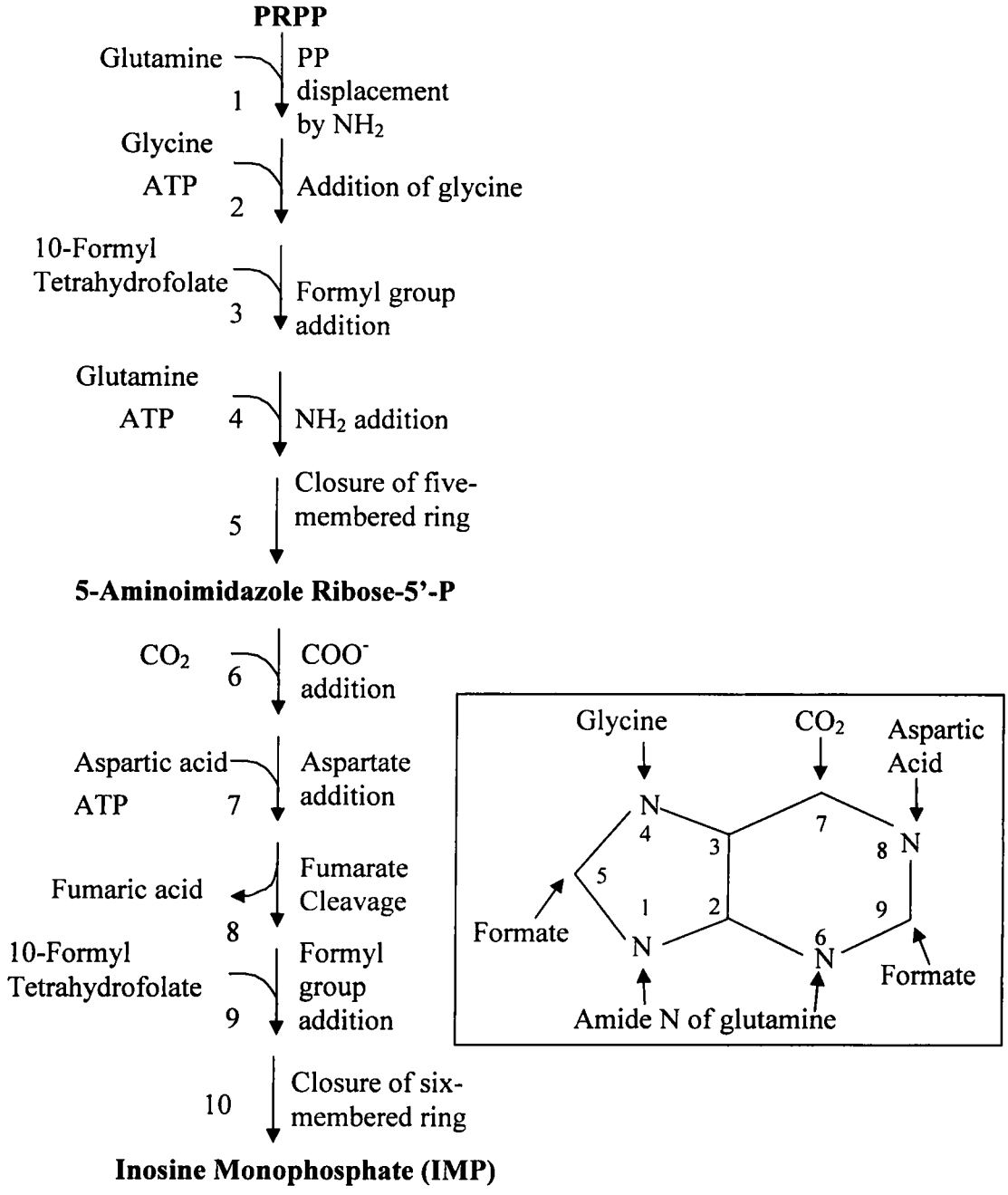

FIGURE 3: Steps one through ten for the synthesis of inosine monophosphate from PRPP.

IMP can then be easily converted into adenosine 5' monophosphate (AMP) and guanosine-5'- monophosphate (GMP) in a short series of reactions. Both reactions occur in two steps. For the conversion of IMP to AMP, the first step, which is catalyzed by adenylosuccinate synthetase, involves the 6-hydroxyl group of the IMP being displaced by an amino group from aspartate. This forms adenylosuccinate and is a nonreversible reaction. The adenylosuccinate then yields fumarate and AMP by the nonhydrolytical cleavage from adenylosuccinate lyase. Energy for the reaction is derived from the cleavage of GTP to GDP and P_i. AMP can be converted to IMP by aminohydrolase. This enzyme has been found in the muscle and other tissues. An absence of this enzyme is usually fatal.

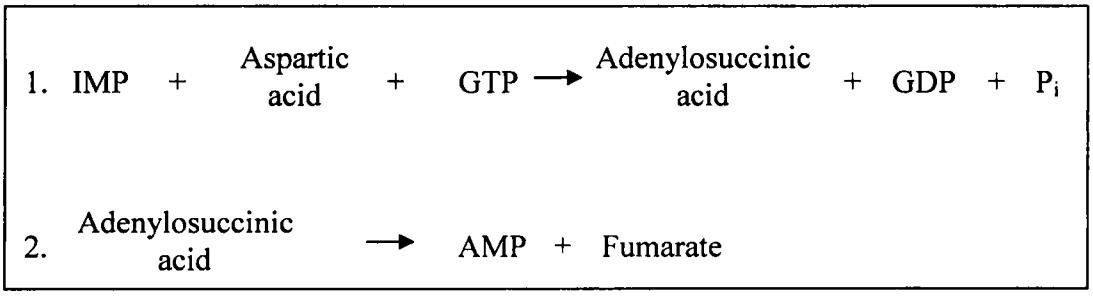

FIGURE 4: The conversion of IMP to AMP. First the IMP is converted to adenyloscuccinic acid by the enzyme adenylosuccinate synthetase. Then adenylosuccinic acid is converted to AMP by adenylosuccinate lyase.

IMP to GMP first requires the dehydrogenation of IMP to xanthosine-5'-phosphate by IMP dehydrogenase. Next, the amine group from a glutamine is transferred to the C-9 of the xanthine ring to yield GMP, using the enzyme GMP synthetase. This reaction uses two ATP. These two reactions are regulated. The

production of GMP requires ATP, and the production of AMP requires GTP.

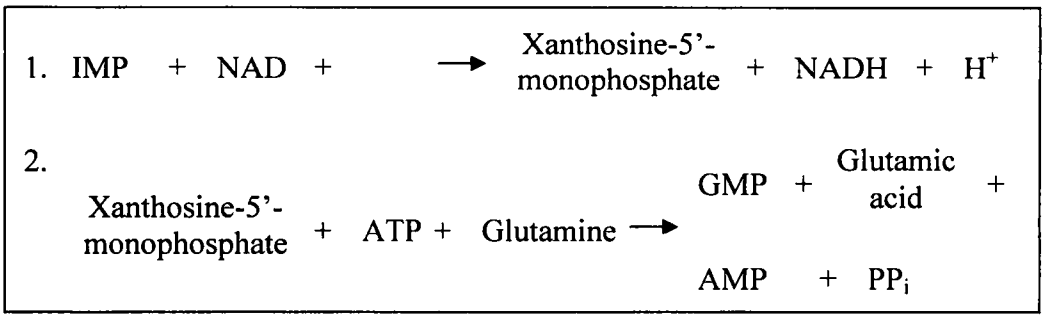

FIGURE 5: The conversion of IMP to GMP. First the IMP is converted to xanthine-5'- monophosphate by the enzyme IMP dehydrogenase. Then xanthine- 5'-monophosphate is converted to GMP by GMP synthetase.

Several enzymes are related to the interconversion purines. Once AMP, IMP and GMP have been formed, next step is removal of P_i. AMP is converted to adenosine, IMP is converted to inosine, and GMP is converted the guanosine by a 5'nucleotidase. This nucleotidase uses water and removes a P_i (*2, 15, 16*). Inosine can be converted to hypoxanthine, and guanosine can be converted to guanine by a purine nucleoside phosphorylase (*16*). Guanine is converted to xanthine by the enzyme guanine aminohydrolase. Guanine aminohydrolase is found in the liver, brain, and other tissues (*2*). Xanthine oxidase converts hypoxanthine to xanthine, and then xanthine to uric acid. Xanthine oxidase is found mainly in the liver and intestinal mucosa. It contains molybdenum, iron, FAD, and acid-labile sulfur. It forms hydrogen peroxide and the superoxide anion, which makes it able to oxidize many compounds (*2, 15, 16*).

Two key enzymes are important for the salvaging of purines. They are HPRT and APRT. HPRT converts guanine and hypoxanthine to guanosine monophosphate and inosine monophosphate respectively. APRT converts adenine into adenosine monophosphate. Both of these enzymes will be further analyzed below.

KEY REGULATORY ENZYMES. While many different enzymes are involved in purine metabolism, three key enzymes regulate the production and degradation of purines. Two of these essential enzymes are HPRT and APRT. These enzymes salvage purines. HPRT catalyzes the formation of IMP from hypoxanthine and the formation of GMP from guanine. During this process one PRPP is utilized, which makes this enzyme dependent on the concentration of PPRP. HPRT is a cytoplasmic enzyme that is in every cell. The highest levels of HPRT are found in the basal ganglia and the testis. APRT converts adenine to AMP. This uses one PRPP also. The other important enzyme is PRPP synthetase. Once PRPP has been produced, it can be converted into IMP, which can later be converted to GMP and AMP. An overactive PRPP synthetase or a deficiency in HPRT and APRT activity has been indicated as possible causes for hyperuricemia.

PRPP synthetase is the enzyme responsible for the production of PRPP. The gene for PRPP synthetase is found in two genes on the X chromosome at Xp 22.2 - 22.3 and Xq 22-24 (*18*). There is evidence of three different isoforms of PRPP synthetase: PRPS1, PRPS2, and PRPS3 (*17, 20*). PRPS1 and PRPS2 are in all tissues on the long and short arms of the X chromosome (*18, 21*). PRPS3 is only detectable in the testes, and it is autosomally encoded (*19*). While normally this PRPP synthetase has enough genetic control points to inhibit overproduction of PRPP, occasionally

certain mutations arise that allow the PRPP synthetase to become hyperactive. The main causes of this hyperactivity are catalytic site alteration and/or regulatory site alteration, which are caused by genetic mutations (*14, 22*). Increased quantities of the synthetase may also be present (*14, 23, 24*). An example of a regulatory defect is the impairment of allosteric control of the PRPP synthetase activity by purine nucleotides and P_i. This defect would cause the amounts of the enzyme to remain the same, but the activity of the enzyme is greater than normal, which is an indication of increased specific activity for the substrate (*23*). Another possibility is that an increased selective PRPS1 transcription, which causes an increase in the concentration of normal PRPS1. An increase in the PRPS1 content and enzyme activity as well as an increased PRPS1 transcript level would suggest a pretranslational defect in the expression of the PRPS1. Patients have been found with point mutations in the PRPS1 cDNA that causes mutant PRPS1 with altered allosteric properties. This helps to confirm the idea that a point mutation can cause PRPP synthetase hyperactivity (*14*). An increase in PRPP synthetase hyperactivity leads to hyperuricemia and uricosuria. It also leads to urinary tract calculi, gouty arthritis, and nephropathy (*22*). The reaction for this can be seen in figure 2.

A recent report by Ahmed *et al.* was done to support to the idea that the accelerated transcription of PRPP synthetase could be causing some patients to have gout. The DNA sequences of a normal patient were compared to an affected patient, and the results showed the PRPS1 gene was normal. Genomic DNA sequencing also found that the transcription structure as well as the transcription initiation structure was normal. A southern blot test was preformed on the restriction enzyme-digested

normal and affected DNA. This test found that the two DNA's were identical in patterns and intensities on the southern blot. These findings rule out the possibility of PRPS1 gene amplification being the culprit. A northern blot test was then run to see if there was an increased concentration of one isoform as compared to another. Relative to the levels of GAPDH transcript and 18S ribosomal RNA in the lymphoblasts, there was found to be a higher concentration of PRPPS1 than PRPPS2 when patients' levels were compared to the control. A higher concentration of PRPPS1 was also observed in the fibroblasts of the patient as compared to normal. A slot-blot analysis, which was of specific RNA labeled in nuclei that were derived from the control and the gout patients, exhibited a difference in the relative rate of labeling the PPRPS1 mRNA. The affected patient's fibroblasts labeling relative to GAPDH transcript labeling was three to four times greater, while in the lymphoblasts the rate was 1.9 to 2.4 times greater in the gout patient. PPRPS2 was found to be the same in both.

The results showed that when compared to the normal, the affected patients had an increase in the relative PRPS1 transcription rate, relative PRPPS transcription rate, PRPP synthetase activity, PRPP generation, total PRPP synthetase isoforms, and rate of purine synthesis *de novo*. These results were found in both the lymphoblasts and the fibroblasts. This evidence supports the idea that an increased selective transcription of PRPPS1 can be the culprit behind the PRPP synthetase superactivity for the affected patients. The genetic cause of this was not been determined in this study as there was no difference between the affected and normal patient's DNA (*14*).

APRT converts adenine to AMP. People with a deficiency of APRT are unable to reutilize adenine (*2*). It does this by first binding the PRPP to the active site, and then binding the purine base to the active site (*25*). A deficiency in APRT has been linked to the formation of 2,8-dihydroxyadenine calculi in kidneys. The calculi are grey in appearance, but once they are crushed, they turn a grey blue color. 2,8-dihydroxyadenine is formed by the catalyst xanthine oxidase with adenine (*2*). The conversion of adenine to adensine monophosphate by APRT is shown below in figure 6.

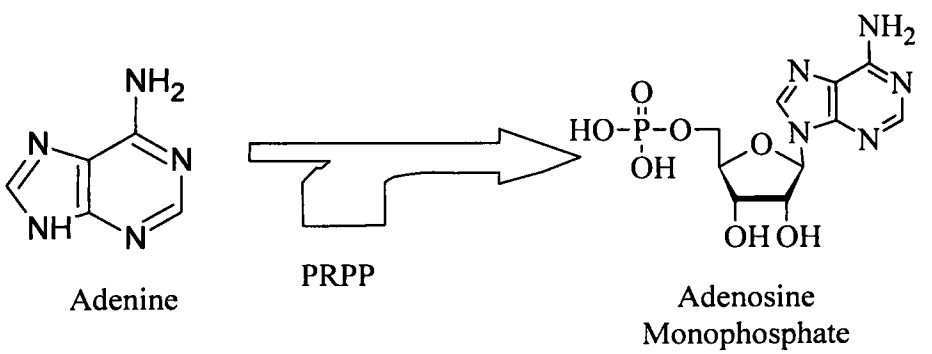

FIGURE 6: APRT converts adenine to adenosine monophosphate. This salvage pathway requires one PRPP.

HPRT catalyzes the formation of IMP from hypoxanthine and the formation of GMP from guanine. The gene for HPRT is located on the long arm of the X chromosome at Xq 26-27. The gene spans 44kb and has 654 nucleotides in 9 exons. There are some diseases associated with the mutation of the gene. The most severe of theses is Lesch-Nyhan syndrome. Gout is also associated with a mutation in the HPRT gene. Over 270 gene mutations have been found with 63% of them are point

mutations. People that are deficient in this enzyme normally have more sever type of gout at an earlier age. There is also a higher incidence of renal stones (*22*). Underutilization and accumulation of PRPP as well as lessened feedback inhibition of amidotransferase may cause the overproduction of uric acid that is associated with a deficiency of HPRT (*26*). A complete deficiency leads to Lesch-Nyhan syndrome (*22*).

HPRT binds the substrate PRPP with hypoxanthine and guanine to form the IMP and GMP. Similar to APRT, it does this by first binding the PRPP to the active site, which is then followed by the binding of the purine base. After the catalysis, the pyrophosphate is released before the nucleotide (*27, 28*). A deficiency in HPRT means that guanine and hypoxanthine are not easily reverted to GMP and IMP. Since GMP is a feedback inhibitor to the production of PRPP, overproduction of PRPP can occur (*1, 2, 13, 22*). This reaction is shown in Figure 7.

PURINE METABOLISM AND REGULATIONS. Purines can be incorporated into the de novo pathway in several ways. Dietary sources are on way the body incorporates purines. Purines can also be salvaged from cells to conserve energy. Once the purines are incorporated into tissues, they can undergo further alterations. Purines can be derived from the diet. Nucleic acids are unaffected by gastric enzymes, but are broken down and processed in the small intestine by a variety of enzymes. The first enzymes the nucleotides meet are the ribonuclease and the deoxyribonuclease I. These enzymes are secreted by the pancreas, and they hydrolyze the nucleic acid to oligonucleotides. Phosphodiesterases, which are also secreted by the pancreas, then hydrolyze the oligonucleotides to 5'- and 3'- mononucleotides. Various group-specific

nucleotidases and nonspecific phosphatases then further hydrolyze the mononucleotides to nucleosides. Further hydrolyzation probably occurs in the tissue (*1, 2, 15*).

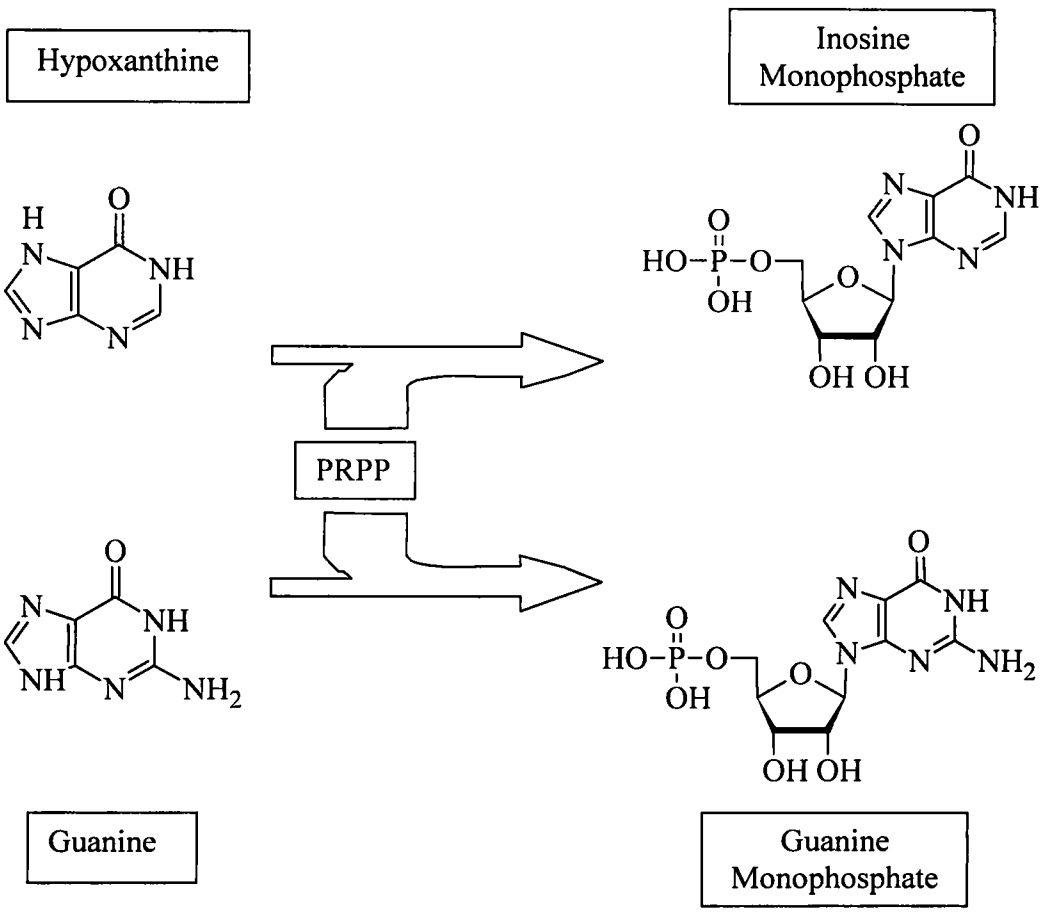

FIGURE 7: HPRT converts guanine to guanosine monophosphate and hypoxanthine to inosine monophosphate. This salvage pathway requires one PRPP.

Nucleotides can also be salvaged from cells by different intracellular nucleotidases. They can be salvaged from dying cells or from purines in the plasma. Intracellular nucleotidases hydrolyze the phosphate ester groups in the nucleotides and

release P_i. It has been suggested that one of the 5'-nucleotidases is located in lysosomes due to its pH optimization of five. Lymphocytes contain two cytoplasmic purine nuleotidatases. One works on ribonucleotides and the other works on deoxyribonucleotides. The ecto-5' nucleotidase dephosphorylates purine and pyrimidine ribonucleosides and deoxyribonucleoside monophosphates to their corresponding nucleosides. This enzyme is found on the outer surface of the plasma membrane. Once the nucleotides have been processed, they are transported into the cell where other enzymes can work on them. Once the nucleosides are out in the tissue, they can undergo further alterations (*1, 2, 15*).

While there seems to be a high possibility for futile cycles using up energy, both the biosynthetic and the catabolic processes are highly controlled to prevent futile cycles. One example of this is the rate-limiting step for the conversion of PRPP to IMP through PRPP amidotransferase. This enzyme commits the PRPP to the production of IMP, but this step is inhibited by purine-5'-nucleotides. Two kinds of inhibitor-binding sites are suggested because a combination of AMP and GMP produces the maximum inhibitory effects. This enzyme is also limited by the amount of PRPP available.

Another regulation is the conversion of GMP and AMP to IMP and vice versa. The conversion of IMP to AMP is an irreversible reaction that produces adenylosuccinate as an intermediate. The conversion of IMP to GMP is also a two-step reaction. The first step of the reaction is irreversible, while the second step of the reaction requires one ATP. One way to regulate the conversion of GMP and AMP to IMP is feedback inhibition. AMP and GMP are inhibitors to their own synthesis. The

other way that these compounds are regulated is through equilibrium. Excess AMP accelerates GMP synthesis while excess GMP accelerates AMP synthesis. The conversion of IMP to GMP and AMP can be seen in figure 4 and 5 respectively (*2, 15, 16*).

Three enzymes use PRPP as a substrate: APRT, HPRT and glutamine amidotransferase. The Km value for each of these enzymes was determined, and APRT was found to have the lowest Km value of 0.033 mM, which means it will have the highest affinity for PRPP. HPRT had the next lowest Km value of 0.074 mM, which means it will have the second highest affinity for PRPP. Glutamine amidotransferase catalyzes the first step for the ten-step conversion of PRPP to IMP. This enzyme was found to have the lowest Km value. This priority allows the cell to save energy by utilizing preformed purines at the cost of two ATP molecules versus the seven needed to synthesize them via the de novo pathway (*2*).

CLASSIC TREATMENTS. There are several possible treatments used for gout. The two drug therapies most often associated with gout are allopurinol and colchicines. Allopurinol is the most common drug used to prevent a gout attack. Allopurinol works by inhibiting xanthine oxidase through a suicide reaction, thus blocking the production of uric acid. Oxypurinol is formed by the enzymatic hydroxylation in position 2, which is shown in Figure 8. Allopurinol is a substrate of xanthine oxidase, but the product formed from it, oxypurinol, binds tightly to the reduced form of xanthine oxidase and complexes with the partially reduced form of the enzyme (*17*). While this does increase the concentration of hypoxanthine and xanthine in blood plasma, both of these compounds are more easily excreted by the

kidney than uric acid. The xanthine can also be salvaged to form other purines. The small increase in concentration of hypoxanthine and xanthine normally does not have a detrimental effect on the patient. This drug is used to prevent renal calculi from forming, although there is a slim chance of xanthine building up in the kidneys. While the kidney more readily absorbs xanthine, uric acid has a higher solubility in urine, which is why sometimes there can be stone formation in the kidneys from the excess xanthine. Since this treatment decreases the total quantity of purines excreted, the treatment is found to be the most suitable for patients with uric acid calculi of the urinary tract, or an excretion of excessive quantities of uric acid in the daily urine as shown by a high uric acid to creatine ratio (*2*).

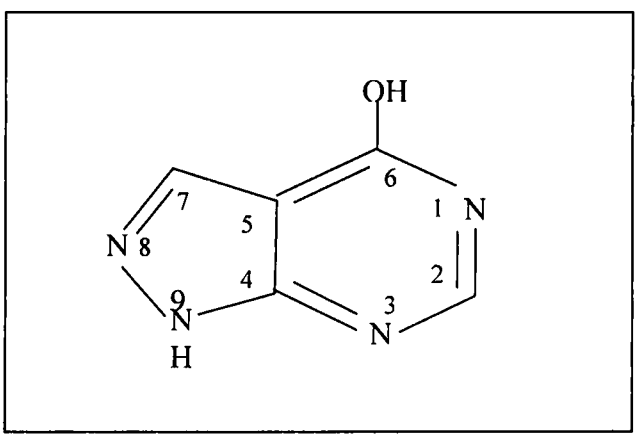

FIGURE 8: The structure and numbering scheme for allopurinol.

Another classical treatment is the use of colchicine. Colchicine works by interfering with the function of neutrophils, which are key part of the inflammatory response. The tubulin-colchicine dimers cap the assembly end of microtubules. This interferes with the cell structure and movement. This also decreases the formation of

digestive vacuoles, the releases of chemotactic factors, cell motility, chemotaxis, and lysosomal degranulation. Colchicine has also been linked with the inhibition of tyrosine phosphorylation and the generation of leukotriene B_4. Colchicine inhibits a gout attack by inhibiting macrophages ability to attack the uric acid crystals (*29*).

EXPERIMENTAL PROCEDURES

MATERIALS. Phosphoribosylpyrophosphate, Tris base, guanine, xanthine, 7-methylxanthine, sodium phosphate, glutatathione, myokinase, ribose-5-phosphate, tetrabutylammonium hydroxide, nicotinamide, Dradkin's Brij-35 solution, $MgCl_2$, and hypoxanthine were purchased from Sigma Chemical Co. Ethyl ether, acetonitrile (HPLC grade), and methanol (HPLC grade) were obtained from EM Science. Ethylenediamine tetraacetic acid (disodium salt) was obtained from Calbiochem. *p*-Nitrobenzoic acid was purchased from Eastman. Perchloric acid and potassium phosphate were purchased from Fischer. Heparinized tubes were purchased from Fischer. All other chemicals used for this study were reagent grade.

INSTRUMENTATION. For all enzyme assays, the HPLC system used consisted of a Varian Model 2510 HPLC pump equipped with an injector with a 20-μL sample loop, a Varian Model 4290 integrator, and a Kratos Model 783 absorbance detector. The column used was a 4.6 x 150 mm partisil ODS (C_{18}) 3 reverse phase column from Whatman, which was attached to a 4.6 x 100 mm partisil ODS (C_{18}) connecting guard column from Alltech. For the determination of uric acid the HPLC system consisted of Beckman SYNCHRON CX-4 system autoanalyzer. For the determination of hypoxanthine and xanthine, the system consisted for a Beckman System Gold 125 solvent module with a 20 20-μL sample loop, and a Beckman System Gold 166 detector. The column used was a 4 x 250 mm Synchropak RPP-100 C18 reverse phase column, which was attached to a 4 x 100 mm Synchropak RPP-100 C18 connecting guard column.

SUBJECTS. This study was approved by the Institutional Research and Ethics Review Committee of the McKay Memorial Hospital in Taipei, Taiwan. Informed consent was obtained from the patient and/or his/her parents depending on their age. The study consisted of 82 subjects who were diagnosed with gout. The mean age of the patients was 44 with a range of 11 to 84 years of age. 58 of these patients were men, whose ages ranged from 11 to 84 years with a mean age of 45. 24 of the patients were women, whose ages ranged from 12 to 66 years with a mean age of 43. These patients were diagnosed with gout according to the American Rheumatism Association criteria.

The diagnosis for gout by the American Rheumatism Association requires either the presence of uric acid crystals in the synovial fluid or the deposition of urates in the tissues, which is demonstrated by chemical or microscopic examination. The presence of two or more of the following criteria can also be used to indicate that the patient has gout: a clear history and/or observation of at least two attacks of painful limb joint swelling, a clear history and/or observation of podagra, the clinically observed presence of a tophus, or a clear history and/or observation of a good response to colchicines, which is defined as a major reduction in objective signs of inflammation within 48 hours of onset of the therapy (*30*).

The study also had a control group of 58 healthy subjects with a range of 8 to 78 years of age and a mean age of 35. This consisted of 33 males, whose ages ranged from 8 to 78 years with a mean age of 35, and of 25 females, whose ages ranged from 8 to 75 years with a mean age of 36. The control group was chosen from a pool of

normals that did not have primary or secondary hyperuricemia in two generations, and did not have any immediate family members suffering from gouty diseases.

SPECIMEN COLLECTION. Fasting blood specimens were drawn from the 82 gout patients and the 58 healthy control subjects between 8:30 and 9:30 am at the out patient clinic of the Mackay Memorial Hospital. A blood sample of 5 mL was collected in a heparinized tube from each individual. This sample was then cooled immediately in an ice bath, and centrifuged within 30 minutes. The red blood cells and the plasma were separated by centrifugation at 3000 x g at 4°C for 10 minutes. The plasma was saved and stored at -20°C immediately until analysis.

PREPARATION OF RED BLOOD CELL ENZYMES. The packed red blood cells (RBC) were washed with cold saline and centrifuged twice at 3000 x g for 10 minutes at 4°C. The packed RBC were the suspended in equal volume of 10mM sodium phosphate buffer at a pH of 7.4, and lysed by freeze/thawing twice at -20°C and 37°C. The sample was then centrifuged at 10,000 x g at 4°C for 10 minutes, and the supernatant (hemolysate) was immediately subjected to HPRT, APRT, or PRPP synthetase assays.

DETERMINATION OF HPRT AND APRT ACTIVITY. In order to determine the HPRT and APRT activity, an assay is preformed at 37°C which contained a mixture of 0.05 M Tris-chloride buffer at pH 7.4, 2.0 mM PRPP, 6.0 mM $MgCl_2$, 1.0 mM hypoxanthine, and 0.2 mM adenine in a total volume of 2 mL. 100μL of the hemolysate is added to initiate the reaction. At 5 minutes and 35 minutes, 0.5mL of the reaction mixture is withdrawn, and then 0.5 mL of 2.0 M Tris-chloride buffer at pH 8.0, 4.0 mM of $MgCl_2$, and 10 units of alkaline phosphatase were added to the

mixture. The mixture was then allowed to incubate for 25 minutes at 37°C. 0.80 mL of 0.60 M of perchloric acid is added to stop the reaction. The precipitate is removed by centrifugation (*30*). 200 µL of the supernatant is mixed with 200 µL of 20 µM *p*-nitrobenzoic acid, which was used as an internal standard, in 1 M potassium phosphate buffer at a pH of 7.5. This was then placed in an ice-bath until the perchlorate salt precipitates. The sample was injected into the high performance liquid chromatography unit (HPLC) with a 20 µL sample loop. The analysis is preformed on a 4 x 250 mm C18 reverse phase column, under isocratic condition with the mobile phase being 10% methanol and 90% 60mM $KHPO_4$ at a pH of 7.9 (*31,32,33*) The enzyme assay is shown in Figure 9.

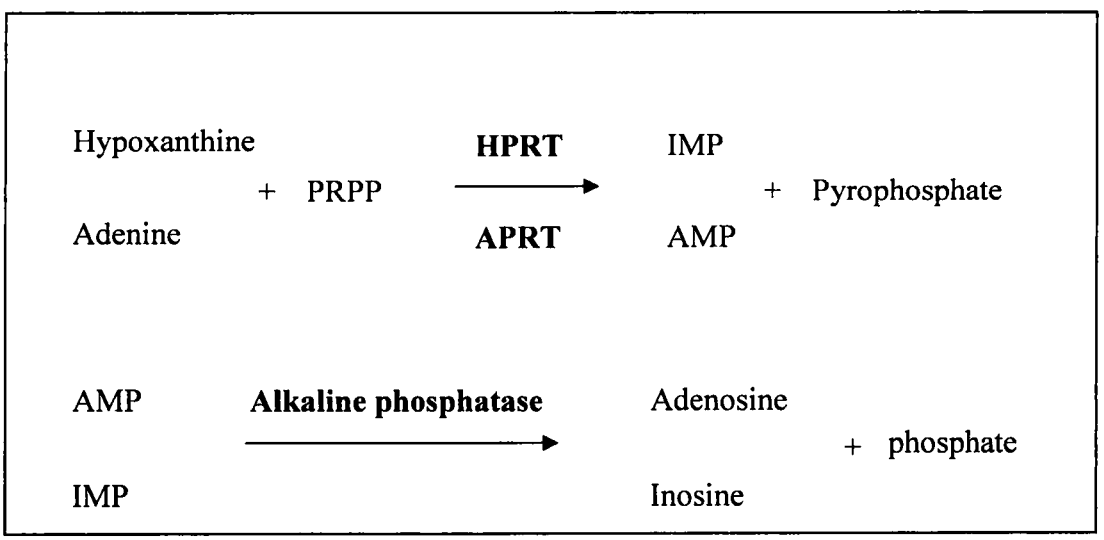

FIGURE 9: The reaction for the HPRT and APRT enzyme assay. HPRT and APRT use one PRPP and convert hypoxanthine and adenine to inosine monophosphate and adenosine monophosphate respectively. Alkaline phosphatase then converts AMP and IMP to adenosine and inosine respectively by cleaving a phosphate.

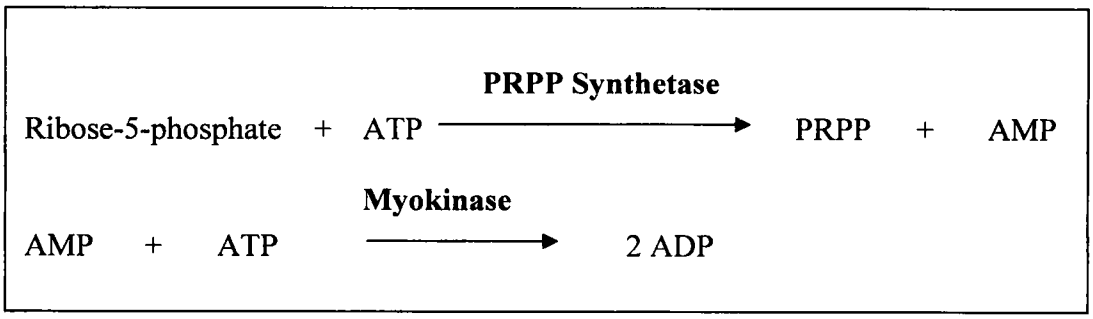

FIGURE 10: The PRPP synthetase enzyme assay reaction. PRPP synthetase converts ribose-5-phosphate to PRPP and AMP. This AMP and an ATP are later converted to 2 ADP by myokinase.

DETERMINATION OF PRPP SYNTHETASE ACTIVITY. PRPP synthetase activity was determined by adapting a method that was previously described by Sakuma and Nishina, which is shown in figure 10 (*34*). The assay was performed at 37°C. The assay mixture contains 0.04 M potassium phosphate buffer at a pH of 7.4, 1.0 mM ribose-5-phosphate, 1.4 mM ATP, 6.0 mM $MgCl_2$, 1.0 mM reduced gluatathione, and 10 units of myokinase in a total volume of 5 mL. 25 µL of the hemolysate is then added to initiate the reaction. At 10 minutes and 40 minutes, 0.5 mL of the reaction mixture is withdrawn and added to the 0.5 mL of the 6.0 M perchloric acid. The sample is stored in an ice bucket. Centrifugation removes the precipitate. 200 µL of the supernatant is mixed with 200 µL of 200 µM nicotinamide, which is used as an internal standard, in 1 M potassium phosphate buffer at a pH of 7.5. The sample is placed in an ice-bath until the perchlorate salt precipitates. The sample is then injected into the HPLC unit with a 20 µL sample loop. The analysis is preformed on a 4 x 250 mm C_{18} reverse phase column under isocratic condition. The mobile phase is 20% methanol and 80% 0.1 M potassium phosphate buffer solution

containing 4 mM of tetrabutylammonium sulfate at a pH of 6.0.

HPLC ANALYSIS OF PLASMA HYPOXANTHINE AND XANTHINE. In order to determine the plasma hypoxanthine and xanthine concentrations, the HPLC system mentioned in instrumentation was employed. The compounds were separated under isocratic conditions with a mobile phase of 4.0 mM potassium phosphate buffer at a pH of 5.8. The plasma, which was previously stored at -20°C, was thawed. 0.5 mL of the plasma sample were pipetted into a polystyrene tube, which was then followed by 1.0 mL of ice-chilled 6% trichloroacetic acid (TCA). The mixture was then allowed to stay on ice for another 5 minutes to ensure max precipitation. The sample was centrifuged at 2000 rpm at 4 °C for 10 minutes to remove the protein precipitate. The supernatant was then separated into a glass tube and washed with 2.5 mL of water saturated diethyl ether twice to remove excess TCA. The sample is then bubbled under a stream of N_2 gas to evaporate the residual ether, and centrifuged at 2000 rpm at 4°C for 10 minutes to remove any particulate matters. 7-methylxanthine is added as an internal standard. The standard curves were obtained by the ratio of peak area of hypoxanthine and xanthine to the peak area of the internal standard, 7-methylxanthine, versus the concentrations of hypoxanthine and xanthine standards. The standard curves were then used to calculate the concentrations of the hypoxanthine and xanthine in the sample. All compounds used were reagent grade, and all solvents were HPLC grades.

DETERMINATION OF PLASMA URIC ACID. The plasma uric acid concentration was determined by the uricase-peroxidase coupling reaction on a Beckman SYNCHRON CX-4 system autoanalyzer.

DETERMINATION OF HEMOGLOBIN. The specific activity of the RBC HPRT was expressed as enzyme unit per mg hemoglobin or nmoles/hour/mg-hemoglobin. This was done by dissolving Drabkin's Reagent in 1000-mL dH_2O with 0.5 mL of Brij-35 solution. The solution is then thoroughly mixed, and insoluble particles are separated using a filter. Hemoglobin standards are then reconstituted using Drabkin's Reagent. They are thoroughly mixed and allowed to stand for at least 30 minutes at room temperature before used. A standard curve was calculated by performing the following assay as shown in Table 1.

The absorbance at 540 nm was then determined and a standard curve was obtained. The patient's samples were treated by adding 1.0 mL of Drabkin's Reagent to 30 μL of patient's charcoal-treated RBC lysate. The sample then stood for 15 minutes at room temperature before taking the absorbance at 540 nm.

Table 1: Hemoglobin Standards

Tubes	**Hemoglobin Standard Solution (mL)**	**Drabkins Reagent (mL)**	**Equivalent to blood hemoglobin (g/dL)**
Blank	0.0	3.0	0.0
Standard 1	0.5	2.5	3.0
Standard 2	1.0	2.0	6.0
Standard 3	1.5	1.5	9.0
Standard 4	2.0	1.0	12.0
Standard 5	2.5	0.5	15.0
Standard 6	3.0	0.0	18.0

DATA ANALYSIS. The results of the analysis are presented as arithmetic mean ± standard deviation. The data were analyzed with standard statistical method from

the Statistical Package for the Social Science (SPSS). Differences were considered statistically significant at $p<0.05$. The means difference of uric acid, hypoxanthine, xanthine, HPRT, APRT, and PRPP synthetase levels between the gout patients and the healthy normal control group was analyzed with the use of paired Student's *t*-test.

RESULTS

HPLC ANALYSIS HPRT, APRT AND PRPP IN RED BLOOD CELLS, AND HYPOXANTHINE AND XANTHINE CONCENTRATIONS IN PLASMA. Figure 11-A and 11-B depict an example of a patient's HPRT and APRT assay after 5 minutes (Figure 11-A) and after 35 minutes (Figure 11-B). During this reaction HPRT and APRT converts hypoxanthine and adenine into inosine monophosphate and adenosine monophosphate. The peak areas of the analytes are compared to the internal standard, nicotinamide, to determine the analytes concentration. From this figure the activities of the enzymes are determined in units of μmoles/hr-mL sample. Figure 12-A and 12-B depict an example of a patient's PRPP synthetase assay after 5 minutes (Figure 12-A) and 35 minutes (Figure 12-B). The PRPP synthetase converts ribose-5-phosphate and ATP to PRPP and AMP. A myokinase then converts the AMP to 2 ADP using an ATP (Figure 10). The ADP and ATP concentrations are compared against the internal standard *p*-aminobenzoic acid, from which the PRPP synthetase activity is derived in units of μmoles/hr-mL sample. Figure 13 shows a typical chromatogram of the separation of hypoxanthine and xanthine in plasma. 7-methylxanthine is used as the internal standard, and the concentrations of hypoxanthine and xanthine are derived by comparing the peak areas. This was done by obtaining the standard curves from the ratio of peak area of hypoxanthine and xanthine to the peak area of the internal standard, 7-methylxanthine, versus the concentrations of hypoxanthine and xanthine standards. The standard curves were then used to calculate the concentrations of the

hypoxanthine and xanthine in the sample. Figure 13-A shows an example of the standard, and Figure 13-B shows an example of a patient's results.

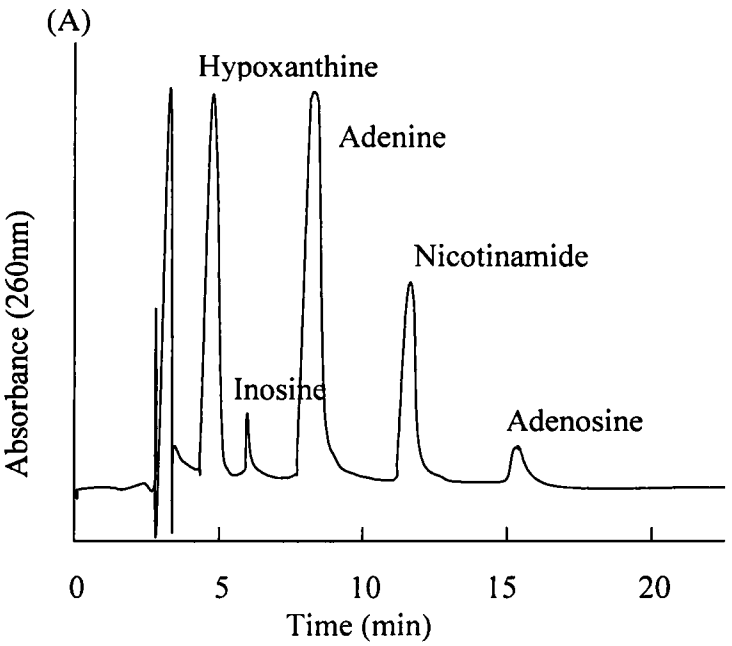

FIGURE 11: HPLC chromatograms of HPRT and APRT assays after (A) 5 and (B) 35 minutes. This figure shows typical chromatograms of the separation of hypoxanthine and adenine (reactants), inosine monophosphate and adenosine monophosphate (products) from HPRT and APRT assay, and nicotinamide (internal standard) at 5 minutes of reaction time (Figure 11-A) and 35 minutes (Figure 11-B).

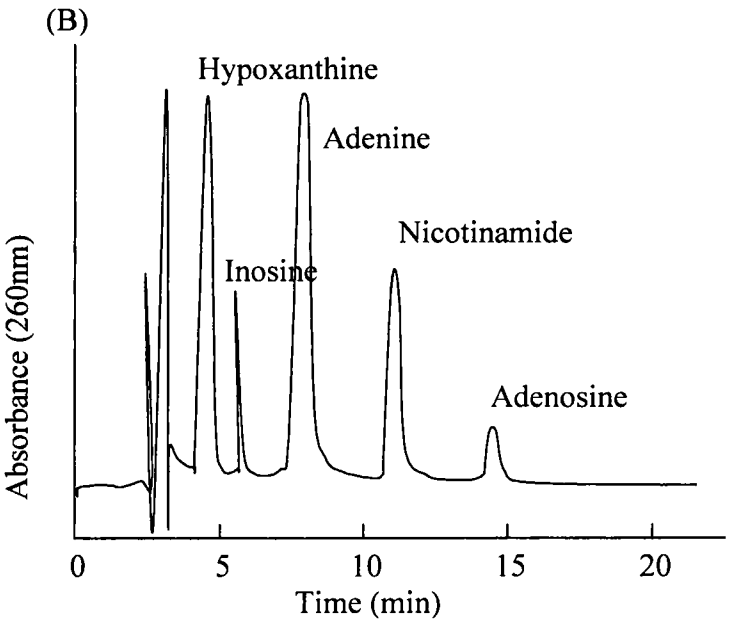

Figure 11: (Continued)

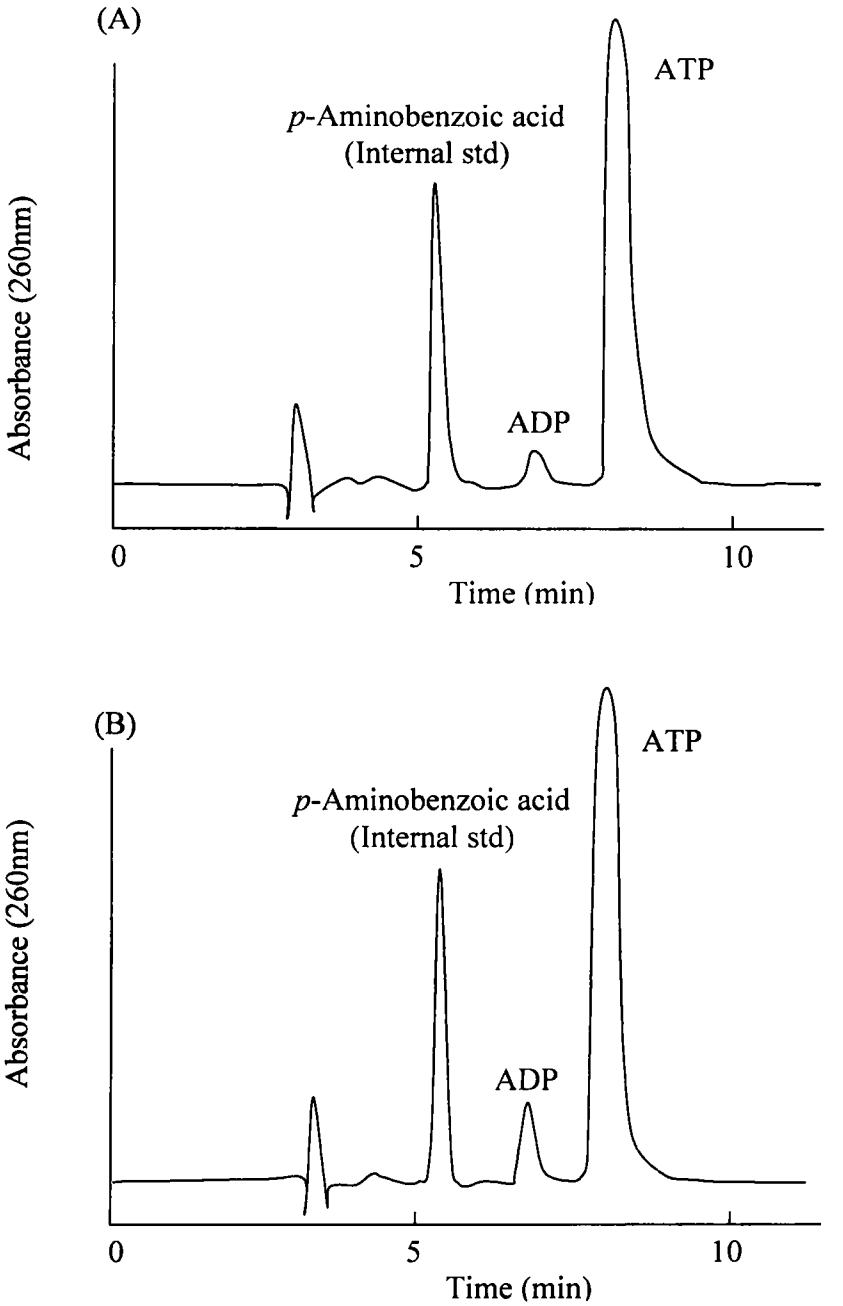

FIGURE 12: PRPP synthetase assays after (A) 5 and (B) 35 minutes. This figure shows typical chromatograms of the separation of ADP (product), ATP (reactant) of a PRPP synthetase assay, and *p*-aminobenzoic acid (internal standard) at 5 minutes of reaction time (Figure 12-A) and 35 minutes (Figure 12-B).

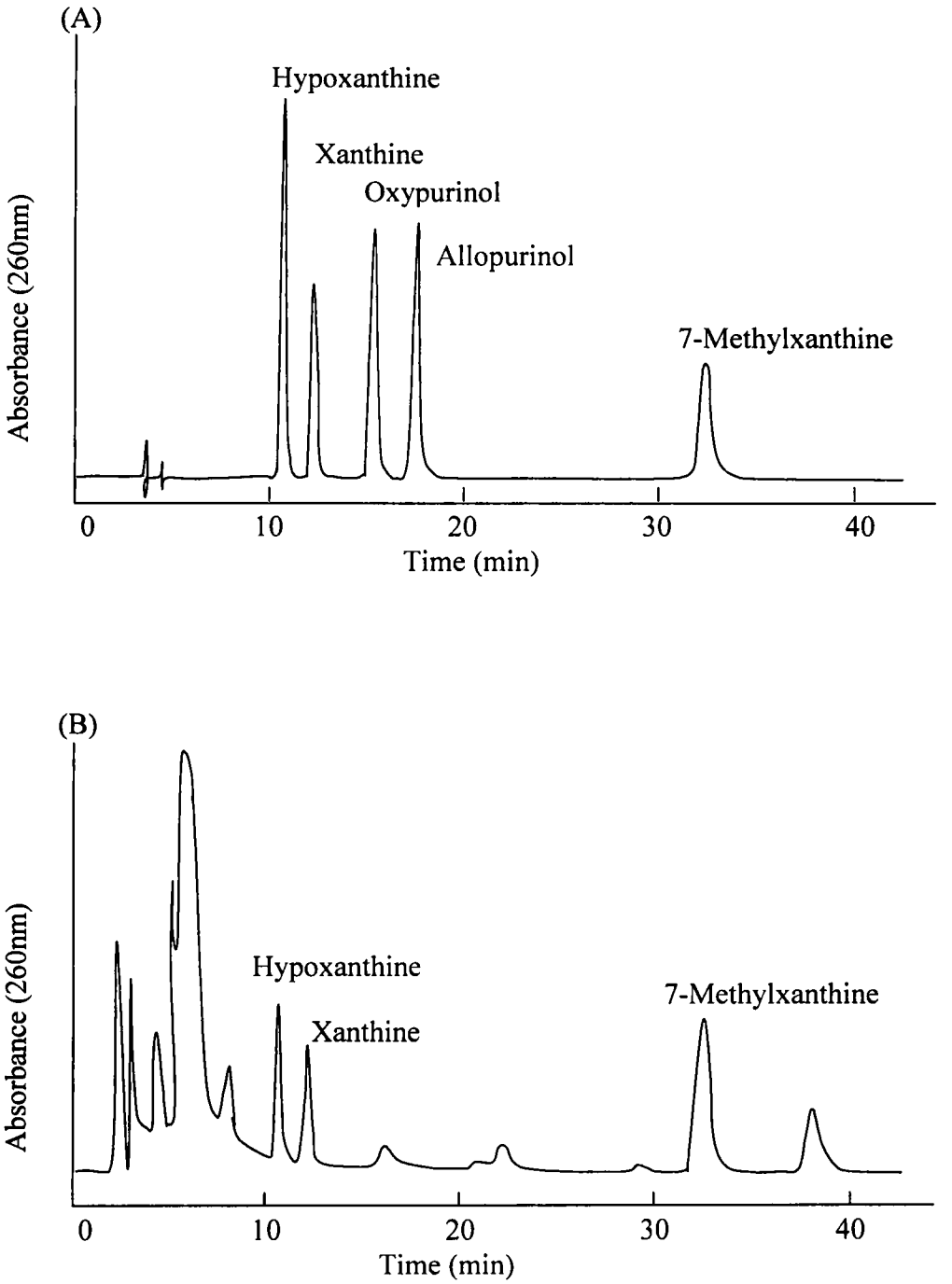

FIGURE 13: Typical chromatograms of the separation of hypoxanthine and xanthine in plasma, and 7-methylxanthine (internal standard). (A) the standard. (B) patient sample.

The mean and standard deviation for the enzymes' concentrations as well as the plasma urate, hypoxanthine, and xanthine concentrations are reported in Table 2. The *p* values for comparison between the two groups are reported in Table 3. A plasma urate level above 7 mg/dL is considered hyperuricemic because this is above saturation level. HPRT and APRT activity levels that were less than 40% of the reference range were arbitrarily considered partially deficient. PRPP synthetase levels above 500% of the reference range were arbitrarily set as elevated. The reference range is taken from the results of the normal healthy control subjects. The values of the reference levels are in Table 2.

GOUT PATIENTS VERSUS CONTROL GROUP. When the gout patients are compared to the control group, the plasma uric acid, hypoxanthine, and xanthine levels were found to be significantly higher than those of the normal control subjects at a *p* rejection level of 0.05. The gouty patients' uric acid levels are 9.08 ± 2.72 mg/dl, compared to the control group, which has levels of 5.90 ± 1.15 mg/dl. The *p* value for this comparison is $p<0.01$. The hypoxanthine levels are 14.8 ± 24.0 μM versus the control groups' average, which is 3.51 ± 2.36 μM. The *p* value for this comparison is $p<0.01$. Xanthine levels are 8.04 ± 9.77 μM for gout patients versus 0.95 ± 0.88 μM for the control group. The *p* value for this comparison is $p<0.01$. The values all agree with the idea that elevated uric acid, hypoxanthine, and xanthine levels correspond with the onset of gout.

When the enzyme activities for HPRT, APRT, and PRPP synthetase of the gout patients are compared to the control group, only the PRPP synthetase has a significant difference between the gouty patients and the control group. The PRPP

synthetase average for the gout group is 59.9 ± 61.0 nmoles/hr-mg hemoglobin. The control groups' PRPP synthetase average is 28.6 ± 25.6 nmoles/ hr-mg hemoglobin.

Table 2: The Plasma Urate, Hypoxanthine, Xanthine and Erythrocyte HPRT, APRT, and PRPP Synthetase Levels of Gout Patients and Control Subjects

	Gout	**Control**
Total	82	58
Urate (mg/dL)	9.08 ± 2.72	5.90 ± 1.15
Hypoxanthine (μM)	14.8 ± 24.0	3.51 ± 2.36
Xanthine (μM)	8.04 ± 9.77	0.95 ± 0.88
HPRT (nmol/hr-mg Hb)	1.96 ± 1.49	2.23 ± 1.16
APRT (nmol/hr-mg Hb)	0.263 ± 0.220	0.305 ± 0.104
PRPP synthetase (nmol/hr-mg Hb)	59.9 ± 61.0	28.6 ± 25.6
Male	58	33
Urate (mg/dL)	9.25 ± 2.72	6.49 ± 1.02
Hypoxanthine (μM)	16.6 ± 28.1	4.11 ± 2.69
Xanthine (μM)	9.31 ±10.9	1.25 ± 1.01
HPRT (nmol/hr-mg Hb)	1.85 ± 1.42	1.92 ± 0.95
APRT (nmol/hr-mg Hb)	0.267 ± 0.247	0.327 ± 0.118
PRPP synthetase (nmol/hr-mg Hb)	64.9 ± 66.6	35.0 ± 29.0
Female	24	25
Urate (mg/dL)	8.75 ± 2.31	5.35 ± 0.99
Hypoxanthine (μM)	10.2 ± 7.39	2.73 ± 1.56
Xanthine (μM)	4.95 ± 5.34	0.56 ± 0.44
HPRT (nmol/hr-mg Hb)	2.22 ± 2.72	2.56 ± 1.29
APRT (nmol/hr-mg Hb)	0.256 ± 0.140	0.277 ± 0.074
PRPP synthetase (nmol/hr-mg Hb)	47.8 ± 43.5	20.3 ± 17.1

The p value for this comparison is $p<0.01$. The HPRT average activity for gout patients is 1.96 ± 1.49 nmoles/ hr-mg hemoglobin versus 2.23 ± 1.16 nmoles/ hr-mg hemoglobin. The APRT average activity for gout patients is 0.263 ± 0.220 nmoles/ hr-mg hemoglobin versus 0.305 ± 0.104 nmoles/ hr-mg hemoglobin. For both

of these comparisons, the *p* value exceeded 0.05 and is therefore deemed insignificant. A reason why there might not be a significant difference between the control groups and gout groups' enzyme activity is because not all gout patients are deficient in HPRT and APRT. Patients that are deficient in HPRT and APRT would have lessened feedback inhibition for the PRPP synthetase, which could explain why the PRPP synthetase activity levels are significantly different between the gout and control group. The *p* value data is in table 3 and table 4 below.

Table 3: The P Values Obtained from the Comparison Between Normal Healthy Control Subjects and Gout Patients

	Urate	**Hypoxanthine**	**Xanthine**	**HPRT**	**APRT**	**PRPP Synthetase**
Total	$p < 0.01$	$p < 0.01$	$p < 0.01$	n.s.	n.s.	$p < 0.01$
Male	$p < 0.01$	$p < 0.05$	$p < 0.01$	n.s.	$p < 0.01$	$p < 0.01$
Female	$p < 0.01$	$p < 0.01$	$p < 0.01$	n.s.	n.s.	$p < 0.05$

Table 4: The P Values Obtained from the Comparison Between Male and Female Control Subjects and Male and Female Gout Patients

	Urate	**Hypoxanthine**	**Xanthine**	**HPRT**	**APRT**	**PRPP Synthetase**
Control	$p < 0.01$	$p < 0.05$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.05$
Gout	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

MALE GOUT PATIENTS VERSUS MALE CONTROL SUBJECTS. The next group to examine is the difference between the male gout patients and male control subjects. In total, there are 58 male gout patients and 33 normal healthy control subjects. All of the defined biochemical parameters are significantly different

between the gout patients and the control group except the HPRT activity. The uric acid levels are 9.25 ± 2.92 mg/dL for the gout patients, and 6.49 ± 1.02 mg/dL for the control group with a *p* value of less than 0.01. The hypoxanthine level for the gout patients is 16.6 ± 28.1 μM and 4.11 ± 2.69 μM for the control group, with a *p* value of less than 0.05. The xanthine average level is 9.31 ± 10.9 μM and 1.25 ± 1.01 μM for the gout and control subjects respectively with a *p* value of less that 0.01. The PRPP synthetase average activity level is 64.9 ± 66.6 nmol/hr-mg hemoglobin for the gout patients, and 35.0 ± 29.0 nmol/hr-mg hemoglobin. The *p* value is less than 0.01. The APRT average activity level for the gout patient is 0.267 ± 0.247 nmol/hr-mg hemoglobin versus 0.327 ± 0.118 nmol/hr-mg hemoglobin with a *p* value less than 0.01. No statistically significant difference is observed between the male gout patients and the male normal healthy control subjects for erythrocyte HPRT levels. The average HPRT activity is 1.96 ± 1.49 nmol/hr-mg hemoglobin for the gout patients versus 2.23 ± 1.16 nmol/hr-mg hemoglobin for the control. The *p* value is above 0.05, which indicates the two means are not significantly different.

FEMALE GOUT PATIENTS VERSUS FEMALE CONTROL PATIENTS. In a comparison between the female gout patients and the female normal healthy control subjects, mean plasma uric acid, hypoxanthine, and xanthine concentrations are all statistically different. The erythrocyte PRPP synthetase level is significantly higher for the female gout patients versus the female control group. The mean plasma uric acid, hypoxanthine, and xanthine concentrations for the female gout patients are 8.75 ± 2.31 mg/dL, 10.2 ± 7.39 μM, and 4.95 ± 5.34 μM respectively. The mean plasma uric acid, hypoxanthine, and xanthine concentrations for the female control subjects

are 5.35 ± 0.99 mg/dL, 2.73 ± 1.56 μM, and 0.56 ± 0.44 μM respectively. The *p* value for all three comparisons is less than 0.01. The mean PRPP synthetase level is 47.8 ± 43.5 nmol/hr-mg hemoglobin for the female gout patients and 20.3 ± 17.1 nmol/hr-mg hemoglobin for the female control subjects. The *p* value is less than 0.05. In contrast, the comparison of HPRT and APRT between the female gout patients and the female control group yields a *p* values greater than 0.05; therefore, there is no significant difference between the two groups. The mean HPRT and APRT levels for the female gout patients are 2.22 ± 1.66 nmol/hr-mg hemoglobin and 0.256 ± 0.140 nmol/hr-mg hemoglobin respectively. The mean HPRT and APRT levels for the female control group are 2.65 ± 1.29 nmol/hr-mg hemoglobin and 0.277 ± 0.074 nmol/hr-mg hemoglobin. The female group consists of 24 gout patients and 25 normal healthy control subjects.

MALE VERSUS FEMALE. The differences in the biochemical parameters between the male and female patients are evaluated. No significant differences are observed between the male and female gout patients. In contrast, significant differences are observed between the female control subjects and the male control subjects in all biochemical parameters. Plasma urate and xanthine concentrations are significantly different with *p* values of less than 0.01. HPRT and APRT levels are also significantly different with *p* values of less than 0.01. The *p* value for the difference between the male and female control group for hypoxanthine and PRPP synthetase levels is less than 0.05. The *p* value data is in Table 3 and Table 4 above.

GROUPING. The gout patients are divided into one of eight groups depending on their enzymatic levels. HPRT and APRT levels below 40% the reference group are

considered to have a partial enzyme deficiency, while PRPP synthetase levels above 500% of the reference range are considered elevated. Based on the criteria, the gout patients are divided into eight groups. Group 1 consists of patients with a partial deficiency of HPRT activity. Group 2 has a partial deficiency of APRT activity. Group 3 is of patients with an overactive PRPP synthetase. Group 4 has a partial deficiency in both HPRT and APRT. Group 5 has a deficiency in HPRT and an elevated PRPP synthetase activity. Group 6 has a partial deficiency of APRT activity and an elevated PRPP synthetase activity. Group 7 consists of patients with a partial deficiency in HPRT and APRT and an overactive PRPP synthetase. Group 8 is possible renal dysfunction and other factors. This conclusion is made because a partial deficiency in APRT and HPRT activity was not observed, nor was an elevated PRPP synthetase activity observed.

The percentages and totals for each group are displayed in Table 6. One interesting observation is other factors accounted for approximately 60% of the patient's cause of gout. A partial HPRT deficiency is the most common cause of gout with approximately 25% having at least a partial HPRT deficiency, with some having multiple enzyme abnormalities. Approximately 13% of the study group has an elevated PRPP synthetase, with some having multiple enzyme abnormalities. At least a partial deficiency in APRT accounts for approximately 12% of the patients' cause of gout. Roughly, 10% of the total gout population has an abnormality in at least two enzymes. One percent of the gout population is deficient in HPRT and APRT and has an elevated PRPP synthetase activity.

Table 5: The Biochemical Profile of Representatives of the Eight Gout Groups.

Group 1 HPRT Partial Deficiency	Patient 2052 (Male)	Patient 2048 (Female)
Uric acid (mg/dL)	6.7	8.2 (Elevated)
Hypoxanthine (μM)	6.47 (Elevated)	8.51 (Elevated)
Xanthine (μM)	9.10 (Elevated)	9.11 Elevated)
HPRT (nmol/hr-mg Hb)	**0.65 (25.5%)**	**0.08 (3.14%)**
APRT (nmol/hr-mg Hb)	0.290 (102.5%)	0.318 (118%)
PRPP synthetase (nmol/hr-mg Hb)	82.8 (267.8%)	53.7 (41.1%)

Group 2 APRT Partial Deficiency	Patient 2046 (Male)	Patient 2013 (Male)
Uric acid (mg/dL)	10.3 (Elevated)	9.0 (Elevated)
Hypoxanthine (μM)	111 (Elevated)	5.67 (Elevated)
Xanthine (μM)	28.9 (Elevated)	6.07 (Elevated)
HPRT (nmol/hr-mg Hb)	4.65 (182%)	1.88 (73.7%)
APRT (nmol/hr-mg Hb)	**0.060 (21.2%)**	**0.093 (32.9%)**
PRPP synthetase (nmol/hr-mg Hb)	119.2 (386%)	15.6 (50.5 %)

Group 3 PPRP Synthetase Elevation	Patient 2035 (Male)	Patient 2021 (Female)
Uric acid (mg/dL)	10.2 (Elevated)	8.4 (Elevated)
Hypoxanthine (μM)	13.48 (Elevated)	10.91 (Elevated)
Xanthine (μM)	2.41	4.62 (Elevated)
HPRT (nmol/hr-mg Hb)	1.89 (74.1%)	3.06 (12%)
APRT (nmol/hr-mg Hb)	0.190 (67.1%)	0.143 (50.5%)
PRPP synthetase (nmol/hr-mg Hb)	**355.9 (1151%)**	**158 (1039%)**

Group 4 HPRT and APRT Partial Deficiencies	Patient 2061 (Male)	Patient 2015 (Female)
Uric acid (mg/dL)	9.9 (Elevated)	9.2 (Elevated)
Hypoxanthine (μM)	4.75 (Elevated)	15.16 (Elevated)
Xanthine (μM)	13.54 (Elevated)	2.17 (Elevated)
HPRT (nmol/hr-mg Hb)	**0.64 (25.1%)**	**0.76 (29.8%)**
APRT (nmol/hr-mg Hb)	**0.030 (10.6%)**	**0.062 (21.9%)**
PRPP synthetase (nmol/hr-mg Hb)	65.9 (213%)	0.92 (6.0%)

Table 5: (Continued)

Group 5 HPRT Partial Deficiencies and PRPP Synthetase Elevation	
	Patient 2036 (Male)
Uric acid (mg/dL)	8.5 (Elevated)
Hypoxanthine (μM)	5.40 (Elevated)
Xanthine (μM)	1.38
HPRT (nmol/hr-mg Hb)	**0.80 (31.4%)**
APRT (nmol/hr-mg Hb)	0.580 (205%)
PRPP synthetase (nmol/hr-mg Hb)	**198.5 (642%)**

Group 6 APRT Partial Deficiencies and PRPP Synthetase Elevation	
	Patient 2062 (Female)
Uric acid (mg/dL)	10.2 (Elevated)
Hypoxanthine (μM)	32.05 (Elevated)
Xanthine (μM)	21.55 (Elevated)
HPRT (nmol/hr-mg Hb)	1.32 (51.8%)
APRT (nmol/hr-mg Hb)	**0.108 (38.2%)**
PRPP synthetase (nmol/hr-mg Hb)	**126.2 (830%)**

Group 7 HPRT and APRT Partial Deficiencies and PRPP Synthetase Elevation	
	Patient 2025 (Male)
Uric acid (mg/dL)	9.1 (Elevated)
Hypoxanthine (μM)	8.59 (Elevated)
Xanthine (μM)	1.39 (Elevated)
HPRT (nmol/hr-mg Hb)	**0.78 (30.6%)**
APRT (nmol/hr-mg Hb)	**0.089 (31.5%)**
PRPP synthetase (nmol/hr-mg Hb)	188.3 (609%)

Table 5: (Continued)

Group 8 Possible Renal dysfunctional (Other Factors)	Patient 2031 (Male)	Patient 2024 (Female)
Uric acid (mg/dL)	10.1 (Elevated)	11.2 (Elevated)
Hypoxanthine (μM)	7.75 (Elevated)	4.00 (Elevated)
Xanthine (μM)	0.85 (Elevated)	0.50 (Elevated)
HPRT (nmol/hr-mg Hb)	3.28 (128.6%)	3.97 (155.7%)
APRT (nmol/hr-mg Hb)	0.241 (85.2%)	0.212 (74.9%)
PRPP synthetase (nmol/hr-mg Hb)	27.9 (90.2%)	13.0 (85.5%)

The first number represents value corresponding to the particular analyte. For uric acid, hypoxanthine, and xanthine, "elevated" means that the concentration of purine is considered elevated compared to normal. For HPRT, APRT, and PRPP synthetase, the number in parentheses indicates the patient's enzyme activity when compared to the control group's average.

There is no significant differences between the female and male gout patients means, but there are some slight difference between the general distribution of the groups and the sexes observed in Table 6. While generally the distribution percentages are similar between the sexes, such as Group 1, some groups have a noticeable difference between the two sexes. Three of the 58 male gout patients for a total of 5.17% are deficient of APRT, but none of the 24 female patients have a partial APRT deficiency. Group 8 represents approximately 62 % of the male gout patients, but only 54% of the female population. While these differences are observed, it is quiet possible that with more subjects the gap between the sexes would diminish.

Table 6: The Numbers of Patients and Percentages in Each Group

Group	***Total (N=82)***	***Male (N=58)***	***Female (N=24)***
1. HPRT partial deficiency	14 (17.1%)	10 (17.2%)	4 (16.7%)
2. APRT partial deficiency	3 (3.66%)	3 (5.17%)	0 (0%)
3. PRPP synthetase elevation	8 (9.76%)	4 (6.90%)	4 (16.7%)
4. HPRT and APRT Partial deficiencies	5 (6.10%)	3 (5.17%)	2 (8.33%)
5. HPRT partial deficiency and PRPP synthetase elevation	1 (1.22%)	1 (1.72%)	0 (0%)
6. APRT partial deficiency and PRPP synthetase elevation	1 (1.22%)	0 (0%)	1 (4.17%)
7. HPRT, APRT partial deficiency and PRPP synthetase elevation	1 (1.22%)	1 (1.72%)	0 (0%)
8. Other factors	49 (59.8%)	36 (62.1 %)	13 (54.2%)

DISCUSSION AND CONCLUSION

The results of this study correspond well to previous reports (*35, 36, 37*). Hypoxanthine, xanthine, and uric acid levels were all found to be significantly higher than the levels of the control group. The elevated concentration of these products can be caused by a deficiency in the HPRT or APRT, an overactive PRPP synthetase, or a combination of the above factors. A decrease in the HPRT or APRT activities could lead to the underutilization and accumulation of PRPP, while an overactive PRPP synthetase would lead to an accumulation of PRPP. PRPP is later converted into inosine monophosphate, which can later be converted to increased levels of hypoxanthine, xanthine, and finally to uric acid leading to gout.

Gout patients in Groups 1, 2, and 4 (Table 6) were found to have a partial deficiency in HPRT, APRT or both enzymes. These results suggest that these enzymes may play an important role in the underutilization of hypoxanthine, which can lead to an overproduction of uric acid. Patients in Group 3 were found to have an elevated PRPP synthetase, which would lead to an accumulation of PRPP. Increased PRPP concentration can ultimately lead to gout. Groups 5, 6, and 7 were found to have a combination of either HPRT or APRT partial deficiency and an elevation of PRPP synthetase activity, which suggests that multiple mechanisms can be involved in the formation of gout. Patients from Group 8 were found to have neither a deficiency in HPRT or APRT, nor an overactive PRPP synthetase. This suggests that renal dysfunction in uric acid clearance or other unknown causes may also be involved in

the onset of gout. While many of the gout patients are thought to have possible renal clearance issues, this study is unable to positively identify if renal dysfunction is the culprit.

When comparing the sexes of the patients, there are no major differences between male and female plasma uric acid levels in gout patients. This observation corresponds with previous studies (*35, 36, 37*). However, there is a significant difference between the male and female plasma uric acid levels in control subjects. It has been suggested that female's renal clearance of uric acid is more efficient during reproductive years as compared to males (*38*). In general, females have a lower incidence of gout, which has been observed in previous studies (*31, 33, 37*), which could be caused by the lower uric acid levels in females versus males. The hormone oestrogen promotes the excretion of urate, which could account for why women are less prone to gout before menopause (*38*).

TREATMENT OPTIONS. Once the underlying cause of the gout is determined, effective treatment options can be developed to treat and prevent gout. While there are no formal guidelines for the treatment of acute gout, normally nonsteriodal anti-inflammatory drugs such as indomethacin, naproxen, and sulindac are used to treat the inflammation and pain. Colchicine is a traditional drug used to treat the initial symptoms of gout, but its use has declined due to renal and gastrointestinal problems associated with the drug. A cyclooxygenase –2-inhibitor call etoricoxib has also been studied as an effective treatment for acute gout (*39*).

After the gout has been treated, the focus turns to the prevention of the next attack by lowering the blood urate levels. It is recommended that patients limit their

purine intake and discontinue the use of diuretic agents (*40*). Gradual weight loss, which may decrease the overproduction of insulin, is also recommended. Allopurinol is a urate-lowering drug that is the most commonly prescribed, which inhibits the synthesis of uric acid by inhibiting xanthine oxidase. While allopurinol is a potent life-long option, it can produce side effects. The most common side effect is pruritus or rash. People who are HPRT deficient may have a decrease effectiveness of allopurinol due to dose-limiting renal failure.

If the patient is sensitive to allopurinol, then other treatment options exist. Losartan and fenofibrate are antihypertensive agents that moderately decrease serum urate levels. These drugs increase the urinary excretion of uric acid; therefore, they can only be used for patients with a negative history of urinary lithasis and normal uric acid levels in urine (*40*). Benzobromarone is a potent and effective drug that can even be used with patients with moderate renal failure. It is currently not used in the European Union due to its association with fatal hepatitis. Another expensive option is uricase therapy. This treatment decreases serum urate levels by degrading urate to allantoin (*40*). Febuxostat is a non-purine inhibitor of xanthine oxidase. While allopurinol can be metabolized by other purine enzymes such as HPRT, febuxostat has a reduced metabolism by other enzymes. This may reduce some of the complications associate with allopurinol (*41*).

Patients whose test indicated that their gout might probably be caused by renal clearance issues are recommended to go talk to a nephrologist. This method for examining the cause of gout is unable to distinguish what may be causing the renal

clearance problems. A nephrologist is better able to determine what exact factor is limiting renal clearance.

METHOD USED. One of the past methods used to determine the cause of patient's gout is through radiochemical techniques. An isotopic analyte would be administered to the patient, and the levels of the isotope would then be analyzed. Other methods preformed assays with isotopically labeled substrates and determined the enzymes activity by observing the concentration of isotopically labeled end product. This can be done using numerous methods. One study used a radioisotopic procedure to determine the activity of PRPP synthetase under various conditions. The PRPP synthetase activity was determined by measuring the amount of ^{14}-adenylates (AMP, ADP, ATP) generated from the assay. The labeling of all of the adenylates must be considered when determining the PRPP activity because the resulting AMP from the assay could react with residual ATP to form labeled ADP and ATP. The resulting concentrations were pooled, and the PRPP synthetase activity was determined (*42*).

Another study injected a single dose of isotopic uric acid into control and gouty human subjects. The uric acid was later collected from serial urine samples. Based on the isotope concentration, the magnitude and turnover of the pool of miscible uric acid were calculated (*43*).

The activity of HPRT and APRT was also determined using isotopically labeled substrates. This method used [8-^{14}C] hypoxanthine and [8-^{14}C] adenine to determine the activity of HPRT and APRT. After the micro-assay was preformed, the isotopically labeled end products, IMP and AMP, are separated using electrophoresis.

The bands from the gel were cut out, and the radioactivity of the samples was determined. The activity of the HPRT and APRT was determined using the radioactivity of the enzymes' respective end products (*44*).

Several studies have used HPLC for detection of analytes relating to gout. Sakum *et al* used HPLC to simultaneously determine the activity of APRT and HPRT from red blood cells. They first preformed a simultaneous enzyme assay, and the reaction mixture was stopped after 5 and 25 minutes. The concentrations of AMP and IMP were then compared to determine enzymes activity. This method could detect 1% of normal APRT activity and 0.3% of normal HPRT, and the method was adopted for the determination of APRT and HPRT activity for this study (*45*). Other studies have used HPLC methods to determine hypoxanthine, xanthine, and uric acid concentrations (*46, 47*), as well as PRPP synthetase activity (*48*).

One of the advantages to using HPLC instead of radiochemical methods is that special equipment is not required. This method can be easily automated, and the procedure is generally less complicated. Some studies have had the subjects ingest isotopically labeled purines to determine purine concentrations. This method is not as invasive because it does not require any special treatments for the patients. The individual analytes can be identified and analyzed as none of their peaks overlaps (*49*).

CONCLUSION. Gout is a disease that is linked to the *de novo* pathway. Abnormalities of HPRT, APRT, and PRPP synthetase in red blood cells have been linked to increased levels of uric acid in plasma. A deficiency in HPRT and APRT causes the underutilization of synthesized purines. This can lead to an accumulation of purines, which can ultimately lead to an elevated uric acid blood plasma

concentration. When the concentration of uric acid exceeds blood saturation level, then a gout attack may precipitate. Hyperuricemia in patients with partial HPRT deficiency or Lesch-Nyhan syndrome has been suggested to be associated with increase *de novo* purine synthesis. This could be due to increased loss of purines, a lessened feedback inhibition of purine synthesis, or a combination of the two (*23*). Based on our results a deficiency in APRT does not seem to be as big of a contributor to gout as compared to HPRT, but it should not be overlooked as a possible cause of gout.

Our findings indicate that there is a significant difference between the activities of the PRPP synthetase for the gout patients versus the healthy control group. An over production of PRPP is one possible cause of gout (*14*). This would lead to an accumulation of PRPP, which could ultimately lead to the over production of uric acid. The over production of uric acid could trigger a gout attack.

Another possible cause of gout is the under excretion of uric acid by the kidneys, which could be caused by many factors. While this study was unable to positively identify if group eight, or the other factors group, is caused by kidney renal clearance problems, renal clearance problems have been linked to gout. The exact mechanism of this is not completely understood.

A determination of urinary total output of purine should be planned for the future study. Patients who have increased purine synthesis or decreased purine reutilization have an increased excretion of uric acid. Increased urinary excretion of xanthine along with hypoxanthine is also observed in the excessive levels of plasma hypoxanthine. Lower levels of AMP, IMP, or GMP, which would reduce the ability

to inhibit purine synthesis, could cause this. An increase in PRPP synthetase activity could also cause an increased excretion of uric acid.

While 40.2% of patients exhibited an erythrocyte enzyme deficiency, 59.8% of the patients had other factors, which could be renal clearance problems. Future studies can be developed to confirm the correlation between gout and renal clearance issues.

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