Synthesis and Biochemical Pharmacology of Oxoazopurines

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SYNTHESIS AND BIOCHEMICAL PHARMACOLOGY OF OXOAZOPURINES

by

Arun L. Jadhav

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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LOYOLA UNIVERSITY NEW CUMBERLAND
SYNTHESIS AND BIOCHEMICAL PHARMACOLOGY OF OXOAZOPURINES

This study was undertaken to investigate the synthesis and chemical and biological properties of a new class of compounds, the oxoaazopurines. 8,8'-Dioxo-6,6'-azopurine monohydrate (DOAP), the parent member of this new class, was synthesized from adenine using sulfuric acid, potassium bromide, potassium permanganate, and hydrogen peroxide. A hydrazo derivative of DOAP, 8,8'-dioxo-6,6'-hydrazopurine monohydrate (DOHP), was synthesized by a partial reduction of DOAP using SnCl₂/HCl at 70°C, and an azoxy derivative of DOAP, 8,8'-dioxo-6,6'-azoxypurine hexahydrate trisodium salt (NaDOAXP), was synthesized from the air oxidation of DOAP in 0.005 N NaOH. Reductive cleavage of DOAP using sodium hydrosulfite and NaOH resulted in 8-hydroxyadenine indicating that DOAP was a purine dimer joined through an azo bond between the C₆ and C₆', positions. The structure of DOAP was further confirmed by enzymatic synthesis of DOAP from the oxidation of 6,6'-azopurine disodium salt (NaAP) by rabbit liver aldehyde oxidase. Since rabbit liver aldehyde oxidase is known to oxidize purines selectively at the C₈ position, the enzymatic synthesis of DOAP confirmed that the oxo groups of DOAP are at the C₈ and C₈', positions. DOAP has also been shown to be a potent inhibitor of xanthine oxidase.
During this investigation it was also observed that 6,6'-azopurine disodium salt (NaAP) was a potent competitive inhibitor of rabbit liver aldehyde oxidase. A 50% inhibition of rabbit liver aldehyde oxidase activity was found to occur at a final concentration of 2.6 \times 10^{-6} \text{ M} NaAP in the \textit{in vitro} reaction mixture. Results of both a Lineweaver and Burk plot as well as a Dixon plot revealed a mean $K_i$ of $3.3 \times 10^{-6}$ M for NaAP while indicating that the nature of the inhibition of rabbit liver aldehyde oxidase by NaAP was competitive. The present finding of such competitive inhibition of rabbit liver aldehyde oxidase suggests that further investigations of substituted azopurines may elucidate their physiologic function.
DEDICATION

I take great pleasure in dedicating this dissertation to my wife, Kumud and my daughter, Leena, in appreciation for their constant encouragement and positive attitude throughout the period of my graduate training.
ACKNOWLEDGMENTS

The preparation of this dissertation would not have been possible without the help and encouragement of many individuals. I am greatly indebted to Professor Joseph R. Davis for directing this study and overseeing my training as a scientist. My sincerest appreciation is expressed to Professor Alexander G. Karczmar, Chairman of the Department of Pharmacology for giving me an opportunity to obtain graduate training in this Department. It is also a pleasure to acknowledge the expert assistance of Dr. David Crumrine in obtaining spectroscopic data.

Thanks are extended to Miss JoAnn Ritter and Mrs. Joan Iwankovitsch for their patience in typing the text. I also appreciate the time my colleague Mr. Michael Avram spent in making many helpful suggestions.
VITA

Arun Laxman Jadhav was born on July 15, 1946, in Eran­
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Publications appearing during the graduate career are
as follows:

Davis, J. R., Fareed, J., Jadhav, A. L. 1972. Synthesis of
hydroxylated azopurine with smooth muscle stimulating


ABBREVIATIONS

A = Absorbance.
DOAP = 8,8'-Dioxo-6,6'-azopurine monohydrate.
DOHP = 8,8'-Dioxo-6,6'-hydrazopurine monohydrate.
HP = 6,6'-Bisadenine monohydrate (6,6'-hydrazopurine, HP).
int. area = integrated area.
ir, IR = Infrared.
M = Molar.
NaAP = 6,6'-azopurine hemihydrate disodium salt.
NaDOAXP = 8,8'-Dioxo-6,6'-azoxypurine hexahydrate trisodium salt.
NaAXP = 6,6'-Azoxypurine dihydrate disodium salt.
N'MN = N'-methylnicotinamide.
nmr, NMR = Nuclear magnetic resonance.
O. D. = Optical density.
ppm = parts per million.
Rf = Rate of flow.
uv, UV = Ultraviolet.
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INTRODUCTION

The ongoing search for new biologically-active purines has led to numerous significant compounds such as 6-mercaptapurine, a purine analogue; methotrexate, a folic acid analogue; azothioprine, an immunosuppressive agent, etc.

This study was undertaken to investigate a new class of purines, the oxoazopurines. The parent member of this new class, an Orange Adenine Chromophore, was first synthesized and isolated by Professor Joseph R. Davis in 1956 during the development of a colorimetric analysis of adenine compounds (Davis and Morris, 1963).

Chemical characterization of the members of this new class of compounds was the first essential step in this investigation. These studies included establishing the molecular structure of the Orange Adenine Chromophore (8,8'-Dioxo-6,6'-azopurine monohydrate, abbreviated as DOAP), preparation of its derivatives, along with studying their physico-chemical characteristics by using various spectroscopic techniques. DOAP is an oxidized purine dimer resulting from the oxidation of a brown adenine chromophore (BAC)
with 30 per cent \( \text{H}_2\text{O}_2 \) in the reaction medium. The structures of these chromophores were established in light of the mechanism of this unique reaction. Physical properties such as molar extinction coefficients, characteristic absorptions of functional groups in the infrared region, chemical shifts of various protons in nuclear magnetic resonance spectra, and \( R_f \) values are discussed in light of the structures of these compounds, and compounds whose structures have already been characterized.

From a mechanistic point of view, the reaction through which these various chromophores are formed is unique, since it involves an entirely new method of synthesizing biologically-active oxopurines.

Previous reports from this laboratory showed that DOAP is a non-competitive inhibitor of bovine milk xanthine oxidase in vitro (Davis et al., 1974). This finding has also been confirmed in vivo.

Many purines act as a substrate for the enzyme aldehyde oxidase (aldehyde: \( \text{O}_2 \) oxidoreductase, EC 1.2.3.1). This enzyme is particularly known to oxidize purines at 8-position. Thus, Bradshaw and Baker showed that, while xanthine oxidase oxidized purine initially to hypoxanthine, purine is converted to 8-hydroxypurine by aldehyde oxidase (Bradshaw and Baker, 1960). Azathioprine is oxidized to 8-hy-
droxyazathioprine by aldehyde oxidase from rabbit and human liver (Chalmer et al., 1969). These findings led us to the enzymatic synthesis of 8,8'-dioxo-6,6'-azopurine (DOAP) from 6,6'-azopurine disodium salt by rabbit liver aldehyde oxidase.

Investigation of the interaction between rabbit liver aldehyde oxidase and 6,6'-azopurine disodium salt showed that this compound is a very potent competitive inhibitor of this enzyme.
CHAPTER II

REVIEW OF THE RELATED LITERATURE
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REVIEW OF THE RELATED LITERATURE

A. HISTORY OF THE DEVELOPMENT OF SYNTHETIC METHODS FOR PURINES

Although Emil Fischer introduced the term "purine" in 1898 (Fischer, E., 1898), the history of purine chemistry is 200 years old. It started when the pioneering chemists began characterizing the naturally occurring purines. Nature contains a wide variety of purines and their derivatives. Uric acid was the first purine to be recognized in nature. It was discovered and obtained in a pure state from gallstones by Scheele and Bergmann in 1776 (Scheele, C. W., 1776 and Bergmann, T., 1776). Liebig and Wohler carried out the pioneering studies on uric acid (Liebig, J., 1834 and Wohler, F. and Liebig, J., 1838a). Mitscherlich and Baeyer established its correct empirical formula (Mitscherlich, 1834 and Baeyer, A., 1864). Various structural formulae were proposed based on these results. The structural formula of uric acid proposed by Fittig had two 5-membered rings with one carbonyl and two imidazole -NH groups and these two rings were linked through a 3-membered
ring with one carbonyl group (Fittig, 1877). Medicus proposed a formula in which a 6-membered ring containing two carbonyl groups is attached through -C=O- to a 5-membered ring containing two imidazole (-NH) groups and one carbonyl group, which is the currently accepted formula of uric acid (Medicus, L., 1875). The final proof for the structure of uric acid was furnished by the long and ingenious work of Fischer in 1895. Fischer and his school (Fischer, E., 1907) synthesized many of the purine bases and demonstrated the conversion of uric acid to a number of naturally occurring purines which included xanthine (1,2,3,6-tetrahydro-2,6-dioxopurine), guanine (2-amino-1,6-dihydro-6-oxopurine), theophylline (1,3-dimethylxanthine), theobromide (3,7-dimethylxanthine), and caffeine (1,3,7-trimethylxanthine).

Adenine and Guanine are the two important constituents of RNA and DNA. Kossel discovered and isolated adenine from beef pancreas by acid hydrolysis (Kossel, A., 1885, 1886). He also converted adenine into hypoxanthine by treating adenine with nitrous acid. Although Medicus proposed the correct cyclic structure for hypoxanthine, the final chemical evidence for the structure of adenine was furnished by Fischer (Fischer, E., 1897).

Guanine was found in 1844 in the excreta (guano) of birds (Unger, B., 1846). The cyclic structure for guanine,
postulated by Medicus was accepted when it was shown that guanine can be converted by nitrous acid to xanthine and can also be oxidized to guanidine and parabanic acid by the action of perchloric acid.

Hypoxanthine was discovered by Scherer in 1850 (Scherer, A., 1850). He found the presence of hypoxanthine in the spleen of many animals including human. He named it hypoxanthine because according to his analytical data, hypoxanthine contained one oxygen atom less than xanthine, and the name hypoxanthine indicated to him a substance in a lower oxidation state than xanthine. A comprehensive work on the structure of hypoxanthine was undertaken by Kruger in 1894 (Kruger, M., 1894). At this time Fischer's work on purines had already made important progress. Kruger prepared several alkyl derivatives, the bromo derivatives, and the oxidation products of hypoxanthine such as alloxan and urea.

Marcet discovered xanthine in 1817 (Marcet, 1817). Wohler and Liebig identified the presence of xanthine in a surgically removed urinary stone (Wohler and Liebig, 1838b). Scherer found the presence of xanthine in pancreas gland and showed that xanthine and hypoxanthine can be distinguished by their color reaction with potassium hydroxide in the presence of nitric acid. Xanthine gives a yellowish-red
coloration which changes to purple on warming while hypoxanthine does not react (Scherer, 1859). Although correct formulae for the structures of xanthine and hypoxanthine were given by Medicus, Fischer developed the currently accepted structural formulae (Fischer, E., 1882).

Thus, Fischer's work laid the groundwork for the purine chemistry. Between the years 1882 and 1906, Fischer and his school published nearly fifty papers describing 150 new purine derivatives.

The synthetic routes devised by Fischer involved pyrimidine precursors and hence were limited in scope. This limitation vanished when Traube devised versatile synthesis of a wide spectrum of purines (Traube, W., 1900). Following this pioneering work, purine chemistry flourished and at the turn of the 19th century the literature is profuse with papers predominantly from the German Schools.

In Traube's method of purine synthesis, a 4,5-diaminopyrimidine was condensed with a one carbon carboxylic acid such as formic acid; and the resulting 5-formamido derivative gave the purine when it was heated at 220°C. The first purine to be synthesized in this manner was guanine which was formed directly by heating 2,4,5-triamino-6-hydroxy-pyrimidine with formic acid at 220°C. 9-alkylpurines were also synthesized using this procedure. 2-hydroxy-6,9-di-
methylpurine was obtained by heating 5-amino-2-hydroxy-4-
methylamino-6-methylpyrimidine with formic acid (Johns, 1912a, 1912b). 8-C-adenine was also synthesized by this route using 4-formylmorpholine as a solvent (Clark, 1950).

If a group capable of providing an alternative route of cyclization is present in the 6-position of the pyrimidine ring, then formic acid cannot be used in the synthesis of purines as described above. For example, 4-amino-5-formamidom-6-mercaptopurine can cyclize to give either thiazolepyrimidine or 6-mercaptopurine according to the cyclization conditions (Elion et al., 1956). Some substituted pyrimidines were also found to be unstable when treated with formic acid. For these reasons a substitute for formic acid was sought for a long time.

Purine nucleosides were synthesized using dithioformic acid instead of formic acid (Baddiley et al., 1943a). Aqueous sodium dithioformate or methanolic dithioformic acid reacts readily with 5-amino-4-alkylamino- or 5-amino-4-glycosylaminopyrimidines to give the corresponding 5-thioformamido compounds which cyclize readily at their melting point or in the presence of a base such as hot pyridine or methanolic sodium methoxide to give 9-substituted purines. Adenine, 2-methylthio-9-methyladenine were synthesized using this technique (Baddiley, Lythgoer, Todd, 1943b).
Dithioformic acid does not provide complete cyclization in certain cases. Use of formamide was introduced by Bendich and co-workers to synthesize purines unsubstituted at 8-position (Bendich *et al.*, 1948). Diaminopyrimidines also afford 8-unsubstituted purines when heated in acetic anhydride with ethyl orthoformate, the N-acetyl derivative initially formed being hydrolyzed by aqueous alkali (Montgomery *et al.*, 1958a). A more unusual reagent, 5-triazine, which reacts only with primary amino groups, is capable of converting 4,5-diaminopyrimidines to purines. Theophylline was obtained in this way in a 99 per cent yield (Grundenmann and Kreutzberger, 1955).

Purines carrying a substituent in the 8-position were synthesized using carboxylic acids other than formic acid to cyclize 4,5-diaminopyrimidines. 3,8-dimethylxanthine and 3-methylxanthine-8-carboxylic acid were synthesized by condensing the appropriate pyrimidine with acetic acid and oxalic acid, respectively (Traube, 1923). 1,3,7,8-tetramethylxanthine was prepared by boiling 1,3-dimethyl-4,5-dimethylaminouracil with acetic anhydride (Bredereek *et al.*, 1953).

4,5-dihydroxypyrimidines were condensed with urea in the classical synthesis of uric acid, and Fischer in many of his synthesis condensed 5-amino-4-hydroxypyrimidines
with cyanate. Imidazoles have also been used to synthesize purines (Prasad and Robin, 1957). This type of synthesis received some attention since it was shown that 4-aminimidazole-5-carboxyamide accumulated in E. coli cultures inhibited by sulforamides (Shire et al., 1947). It is now known that the main biosynthetic route to purine is through imidazole derivatives.

Other minor historical approaches to purine synthesis include those from acyclic precursors. Shaw prepared hypoxanthine by heating the substituted formamidomalonamidinecarboxyamide with formamide (Shaw, 1950). Taylor prepared 2,8-dialkylpurines by the reaction of ortho esters in acetic anhydride or acetonitrile with aminomalonamidinecarboxyamide (Taylor, 1957). 6-aminopurines have been prepared by converting silver salt of oximinomalonitrile into 4,6-diamino-5-nitrosopyrimidines by means of amidines which, with formamide and formic acid in the presence of sodium hydrosulfite gives 6-aminopurines (Vogl and Taylor, 1957).

Purines have also been prepared from imidazole precursors. Workers in the field of nucleoside synthesis approached the synthesis of purine glycosides through this route. In alkaline potassium hypobromite solution at 0°C, 4,5-dicarbamoylimidazole undergoes a Hofmann reaction from which xanthine is obtained in good yield (Baxter and Spring,
1944). The nucleoside, xanthosine, having the ribose moiety in the furanose form, was also obtained in the same way (Howard et al., 1949). Sarasin and Wegmann were the first to demonstrate a purine synthesis from an imidazole. They obtained 7-methylxanthine by heating 4-amino-5-carbamoyl-1-methylimidazole with ethyl carbonate (Sarasin and Wegmann, 1924). Various agents like a mixture of formic acid and acetic acid, alkyl esters and trialkyl orthoesters, amides, urea, isothiocyanates, chlorocarbonic esters, diethyl carbonate, carbon disulphide, thiophosgenes, and thioesters have been used for cyclization of imidazoles in the synthesis of various purine derivatives.

B. BIOCHEMICAL EVALUATION OF PURINES: IMPACT ON SYNTHETIC METHODS

The synthetic work has advanced mutually interdependent with the advances in the biochemistry of purines. This relationship between the chemistry and the biochemistry of purines started with the pharmacological evaluation of N-methylated purines especially with respect to their stimulating and diuretic actions of N-methylated purines. This study was then pursued by Scott and Chen and they showed that caffeine and theobromine stimulated the central nervous system, produced convulsions, and also stimulated the
respiratory system of mice. They also showed that caffeine and theobromine inhibited weight gain of young rats when fed for 28 days in 0.3-0.5% of food, stimulated the motor activity of rats in spring-suspended cages, and had a diuretic ratio of 2.45 at the dosages of 0.2 millimole/Kg body weight (Scott and Chen, 1944 and 1946). In another study, testing of 40 xanthine derivatives revealed that these compounds, at minimum effective dosages, reduced pulmonary arterial pressure due to local vasodilatation, increased pulmonary blood flow, increased force of myocardial contraction, and also produced aortic hypotension (Quimby et al., 1958).

The search for substances which can act as antagonists for the constituents of nucleic acids started in the early fifties, and led naturally to the synthesis and study of a large number of purines. Studies on the specificity of the purine requirements of Lactobacillus casei were then undertaken to investigate either the growth promoting or the inhibitory activities of various derivatives of purines. It was hoped that structural modifications of these compounds might lead to the discovery of new inhibitors of the growth-promoting substances, or modify the activity of known antagonists. The activity of 2,6-diaminopurine against leukemia and sarcoma 180 in tissue cultures (Burchenal et al.,
1949 and Biesele et al., 1951) supported the view that such antagonists might be expected to be useful against neoplastic diseases.

Thus, 100 purines representing a variety of structural changes of the physiological purine bases were tested for their effects on the growth of Lactobacillus casei in a variety of media by Elion, Hitchings, and Vanderwerff (Elion et al., 1951). In these studies, a diminution of the growth promoting or inhibitory activity of the parent compound 2,6-diaminopurine resulted when certain structural changes, as the introduction of an 8-hydroxyl or 8-chloro group and methylation of the imidazole nucleus and alkylation of the amino groups were made.

Many programs of purine synthesis were initiated to design antimetabolites capable of interfering with nucleic acid replication in malignant cells, as the role played by nucleic acids in various cell functions became better understood. This resulted in the synthesis and clinical application of 6-mercaptopurine. It was synthesized by Elion and co-workers by treating hypoxanthine with phosphorous pentasulfide under specified conditions in 1952. The studies which began with the investigation of mercaptopyrimidines eventually led to the synthesis of 6-mercaptopurine. Thus, thiouracil and thiothymine were found to be competitive
antagonists of uracil and thymine (Strardskov, 1945).

Clinical trials of Burchenal and co-workers finally proved that 6-mercaptopurine produced good clinical and hematologic remission of acute leukemia in children (Burchenal et al., 1953).

This became the turning point in the history of purine chemistry; various compounds were synthesized in part because they bore a structural resemblance to purines which had shown a particular biological effect. Thus, 6-methylpurine was found to be extremely toxic to mice and rats by Philips and his co-workers (1954), so it was decided to prepare 6-trifluoromethylpurine. Well-known inhibitory activities shown by analogues of adenine as studied by Stock in 1954, prompted the preparation of 6-N-hydroxylaminopurine and azidopurine.

Using the wealth of information available by then, attempts were made to synthesize specific biologically active purine derivatives; thus, purine-6-carboxaldehyde and the related derivatives were synthesized when they were shown to be the oxidation products of carcinolytic and extremely toxic 6-methylpurine (Giner-Sorolla et al., 1959 and Clarke et al., 1954). In 1954, Rutman, Cantarow, and Paschkis reported an increased utilization of labeled uracil in primary induced rat hepatoma as compared with normal liver. Similar
observations were reported with the transplanted Flexner-Jobling carcinoma, and the mechanism of this effect was investigated by Heidelberger and co-workers in 1957. These studies suggested the synthesis of a blocking agent very closely related to uracil. The profound alterations in biological effects brought about by fluorine substitution in various unrelated series of compounds such as the high toxicity of fluoroacetate, the amino acid-inhibiting properties of p-fluorophenylalanine, the increased biological potency of fluorinated steroids and fluorinated azo dye hepatocarcinogens, the nerve toxicity of diisopropylfluorophosphate, and related compounds suggested that the replacement of a hydrogen atom by a fluorine atom in the pyrimidine ring would lead to an analogue of considerable potency. Thus, the activity of a series of fluorinated pyrimidines was screened against several transplanted tumors. In these studies Heidelberger and co-workers found that 5-fluoropyrimidines and particularly 5-fluorouracil possessed marked tumor-inhibiting properties. The activity of 5-fluorouracil was found to be considerably greater than that of 6-mercaptopurine on the Ehrlich ascites carcinoma, Novikoff hepatoma, and L1210 leukemia (Heidelberger et al., 1958). In the continuing search of anticancer agents, this finding prompted the synthesis of 2-fluoropurines (Montgomery and
One aspect of the chemotherapeutic attack on malignant growth is the search for a means of interrupting the production of certain nucleic acids which may be characteristic for the tumor cell. The Watson-Crick hypothesis for DNA postulated a twin helical structure in which certain of the purine and pyrimidine bases in each helix are cross linked by hydrogen bonds. These bonds break momentarily at the time of replication and it was suggested that one possible means of stopping further replication might be to use synthetic polymers for blocking the polynucleotide synthesis on a template. Through this hypothesis emerged the synthesis of polypurines (Lister, 1960). Taylor, Barton, and Paudler (1961) synthesized 9-aminopurines, a class of purine derivatives potentially capable of transformation into interesting types of "pseudo" nucleosides, which was then followed by Montgomery and Temple (1960b) who obtained 9-aminohypoxanthine and related compounds. When Gierer and Mundry in 1958 showed that nitrous acid, when applied to nucleic acids, was a potent mutagen, Shapiro (1964) studied this reaction and isolated an interesting purine derivative, 2-nitropurine. Hydrazinolysis of purine and pyrimidines was carried out by Hayes and Hayes-Baron (1967). 3-Hydroxyxanthine and 3-hydroxyguanine are carcinogens comparable in
potency to the oncogenic arylamines or hydrocarbons; but the isomeric 1-hydroxyxanthine induces inflammations and granulomas which rarely develop into tumors as was shown by Brown et al., in 1965, Teller et al., in 1970, and Sugiura et al., in 1970. This distinct difference in the biological responses prompted the synthesis of 7- and 9-isomers and thus Watson et al. (1972) obtained 9-hydroxy-8-methylhypoxanthine, -xanthine, and -guanine.

The first attempt to diazotize a purine unit at 8-position was that of Gomberg when in 1901 he reported the diazotization of an amino group of 8-aminocaffeine with nitrous acid yielding a very unstable substance which he called "diazocaffeine". This substance was obtained only in solution and was stable only at a low temperature (Gomberg, 1901). Woodhouse in 1950 devised a method for the colorimetric determination of adenine, in which he prepared a diazotized complex of adenine for the microdermination of substituted purines in pure solutions and in hydrolyzates of nucleosides or nucleic acids; but the isolation and characterization of the azo complex was not reported. In another method of colorimetric determination of purines in biological fluids, the purines were reduced by zinc amalgam in dilute acid to a diazotizable amine, and was then diazotized and coupled with N-(1-naphthyl)-ethelenediamine to
give an intensely colored azo dye (Loo and Michael, 1958). Various purine derivatives including adenine, guanine, 6-mercaptopurine, xanthine, and uric acid were successfully diazotized and the formation of azo complexes was achieved; but again, they were not isolated; nor were they characterized.

Successful attempts for isolating and characterizing diazopurines were made by Jones and Robins when they isolated a number of 8-diazopurines. Thus, they prepared 8-diazo adenine, 8-diazo-6-hydroxy-2-methylaminopurine, 8-diazo theophylline, 8-diazohypoxanthine, 8-diazoxanthine, 8-diazoguanine, and numerous other compounds (Jones and Robins, 1960). Feldman (1962), condensed adenine and adenosine through methylene linkage by treating them with formaldehyde. Alfred Giner-Sorolla, while synthesizing N-oxides, first reported the formation of a purine dimer through an azo linkage (Giner-Sorolla, 1969 and 1970). In the light of this history, the synthesis of oxoazopurine dimers in our laboratory is quite novel.

C. ANALYTICAL AND SPECTROSCOPIC STUDIES OF PURINES

Numerous methods have been tried for the detection and quantitative analysis of purines in different biological fluids. The early methods involved a preliminary separa-
tion of uric acid by precipitation of its silver, magnesium, ammonium, cuprous or cupric salts. These methods, however, lacked specificity, and the solubility of these salts usually introduced large errors. The reduction of phosphotungstic acid was introduced and the intensity of the blue colored complex of uric acid with phosphotungstic acid was used as a quantitative measure of uric acid in urine and protein-free filtrates. Hutchings et al. (1947), described a method for the estimation of folic acid by reduction with zinc in acid solution, diazotizing the resulting amine and coupling it with the reagent of Bratton and Marshall. When this reaction was applied to tissue extracts by Glazko and Wolf (1949), it was observed that the presence of adenine introduced an interfering color while various other purine and pyrimidines did not give any colored solutions. Woodhouse (1950), took advantage of this observation and devised a colorimetric method for the detection and microdetermination of adenine. Differential spectroscopy has been used by Kalckar (1947) to differentiate a number of purine compounds by changes in the ultraviolet absorption of these compounds resulting from actions of specific enzymes. A method developed by Davis (Davis and Morris, 1963) for the rapid colorimetric determination of adenine compounds was an important advance since it was
specific, rapid, and could be used for determination of a number of adenine nucleosides and nucleotides without prior hydrolysis of these compounds. Cation-exchange chromatography of purines was developed by Cohn (1949) and has been employed for the isolation and identification of various purines from human urine (Weissman et al., 1957; Wyngaarden et al., 1958; Ayvazian et al., 1965). However, this method required a preliminary purification involving the precipitation of purines as silver salts. Sweetman and Nyhan (1969) simplified this procedure and used their modification for direct quantitation of different oxypurines in urine, serum, and cerebrospinal fluid. In addition, a method for the direct enzymatic determination of xanthine and hypoxanthine was developed by Jorgensen and Poulsen (1955). This method can be used for in vivo as well as in vitro studies of inhibition of xanthine oxidase. A combination of two dimensional thin-layer high-voltage electrophoresis and chromatography for the separation of urinary purines, pyrimidines, and pyrazolopyrimidines was devised by Simmonds (1969a), and was used to study the urinary excretion of these substances in patients treated with allopurinol or oxipurinol (Simmonds, 1969b). This technique was also used to investigate the purine and pyrimidine excretion in psoriasis (Simmonds and Bowyer, 1974).
Partition chromatography on paper was developed by Markham and Smith (1949), which afforded the possibility of the separation of mixture of purine and pyrimidine derivatives in minute amounts. Once a reasonable separation is achieved, the spots are cut out, extracted in a suitable solvent, and are estimated by ultraviolet spectroscopy. Filter paper chromatographic data in ten solvent systems were reported by Fink and Adams in 1966 for several classes of compounds, including amino acids, purines, pyrimidines, sugars, compounds reacting with p-dimethylaminobenzaldehyde, and ultraviolet absorbing or fluorescing compounds.

The absorption spectra of purines are invaluable for identification when they are present in minute amounts. The usually, relatively simple spectra of purines often comprise two main bands, e.g. purine itself absorbs just below 220 and 263 nm in neutral solution. Since the imidazole ring does not have characteristic ultraviolet light absorption, the spectra of analogous purines and pyrimidines show resemblance, for example the 2,6-disubstituted purines resemble the 2,6-disubstituted 4,5-diaminopyrimidines (Cavalieri et al., 1948). The position and intensity of the peaks is often affected by pH since many derivatives are amphoteric. The position of substitution also affects the spectrum, the order of increasing bathochromic shift.
usually being 6 8 2 and of increasing hyperchromic effect 2 6 8 for the longer wavelength band.

J. M. Gulland and his collaborators were the first to use ultraviolet light absorption to elucidate the structure of purine derivatives. A comparison of the spectra of natural and synthetic derivatives of adenine, xanthine, isoguanine, and uric acid showed that the natural glycosides carried the carbohydrate moiety in the 9-position and similar studies in the theophylline series indicated that the synthetic theophylline glycosides were substituted at position 7 (Gulland et al., 1934).

The spectra of purines are often markedly affected by pH. The ionization constants and the order in which ionization occurs in polysubstituted compounds have been studied by spectroscopic methods by Cavalieri (1954), and Bergmann and Dikstein (1955). Ultraviolet spectra have also been used to determine structures of predominant tautomeric forms of hydroxy, mercapto, and aminopurines. For example, Mason (1954) concluded that 2-, 6-, and 8-amino-substituted purines exist predominantly in the amino- and not the imino forms. In the case of hydroxypurines, the evidence from ultraviolet spectra alone is sometimes ambiguous; for example, 6-methoxypurine, hypoxanthine, and 1-methylhypoxanthine have similar ultraviolet absorption spectra, and as-
signments of the predominance of the oxo as opposed to the hydroxy form rests on infrared spectral evidence (Mason, 1957).

Emission (Fluorescence and Phosphorescence) spectra of purines are at longer wavelengths than the excitation (Ultraviolet, Visible, and Infrared) spectra. Purine exhibits a low fluorescence intensity as a neutral molecule, but this increases markedly on either acidification or alcalinization (Borrensen, 1963). Appreciable fluorescence at room temperature can be observed for the cation of adenine. Phosphorescence of purine is readily observed at low temperatures, and detailed emission spectra in glasses of appropriate composition to retain purine in solution at -196°C have been recorded by Drobnik and Augenstein (1966).

Raman spectras of aqueous solutions of purines were studied by Malt in 1966, Lord and Thomas in 1967, and Lautie and Novak in 1968.

Due to the low volatilities and low solubilities in nonpolar solvents, infrared spectroscopy has limited application in purine chemistry. Even then, useful studies in this field have been carried out by studying the spectra of solids in the form of mulls or pastes in nujol or perfluorokerosene and as potassium bromide discs.

The infrared spectrum of an organic compound is its
most characteristic physical property. The various bands in the spectra arise from the normal modes of vibration of a molecule; these modes being dependant upon the molecule's constituent atomic masses, the bond strengths, and the geometry of the molecule as a whole. The infrared spectra, therefore, has a distinctive character, especially in the region below 1500 cm$^{-1}$. The infrared spectra of adenine, guanine, xanthine, and hypoxanthine show some similarities near 1600 cm$^{-1}$, but show distinctive differences in the region of 957 to 935 cm$^{-1}$. Willits and co-workers (1955) have investigated the variability of purine spectra below 1000 cm$^{-1}$ and have also resolved into several components and a doublet near 800 cm$^{-1}$. Friedlander and DiPietro (1962) have examined purine and a number of its derivatives in the region 800 to 650 cm$^{-1}$ using potassium bromide disc technique. Monosubstituted purines, adenine, and hypoxanthine show one absorption below this value, but purine itself doesn't absorb below 791 cm$^{-1}$. Guanine and xanthine, the dissubstituted purines, on the other hand, show three distinct bands. These different spectral features can be used to identify these compounds either separately or in a mixture.

Montgomery and co-workers applied infrared spectroscopy for structural identification of a number of deriva-
atives of purine (Montgomery et al., 1956c, 1957, and 1958). Hydrazino derivatives give spectra similar to those of amino purines. Both show broad NH stretching (NH) bands between 2900 and 2400 cm^{-1}, and the 1750 to 1500 cm^{-1} region can be used in either case to differentiate between 2-monosubstituted, 6-monosubstituted, and 2,6-disubstituted purines (Montgomery and Holum, 1957).

The nature of the predominating tautomeric forms of oxo- and aminopurines has been the center of the long argued speculations in purine chemistry. It has been suggested that 2- and 6-oxopurines exist in the keto form because of the absence of OH stretching bands (OH) near 3400 cm^{-1} in the solid state spectra; but it is very difficult to differentiate between (OH) and (NH) bands in the condensed phase spectra. Deuteration studies of solid guanine in the 3300^{-1} cm region support the oxo-amino structure for these compounds (Angell, 1961).

In the initial attempts to correlate theoretical calculations of charge densities and related physical properties of the ring atoms with the order and position of the resonance peaks, magnetic resonance spectroscopy was used in the studies of purines. The three types of the magnetic resonance spectroscopy i.e., proton magnetic resonance (pmr or nmr), electron spin resonance (esr), and carbon-13 res-
Resonance spectroscopy have recently been used very extensively to elucidate the structures of various purines.

Applications of nuclear magnetic resonance techniques to establish chemical structure of different purines began a decade ago (Jardetzky and Jardetzky, 1962), and significant results were obtained by a number of investigators such as Jardetzky (1960), Kokko et al. (1961), Gatlin (1962), Miles (1963), Katritzky (1963), Becker et al. (1965), Montgomery et al. (1966), Chan et al. (1964), and Coburn et al. (1965).

Freese's hypothesis (1959) that mutagenesis may result from mistaken base pairings in nucleic acids due to the tautomeric behavior of various purines created a great deal of interest in the study of tautomerism and charge distribution in purine derivatives and their nucleosides. Nuclear magnetic resonance spectroscopy has been a very useful technique for these studies. The usefulness of this method, however, depends on the accuracy of the assignments of the peaks arising from the protons attached to the purine nucleus. 2,6-Disubstituted purine derivatives such as xanthine and guanine do not present special problems in assigning the peaks, however, derivatives such as adenine and hypoxanthine contain two ring protons situated in very similar chemical environments and the attempts to assign the
peaks unequivocally have been unsuccessful.

Proton magnetic resonance spectra of purine shows three peaks (Matsuura and Groto, 1963; Schweizer et al., 1964; Bullock and Jardetzky, 1964), assigned to the three ring protons.

![Purine](image)

Using isotope labeling and effect of substituents to distinguish between the two peaks, Jardetzky and Jardetzky (1960) successfully made proton magnetic resonance assignments for purine and some of its derivatives. In their studies, they observed that the effect of substituting the ribofuranosyl group at N-9 in guanine (2-amino-6-hydroxypurine), and in xanthine (2,6-dihydroxypurine) was to shift the peak arising from the proton at C-8 down field by 0.3 δ ppm in both cases. The effect of substituting the ribofuranosyl group at N-9 in hypoxanthine (6-hydroxypurine), and in adenine (6-aminopurine) was to shift the high-field
peak down-field by 0.3 p.p.m. These observations enabled them to assign the high-field peak in the spectra of hypoxanthine and of adenine to the proton at C-8 and the high-field peak in the spectrum of adenosine to the proton at C-2. In inosine, peaks arising from the protons at C-2 and C-8 are superimposed. In the spectrum of purine, since the calculations of the charge densities indicated that the charge densities at C-6 were greater than at C-8 (Mason, 1957), the high-field peak was assigned to C-6, the middle peak to proton at C-8, and the low-field peak to proton at C-2. The following interesting facts were observed during these investigations.

a. Unsubstituted purines have the least shielded protons while substituted purines have the most shielded protons.

b. The shielding is not proportional to the number of negative charges, due to the dissociated groups as seen by comparing the shifts in adenine and 2,6-diaminopurine, both of which have the imidazole proton dissociated, with the shifts in hypoxanthine which bears an additional negative charge due to the dissociation of the H-N-C=O group.

c. There is no difference in shielding of the C-8 proton by an oxy or an amino group.

d. Substitution of a dissociated oxy by an amino
group in the sixth position results in unshielding of the C-8 proton by 0.13 ± 0.03 6 ppm. as indicated from the shift in guanine and 2,6-diaminopurine, as well as xanthine and isoguanine.

Coburn, Thorpe, Montgomery and Hewson (1965a & 1965b) correlated the proton magnetic resonance chemical shifts of substituted purines with their reactivity parameters. Their studies of proton magnetic resonance spectra of 6-substituted and 2,6-disubstituted purines in dimethyl sulfoxide solution showed a linear correlation of the chemical shift of the C-8 proton with the reactivity parameter (Brown and Okamoto, 1958).

The substitution of deuterium for a hydrogen is the most accurate method for making proton magnetic resonance assignments. Using this technique, Matsuura and Goto (1963) reported the first truly unequivocal assignment of the proton magnetic resonance spectrum of purine. They prepared purine-2-d by reduction of 2-chloro-4,5-diaminopyrimidine with deuterium followed by ring closure with formic acid. Purine-6-d was similarly prepared from 6-chloro-4,5-diaminopyrimidine. The spectra of these two compounds were run in acidic, basic and neutral solutions of deuterium oxide. Studying these spectra Matsuura and Goto assigned the low-field peak to the C-6 proton, the middle peak to
the C-2 proton, and the high field peak to the C-8 proton.

Specific base-pairing between purines and pyrimidines was studied by Shoup and co-workers by employing the NMR technique (1966). They reported that the mixtures of simply substituted purines and pyrimidines in DMSO solution interact to form a base-paired complex and the specificity of pairing appeared to be similar to that observed for polymer-polymer or monomer-monomer interactions in aqueous solutions. The purine and pyrimidine bases are paired through hydrogen bonding and it has been shown that hydrogen-bonding results in the shift of the proton signal to down field (Pople, Schneider and Bernstein; 1959). Although the organic solvents used (DMSO and DMF) were known to be hydrogen bond acceptors (Gramstad, 1963), the reference state was designated as the one in which purine and pyrimidine is bounded to solvent and further downfield shifts of the NH and NH₂ protons upon mixture were observed.

Fox (1965) made specific spectral assignments of the C-2 and C-8 proton peaks in the nuclear magnetic resonance spectra of the free bases and hydrochlorides of adenine, 6-benzylaminopurine, 1-benzyladenine, 3-benzyladenine, 7-benzyladenine, 9-benzyladenine, 9-methyladenine, adenosine, 6-dimethylaminopurine, hypoxanthine, 6-chloropurine, and 6-mercaptopurine and these were confirmed by unequivocal
synthesis of the corresponding 8-\text{d} derivatives.

Hruska and co-workers studied the effect of solvents on the NMR spectra of purines (1968). They studied the proton chemical shifts of purine, 6-methylpurine, and 9-ribosylpurine at several concentrations in a series of solvents and binary solvent mixtures with varying proton acceptor strength and dielectric constants. In an aqueous media large variations in the chemical shift of C-8 proton were observed, whereas the C-2 and C-6 protons were essentially solvent independent. This shift was attributed to hydrogen bond formed between C-8 proton and the proton acceptor group of the solvent molecule. They also observed the largest deshielding for C-8 proton and largest shielding for C-2 and C-6 protons; which was again attributed to the hydrogen bonding of C-8 proton and also to the base-stacking interactions.

Reddy, Mandell and Goldstein (1963), synthesized a number of N-acetyl derivatives of imidazoles, purines, and benzimidazoles and studied the nuclear magnetic resonance spectra of these N-acetyl products in chloroform solution. They made several spectral assignments by comparing these spectra with those of the corresponding parent heterocyclic compounds.

Assignments for the proton magnetic resonance spectra
of several purines and their nucleosides were made by exchanging a facile hydrogen for deuterium in the purine nucleus (Bullock and Jardetzky, 1964). They achieved an unambiguous synthesis of 6- and 8-deuteriopurine. Jardetzky also studies the nucleotide interactions of purine and pyrimidine derivatives using NMR spectroscopy (Jardetzky, 1964). His findings indicated that the nitrogenous bases of nucleic acids and the majority of their derivatives tend to form vertical stacks in aqueous solution. The distribution of bases was found to be skewed toward the monomer or the smaller polymer.

It is well known that aromatic character (ability to sustain a ring current) (Davies, 1965) of a ring affects the shielding of ring protons. Thus, Baur and co-workers (1965) observed that the chemical shifts (going upfield) for 4-pyrimidones and 4-pyrimidthiones appeared to be in the order of C-2 proton, C-6 proton and C-8 proton, but were somewhat upfield as compared to the pyrimidines. This was attributed to the greater degree of shielding of corresponding protons in pyrimidones and thiones due to their lesser aromatic character.

Structures of nucleoside antibiotics like Formycin, Formycin B, and Laurusin were studied by Robbins and co-workers (1966).
Tautomerism of purines and pyrimidines is of fundamental biological importance; and has been implicated in the mutation (Watson and Creek, 1953; Freese, 1959). The existence of these bases in tautomeric forms, imine and lactim, instead of the usual amine and lactam ones, may lead to miscouplings of the bases and thus through the perturbation of their sequence and the DNA axis, to a perturbation of the genetic code. The essential energetic and electronic aspects of such miscouplings were investigated by Pullman (1964). The discovery that the crystal of purine (Watson, Sweet and Mars, 1965) involves long chains of molecules, linked together by single hydrogen bonds aroused new interest in phenomenon of tautomerism of purines. Pullman also studied the electronic properties of the purines and suggested that a proton is attached to N-7 of the imidazole ring of purine rather than to the N-9 (Pullman, 1969). Lichtenberg, Bergmann and Neiman investigated the phenomenon of tautomerism of purines by the NMR technique (1971). They found that the chemical shifts of the C-8 protons in xanthines with an unsubstituted imidazole ring and in 7-methylxanthines were very similar; however, the C-8 proton signals for 9-methyl derivatives occur at higher field. From this observation they concluded that the xanthines with an unsubstituted imidazole ring must be essentially
present as the N-7 proton tautomers.

Specific assignments for N-H protons in the purine ring were not made until recently when Twanmoh, Wood, Jr., and Driscoll (1973) reported their work on purine derivatives. Until then no systematic survey of N-H absorptions in purines was available. They observed the N-H absorptions in various purine derivatives as broad signals at low field, usually between 10.7 - 14.2 δ ppm. The signals from some of the derivatives containing two N-H protons appeared as a singlet. These protons were characterized by the proper integral intensities and by deuterium oxide exchange experiments.

During these studies it was observed that the moisture content of the solvent (DMSO-d6) had a significant effect on the N-H absorptions. In 1,3-dimethylxanthine, the N7-H gave a signal at 13.5 δ with Aγ16 Hz in very dry solvent only. Two N-H signals at 10.8 δ and 11.4 δ were observed for 7-methylxanthine in molecular sieve dried solvent, while only one signal was observed in undried DMSO-d6. During the investigation of NMR spectra of a series of 5-azapurines, it was also confirmed that all the N-H signals were seen only in carefully dried solvent (Hirata et al., 1972). From the studies designed to confirm the extent of water content of the solvent that would lead to the non-observa-
tion of the N-H signals, it was concluded that as a general rule, when the moisture content of the DMSO-d$_6$ sample solution reached 0.15%, one of the N-H signals could not be detected. The proton exchange between the sample molecule and traces of water in the solvent is the major cause for non-observation of N-H signals. The N$_7$-H was found to be more susceptible to the exchange and N$_1$-H was the least liable (Twanmoh, 1973).

The positive shielding effect of the neighboring carbonyl groups also has a significant effect on the N-H absorptions (Jackman, 1964). The N$_1$-H signal of 9-methylxanthine is influenced by two adjacent carbonyl groups and hence is shifted in the higher field, while the N$_3$-H is slightly affected by only one carbonyl group and hence is moved downfield. Similar situation was observed for the methyl absorptions frequencies in methyl xanthines by Lichtenberg and his co-workers (Lichtenberg et al., 1971a). The imidazole ring also provides the deshielding ring current of the 5-membered ring to N$_3$-H which is one bond closer than N$_1$-H.

A C=S group is more deshielding than a C=O group (Lichtenberg et al., 1971b; Baur et al., 1965). The N$_1$-H signal of xanthine was observed at 10.8 $\delta$ but that of 2,6-dithioxanthine was observed at 13.3 $\delta$. In 2-thioxanthine and 6-
thioxanthine where $N_1$ is flanked by one $C=O$ and $C=S$ group, the $N_1-H$ signal appeared at an intermediate position of 12.00 $\delta$ in both compounds.

The chemical shifts were also found to be dependent on the concentration of the purine compound in DMSO-$d_6$. For purine and 6-mercaptopurine 10 Hz upfield shift was observed over a concentration range of 12% to 1% (Twanmoh, 1973). Montgomery et al. (1966) also observed a sizable shift of N-H signals when the concentration of the purine compounds was changed.

A change in the temperature also has a predominant effect on N-H line broadening and has been investigated by Roberts (1956) and Twanmoh (1973). A study of a number of factors influencing proton chemical shifts of purine showed that, aside from different electron densities at the various protons, the following perturbing effects are also expected to contribute to the proton shifts in purine: (a) the ring current effect associated with the mobile $\pi$-electron cloud; (b) the magnetic anisotropy of the hetero atoms in the aromatic rings; (c) solvent effects; and (d) solute-solute interactions (Reddy et al., 1963 and Schweizer et al., 1964). It has now been shown that these are the main intrinsic properties of a molecule which are responsible for the main resonance peaks of purine.
D. BIOLOGICAL APPLICATION OF NMR SPECTROSCOPY

Direct application of physicochemical methods to human pathology was the most significant step taken during the early seventies. Workers in the field of purine chemistry and nuclear magnetic resonance spectroscopy have developed nmr as a new tool in cancer research. Damadian and his co-workers have screened human tumors by nmr and came up with very important findings. During the initial investigation, using laboratory animals, it was found that magnetic relaxation parameters $T_1$ and $T_2$ (Spin-lattice Relaxation) were considerably altered in malignant tissues. For Novikoff's hepatoma, a malignant tumor of the liver, the $T_1$ values were more than two and one half times greater than those for the normal liver (Damadian et al., 1973). Thus, nmr spectroscopy has been extensively explored as a possible diagnostic tool in cancer research. This application enables more accurate and specific study of malignant tissues on molecular level; reclassification of tumors according to their chemical nature rather than morphologic appearance; and also early detection and on-line discrimination of biopsy materials within the operating room.

The application of nuclear magnetic resonance to cancer research originated in the Ion Exchange Resin Theory of
biological structures (Damadian, 1971a; Damadian, Goldsmith and Zaner, 1971; Damadian, 1971b). Damadian and co-workers showed that biological selectivity varies with medium osmotic strength and temperature indicating that intracellular hydration and endosolvent (intracellular water) structure are important determinants in selectivity. Using steady-state nuclear magnetic resonance they concluded that changes in the ionic composition of Escherichia coli cells are accompanied by changes in endosolvent structure.

Using these concepts and nmr spectroscopy Damadian et al (1973) studied magnetic relaxation parameters $T_1$ and $T_2$ of malignant tissues from laboratory animals and human tumors. They found that considerable alteration of the magnetic relaxation parameters, $T_1$ and $T_2$ accompanied the malignant transformation. These findings were confirmed by many other laboratories. Hollis et al (1973) found that the magnetic relaxation parameters were longer for tumors and cultured tumor cells as compared with normal tissues. Frey et al (1972) measured the magnetic relaxation parameters and showed that many nonmalignant tissues (spleen, kidney, liver and heart) in mice with a tumor on the hindleg had significantly longer relaxation times than the corresponding tissues in healthy mice. $T_1$ values for animals with a recent (2 days) injection of tumor cells, or with
Salmonella infections, were not significantly different from values for the healthy controls in any tissue tested. Measurements of $T_1$ values of blood serum and liver homogenates, determined at various times after Ehrlich-Lettre ascites and dead ascites cell inoculation of mice showed that the $T_1$ of both blood serum and liver homogenate of animals inoculated with live or dead cancer cells found that cancer development is sufficient to induce $T_1$ changes; however, it is not necessarily the only condition that will induce these modifications; thus emphasizing the need for more work to implement an understanding of conditions that induce changes in $T_1$ (Floyd et al., 1974). Inch et al. (1974) found that large, slow-growing tumors interfere with induction of longer $T_1$ values. They also found that fetal and regenerating liver had longer $T_1$ values than normal liver.

Phosphorus-13 and Potassium-39 are also being explored as the nuclear probe for malignant tissues (Zaner and Damadian, 1975).

E. ALDEHYDE OXIDASE

The presence of aldehyde oxidase in the liver was postulated by Lemberg et al., 1936. Gorden, Green, and Subrahmanyan confirmed this postulation and isolated partially
purified aldehyde oxidase in 1940. Mahler and co-workers further purified it and reported the presence of non-heme iron and molybdenum as well as flavin adenine dinucleotide (Mahler et al., 1954).

Through the ingenious work of Rajagopalan this enzyme has been systematically characterized as the aldehyde oxidase (aldehyde:02 oxidoreductase, EC 1.2.3.1).

It was shown that each mole of rabbit liver aldehyde oxidase contains 2 moles of FAD, approximately 2 moles of coenzyme Q$_{10}$, 8 atoms of non-heme iron, and 2 atoms of molybdenum (Rajagopalan, 1962). He also established that the enzymes called "the quinine oxidase" (Knox, 1946), "a form of xanthine oxidase" (Igo and Mackler, 1960), "purine oxidase" (Chalmers et al., 1969), "methotrexate oxidase" (Johns et al., 1965), and aldehyde oxidase are the same protein. Purified rabbit liver aldehyde oxidase reduces oxygen, ferricyanide, 2,6-dichlorophenolindophenol, methylene blue, phenazine methosulfate, siliconolybdate, and cytochrome c with either aldehydes or N$^1$-methylnicotinamide as substrate. NAD$^+$ does not serve as an electron acceptor either aerobically or anaerobically.

The markedly different effects of several inhibitors on the reduction of various electron acceptors shows the existence of multiple sites of action on this enzyme. Thus,
reduction of 2,6-dichlorophenolindophenol is sensitive only to cyanide and mercuribenzoate, while amytal inhibits reduction of all acceptors other than 2,6-dichlorophenolindophenol. Antimycin A and ascosin inhibit reduction of trinitrobenzenesulfonic acid, nitro blue tetrazolium, oxygen, and cytochrome c; but are without effect on other activities. From these observations Rajagopalan postulated four different sites of action corresponding to the four different carriers; the flavin, iron, molybdenum, and the co-enzyme Q_{10} (Rajagopalan and Handler, 1964a).

Presence of a quaternary nitrogen greatly facilitates the rate of oxidation of heterocyclic substrates. The product of oxidation of N^{1}-methylnicotinamide by aldehyde oxidase is N^{1}-methyl-2-pyridone-5-carboxamide. The absorption spectrum of a standard sample of N-methyl-2-quinolone was identical to that of the enzymatic oxidation product of N-methylquinolinium, indicating that the site of attack by the enzyme is the carbon atom alpha to the ring nitrogen.

Hypoxanthine is oxidized by aldehyde oxidase to xanthine but 7-methylhypoxanthine is a better substrate than hypoxanthine, and is oxidized to 7-methylxanthine. Purine itself is readily oxidized to 8-hydroxypurine (Rajagopalan, 1964b). While 6-methylmercaptopurine is oxidized to 8-hydroxy-6-methylmercaptopurine by liver aldehyde oxidase
(Loo et al., 1967) and azathioprine is also oxidized to 8-hydroxyazathioprine (Chalmers et al., 1969). Thus purines are oxidized predominantly at the C-8 position. The functional arrangement at the enzymatic binding site, and the manner in which this interacts with substrate, determines the ultimate product of enzymic purine oxidation.

Rajagopalan has also postulated that both aldehyde oxidase and xanthine oxidase can be regarded as hydroxylases. Therefore, the mechanism of action of these enzymes can be viewed as the simultaneous removal of a hydride ion from the carbon atom, to be oxidized with replacement by a hydroxyl ion from the medium. These enzymes facilitate such an attack by providing a group which could serve as a Lewis acid. Thus, the quaternary nitrogen adjacent to the carbon atom which is attacked, becomes a strongly positive nitrogen and withdraws electrons from the adjacent carbon atom, thereby facilitating attack by a hydroxyl ion with departure of a hydride ion as shown on the following page.
Me = a group on aldehyde oxidase acting as a Lewis acid.

Molybdenum atom at the functional site of the enzyme has been assigned the role of the Lewis acid.

Aldehyde oxidase and xanthine oxidase are inactivated by methanol, and this inactivation is retarded by excess substrate and competitive inhibitors. These observations suggest that methanol exerts its effect at the substrate-binding site. Nitrate reductase, a molybdenum containing enzyme is also inhibited by methanol. Thus, the inactivation of all these three molybdenum-containing enzymes by methanol suggests that the molibdenum component may be the site of methanol action. Another observation which gives strength to the above hypothesis is that methanol-metal complexes have been reported to be formed during non-enzy-
matic oxidation-reduction reactions (Fraser et al., 1959).

The non-heme Fe\(^{3+}\) ions, eventhough Lewis acids, do not participate in this oxidation-reduction reaction because they are the components of the electron transport sequence of these enzymes which are far from the active site. Electron paramagnetic spectrometric studies have indicated that the molybdenum, Mo\(^{6+}\) is reduced to the Mo\(^{5+}\) in the presence of the substrate (Handler et al., 1964). This hypothesis was finally confirmed in 1968 when the electron paramagnetic resonance data proved the intramolecular passage of electrons from substrate via molybdenum to flavin and iron complex. These studies showed that at equilibrium, the molybdenum component was found to be reduced at least as fast as, or faster than, the other carriers (Rajagopalan et al., 1968a and 1968b). Similar studies on xanthine oxidase have also established that the molybdenum centers of xanthine oxidase are the sites where the active hydroxylation of substrate is accomplished. Xanthine oxidase activity is abolished following the production of molybdenum deficiency in rats by feeding tungsten (Johnson et al., 1974).

Aldehyde oxidase have also been detected in potato juices and was found to transfer hydrogen to several redox dyes including 2,6-dichlorophenolindophenol (Bach, 1913). Two isoenzymes of aldehyde oxidase have been isolated by
Rothe from potato tubers (Solanum tuberosum). They are similar to the enzyme from rabbit liver in their pH optima and electron acceptors. The difference between these enzymes from two sources is that the enzymes from potato tuber cannot use ferricyanide and 2,6-dichlorophenolindophenol as an electron acceptor; nor can they oxidize quinine alkaloids, but oxidize aliphatic aldehydes preferentially (Rothe, 1974).

The presence of the coenzyme Q₁₀ in aldehyde oxidase has been debated. Rajagopalan (1962), Weidenbach and Folkers (1971), and Weidenbach (1973) have shown the presence of the coenzyme Q₁₀, while Felsted et al. (1973) could not detect the coenzyme Q₁₀ in hog or rabbit liver preparations, and Romeo and co-workers showed its absence in rabbit liver preparation (Romeo et al., 1974).

Johns isolated aldehyde oxidase from human liver. This enzyme differed from the rabbit liver aldehyde oxidase with respect to the aerobic hydroxylation of phenazine methosulfate and N¹-methylnicotinamide chloride. The aerobic hydroxylation of these two substances is inhibited only by agents with affinity for the substrate-binding site such as cyanide and N-alkylphenothiazines, and not by the agents which inhibit the "internal electron transport chain" of the enzyme such as menadione and diethylstilbestrol.
The inhibition of oxidation was affected by charged and uncharged substrates (Johns, 1967).

Methotrexate and its dialkyl and dichloro derivatives have been shown to be very potent substrates for aldehyde oxidase with $K_m$ values significantly below 0.01 nM (Johns et al., 1973).

Oral ingestion of the immunosuppressive agent azathioprine is followed by rapid urinary excretion of 6-thiouric acid (Chalmers et al., 1969), which is also the main urinary metabolite of azathioprine in a number of mammalian species (Elion, Callahan, Bieber, Hitchings and Rundles, 1961). It has also been noted that 6-methylmercaptopurine was oxidized to 8-hydroxy-6-methylmercaptopurine by liver aldehyde oxidase (Loo et al., 1967). These observations suggested that azathioprine could be metabolized in vivo through a pathway involving aldehyde oxidase since azathioprine is also a 6-S-substituted derivative of 6-mercaptopurine and, therefore, a potential substrate for the enzyme. The successive steps of 8-hydroxylation and thiolysis would convert azathioprine into 8-hydroxy-6-mercaptopurine which is known to be readily oxidized to 6-thiouric acid by xanthine oxidase (Bergmann and Ungar, 1960).

Chalmers and his co-workers proposed a pathway for the metabolism of azathioprine, i.e., azathioprine $\rightarrow$ 8-hy-
droxyazathioprine $\rightarrow$ 8-hydroxy-6-mercaptopurine $\rightarrow$ 6-thiouric acid (Chalmers et al., 1969). This pathway bypasses 6-mercaptopurine. This alternative pathway of azathioprine degradation is of potential physiological significance. Inhibitors of aldehyde oxidase and xanthine oxidase, either endogenous or therapeutically administered, may change the patterns of conversion of azathioprine into active and inactive metabolites. This becomes a significant factor for development of treatment schedules that will give the most favorable balance between therapeutic effect and drug toxicity.

Tsukada and co-workers postulated that aldehyde oxidase oxidizes formycin B to oxoformycin B (Tsukada et al., 1969). The conclusive evidence for this postulation was offered by Sheen and co-workers. Their studies demonstrated that hepatic aldehyde oxidase oxidizes formycin B, an inosine analogue, to oxoformycin B, a xanthosine analogue (Sheen et al., 1970). These findings were very interesting since formycins A and B closely resemble the natural nucleosides, adenosine and inosine, in their ability to interact with various enzymes of nucleoside metabolism (Sheen et al., 1968; Ward et al., 1969; Henderson et al., 1967). However, inosine nor adenosine inhibited enzyme aldehyde oxidase nor did they interact with xanthine oxidase. Sheen's studies
showed that formycin B, a structural analogue of inosine, is an excellent substrate for liver aldehyde oxidase, and both formycins A and B are competitive inhibitors of milk xanthine oxidase. These observations cannot be attributed wholly to the stereochemical differences between these molecules and a more satisfying explanation of these discrepancies is still being sought.

One of the physiological roles attributed to aldehyde oxidase is the oxidation of \( N^1 \)-methylnicotinamide. The products of oxidation absorb light at 290 nm and this principle is used for the assay of aldehyde oxidase. There are two products of the oxidation of \( N^1 \)-methylnicotinamide catalyzed by aldehyde oxidase, \( N^1 \)-methyl-4-pyridone-3-carboxamide (4-pyridone) and \( N^1 \)-methyl-2-pyridone-5-carboxamide (2-pyridone), Huff et al. (1967); and Felsted et al. (1967). A second physiological role has also been ascribed to aldehyde oxidase, namely, the oxidation of pyridoxal to 4-pyridoxic acid (Shwartz and Kjeldgaard, 1951).

Pyridoxic acid has long been recognized as the principle excretory product of vitamin B\(_6\) in man (Huff and Pelzweig, 1944; Robinowitz and Snell, 1949; Reddy et al., 1958). These contentions were conclusively proven by Stanulovic and Chaykin and it was established that aldehyde oxidase is the physiological catalyst of pyridoxal oxidation in vivo.
(Stanulovic and Chaykin, 1971a and 1971b).

F. ALDEHYDE OXIDASE AND XANTHINE OXIDASE:
A COMPARISON

The possibility of having a common origin and the metabolic functions of aldehyde oxidase and xanthine oxidase is a continuing conundrum. The similarity in the properties of the two enzymes rather than their differences are most striking. A common evolutionary origin for both of the enzymes has been speculated. Another fascinating concept of this hypothesis is that, aldehyde oxidase is viewed as the enzyme in transition (Stanulovic, 1971b). Both enzymes show preference for the substituted pyrimidines and purines. Significant differences among the specificities were the effects of the number and position of the ring substituents. While only xanthine oxidase rapidly oxidizes C-disubstituted derivatives of different heterocycles, both enzymes oxidized a variety of unsubstituted and C-monosubstituted heterocycles. N-substitution in many cases dramatically increased the substrate activity with aldehyde oxidase (Krenitsky et al., 1972).

In general, both enzymes are capable of hydroxylating substituted pyrimidines and purines which might be endogenously produced or ingested from exogenous sources by the
organism. Mammals have a DPNH-dependent aldehyde oxidase which has a much higher affinity for aldehydes than does aldehyde oxidase (Maxwell et al., 1961; Deitrich et al., 1962), therefore, the oxidation of aldehydes cannot be the principle physiological function of these enzymes.

Unlike xanthine oxidase, aldehyde oxidase did not use NAD$^+$ as an electron acceptor (Krenitsky, 1973). Another noticeable difference is that aldehyde oxidase converts vanillin to vanillic acid while xanthine oxidase does not (Wurzinger and Hartenstein, 1974).

Xanthine oxidase and aldehyde oxidase were found in higher concentrations in mammalian liver and small intestine. The levels of aldehyde oxidase in guinea pigs and rabbits were found to be higher than those in mice, rats, cats, dogs, monkeys and humans. Aldehyde oxidase is also found in many non-mammalian vertebrates (Hartenstein, 1973 and Lakshmanan et al., 1964), and in the primitive invertebrates like molluscs, arthropods and crustacea (Wurzinger and Hartenstein, 1974).

In the studies of phylogenetic relationship between aldehyde oxidase, xanthine oxidase, xanthine dehydrogenase and peroxidase using 79 animal species, Wurzinger and Hartenstein concluded that aldehyde oxidase and xanthine oxidase was not measurable in phylogenetically primitive spe-
cies. They showed that the order of phylogenetic primitivity is aldehyde oxidase → xanthine dehydrogenase → xanthine oxidase.

Aldehyde oxidase thus appears rather widely distributed in the animal kingdom, which suggests that the primary metabolic function of aldehyde oxidase is of a fundamental nature rather than a highly specialized one.
CHAPTER III

MATERIALS AND METHODS
CHAPTER III

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A. SYNTHESIS OF
8,8'-DIOXO-6,6'-AZOPURINE (DOAP)

A 6.0 mg/ml solution of adenine was prepared employing a special technique described below; the low solubility of adenine in 1 N \( \text{H}_2\text{SO}_4 \) made it necessary to design this technique. 15.00 g of adenine, obtained from Aldrich Chemical Company, was weighed out in a weighing boat, and small portions were added to a glass homogenizer tube containing 10.0 ml of 1 N \( \text{H}_2\text{SO}_4 \). It was homogenized with a teflon pestle six times up and down. This suspension was then poured into a large glass reaction vessel containing 1 liter of 1 N \( \text{H}_2\text{SO}_4 \) which was constantly being stirred with a magnetic stirrer. In this way, the 15.00 g of adenine was dissolved into a total of 2.5 liters of 1 N \( \text{H}_2\text{SO}_4 \). This solution was then filtered through Whatman No. 3 filter paper. To this filtrate, 120 ml of a 15 mg/ml solution of KBr in distilled H\(_2\)O was added. The mixture was then stirred with a magnetic stirrer for 1 hr. The solution remained clear and colorless after 1 hr; 800 ml of 1 N KMnO\(_4\) was then
added, and the reaction mixture was a brownish-purple suspension. After 1 hr of stirring, a total of 50 ml of 30% \( \text{H}_2\text{O}_2 \) was added, slowly, in 10 ml portions, with stirring. The reaction mixture gradually decolorized, resulting in a bright orange precipitate. It was then stirred for 1 hr at room temperature, and was allowed to stand in the dark for 16 hr at 4°C. The resulting orange precipitate of DOAP was collected by centrifugation at 600 x g for 15 min, employing four 150-ml capacity glass centrifuge bottles. The DOAP solid present in each of the four centrifuge bottles was successively washed 12 times with 150 ml portions of 4 N \( \text{H}_2\text{SO}_4 \) and collected by centrifugation at 600 x g for 15 min until no absorbancy at 327 nm could be detected in the decanted acid wash. This was followed by six successive washes with 150 ml portions of distilled \( \text{H}_2\text{O} \). During these washes it was necessary to centrifuge at 600 x g for 1 hr or longer because of the microcrystalline nature of DOAP. The DOAP microcrystalline precipitate in each bottle was then washed with four 150 ml portions of acetone and collected by centrifugation at 600 x g for 1 hr. The final washed DOAP solid was combined from all four centrifuge bottles as an acetone suspension, allowed to evaporate to dryness on a large watch glass at room temperature, and then placed in a drying oven at atmospheric pressure for
15 hr at 75°C. The dried bright orange microcrystalline product was then transferred into a clean dry glass vial, placed in a brown plastic container, and stored in a vacuum dessicator (Chart 1).

B. SYNTHESIS OF 8,8'-DIOXO-6,6'-HYDRAZOPURINE (DOHP)

To an Erlenmeyer flask containing 10 ml of 1 N HCl, 1.5 g of DOAP and 3.0 g of solid SnCl₂·2H₂O were added and the flask was heated in a water bath at 70°C for 20 min, using an air condensor. The resulting yellow solution was diluted with equal volume of distilled H₂O (final normality of HCl 0.5 N), was allowed to stand at room temperature for 3 hr, and in an icebath of 16 hr. The yellow-green precipitate observed after 16 hr was collected by centrifugation at 600 x g for 10 min, washed twice with 10 ml of 0.5 N HCl, and distilled H₂O. It was then crystallized from 700 ml of 0.5 N HCl, heated at 70°C in a water bath for 20 min, and recrystallized by repeating the same procedure. The yellow-green crystals obtained after recrystallization were washed six times with 150 ml of distilled H₂O to remove any acid; the acid-free crystals were washed six times with 150 ml portions of acetone to remove water, transferred to a clean watch glass with a few mls of acetone,
allowed to evaporate to dryness, and finally dried in a
drying oven at 75°C for 16 hr to obtain tan-yellow crystals
(Chart 1).

C. SYNTHESIS OF

8,8'-DIOXO-6,6'-AZOXYPURINE (NaDOAXP)

Method a (from DOHP): 0.60 g of DOHP were dissolved in
125 ml of 0.5 N NaOH by homogenization using a teflon pes-
tle, and air was bubbled through it for 2 hr. The brick
red product was allowed to precipitate slowly over 16 hr by
letting it sit in an icebath at 4°C, and was then collected
by centrifugation at 600 x g for 10 min. It was washed
three times each with 200 ml portions of 90% EtOH, absolute
EtOH, and acetone, in that order; it was transferred to a
clean watch glass with a few ml of acetone, allowed to air
dry to complete dryness, dried in a drying oven at 50°C for
1 hr, and then in a dessicator until constant weight was
reached.

Method b (from DOAP): 0.1 g of DOAP was dissolved by
taking small portions each time and homogenizing in 10 ml
portions of 0.005 N NaOH, requiring a total of 125 ml of
0.005 N NaOH to get a red solution. It was then filtered
through Whatman qualitative filter paper to remove the few
particles from it, and the clear red solution was placed in
an icebath at 4°C for 60 hr which resulted in a cherry red precipitate. The precipitate was collected by centrifugation at 600 x g for 10 min. It was washed with 200 ml portions of 90% EtOH, absolute EtOH, and acetone. The precipitate was then transferred to a clean watch glass with a few ml of acetone, allowed to evaporate to dryness and finally dried in a drying oven at 50°C for 1 hr, and then in a dessicator until constant weight was obtained.

D. ELEMENTAL ANALYSES

All the elemental analyses were carried out by Schwarzkopf Microanalytical Laboratories, Woodside, New York. The analyses were carried out under two different conditions for each compound in order to determine the number of moles of water of hydration for each compound. The samples were analyzed as they were received, without any prior drying. The values obtained from this analysis gave the percentages of all the elements present in the sample. The samples were then dried at 100°C under a stream of nitrogen for 1 hr to remove all of the water associated with the molecule and were again analyzed for the elements they contained. Subsequent drying of the samples for 1 hr at 150 and 200°C did not result in any further change in the analyses. Empirical formulae from these two analyses were obtained, and
the number of moles of water of hydration were obtained by subtracting the empirical formula of the dried (anhydrous) compound from that of the non-dried (hydrous) compound.

The water of hydration was also determined directly by measuring the sample weight loss and the vaporized water absorbed in magnesium perchlorate and the increase in weight of the drying agent.

E. UV, IR, AND NMR SPECTRAL STUDIES

A Beckman DB-G recording spectrophotometer was used to obtain the uv spectra of all the compounds. To determine the ultraviolet absorption characteristics and to calculate the molar extinction coefficients, a pre-weighed sample of a compound was dissolved in appropriate solvent by homogenization in a glass tube with a teflon pestle. A 3.00 ml sample of the solution was then placed in a silica cuvette with 1.0 cm path length, and read against an appropriate blank. The concentrations of the solutions were so adjusted that the optical densities would be between 0.3 and 0.9 because of the higher sensitivity of the spectrophotometer in this region.

A similar procedure was used to investigate the stabilities and absorption characteristic of the compounds utilized for various experimental purposes. To study the
biochemical and pharmacological properties of 8,8'-dioxo-6,6'-azopurine (DOAP) and a structurally similar compound, 6,6'-azopurine disodium salt, abbreviated as NaAP, the effect of pH on the stability of these compounds in 0.05 M potassium phosphate buffer at room temperature was investigated. 6,6'-Azopurine disodium salt (NaAP) was obtained as a generous gift from Dr. Alfred Giner-Sorolla of the Sloan-Kettering Institute for Cancer Research, New York, New York. Potassium phosphate buffer solutions (0.05 M) of pH 6.2, 6.6, 7.0, 7.4, 7.8 and 8.2 were prepared. 2.85 ml of the buffer at the desired pH was placed in a silica cuvette and 150 μl of 500 μg/ml solution of DOAP in 0.005 N NaOH was added to obtain a final concentration of 8.0 x 10^{-5} M; this solution was scanned vs 3.0 ml buffer of the same pH. Similar scans were obtained at all the pHs. From these scans it was inferred that a change in O.D. at 455 nm can be used to monitor the stability of DOAP in 0.05 M potassium phosphate buffer at these pHs.

Thus, in another series of experiments, 2.85 ml of 0.05 M potassium phosphate at the desired pH was placed in a silica cuvette and 150 μl of 500 μg/ml solution of DOAP in 0.005 N NaOH was monitored for 60 min using a chart speed of 0.1 inch/min. Thus, in these two sets of experiments, it was confirmed that DOAP solutions in 0.05 M po-
tassium phosphate buffer were stable up to 3.0 minutes at pH 7.4 and 7.8. The solution at pH 8.2 was stable up to 10 min.

Similar sets of experiments were performed using NaAP and it was confirmed that NaAP solutions in 0.05 M potassium phosphate were stable up to 3.0 minutes at pH 7.0. The solutions at pH 7.0, 7.4, 7.8, and 8.2 were stable up to 10 min.

The infrared spectra were obtained on a Beckman Acculab 1 grating infrared spectrophotometer. It was standardized to be within 15 cm\(^{-1}\) between 4000 and 2000 cm\(^{-1}\), and 8 cm\(^{-1}\) between 2000 and 600 cm\(^{-1}\). The recorded spectra of the commercially available standard compounds such as adenine, isoguanine sulfate, 2,8-dioxa adenine, xanthine, hypoxanthine, and uric acid were in good agreement with their reported spectra. The spectral assignments were made by using several standard compilations such as the Aldrich Infrared collection, Katritzky (1963 and 1971), and the catalog of Infrared Spectrograms by Sadtler Research Laboratories.

The nmr spectra were determined in DMSO-d\(_6\) solvent at 60 MHz with a Varian A-60A spectrophotometer. Tetramethylsilane (TMS) was used as an internal standard; and the chemical shift values are expressed in \(\delta\) ppm relative to
the TMS reference. All of the compounds were dissolved to a concentration of approximately 20 mg/ml by homogenizing in DMSO-d₆ using a teflon pestle and a glass tube. The spectra were run at the probe temperature. The NH protons were identified by D₂O-exchange.

During this investigation it was observed that the moisture content of the DMSO-d₆ solvent was a very critical factor influencing particularly labile protons like the N-H protons. In order to detect the N-H protons, the solvent had to be free from moisture. A fresh bottle of DMSO-d₆, obtained from Bio-Rad Laboratories, Richmond, California, was used each time in order to eliminate the picking up of moisture; its moisture content was determined by scanning the DMSO-d₆ alone. Only the solvent containing a minimum amount of moisture was used to dissolve the compounds investigated.

The deuterium oxide used in the D₂O-exchange studies was 99.87% mole D₂O and was also obtained from Bio-Rad Laboratories. 25 µl of the D₂O was added to the nmr tube containing the solution of a compound, shaken well and scanned.

F. PAPER CHROMATOGRAPHY

After investigating a number of solvent systems, four of the following were found to be excellent for the paper
chromatography of oxoazopurines. They are:

1. abs. EtOH: Pyridine: Distilled water
   67: 20 : 13

2. n-Propanol: Conc. NH₄OH: Distilled water
   60: 30 : 10

3. n-Butanol: Glacial acetic acid: Distilled water
   80: 20 : 20

4. abs. EtOH: 0.5 M Ammonium acetate
   5: 2

500-600 ml of each solvent system were placed in a tightly closed tall glass jar and the system was allowed to equilibrate at room temperature for 2 hr. The compounds were dissolved in appropriate solvents and were spotted so that the final concentration of each spot would be 4 μg. Whatman chromatographic paper grade 1 was cut into strips of 8" x 17 3/8" and the compounds were spotted on a base line drawn 2" from the bottom of the strip. The solvent was then allowed to run for 16-20 hr. The chromatograms were dried at room temperature and then in an oven at 60°C for 10 min. The spots were marked visually and also under 262 nm and 360 nm ultraviolet lamps.
G. COLUMN CHROMATOGRAPHIC SEPARATION OF PRODUCTS FORMED DURING THE SYNTHESIS OF DOAP

The synthesis of DOAP was carried out as described in Section A. The resulting reaction mixture was centrifuged at 600 x g for 15 min and a clear yellow supernatant was obtained. The supernatant was adjusted to 0.5 N H₂SO₄ by adding equal volume of distilled H₂O to a final volume of 636 ml.

AG 50W-X8 (H⁺ form), 200-400 mesh cation exchange resin, purchased from Bio-Rad Laboratories, Richmond, California, was used for the column chromatography. This resin was regenerated by taking the following steps:

1. In a brown bottle, 1 lb of the resin was stirred with 5 liters of distilled H₂O, allowed to sit for 20 min and then decanted. This was repeated a second time.

2. The resin was then stirred with 1 liter of 5 N HCl, mixed for 2 hr and decanted; this was repeated once.

3. Next the resin was washed with 5 liters of distilled H₂O, allowed to sit for 20 min decanted and repeated 4 times to neutrality.

4. It was then stirred with 5 liters of 1 N NaOH, al-
lowed to sit for 20 min and decanted.

5. The resin was then washed with 5 liters of distilled H₂O, allowed to sit for 20 min decanted and repeated 4 times to neutrality.

6. It was next stirred with 5 liters of 1 N HCl, allowed to sit for 20 min, and decanted.

7. Next, the resin was washed with 5 liters of distilled H₂O, allowed to sit for 20 min, decanted and repeated to neutrality.

8. Subsequently, it was stirred with 5 liters of 1 N NaOH, allowed to sit for 20 min and decanted.

9. The resin was then washed with 5 liters of distilled H₂O, allowed to sit for 20 min, decanted and repeated to neutrality.

10. It was next stirred with 5 liters of 1 N HCl, allowed to sit for 20 min, and then decanted.

11. Finally the resin was washed with 5 liters of distilled H₂O, allowed to sit for 20 min, decanted and repeated to neutrality.

The resin is stored in a brown bottle filled with distilled H₂O. The resin was washed twice with distilled H₂O prior to use on the day the resin was used.

The column was fitted with a glass wool plug at one end and was filled with distilled H₂O to 1/3 of its length.
A slurry of the resin in distilled H₂O was then poured into the column and 5 psi of air pressure was applied. By repeating this procedure the column was filled with resin. The resin in the column was kept submerged under distilled H₂O so that air would not get into the resin. The column was then washed by passing 400 ml distilled H₂O through it using 5 psi air pressure. 636 ml of the sample was then applied to the column and 640 ml of neutral eluent was collected in 25 ml portions.

The column was washed again by passing distilled H₂O through it until the O.D. at 260, 280, 305, 330 and 420 nm was zero. The column was then connected to a gradient of HCl with a reservoir containing 4500 ml of 5 N HCl and a mixing chamber containing 1000 ml of distilled H₂O. The flow rate was adjusted by changing the air pressure to 2 psi which eluted 6.0 ml/tube/min; or 5 drops/10.5 sec. Thus 810 tubes were collected and the O.D. at 260, 280, 305, 330 and 420 nm were recorded using a Beckman DB-G spectrophotometer.

A graphical plot of O.D. vs eluted fraction showed the presence of 10 different products in the reaction mixture. The peak tubes were pooled together and were flash evaporated at 50°C for 30 min. The solid product obtained after flash evaporation was transferred into a test tube with 5
ml of distilled H₂O; and was washed with 10-ml portions of distilled H₂O for 6 times, followed by 10-ml portions of acetone for 4 times. It was then air dried and the dried solid was stored in a dessicator.

The products were identified by comparing their uv and ir absorptions with those of the authentic compounds. Hypoxanthine and adenine were found to be the major products in the final reaction mixture. The other seven compounds are yet to be identified.

H. COLUMN CHROMATOGRAPHY OF OXOPURINES

For column chromatography, uric acid xanthine, hypoxanthine, and adenine were obtained from Sigma Chemical Co., St. Louis, Missouri. 2-Hydroxyadenine and 2,8-dihydroxyadenine were purchased from Aldrich Chemical co., Milwaukee, Wisconsin. 8-Hydroxyadenine sulfate was a generous gift from Dr. Bernard Regan of Baxter Laboratories, Morton Grove, Illinois.

The cation exchange resin used was AG 50W-X8 (H⁺ form), 200-400 mesh, purchased from Bio-Rad Laboratories, Richmond, California. One pound of resin was washed by suspension, settling, and decanting with two volumes of the following solutions, using 5 liters of each for every washing,
distilled water, 5 N HCl; distilled water, 1 N NaOH; distilled water, 1 N HCl; distilled water, 1 N NaOH; distilled water, 1 N HCl; distilled water. The resin was washed between each acid and alkali wash until the pH of wash water is the same as that of distilled water. Resin generated this way can be stored in a brown bottle. On the day of its use, it was washed twice with distilled water (the procedure is described in detail in Section G).

The resin was packed as a distilled water suspension in a 40 x 0.9 cm pyrex glass column with 5 psi air pressure with a glass wool plug at the bottom and 50 ml of 0.5 N HCl was passed through it. The sample was applied using 2 psi air pressure. After application of the sample, 1 ml of distilled water was passed through the column and was then connected to gradient system having a reservoir containing 2000 ml of 5 N HCl and a mixing chamber with 500 ml of distilled water. The running pressure was adjusted to give a constant flow rate of 1 tube containing 3 ml of eluent per 5 min (0.6 psi); this was also equal to 2 drops dripping from the column per 9.5-10 sec. A total of 350 tubes were collected on an automatic fraction collector. Optical densities of these tubes were read at 208, 215, 250, 260, 280, and 305 nm, using a Beckman DB-G recording spectrophotometer.
I. REDUCTION OF
8,8'-DIOXO-6,6'-AZOPURINE (DOAP) WITH
SODIUM HYDROSULFITE

25.0 mg of 8,8'-dioxo-6,6'-azopurine were dissolved in 150 ml of 1 N NaOH by homogenizing it with a teflon pestle, which resulted in a dark red solution. It was heated at 65°C for 15 min and 1.0 gm of sodium hydrosulfite (Na₂S₂O₄) was added while stirring the solution with a magnetic stirrer. The original dark red solution turned yellow upon addition of sodium hydrosulfite. This clear yellow solution was then refluxed in a water bath at 80°C for 1 hr, and cooled to room temperature. A 4 ml aliquot of the clear yellow solution was neutralized to pH 7.0 with 1 N HCl, adjusted to equivalent of 0.5 N HCl, and was then applied to a 21.0 X 0.9 cm column of AG 50W-X8 200-400 mesh resin in H⁺ form, using 2 psi air pressure. The column was then eluted with a gradient of HCl equipped with a reservoir containing 2000 ml of 5 N HCl and a mixing chamber containing 500 ml of distilled water. The running pressure was adjusted to 0.6 psi which gave a constant flow rate of 0.6 ml/min. Fractions of 3 ml were collected every 5 min for a total effluent volume of 1050 ml. The optical density of each tube was determined at 208, 215, 250, 260, 280, and
305 nm, using a Beckman DB-G spectrophotometer. The uv characteristics of tubes 123-130 exactly matched those of an authentic sample of 8-hydroxyadenine sulfate. The effluent from these tubes was collected and dried in a 25 ml tube placing it in a water bath at 40°C and passing gaseous nitrogen through it to obtain a yellow solid. Uv spectral characteristics of the yellow solid exactly matched those of the authentic 8-hydroxyadenine sulfate in 1 N HCl, 1 N NaOH and dimethylsulfoxide.

J. STUDIES OF THE IN VIVO INHIBITION OF XANTHINE OXIDASE AND THE METABOLISM OF DOAP

A previous report from this laboratory has shown that DOAP is an extremely potent inhibitor of bovine milk xanthine oxidase in vitro (Davis, et al., 1974). This finding was confirmed in vivo by studying the urinary excretion of xanthine, hypoxanthine, uric acid, and creatinine in male Sprague-Dawley rats between the ages of 63 and 110 days, weighing 235 to 380 gm, injected subcutaneously with a single dose of 25 mg/kg body weight of DOAP dissolved in 0.01 N NaOH, behind the nape of the neck. The rats were placed in Holdge Metabolic Cages. The collected urine was used for determination of xanthine, hypoxanthine, uric acid and
creatinine. Attempts were also made to isolate the products of the metabolic degradation of DOAP.

a. Collection of Rat Urine

A rat was placed in a Holdge Metabolic Cage, fed mushed Purina Lab Chow (a thick paste of ground Purina Lab Chow obtained by mixing it with a small amount of water), and given tap water to drink. The outlet for urine and feces was plugged with a glass wool to separate the urine from feces and food particles. The urine would then flow through the spout into a funnel and then into a test tube placed on an automatic fraction collector. The lower conical part of the Holdge Metabolic Cage was cleaned thoroughly every 2 hr to eliminate prolonged contact of the urine with the feces.

The automatic fraction collector was set to change the tube every 2 hr; thus, the urine for the desired period was collected in 2-hr fractions. One drop of 5 N HCl was then added to each tube to change the pH of the urine to 2.0. This was necessary to precipitate the dissolved DOAP in the urine. The amount of 5 N HCl thus added to all tubes was recorded and then subtracted from the final volume of all the tubes to obtain the true volume of the urine collected. After adding the drop of 5 N HCl, each tube was centrifuged
at 600 x g for 10 min. The urine collected after the subcutaneous injection of DOAP gave an orange precipitate and a clear yellow supernatant.

The orange precipitate was then analyzed for the amount of DOAP and the clear yellow supernatant was analyzed for xanthine, hypoxanthine, uric acid, and creatinine and also any possible metabolites of DOAP.

b. Analysis of DOAP Excreted in Rat Urine After a Single Subcutaneous Injection.

The orange precipitate obtained as described above was washed 3 times with distilled H₂O. The uv scans of these water washes did not show presence of any uv light absorbing material. Then the washed orange precipitate was dissolved in 3.00 ml of 0.1 N KOH. There was no insoluble material left in the tubes after dissolving the orange precipitate in 0.1 N KOH. The O.D. of this solution was then read at 513 nm and the amount of DOAP calculated from a calibration curve. The calibration curve was prepared by measuring the O.D. at 513 nm of 2.5, 5.0, 10.0, 15.0, 20.0, and 22.0 µg/ml solutions DOAP in 0.1 N KOH.

c. Determination of Xanthine and Hypoxanthine in Rat Urine.

Xanthine and hypoxanthine were determined by using a
modification of the method of Jorgensen and Poulsen (1955). In this method, pre-existing uric acid in rat urine is destroyed by uricase; xanthine and hypoxanthine are then converted to uric acid by the action of xanthine oxidase. This newly formed uric acid is then destroyed by uricase and the change in O.D. at 292 nm, which is proportional to the amount of uric acid degraded is measured. Uricase is a highly specific enzyme for uric acid and even methyl derivatives are not attacked by this enzyme. Therefore, this method is very sensitive and accurate when compared with other methods. The procedure and the reagents added to two silica cuvettes marked "blank" and "test" are:

1. 500 μl of urine
2. 1500 μl of M/15 (0.066 M) glycine buffer, pH 9.30
3. Measure pH = 9.25
4. 25 μl of uricase
5. Incubate at room temperature for 1 hr
6. 200 μl of 1.5 N NaOH (pH after adding NaOH = 11.60)
7. Wait 15 min at room temperature
8. 200 μl of 1.6 N HCl
9. 300 μl of 0.6 M glycylglycine buffer, pH 8.20
10. Measure pH = 8.25
11. 25 μl of xanthine oxidase (25 μl of dist H₂O to
12. Incubate at room temperature for 1 hr
13. 200 μl of 0.6 N NaOH
14. 500 μl of 0.7 M glycine buffer, pH 9.4
15. Read O.D. at 292 μm vs blank
16. 25 μl of uricase (25 μl dist H₂O to blank)
17. Incubate at room temperature for 1 hr
18. Read O.D. at 292 μm vs blank

μg/ml of uric acid due to xanthine and hypoxanthine in rat urine is calculated by Praetorius' and Poulsen's formula as:

\[
\text{μg/ml of Uric Acid} = \frac{\Delta A_{292 \text{nm}}}{0.0745} \times \frac{m+b}{m}
\]

Where,
\[\Delta A_{292 \text{nm}}\] is the difference of O.D.s before and after adding uricase in step 16.
0.0745 is the decrease in extinction when 1 μg of uric acid is destroyed by uricase.
\[m + b = \text{total vol. of the contents of the cuvette}.
\[m = \text{vol. of urine present in the cuvette}.

The urine used in step 1 was the clear yellow supernatant of the collected urine sample as described in Section K (a). Glycine, glycylglycine, bovine milk xanthine oxi-
dase containing 6.9 units/ml, and uricase 0.4 units/ml were obtained from Sigma Chemical Co., St. Louis, Missouri.

1/15 M (0.066 M) glycine buffer was prepared by dissolving 2.5033 gm of solid glycine in 200 ml of triple distilled water. To this solution 12.0 ml of 1 N NaOH were added to obtain a pH of 9.30 and the whole solution was diluted to a final volume of 500 ml by triple distilled water. 0.6 M Glycylglycine buffer, pH 8.2 was prepared by dissolving 7.9260 gm of glycylglycine into 10.0 ml of 1 N NaOH and was diluted to 75 ml with triple distilled water. The pH of this solution was then adjusted to 8.2 by adding 19.0 ml of 1 N NaOH and was again diluted to a final volume of 100 ml with triple distilled water. The solution was filtered through double Whatman filter paper. The final pH was 8.20.

d. Determination of Uric Acid in Rat Urine.

A combination of the procedures described by Blauch and Koch (1939); Henry et al. (1957), and Sigma Technical Bulletin No. 680 (Sigma, 1972) was used to determine the amount of uric acid in rat urine. Uric acid has maximum absorption at 292 nm, and, when degraded by the enzyme uricase, the change in O.D. at 292 nm is proportional to the amount of uric acid degraded. A calibration curve is prepared by measuring the change in O.D. at 292 nm produced by
degrading 5, 10, 20, 30, 40, and 50 μg of uric acid by uricase. The procedure used is outlined in the following steps:

1. Take 1.0 ml of the clear yellow supernatant of the collected urine as described in Section K (a); and add 9.0 ml of distilled H₂O to it.
2. Take 0.5 ml of this diluted urine.
3. Add 1.0 ml of 0.7 M glycine buffer, pH 9.40.
4. Add 5.7 ml of distilled H₂O (final volume of this solution for preparing the calibration curve and also during the assays was 7.20 ml).
5. Take 3.0 ml of this solution and read O.D. at 292 nm vs 3.0 ml of distilled H₂O.
6. Add 10 μl of Uricase (Sigma, 0.4 units/ml).
7. Read O.D. at 292 nm after 15, 30, 45, 60, 120, and 240 min. The O.D. did not change after 30 min, indicating that the reaction was completed within 30 min.

Exactly the same procedure was followed for the preparation of the calibration curve.

Inherent absorption of Uricase

To find the inherent absorption of uricase, 10 μl of the enzyme were added to 3.0 ml of 0.7 M glycine buffer, pH
9.4, and the O.D. at 292 nm was measured. It was found to be 0.00, indicating that the enzyme uricase did not have any inherent absorption under the experimental conditions.

The amount of uric acid in the urine was calculated using the following formula. The change in O.D. at 292 nm, as measured in step 7, was used to obtain the concentration of uric acid in \( \mu g/ml \) of the diluted urine from the calibration curve. It was multiplied by the dilution factor 10 which gave \( \mu g \) of uric acid in urine.

\[
\text{\( \mu g/ml \) of Uric Acid in diluted urine obtained from calibration curve} \times \text{dilution factor (10)} = \mu g \text{ of uric acid/ml of urine}
\]

\[
\mu g \text{ of uric acid/ml} \times 100 = \text{mg\% of uric acid.}
\]

e. Determination of Creatinine in the Rat Urine.

A spectrophotometric adaptation of the methods and modifications of Folin and Wu (1919), Hoffman (1941), Peters (1942) and Phillips (1946) was used to determine the amount of creatinine in rat urine. Creatinine in a protein-free filtrate was treated with picric acid in alkaline solution to produce a deep red complex of creatinine picrate. The procedure is outlined as follows:

1. Take 1.0 ml of clear yellow supernatant of the collected urine as described in Section K (a) and
add 9.0 ml of distilled H$_2$O

2. Take 1.0 ml of this diluted urine and place it in a 50-ml Erlenmyer flask containing 8.0 ml of 1/12 N (0.083 N) H$_2$SO$_4$, mixing by rotation.

3. Add 1.0 ml of 10% sodium tungstate, drop by drop, while gently but constantly rotating the flask. A turbid solution is obtained.

4. Centrifuge this solution at 600 x g for 10 min.

5. Take 5.0 ml of the supernatant in a test tube and 5.0 ml of distilled H$_2$O in another test tube marked "blank".

6. Add 2.5 ml of freshly prepared alkaline picrate reagent and mix.

7. Incubate in a waterbath at 25°C for 1 hr.

8. Measure O.D. vs the blank at 520 nm.

Preparation of alkaline picrate reagent:

One percent Picric acid solution was prepared as follows: 12.00 gm of picric acid (J. T. Baker Chemical Co., Phillipsburg, New York) were dissolved in 200 ml of distilled water and diluted to 1000 ml. 20.0 ml of this solution was titrated against 0.1 N NaOH using phenolphthalein as an indicator. It required 8.65 ml of 0.1 N NaOH. (20.00 ml of the picric acid solution in distilled water should
require 8.61 to 8.81 ml of 0.1 N NaOH. If it did not require this amount of titrant, the concentration of picric acid was adjusted accordingly and retitrated.) This solution was stored in a brown bottle.

5.0 ml of 1.0% picric acid was added to an Erlenmyer flask containing 2.5 ml of 2.5 N NaOH. The contents were mixed by gentle but constant rotation of the flask and the solution was diluted to 25 ml with distilled H₂O. This solution was prepared fresh every time and was used within 5 min after its preparation.

A calibration curve was prepared by using 10, 20, 30, 40, 60, 80, and 100 µg of creatinine from a stock solution and following the procedure outlined above. The stock solution of creatinine (1.0 mg/ml) was prepared by dissolving 160.24 mg of creatinine zinc chloride, (C₄H₇N₃O)₂ZnCl₂, in 100 ml of 0.1 N HCl.

f. Metabolites of DOAP.

A single subcutaneous injection of 25 mg/kg of DOAP, dissolved in 0.01 N NaOH was given to an adult male rat. The urine was collected over a period of 24 hr, and the excretion DOAP was precipitated by adding a few drops of 5 N HCl. The urine was then centrifuged at 600 x g/10 min and the clear yellow supernatant obtained by centrifuging the
collected urine was analyzed using column chromatography. A sample of 2.0 ml of the clear yellow supernatant was applied to a cation exchange column as described in Section G. The flow rate was 3 ml/15 min. The eluent showed the presence of a compound having absorption characteristics similar to those of DOHP.

Presence of DOHP, which is a hydrazo derivative of DOAP, in the urine of a rat injected with DOAP, which is an azo compound, indicates that the liver azoreductase system plays a major role in metabolizing DOAP.

K. ISOLATION AND PURIFICATION OF ALDEHYDE OXIDASE FROM RABBIT LIVER

Aldehyde oxidase was isolated from the livers of young adult male New Zealand rabbits. Two rabbits weighing 3.2 and 3.4 Kg were sacrificed by decapitation. The livers were removed, washed twice with distilled H₂O, dried by blotting over paper towels and weighed. 240 gm of livers were then chopped into smaller pieces and were divided into 4 equal portions, each weighing 60 gm. Each portion was then homogenized in 300 ml of distilled H₂O (1:5, W:V) in a Waring blender at room temperature for 3 min. The final volume of the total homogenate was 1450 ml.
This homogenate was then rapidly heated with continuous stirring to 70°C in a boiling water bath and was allowed to remain at 70°C for 2 min. The homogenate was then rapidly cooled to 55°C by adding ice to the water bath. It was transferred to a beaker kept in an ice bath and was cooled to 12°C; stirring occasionally. The homogenate was then centrifuged at 8000 rpm (6500 x g) for 20 min in a refrigerated centrifuge at 4°C. The sediment was discarded and the supernatant was filtered through glass wool and a total of 1175 ml of the supernatant was stored in a cold room at 4°C overnight. All the steps from this stage on were performed in a cold room at 4°C.

The next morning, the supernatant was brought to a 50% saturation of (NH₄)₂SO₄ by adding 313 gm of solid ammonium sulfate per liter of the supernatant. A total of 367.78 gm of solid ammonium sulfate were added, slowly, with continuous stirring, to 1175 ml of the supernatant to obtain the 50% saturation of ammonium sulfate. The supernatant was then stirred very slowly with a mechanical stirrer for 1 hr. The light-brown precipitate obtained after 1 hr was collected by centrifugation at 8000 rpm for 20 min in a refrigerated centrifuge at 4°C.

The precipitate was then dissolved in 15.0 ml of 0.05 M potassium phosphate, pH 7.8, containing 0.005% EDTA. The
pH of this solution dropped below 7.8, hence, was adjusted by adding a few drops of 1 N NaOH very slowly while the solution was stirred continuously. Thus, the final solution of pH 7.8 was then diluted to 20.0 ml by adding the buffer. This solution was then dialyzed overnight against 3750 ml of distilled H₂O containing 0.005% EDTA at 4°C. The cellulose dialysis bags were obtained from Sigma Chemical Co., St. Louis, Missouri, and were boiled in 1.0 mM EDTA solution for 15 min prior to their use. The volume of the dialyzate was found to increase from 20 ml before dialysis to 32.3 ml after dialysis. This solution was used as the source of aldehyde oxidase in all the experiments.

L. DETERMINATION OF PROTEIN
IN THE RABBIT LIVER HOMOGENATE

The protein content of the rabbit liver homogenate, used as a source of aldehyde oxidase, was determined by using the spectroscopic method of Waddell (1956). The peptide bonds of a protein absorb at the shorter wavelengths than the aromatic amino acids which usually absorb at 280 nm; the presence of nucleic acids interferes the measurement of proteins by a method based on the absorption at 280 nm.

Waddell's method is based on the measurement of the
difference of absorption between 215 nm and 225 nm by proteins. The use of the difference rather than the absorption at a single wavelength minimizes the error introduced by non-protein constituents of the sample. At 215 nm the protein solutions conform closely to Beer's Law as long as the absorption is less than 2.0. Another advantage of this method is that the absorption of protein in this spectral region is practically independent of pH over the range of 4 to 8; hence it is unnecessary to use a buffer as diluent. The accuracy of this method was checked by measuring a protein concentration of a solution of human serum albumin in our laboratory.

The dialyzed rabbit liver homogenate was diluted 1:250, 1:500, 1:1000 and 1:2000 by a 9.0 gm/liter solution of NaCl in distilled H₂O (0.154 M). Optical densities of these solutions was measured at 215 and 225 nm. The O.D. at 215 nm was subtracted from that at 225 nm. This difference, multiplied by 144, gave the protein concentration in diluted solutions expressed in micrograms per milliliter. The concentration of protein in the stock solution is obtained by multiplying with the dilution factor i.e. 250, 500, 1000, or 2000.

\[ \Delta \text{O.D.} \times 144 = \mu g/\text{ml of protein in the diluted solution} \]

\[ \Delta \text{O.D.} \times 144 \times \text{dilution factor} = \mu g/\text{ml of protein in} \]
original solution

The factor $144$ is the ratio of $\mu g/ml$ of protein and the difference in O.D. at 215 and 225 nm.

$$144 = \frac{\mu g/ml \text{ of protein in the diluted solution}}{\Delta \text{O.D.}}$$

It was calculated from a series of solutions of known protein concentration.

M. ASSAYS OF ALDEHYDE OXIDASE IN RABBIT LIVER HOMOGENATES

Aldehyde oxidase activity of the rabbit liver homogenate was assayed using $N^1$-methylnicotinamide chloride, also called 1-methylnicotinamide chloride, as the substrate; this compound should not be confused with the $N'$-methylnicotinamide, the difference being that $N^1$-methylnicotinamide or 1-methylnicotinamide has the methyl group on the $N_1$ quaternary ring nitrogen, while the $N'$-methylmethylnicotinamide has the methyl group on a tertiary nitrogen in the side chain. Menadione was used as an inhibitor of the $N^1$-methylnicotinamide oxidase activity (aldehyde oxidase activity). $N^1$-methylnicotinamide chloride and menadione sodium bisulfite salt were purchased from Sigma Chemical Co., St. Louis, Missouri.

A modification of the spectrophotometric methods of
Rajagopalan (1964) and Sheen et al. (1970) was used to assay the aldehyde oxidase activity of the rabbit liver homogenate. The method is based on the principle that $N^1$-methylnicotinamide chloride (NMN) has a maximum absorption at 300 nm and, when it is oxidized to 2-pyrone, the O.D. decreases. The change in O.D. at 300 nm thus gives the rate of this oxidation reaction since the product of the oxidation does not absorb at this wave length. The procedure used is tabulated as follows:

Each experimental cuvette contained 2.25 ml of 0.05 M potassium phosphate containing 0.005% EDTA, pH 7.8, 0.25 ml of 0.06 M solution of $N^1$-methylnicotinamide chloride, and 0.25 ml of 2.5 mg/ml solution of NaAP in 0.05 M potassium phosphate buffer, pH 7.8. After a 5 min equilibration period at 25°C, 0.25 ml of 5.04 mg protein/ml rabbit liver aldehyde oxidase was added to the cuvette. The cuvette contents were rapidly mixed, and $A_{300}$ was recorded with time against a reference cell from which the substrate had been omitted. The $\Delta A_{300}/\text{min}$ was obtained from the linear portion of the curve of decreasing extinction in the presence and absence of NaAP (Chart 2).

The optimum substrate concentrate and the amount of enzyme used in the assay procedure was also determined using this method. The xanthine oxidase activity of this
suspension was found to absent as a similar assay procedure ($\Delta A_{290}$, Davis et al., 1974) employing xanthine as a substrate showed the absence of any xanthine oxidase activity in the final dialyzed rabbit liver homogenate. Oxidation of $N^1$-MN is also a very specific reaction and only aldehyde oxidase can use it as a substrate while xanthine oxidase is unable to oxidize it.

N. INHIBITION OF ALDEHYDE OXIDASE BY NaAP

The assay procedure used to study the inhibition of aldehyde oxidase in rabbit liver homogenate has been described in Section M. Similar experiments were performed using menadione, to compare the inhibition of NaAP vs menadione. DOAP, an extremely potent inhibitor of bovine milk xanthine oxidase was also screened for possible rabbit liver aldehyde oxidase inhibitory activity using the same procedure outlined earlier. The investigation of the stability of DOAP in 0.05 M potassium phosphate, as described in Section E, revealed that DOAP solution was relatively unstable at pH 7.8 but was stable at pH 8.2 up to 10 min. Therefore, the assays employing DOAP, NaAP and menadione were carried out at pH 7.8 and also at pH 8.2.
0. ENZYMATIC SYNTHESIS OF DOAP FROM NaAP

During the studies of inhibition of aldehyde oxidase by NaAP, it was observed that the product of the interaction between NaAP and rabbit liver aldehyde oxidase was DOAP. This finding was confirmed as follows.

2.5 mg of NaAP was dissolved in 10 ml of distilled H₂O to obtain a clear yellow solution. To this solution, 7.5 ml of 0.05 M potassium phosphate buffer containing 0.005% EDTA at pH 7.8 and 2.5 ml of 5.04 mg protein/ml of the aldehyde oxidase suspension were added. A control reaction was carried out using the same reactants with the exception that, instead of the aldehyde oxidase suspension, 2.50 ml of the buffer was added in the last step. Both reaction mixtures were incubated at room temperature for 15 min. At the end of 15 min, both solutions were brought to a 50% ammonium sulfate saturation by adding 313 gm of solid ammonium sulfate per liter of the reaction mixture. The solutions were then centrifuged at 8000 rpm for 20 min at 4°C. A brown precipitate was obtained from the reaction mixture with the enzyme suspension while a yellow precipitate was obtained from the control reaction mixture. Both precipitates were dissolved in 25 ml of distilled H₂O separately and, to each solution, 2.0 ml of 5 N HCl were added. This
solution was centrifuged at 8000 rpm for 20 min at 4°C and the precipitates obtained were treated with the same procedure once again.

At the end, a bright orange precipitate was obtained from the reaction mixture containing the enzyme suspension while a yellow precipitate was obtained from the control reaction. The orange precipitate was identified to be the DOAP since it had exactly the same Rf values in three different solvent systems (Table 35) and also uv characteristics as that of authentic DOAP. The yellow precipitate was identified as NaAP. NaAP was found to be unchanged under the same conditions even after 6 hr in the absence of enzyme. DOAP was also obtained from NaAP using the same procedure except using 5 N HCl and incubation in a boiling water bath for 10 min instead of using 50% saturation of ammonium sulfate to precipitate out the brown-orange precipitate of DOAP:protein complex.
CHAPTER IV

RESULTS
CHAPTER IV

RESULTS

A. STRUCTURE OF 8,8'-DIOXO-6,6'-AZOPURINE (DOAP)

The elemental analysis at room temperature and following drying at 100°C for 1 hr under nitrogen was found to be within the acceptable experimental error of ±0.4% as shown in Table 1. These analyses gave the empirical formulae of DOAP as $C_{10}H_6N_{10}O_3$ at room temperature and $C_{10}H_6N_{10}O_2$ after drying (Table 2). The difference in the two empirical formulae was found to be a mole of water which indicated the presence of a mole of water of hydration per mole of DOAP. The presence of 1 mole of hydration per mole of the DOAP solid was confirmed by drying the sample under a stream of nitrogen for 1 hr at 100°C and trapping the evolved $H_2O$ in magnesium perchlorate. In this manner, the trapped $H_2O$ was found to account for 5.41% of the undried molecular weight, corresponding to a theoretical value of 5.7% (Table 3).

The number of carbon and nitrogen atoms in the anhydrous empirical formula of $C_{10}H_6N_{10}O_2$ was double that of the starting material, adenine. This indicated that the
compound was a purine dimer. The nuclear magnetic resonance (nmr) spectrum of DOAP at a concentration of 20 mg/ml in DMSO-d$_6$ was obtained from a Varian A-60A nmr spectrophotometer, using TMS as an internal standard (Figure 15). Three single peaks were found at δ 8.84, 12.05, and 12.64 ppm relative to the TMS reference (Table 12). Integration of the three proton peaks revealed an integral ratio of 1:1:1, confirming the presence of six hydrogen atoms in the DOAP molecule. Peak assignments were made on the basis of D$_2$O exchange and the ranges of known δ ppm values (Fox, 1965; Shoup et al., 1966; Robins et al., 1966; Chan et al., 1972; Twanmoh et al., 1973). These assignments indicated the presence of two C$_2$-H protons (8.84 ppm), two amide N$_9$-H protons (12.05 ppm), and two imidazole N$_7$-H protons (12.64 ppm). Since the two protons originally present on the nitrogen attached to C-6 of adenine were found to be absent in the nmr spectrum of DOAP, it appeared that the dimerization of adenine occurred through the formation of an azo linkage resulting in an oxoazopurine which is shown in Figure 1, having the chemical structure of 8,8'-dioxo-6,6'-azopurine monohydrate, abbreviated as DOAP.

Additional evidence for the oxo groups at the C$_8$ and C$_8'$ positions was obtained from the infrared spectrum of DOAP which showed the presence of a strong and broad C=O
absorption band at 1760 cm$^{-1}$. Oxopurines are known to form keto and enol tautomers, depending on their chemical environment (Lichtenberg, 1973; Mason, 1957). The keto-enol tautomerism of DOAP is shown in Figure 2.

The synthesis of DOAP from the oxidation 6,6'‐azopurine disodium salt by rabbit liver aldehyde oxidase and reductive cleavage of DOAP by sodium hydrosulfite to 8‐oxoadenine confirmed the structure of DOAP. The results of these two observations are described in detail in Sections H and J.

B. STRUCTURE OF 8,8'-DIOXO-6,6'-HYDRAZOPURINE (DOHP)

The elemental analysis at room temperature and following drying at 105°C for 1 hr under nitrogen was found to be within the experimental error of ±0.4%, as shown in Table 3. These analyses gave the empirical formulae of DOHP as $\text{C}_{10}\text{H}_{10}\text{N}_{10}\text{O}_{3}$ at room temperature and $\text{C}_{10}\text{H}_{8}\text{N}_{10}\text{O}_{2}$ after drying (Table 4). The difference between the two empirical formulae was found to be a mole of water, which indicated the presence of a mole of water of hydration per molecule of DOHP. The presence of one mole of water of hydration per mole of DOHP was confirmed using the procedure described for DOAP.
The structure of DOHP was obtained by studying its nmr spectrum and also its alcoholic oxidation. The nmr spectrum of DOHP (Figure 18) at a concentration of 20 mg/ml in DMSO-d$_6$ was obtained using a Varian A-60A (6). Four single peaks were found at $\delta$ 8.34, 8.92, 10.42, and 11.88 ppm relative to the TMS reference. Integration of the four proton peaks revealed an integral ratio of 1:1:1:1, confirming the presence of eight hydrogen atoms in the molecule of DOHP as shown in Table 12. Peak assignments were made on the basis of D$_2$O exchange and ranges of known $\delta$ ppm values (Fox, 1965; Shoup et al., 1966; Robins et al., 1966; Chan et al., 1972; Twamoh et al., 1973). These assignments indicated the presence of two C$_2$-H protons (8.34 ppm), two amide N$_9$-H protons (10.42 ppm), and two imidazole N$_9$-H protons (11.88 ppm). The assignment of the hydrazo N-H protons at 8.92 ppm was confirmed by obtaining the nmr spectrum of 6,6'-bisadenine under the similar experimental conditions. The hydrazo protons of 6,6'-bisadenine were found to be in the same range as that of DOHP.

The fact that DOHP is a partially reduced, i.e. a hydrazo, derivative of DOAP was confirmed by studying its slow oxidation to DOAP. DOHP has a maximum absorption at 282 nm in absolute ethanol, while DOAP has a maximum absorption at 442 nm. The oxidation of DOHP to DOAP was followed by mon-
itoring the decrease in the O.D. at 282 nm of a 10 µg/ml solution of DOHP in absolute ethanol at room temperature. No significant change in the optical density at 282 nm was found up to 30 min. After 30 min, the O.D. at 282 nm started to decrease while that at 442 nm started to increase (Figure 5). As shown in Table 8, over a period of 24 hr, the O.D. at 282 nm disappeared completely while that at 442 nm reached a peak which was identical to the absorption maxima of DOAP.

Thus, these experiments confirmed the fact that DOHP is a hydrazo derivative of 8,8'-dioxo-6,6'-azopurine having the chemical structure of 8,8'-dioxo-6,6'-hydrazopurine monohydrate as shown in Figure 3, which is abbreviated as DOHP.

C. STRUCTURE OF 8,8'-DIOXO-6,6'-AZOXYPURINE TRISODIUM SALT (NaDOAXP)

The elemental analyses and the determination of six moles of water of hydration per mole of NaDOAXP was carried out as described for DOAP and DOHP. The results are shown in Tables 5 and 6. The empirical formula at room temperature was found to be $\text{C}_{10}\text{H}_{15}\text{N}_{10}\text{O}_9\text{Na}_3$ while after drying, it was found to be $\text{C}_{10}\text{H}_{9.3}\text{N}_{10.3}\text{O}_9\text{Na}_3$. 
The structure of NaDOAXP was arrived at by studying its nmr spectrum and also its reduction to DOAP under acidic conditions. The nmr spectrum of NaDOAXP at a concentration of 20 mg/ml was obtained using a Varian A-60A (δ) and was found to contain two single peaks at δ 8.56 and 11.78 ppm, relative to the TMS reference. Integration of the two proton peaks revealed an integral ratio of 2:1, respectively, confirming the presence of three hydrogen atoms in the NaDOAXP molecule. Peak assignments were made on the basis of D₂O exchange and the sources quoted previously. These assignments indicated the presence of two C₂-H protons (8.56 ppm) and one imidazole N₇-H proton (11.78 ppm).

The anhydrous empirical formula of NaDOAXP of C₁₀H₃N₁₀O₃Na₃ contained three hydrogen atoms fewer and one additional oxygen atom than the anhydrous empirical formula of DOAP, C₁₀H₆N₁₀O₂, in addition to the three sodium atoms. This indicated that the three hydrogen atoms in DOAP had been replaced by three sodium atoms when it was converted to NaDOAXP during its synthesis in NaOH as described in Chapter III, Section C. The fact that NaDOAXP was obtained by air oxidation of DOAP in NaOH also explained the presence of an additional atom of oxygen in azoxy form. These observations indicated that NaDOAXP is a sodium salt of the azoxy derivative of DOAP. This was confirmed by studying
the reduction of NaDOAXP to DOAP (i.e. azoxy → azo) under aqueous as well as under acidic conditions. It was found that an aqueous solution of NaDOAXP slowly, over a period of 72 hr, gave a precipitate of DOAP. This reduction was instantaneous when the pH of the aqueous solution was changed to 2.0 by adding 5 N HCl. This is a characteristic behavior of an azoxy compound, hence, it was concluded that NaDOAXP was a sodium salt of the azoxy derivative of DOAP, having a chemical structure of 8,8′-dioxo-6,6′-azoxypurine hexahydrate trisodium salt as shown in Figure 4, abbreviated NaDOAXP.

D. CHEMICAL PROPERTIES OF DOAP, DOHP AND NaDOAXP

8,8′-dioxo-6,6′-azopurine monohydrate (DOAP) was obtained as a brilliant orange microcrystalline solid. The yield on a preparative scale was found to be 5.2%. Because of its hydrophobic nature, DOAP was found to be practically insoluble in distilled water, dilute HCl, dilute H₂SO₄, and non-polar organic solvents such as CHCl₃, CCl₄, benzene, toluene, cyclohexane, xylene, etc. It is soluble in NaOH at different concentrations depending on the normality of NaOH. It is also soluble in polar organic solvents such as absolute ethanol, n-butanol, and dimethylsulfoxide. It is soluble in Tris-HCl buffer above pH 6.6. Its solubility
characteristics in 0.05 M potassium phosphate buffer at various pHs, at a concentration of \(8.0 \times 10^{-5}\) M, at room temperature, is shown in Figure 35. In this buffer, the DOAP solution is not soluble at pH 6.2, 6.6, and 7.0 and it precipitates out within one minute. At pH 7.4 and 7.8, the DOAP solution is fairly stable for up to 3.0 min. At pH 8.2, the solution is adequately stable for up to 10 min (Figure 37).

8,8'-dioxo-6,6'-hydrazopurine monohydrate (DOHP) was isolated as a tan-yellow solid. It was obtained from partial reduction of DOAP at a 75% yield. DOHP is soluble at smaller concentration in dilute HCl and dilute \(\text{H}_2\text{SO}_4\). It is not soluble in non-polar organic solvents but is soluble in absolute ethanol, \(n\)-butanol, and dimethylsulfoxide. As was mentioned earlier, it was oxidized to DOAP in absolute ethanol after 30 min of standing at room temperature.

8,8'-dioxo-6,6'-azoxypurine hexahydrate trisodium salt (NaDOAXP) was obtained as a cherry-red solid. It was obtained from the air oxidation of DOAP in NaOH as described in Chapter III, Section C. Because of its polar nature, NaDOAXP is very soluble in water. In water solution, NaDOAXP is slowly reduced to DOAP while in acidic solution it is instantly reduced, as described earlier.

In a series of experiments designed to determine the
melting points of DOAP, DOHP, and NaDOAXP, using Fisher-Johns melting point apparatus, it was observed that none of these compounds melted below 300°C. DOHP started to change its tan-yellow color to tan-brown at 285°C and this color change was completed at 290°C, however, it did not melt below 300°C. The cherry-red color of NaDOAXP deepened slowly but it did not melt below 300°C.

Figures 29-36 and Table 9, shows the uv characteristics of these compounds in various solvents. Table 11 shows their Rf values in three different solvent systems.

E. CHEMICAL PROPERTIES OF HP, NaAP, AND NaAXP

6,6'-bisadenine monohydrate (C_{10}H_8N_{10}H_2O), 6,6'-azopurine hemihydrate disodium salt (C_{10}H_4N_{10}Na_2.H_2O), and 6,6'-azoxypurine dihydrate disodium salt (C_{10}H_4N_{10}ONa_2.2H_2O) were obtained as a generous gift from Dr. A. Giner-Sorolla of the Sloan-Kettering Institute for Cancer Research, New York, New York.

6,6'-bisadenine monohydrate, abbreviated as HP (indicating that it is a hydrazopurine for comparison with 8,8'-dioxo-6,6'-hydrazopurine, DOHP), was obtained as a yellow microcrystalline solid. Its solubility characteristics were found to be similar to those of DOHP.

6,6'-azopurine hemihydrate disodium salt, abbreviated
as NaAP, was obtained as a brick-red crystalline solid.
NaAP is soluble in water and aqueous solvents but these solutions were reported to decompose when treated with dilute acid (Giner-Sorolla, 1970). For our experimental purposes, the solubility and the stability of NaAP in 0.05 M potassium phosphate buffer, at room temperature at a concentration of 8.0 \times 10^{-5} \text{M} at various pHs was studied. At pH 6.2 and 6.6, NaAP solutions in this buffer were found to be unstable. They were fairly stable at pH 7.0 for up to 3 min and were practically stable at pH 7.4, 7.8, and 8.2 for up to 10 min.

6,6'-azoxypurine dihydrate disodium salt, abbreviated as NaAXP, was obtained as a red solid. Its solubility characteristics were found to be similar to those of NaDOAXP.

Figures 29-36 and Table 9 shows the uv characteristics of these compounds in various solvents. Table 11 shows their \( R_f \) values in three different solvent systems.

F. NMR AND IR SPECTROSCOPIC STUDIES OF OXOAZOPURINES

The nmr spectra of oxoazopurines, azopurines, and purine derivatives are tabulated in Table 12. The two amino \( \text{NH}_2 \) protons of adenine were found at \( 6 \) 7.36 ppm, however, when two molecules of adenine were dimerized through a hy-
drazo linkage on C₆ and C₆', as in 6,6'-bisadenine (HP), the N-H protons of the hydrazo linkage were found to be shifted downfield to δ 10.04 ppm. The shielding effect of carbonyl groups at the 8 and 8' positions in 8,8'-dioxo-6,6'-hydrazopurine (DOHP) was found to have a considerable effect on the hydrazo N-H proton of DOHP and were found upfield at δ 8.92 ppm. The shielding effect of the adjacent carbonyl group was also observed when the δ ppm values of the amide N-H protons in xanthine, hypoxanthine, 8,8'-dioxo-6,6'-azo-purine (DOAP), and DOHP were compared. The amide N-H protons of hypoxanthine were found to be δ 12.88 ppm, while those of xanthine were moved upfield at δ 11.92, and 11.20 ppm, those of DOAP to δ 12.22 ppm, and those of DOHP to be δ 10.42 ppm. The imidazole N₇-H proton in adenine was found at δ 13.28 ppm. The azo, hydrazo, and azoxy linkages of DOAP, DOHP, and NaDOAXP, in addition to the carbonyl groups at the 8 and 8' positions, increased the shielding of N₇-H imidazole protons which were thus shifted upfield to δ 12.84, 11.88, and 11.78, respectively. In the case of 6,6'-bisadenine, in spite of the presence of a hydrazo linkage, there was no effective shielding because of the absence of the carbonyl groups at the 8 and 8' positions, therefore, the N₇-H imidazole protons moved downfield to δ 12.42 ppm. This was found to be in agreement with the
strong shielding effects of carbonyl groups and thiol
groups reported by Baur et al. (1965) and Lichtenberg et
al. (1971). The C8-H of purine is less shielded than the
C2-H which, in turn, is less shielded than the C6-8 (Fox,
1965). Insertion of C=O groups at the 8 and 8' positions
reduces the aromatic character of the imidazole ring as it
does that of pyrimidine ring. This decreases the ring cur­
rent which, in turn, has a shielding effect on the N7-H
protons.

Standard compilations were used to analyze the ir
spectra of oxoazopurine monomers (Sadtler Research Labora­
tories, American Petroleum Institute; Kitritzky, 1963 and
1971). The frequencies for oxoazopurines and the purine
monomers are tabulated in Tables 13 and 14, respectively.

The region from 3700 to 2200 cm⁻¹ was found to be
characterized by very broad hydrogen bonded peaks which
nearly obscured the C-H absorptions between 3100 and 3000
cm⁻¹. All oxoazopurines, with the exception of NaDOAXP,
exhibited a free N-H stretch in the 3200-3500 cm⁻¹ region.
Broad absorptions with decreasing intensities were found
in the region between 3000 and 2200 cm⁻¹, characteristic of
N7-H stretch. All compounds except NaDOAXP showed this
feature, indicating that N7-H has formed a hydrogen bond
with the azoxy oxygen.
Carbonyl absorptions were found as strong but broad peaks at 1760 cm$^{-1}$ in DOAP, and at 1750 cm$^{-1}$ in DOHP. No carbonyl absorption for NaDOAXP was found because both carbonyls exist predominantly in the enolate configuration with the electron density on the oxygen (Spinner, 1960). The N=N stretch of the azoxy groups of NaDOAXP and NaAXP was found at 1545 and 1550 cm$^{-1}$, respectively. The major difference between the spectrum of adenine and 6,6-bisadenine (HP) was the loss of the adenine NH$_2$ scissors at 1720 cm$^{-1}$; in the dimer, this was accompanied by the appearance of a more prominent 1580 cm$^{-1}$ shoulder on the 1600 cm$^{-1}$ ring mode.

The oxopurines showed characteristic broad peaks between 1500 and 1200 cm$^{-1}$. The carbonyl group absorptions in the purine monomers were found between 1600 and 1750 cm$^{-1}$. Carbonyl group at the C$_2$ position was found in the region between 1680 and 1710 cm$^{-1}$, at C$_6$ position they were found between 1600 and 1960 cm$^{-1}$, and at C$_8$ position they were found between 1700 and 1750 cm$^{-1}$.

G. COLUMN CHROMATOGRAPHIC SEPARATION OF OXOAZOPURINES

Figure 9 shows the technique for chromatographic separation and identification of oxopurines. Table 15 lists the peak tube number and their uv characteristics.
Among the numerous chromatographic methods available for the separation of purines (Wall, 1953; Weissman et al., 1957; Wyngaarden et al., 1958; Ayvazian et al., 1965), a modification of the method described by Sweetman and Nyhan (1969), was found to be very efficient for the separation of oxopurines and is described in detail in Chapter III, Section G. For the chromatographic analysis, three purines with the 6, 2,6, and 2,6,8 positions oxidized (i.e. hypoxanthine, xanthine, and uric acid) and three derivatives of adenine with the 2, 8, and 2,8 positions oxidized (i.e. 2-oxoadenine, 8-oxoadenine, and 2,8-dioxyadenine) were chosen.

As shown in Figure 9, the three oxidized purines were eluted in a definite order, the most oxidized (uric acid) being eluted first, followed by the less oxidized (xanthine and hypoxanthine). These were followed by adenines, again in the same order, the most oxidized 2,8-dioxyadenine (2,8-DOA) was followed by 8-oxoadenine (8-OA) and then by 2-oxoadenine (2-OA). A pure purine base like adenine, which was not oxidized, was eluted last. The elution time was further enhanced by dimerization of two purine units, as is shown by the position of 8,8'-dioxyo-6,6'-hydrazo purine (DOHP) on the chromatogram.
H. REDUCTIVE CLEAVAGE OF 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) BY SODIUM HYDROSULFITE

As shown in Figure 10, the product of the reductive cleavage of 8,8'-dioxo-6,6'-azopurine (DOAP) was identified as a single large peak of absorbance at an effluent volume of 381 ml with two $\lambda_{\text{max}}$ values of 280 and 267 nm, having an $A_{280}/A_{267}$ ratio of 1.04 which matched the chromatographic position and spectral characteristics of the authentic sample of 8-hydroxyadenine sulfate.

The tubes representing the single large peak of absorbance (fraction No. 123-130) were pooled in a 25-ml tube and gaseous nitrogen was passed through it for 20 hr; the tube was kept in a water bath at 4°C. A yellow solid was obtained at the end of the 20 hr period. Uv spectra of the yellow solid were obtained in 1 N NaOH and dimethylsulfoxide (Figure 14) which exactly matched those of the authentic 8-hydroxyadenine sulfate sample (Figure 13).

I. STUDIES OF \textit{in vivo} INHIBITION OF XANTHINE OXIDASE AND THE METABOLISM OF DOAP IN AN ADULT MALE RAT

a. Urinary Excretion of DOAP

A single subcutaneous injection of 8,8'-dioxo-6,6'-azopurine (DOAP) was given to a rat behind the nape of the
neck. The dose used was 25 mg of DOAP dissolved in 0.01 N NaOH/kg (Table 16). The urine was collected in 2 hr samples over a period of 24 hr, using an automatic fraction collector. The DOAP excreted in each 2 hr urine sample was precipitated by adding one drop of 5 N HCl and was washed and dissolved in 0.1 N KOH. The optical density of this solution was measured at 513 nm and the concentration of DOAP in µg/ml (Table 18) was obtained, for each of the 2 hr samples, from the calibration curve (Figure 56). This procedure is described in detail in Chapter III, Section J (b). A graphical plot of µg/ml of DOAP per 2 hr urine sample vs hours after injection (Figure 57), showed that the rate of urinary excretion of DOAP after a single subcutaneous injection of 25 mg/kg in an adult male rat may be biphasic. Two hours after injection, 4.5 µg of DOAP/ml were found in the excreted urine; the peak of the first phase was reached at 6 hr after injection when 20.8 µg of DOAP/ml were excreted. The rate of excretion of DOAP was then reduced and at 14 hr after injection it was found to be 18.9 µg of DOAP/ml. The peak of the second phase was reached at 18 hr after the injection when 30.9 µg of DOAP/ml were excreted. At 24 hr after the injection, the rate of excretion diminished to 2.7 µg of DOAP/ml. A total of 0.3837 mg of DOAP were excreted over the 24 hr period after
the injection which amounted to 6.73% of the injected dose of DOAP. DOAP was not found in the urine samples collected at 26, 28, or 30 hr after the injection of DOAP. This indicated that 93.27% of the injected DOAP remained in the body of the rat 24 hr after the single subcutaneous injection. DOAP might have been excreted in smaller amounts 24 hr after the injection but the technique used to analyze DOAP in rat urine was unable to detect this amount.

The urine output after injecting 25 mg/kg DOAP increased slowly (Table 19) until it reached a peak at 16 hr after injection. The output of urine was also found to be increased 16 hr after injecting 0.01 N NaOH. The amount of 0.01 N NaOH injected was the same as used to dissolve the DOAP. The increase in the urine output, 16 hr after the injection of 25 mg/kg DOAP was twice that found after injecting the equal volume of 0.01 N NaOH (Figure 58 and Table 19). Thus, the relatively moderate increase in urine output after the injection of DOAP indicates that DOAP may have diuretic properties. The urine volume remained at a higher level until 5 days after injecting DOAP but returned to normal, as shown in Figure 59.

b. Effect of a Single Subcutaneous Injection of 25 mg/kg DOAP on Urinary Excretion of Xanthine and Hypoxanthine
Xanthine and hypoxanthine were determined enzymatically, using a method modified from that of Jorgensen and Poulsen (1955). As described in Chapter III, Section J (c), the pre-existing uric acid in rat urine is destroyed by uricase, following which, xanthine and hypoxanthine are converted to uric acid by the action of xanthine oxidase. This newly formed uric acid is then destroyed by uricase again and the change in O.D. at 292 nm is determined; this is proportional to the amount of measured uric acid degraded (Table 22). Figure 60 shows the effect of 25 mg/kg DOAP on urinary excretion of xanthine and hypoxanthine in an adult male rat.

The level of uric acid due to xanthine and hypoxanthine (0.22 mg%) was found to be unaffected by the dose of an equal volume of 0.01 N NaOH (0.21 mg%). Uric acid due to xanthine and hypoxanthine increased to 0.67 and 0.99 mg% at 24 hr and 48 hr after injecting 25 mg/kg DOAP, respectively. The level of uric acid due to xanthine and hypoxanthine remained slightly elevated on the 3rd, 4th, and 5th day after the injection of DOAP. The level returned to normal on the 6th day after the injection of DOAP.

c. Effect of a Single Subcutaneous Injection of 25 mg/kg DOAP on Urinary Excretion of Uric Acid

The determination of uric acid in rat urine is de-
scribed in Chapter III, Section J (d), and the calibration curve is shown in Figure 62. The uric acid level in the urine of a normal adult male rat (0.77 mg%) was found to be unaffected by the injection of equal volume of 0.01 N NaOH (0.75 mg%). The uric acid levels dropped to 0.25 mg% 24 hr after the injection of 25 mg/kg DOAP (Table 21) and returned to 0.70 mg% at the end of 48 hr. The level of uric acid dropped again to 0.45 mg% at the end of the 4th day after the injection of DOAP and then returned to normal. Thus, the excretion of uric acid after the injection of 25 mg/kg DOAP may also be following biphasic pattern.

d. Effect of a Single Subcutaneous Injection of 25 mg/kg DOAP on Urinary Excretion of Creatinine

Creatinine levels in the urine of an adult male rat injected with 25 mg/kg DOAP, were monitored in order to investigate the effect of DOAP on renal function. The level of creatinine (Table 24) in the urine of a normal adult rat (5.77 mg%) was not altered after the injection of an equal volume of 0.01 N NaOH (5.80 mg%). The creatinine level dropped to 1.35 mg% 24 hr after the injection of 25 mg/kg DOAP (Figure 63). The level of creatinine slowly returned to normal at the end of the 5th day after the injection of DOAP. This indicated that one of the toxic effects of DOAP was its nephrotoxicity.
e. Metabolites of DOAP

As described in Chapter III, Section J (f), the metabolites of DOAP in rat urine, after a single subcutaneous injection of 25 mg/kg DOAP, were separated by using column chromatography. The fraction eluted in tube 196 showed the exact same characteristics of that of authentic DOHP in 1 N HCl (Figure 66). It was concluded that one of the possible metabolites of DOAP in the rat was its hydrazo derivative, DOHP.

J. INHIBITION OF RABBIT LIVER ALDEHYDE OXIDASE BY 6,6'-AZOPURINE DISODIUM SALT (NaAP)

The isolation and purification of aldehyde oxidase from rabbit liver is described in Chapter III, Section K. The protein content of the rabbit liver aldehyde oxidase was determined by using Waddell's method and results are shown in Table 25. The aldehyde oxidase suspension was found to contain an average of 5.04 mg protein/ml.

Oxidation of $5.0 \times 10^{-3}$ M $N^1$-methylnicotinamide chloride by rabbit liver aldehyde oxidase is shown in Figure 43. The rate of oxidation increased linearly as the amount of enzyme used was increased from 50 μl to 250 μl. Figure 44 shows the optimal substrate concentration ($5.0 \times 10^{-3}$ M) and optimal enzyme concentration (250 μl of 5.04 mg pro-
tein/ml). Inhibition of rabbit liver aldehyde oxidase was measured using the $\Delta A_{300}$ method (Figure 42). It was decided to compare the inhibition of aldehyde oxidase by 6, 6'-azopurine disodium salt (NaAP) to that by 8,8'-dioxo-6,6'-azopurine (DOAP). As described earlier, DOAP at a final concentration of $8.0 \times 10^{-5}$ M in 0.05 M potassium phosphate buffer, pH 7.8, was unstable at room temperature but at pH 8.2, it was found to be adequately stable for up to 3 min. A series of experiments were designed to investigate the inhibition of rabbit liver aldehyde oxidase by NaAP at pH 7.8 and at pH 8.2 and that by DOAP at pH 8.2. Figure 45 shows the concentration of inhibitor (NaAP) that was found to cause a 50% inhibition of the rabbit liver aldehyde oxidase $(I)_{50}$. The $(I)_{50}$ at pH 7.8 was found to be $2.6 \times 10^{-6}$ M NaAP. The $(I)_{50}$ at pH 8.2 was found to be $3.4 \times 10^{-6}$ M NaAP (Figure 46).

The inhibition of rabbit liver aldehyde oxidase was also compared with a non-competitive inhibitor, i.e. menadione. Menadione influences the internal electron transport chain and does not bind at the substrate site. The $(I)_{50}$ at pH 7.8 for menadione was found to be $1.1 \times 10^{-7}$ M (Figure 47).

The inhibition of rabbit liver aldehyde oxidase by NaAP was found to be competitive, as shown by the Line-
weaver-Burk plot (Figure 49) which showed the characteristic straight lines with differing slope intersecting at a common intercept on \( \frac{1}{V} \) axis and \( \lambda_{\text{max}} \) unaffected by the increasing concentration of NaAP. This finding was further confirmed by plotting a Dixon plot (Figure 50). The inhibitor constant (\( K_I \)) for the inhibition of rabbit liver aldehyde oxidase by NaAP was found to be \( 3.3 \times 10^{-6} \) M. The inhibition of rabbit liver aldehyde oxidase by menadione was found to be non-competitive (Figure 51).

K. ENZYMATIC SYNTHESIS OF 8,8'-DIOXO-6,6'-AZOPURINE (DOAP)

The investigation of the interactions between 6,6'-azopurine disodium salt (NaAP) and rabbit liver aldehyde oxidase showed that 8,8'-dioxo-6,6'-azopurine was the product of oxidation of NaAP by the aldehyde oxidase.

When rabbit liver aldehyde oxidase was added to the solution containing NaAP (Chapter III, Section 0), a brown-orange precipitate of the complex of aldehyde oxidase and DOAP (AO:DOAP) was formed in the solution. The AO:DOAP complex was then broken up by dissolving it in distilled \( \text{H}_2\text{O} \) and acidifying the solution with 5 N HCl to pH 2.0. The resulting solution was centrifuged and DOAP was isolated as a bright-orange precipitate (Charts 3 and 4).
The enzymatically synthesized DOAP and chemically synthesized DOAP showed the exact same uv characteristics (Figure 55) and $R_f$ values in three solvent systems (Table 35). The uv characteristics of the AO:DOAP couple is shown in Figure 53.
CHAPTER V
DISCUSSION
Synthesis of new, biologically active purines has long been an object of research because of their obvious biochemical properties.

This investigation has established the chemical structures, physical properties and some preliminary biological properties of purine dimers.

The parent member of a new class of purine compounds, which have been named the oxoazopurines, was first isolated as an orange adenine chromophore in 1956 by Professor Joseph R. Davis. The unsubstituted azopurine itself was subsequently synthesized in 1970 by A. Giner-Sorolla, Sloan-Kettering Institute of Cancer Research, New York.

Employing uv, ir, and nmr spectroscopic techniques and preparing functional group derivatives, the structure of the orange adenine chromophore as shown in Figure 1 was confirmed to be 8,8'-dioxo-6,6'-azopurine monohydrate (DOAP). Partial reduction of this azopurine gave a hydrazo derivative having the chemical structure (Figure 3) as 8,8'-dioxo-6,6'-hydrazopurine monohydrate (DOHP). Complete
reduction of DOAP with sodium hydrosulfite/NaOH gave 8-hydroxyadenine, thus confirming that DOAP was a dimer containing two molecules of 8-hydroxyadenine joined by an azo bond between C₆ and C₆'. The structure of DOAP was further confirmed by preparing a sodium salt of the azoxy derivative of DOAP, 8,8'-dioxo-6,6'-azoxypurine hexahydrate trisodium salt (NaDOAXP) shown in Figure 4.

Additional evidence for the structure of DOAP was provided by its enzymatic synthesis (Figure 52) from 6,6'-azopurine disodium salt (NaAP). Rabbit liver aldehyde oxidase is known to oxidize purines selectively at the C₈ position. Thus, the carbon atoms at the 8 and 8' positions of NaAP are oxidized by rabbit liver aldehyde oxidase to give DOAP, confirming the fact that the oxo groups in the molecule of DOAP are on the C₈ and C₈'.

The structure of DOHP was confirmed on the basis of its synthesis by the partial reduction of DOAP, and its slow oxidation to DOAP in absolute ethanol (Figure 5). Similarly, the structure of NaDOAXP was confirmed on the basis of its synthesis by oxidation of DOAP, and its slow reduction to DOAP in acidic as well as in aqueous solution (Figure 15).

The IR spectroscopic investigation of the purine monomers and dimers showed that the C-H absorption in the re-
region from 3700 to 2200 cm\(^{-1}\) was almost obscured by very broad hydrogen bonded peaks (Tables 13 and 14). The free N-H stretch in the region between 3200 and 3500 cm\(^{-1}\) was found to be present in all the oxoazopurines except NaDOAXP. Broad absorptions with decreasing intensities were found in the region between 3000 and 2200 cm\(^{-1}\), characteristic of N\(_{7}\)-H stretch. All compounds except NaDOAXP showed these features, indicating that N\(_{7}\)-H has formed a hydrogen bond with the azoxy oxygen. Carbonyl absorptions were also found to be absent in the ir spectrum of NaDOAXP because both of the carbonyls at C\(_{8}\) and C\(_{8}'\), exist predominantly in the enolate configuration with the electron density on the oxygen (Spinner, 1960).

Carbonyl absorptions for the purine derivatives, azopurines, and oxoazopurines were found between 1600 and 1760 cm\(^{-1}\), depending on the position of the carbonyl group. The carbonyl groups at C\(_{2}\) in the molecules of 2-oxoadenine, 2,8-dioxoadenine, xanthine (2,6-dioxopurine), and uric acid (2,6,8-trioxopurine) were found between 1680 and 1710 cm\(^{-1}\) (Table 14), while those at C\(_{6}\) were found between 1600 cm\(^{-1}\) and 1660 cm\(^{-1}\). The carbonyl groups at C\(_{8}\) in the molecules of 2,8-dioxoadenine, uric acid, 8,8'-dioxo-6,6'-azopurine (DOAP), and 8,8'-dioxo-6,6'-hydrazopurine (DOHP) were found at 1750, 1700, and 1750 cm\(^{-1}\), respectively. Thus, the ir
frequencies of the carbonyl groups were found to increase in an order as: $\ce{C_6=O}$ (1600-1660 cm$^{-1}$), $\ce{C_2=O}$ (1680-1710 cm$^{-1}$), and $\ce{C_8=O}$ (1700-1760 cm$^{-1}$).

It has been shown that in aromatic azo compounds with the trans configuration, the N=N band occurs between 1410 and 1440 cm$^{-1}$ and, for those with the cis configuration, it occurs between 1511 and 1576 cm$^{-1}$ (Bellamy, 1968). Both 8,8'-dioxo-6,6'-azopurine (DOAP) and 6,6'-azopurine disodium salt, have an N=N band at 1590 and 1535 cm$^{-1}$, respectively, indicating that both of these compounds exist in the cis configuration.

The nuclear magnetic resonance spectra of the purine monomers and dimers studied during this investigation consisted of one or two sharp absorption signals for the aromatic C$_2$-H protons between $\delta$ 8.34 and 9.08 ppm, while those for C$_8$-H protons were between $\delta$ 8.26 and 8.64 ppm (Table 12). This is consistent with the literature data (Schweizer, et al., 1964; Coburn, Jr., et al., 1965; Hruska, et al., 1968, and Twamoh et al., 1973). The imidazole N$_7$-H signals for DOAP, DOHP, and NaDOAXP appeared as broad signals at low field between $\delta$ 11.78 and 12.84 and for HP it was found at $\delta$ 13.42 ppm. The imidazole N$_7$-H signal for adenine (6-aminopurine), hypoxanthine (6-oxopurine), and xanthine (2,6,-dioxopurine) were found between $\delta$ 13.28 and
13.76 ppm. Twanmoh (1973) reported the imidazole N-H signals for a series of purine derivatives between δ 13.2 to 13.5 ppm which was found to be consistent with our data. The signals from some of the purine dimers containing two imidazole N₇-H protons appeared as a singlet. These protons were characterized by the proper integral intensities and by deuterium oxide exchange experiments.

The two amino NH₂ protons of adenine were found at δ 7.36 ppm, however, when two molecules of adenine were dimerized through a hydrazo linkage on C₆ and C₆', as in 6,6'-bisadenine (HP), the N-H protons of the hydrazo linkage shifted downfield to δ 10.04 ppm. (The shift of a proton signal to a lower value of δ ppm is called upfield shift, while that towards a higher value of δ ppm is called the downfield shift.) The carbonyl group is known for its positive shielding effect (Jackman, 1964; Lichtenberg et al., 1971, and Twanmoh et al., 1973) and moves the adjacent N₇-H signal upfield. Thus, the shielding effect of carbonyl groups at the 8 and 8' positions in the molecule of DOHP was found to have considerable effect on the hydrazo N-H protons of DOHP and were found upfield at δ 8.92 ppm. The amide N₁-H proton of hypoxanthine (6-oxopurine) was found at δ 12.88 ppm, however, when an additional carbonyl group is added, as in xanthine (2,6-dioxopurine), the amide N₁-H
moves upfield to $\delta$ 11.20 ppm. In the molecule of 8,8'-dioxo-6,6'-azopurine (DOAP), the amide $N_9$-H protons were found at $\delta$ 12.22 ppm but in 8,8'-dioxo-6,6'-hydrazopurine (DOHP), they were found to be shifted upfield to $\delta$ 10.42 ppm, as a result of deshielding by the hydrazo N-H protons.

During these studies, it was observed that the moisture content of the solvent (DMSO-d$_6$) had a significant effect on the N-H signals. A fresh bottle of DMSO-d$_6$ was used for each experiment and the moisture content of the solvent was determined by scanning it first. Only the solvent with a minimum amount of moisture was utilized to dissolve the compounds.

It has been shown that the orange adenine chromophore (i.e. DOAP), can only be obtained from adenine and adenine nucleoside and nucleotides and that the formation of the orange adenine chromophore was dependent on the concentration of KBr, $H_2$SO$_4$, and KMnO$_4$ in the reaction medium (Davis and Morris, 1963). Attempts to synthesize DOAP from 2-oxoadenine, 8-oxoadenine, 2,8-dioxoadenine, and adenine-$N^1$-oxide have failed. These observations indicate that the presence of an amino group at the C$_6$ position and the absence of electron withdrawing groups (i.e. oxogroup) at the $N_1$, C$_2$, and C$_8$ positions of the purine ring are essential for the formation of DOAP.
The mechanism of the reaction, through which DOAP was synthesized, seems unique. Oxidation of adenine by KMnO$_4$ with KBr, in acidic medium, appears to be most likely at the C$_8$ position in view of the fact that purines are known to selectively form highly reactive C$_8$ halogen atoms that can give rise to the 8-oxo derivative via acid hydrolysis (Brown, 1971). Manganese dioxide (MnO$_2$) has been shown to cause the dimerization of two molecules of aromatic primary amine through an azo bond (March, 1968). Thus, the oxidation of the amino groups at the C$_6$ position of two molecules of adenine, most likely by MnO$_2$ formed in the reaction medium, and the subsequent dimerization, may form the 8,8'-dioxo-6,6'-azopurine (DOAP) molecule.

Urinary excretion of 8,8'-dioxo-6,6'-azopurine (DOAP) after a single subcutaneous injection of 25 mg/kg, was apparently biphasic. The apparent biphasic nature of the excretion of DOAP in rat urine indicates that DOAP binds with protein in vivo. It has been demonstrated, in our laboratory, that DOAP binds with bovine serum albumin, human plasma proteins, Baker's yeast RNA, and calf thymus DNA in varying degrees, in vitro (Fareed et al., 1975). The unbound DOAP is excreted during the first phase which ends at 14 hr after the subcutaneous injection.

At the end of the first phase, the concentration of
DOAP in the body of the rat is very low and the DOAP-protein complexes dissociate releasing free DOAP. This newly released DOAP makes more DOAP available for excretion, hence is responsible for the second phase which reaches a maximum at 18 hr after the injection.

It was observed that 8,8'-dioxo-6,6'-hydrazopurine (DOHP) appears in the rat urine within 24 hr after a single subcutaneous injection of 25 mg/kg 8,8'-dioxo-6,6'-azo-purine (DOAP). Thus, it can be concluded from the studies of the metabolism of DOAP in rat that, after a single subcutaneous injection of 25 mg/kg, 1) DOAP may bind with serum and other proteins, and 2) DOAP is partially reduced to a hydrazo derivative, DOHP, therefore, indicating that the rat liver azoreductase system is playing a significant role in metabolizing DOAP.

DOHP may not be the final metabolite of DOAP and could be further degraded to 8-hydroxyadenine by reductive cleavage in vivo. This possibility can be explored by examining urinary excretion of 2,8-dihydroxyadenine, since it has been shown that 8-hydroxyadenine is oxidized to 2,8-dihydroxyadenine by xanthine oxidase (Wyngaarden and Dunn, 1957).

The effect of 8,8'-dioxo-6,6'-azopurine (DOAP) on the urinary excretion of xanthine and hypoxanthine, as shown in
Figure 60, confirms the observation that DOAP is an inhibitor of xanthine oxidase in vivo. The levels of xanthine and hypoxanthine in the rat urine remained elevated until 48 hr after the subcutaneous injection of 25 mg/kg DOAP.

The mechanism of the inhibition of xanthine oxidase by DOAP in vivo can be postulated to be: 1) DOAP may be binding to xanthine oxidase in vivo, and thus, inactivating it, 2) 8-hydroxy adenine, formed from the reductive cleavage of DOAP, may be competing with xanthine and hypoxanthine for the substrate sites on the enzyme. As indicated above, these postulations can be further confirmed by examining urinary excretion of 2,8-dihydroxyadenine.

The excretion of uric acid does not follow the same as that pattern followed by the excretion of xanthine and hypoxanthine after the injection of DOAP. The uric acid excretion in the rat urine decreased until 24 hr after the injection but returned to normal at the end of 48 hr. The excretion of uric acid dropped again, to a lesser extent, until the end of 96 hr and then returned to normal. This fall and rise in the uric acid excretion indicates that, in addition to the inhibition of xanthine oxidase, DOAP may have the same effect on the enzyme uricase. Effect of DOAP on the metabolism of xanthine and hypoxanthine in rat can be summarized as follows, where X indicates inhibition:
The extent of the inhibition of xanthine oxidase may be greater than that of the inhibition of uricase until 24 hr after the injection of DOAP, thus, the levels of xanthine and hypoxanthine were found to be increased at this time. However, after 24 hr, the extent of inhibition of uricase may be greater than that of xanthine oxidase. This may compensate for the loss of uric acid, bringing its levels back to normal.

Urinary excretion of creatinine decreased, while the urine output increased after the injection of DOAP, indicating that DOAP affects the glomerular filtration rate.

Therefore, these preliminary studies indicate that, a detailed study of the metabolism of DOAP in rat will elucidate the mechanism of inhibition of xanthine oxidase and uricase in vivo, identify the azoreductase system responsible for the metabolism of azopurines which in turn will lead to the development of DOAP or its derivatives as ther-
apeutic agents used for the treatment of a number of diseases involving impaired purine metabolism.

Aldehyde oxidase appears only in the soluble fraction of a liver homogenate, hence is of cytoplasmic origin (Lemberg, 1936, Mahler et al., 1954, and Rajagopalan et al., 1962). It was shown that each mole of rabbit liver aldehyde oxidase contains 2 moles of FAD, approximately 2 moles of coenzyme Q₁₀, 8 gm-atoms of non-heme iron, and 2 gm-atoms of molybdenum (Rajagopalan et al., 1962). The physiological compound most rapidly oxidized by rabbit liver aldehyde oxidase is N¹-methylnicotinamide (Knox, 1946). The products of the N¹-methylnicotinamide oxidation by liver aldehyde oxidase in a number of mammals have been found to be (Felsted and Chaykin, 1967) N¹-methyl-4-pyridone-3-carboxamide (4-pyridone) and N¹-methyl-2-pyridone-5-carboxamide (2-pyridone). The oxidation of both N¹-methylnicotinamide and pyridoxal is the physiological function of a single liver enzyme, aldehyde oxidase (Stanulovic and Chaykin, 1971b).

The substrate specificity and the manner in which individual purines are oxidized by the three molybdenum containing flavoproteins, i.e. aldehyde oxidase, xanthine oxidase, and xanthine dehydrogenase, depend on the electronic structures of the purines as well as on the environment of
the substrate-binding site of the enzyme. Thus, whereas purine is initially oxidized to hypoxanthine by xanthine oxidase, it is converted to 8-hydroxypurine by aldehyde oxidase and xanthine dehydrogenase. Xanthine is the most efficient substrate for bovine milk xanthine oxidase and xanthine dehydrogenase but is not a substrate for aldehyde oxidase. Hypoxanthine is oxidized to xanthine by aldehyde oxidase and to 6,8-dihydroxypurine by xanthine dehydrogenase, whereas 7-methylhypoxanthine is oxidized to 7-methylxanthine by both enzymes. These examples indicate that the manner of attack and the ultimate product of the enzymic purine oxidation are largely determined by the functional arrangement at the enzymatic binding site and the manner in which this interacts with substrate.

During the investigation of the structure and function of iron-flavoproteins, Handler, Rajagopalan, and Aleman (1964), demonstrated that the pathway of internal electron transport in aldehyde oxidase as:

\[
\text{SUBSTRATE} \rightarrow Mo^{+6} \rightarrow + \rightarrow FAD \rightarrow CoQ_{10} \rightarrow \text{Fe} \rightarrow O_2
\]

Electron paramagnetic resonance studies showed that, the iron atoms behave as an "electron sink", and it was also concluded that, in the internal electron transport sequence of the rabbit liver aldehyde oxidase, iron is the
carrier closest to the site of oxygen reduction while molybdenum is the most remote (Greenlee et al., 1962). The sequence of the electron transport was further confirmed by Palmer et al. (1963).

The fact that the molybdenum atoms of the xanthine and aldehyde oxidase are the first components of these enzymes to be reduced explains the manner of their oxidizing attacks on their substrates.

It has been suggested that both aldehyde oxidase and xanthine oxidase may properly be regarded as hydroxylases rather than dehydrogenases (Perault et al., 1961; Handler et al., 1964, and Rajagopalan et al., 1964b). The "hydroxylase" reactions can be visualized as the simultaneous removal of a hydride ion from the carbon atom which is oxidized by the replacement of a hydroxyl ion from the reaction medium. Presence of a group on the enzyme which could serve as a Lewis acid would facilitate such an attack.

This role is played by molybdenum (Greenlee and Handler, 1963). Competitive inhibitors of rabbit liver aldehyde oxidase such as phenazine methosulfate, 1-(4-pyridyl)pyridinium, and formycin B also serve as substrates. Phenazine methosulfate is oxidized to 3-hydroxyphenazine methosulfate (Johns, 1967), 1-(4-pyridyl)pyridinium is oxidized to 1-(4-pyridyl)-4'-pyridone (Rajagopalan and Handler, 1964b), and
formycin B is oxidized to oxoformycin B (Tsukada et al., 1969).

6,6'-Azopurine disodium salt was found to be a competitive inhibitor of the $N^1$-methylnicotinamide oxidase activity of the rabbit liver aldehyde oxidase and was also found to act as a substrate. This finding indicates that $N^1$-methylnicotinamide and 6,6'-azopurine disodium salt compete for a same site on the enzyme. The competitive inhibitors of rabbit liver aldehyde oxidase react with sulfhydryl groups near the substrate-binding site of the enzyme (Rajagopalan and Handler, 1964b). Thus, the flow of the electrons during the oxidation of 6,6'-azopurine disodium salt (NaAP) by rabbit liver aldehyde oxidase can be visualized as:

$$
\text{NaAP} \rightarrow \text{Mo}^{4+} + \text{FAD} \rightarrow \text{CoQ}_{10} \rightarrow \text{Fe} \rightarrow \text{O}_2
$$

Aldehyde oxidase has also been isolated from human liver. Human liver aldehyde oxidase activity for the hydroxylation of nonaldehydic heterocyclic compounds is lower than that in the rabbit or guinea pig liver aldehyde oxidase. Like the rabbit enzyme, the human enzyme catalyzes the 3-hydroxylation of phenazine methosulfate and the 6-hydroxylation of $N^1$-methylnicotinamide but the mechanism of hydroxylation of both of these compounds is different for
the human enzyme. The hydroxylation of phenazine methosulfate and N\textsuperscript{1}-methylnicotinamide by human liver aldehyde oxidase is inhibited only by agents with affinity for the substrate-binding site such as cyanide and N-alkylphenothiazines, and not by agents which inhibit the internal electron transport chain of the enzyme such as menadione and diethylstilbestrol (Johns, 1967). This mode of oxidation is unique to substrates with a positively charged nitrogen. The hydroxylation of other nonaldehydic heterocyclic substrates follow the conventional mechanism as discussed earlier.

Aldehyde oxidase has also been isolated from potato tubers (Solanum tuberosum) by Rothe (1974) and from Avena coleoptile (Rajagopal, 1971). The difference between the aldehyde oxidase from plants and liver tissues of animals is that the enzyme from livers can use ferricyanide and 2,6-dichlorophenol indophenol as an electron acceptor, while the enzyme from plants cannot.

The investigation of the genetic basis of the variation in activity of the mammalian liver aldehyde oxidase showed that, in mice, the magnitude of the enzyme activity is controlled by autosomal alleles (one of a pair, or any one of a series, of genes having the same locus on homogeneous chromosomes) acting in an additive manner. The ac-
tivity of enzyme was found to be under genetic control, while the level of the enzyme activity was found to be regulated by the endocrine system (Gluecksohn-Waelsch et al., 1967, and Huff and Chaykin, 1967).

6,6'-azopurine disodium salt (NaAP) is the first example of a purine dimer that can react with rabbit liver aldehyde oxidase. This is a very significant finding because it has been suggested that controlled inhibition of aldehyde oxidase will reduce the cytotoxic effects of the immunosuppressive agent azathioprine and modify its chemotherapeutic effects in order to develop more effective treatment schedules (Chalmers et al., 1969). It can be postulated that in vivo investigation of the inhibition of mammalian aldehyde oxidase by 6,6'-azopurine disodium salt will provide a beneficial approach to find such an agent.

Because of the significance of aldehyde oxidase in the metabolism of a variety of biologically active N-heterocyclic compounds, 6,6'-azopurine disodium salt can be used in the investigation of the mechanism of the action of these compounds.

It has been shown in our laboratory that 8,8'-dioxo-6,6'-azopurine (DOAP) inhibits bovine milk xanthine oxidase and forms a blue chromogen. This interaction of DOAP with xanthine oxidase does not produce any change on DOAP mole-
cule and the chromogenic coupling may involve a non-protein component of xanthine oxidase. DOAP has also been found to bind with bovine serum albumin (BSA).

6,6'-Azopurine disodium salt, when oxidized by rabbit liver aldehyde oxidase, forms a brown-orange complex. This complex contains DOAP, possibly bound to the protein, and DOAP was isolated from this complex by dissolving it in distilled water. The structure of the protein complex of DOAP would also be very interesting as compared to the structure of the blue chromogen of DOAP and xanthine oxidase, and the DOAP-BSA complex. The elucidation of the structures of these complexes would 1) establish the conformation of the binding sites involved, 2) explain the nature of the interactions between oxoazopurines and molybdenum containing metalloflavo proteins, 3) throw some light on the mechanism of the reaction of oxoazopurines with these macromolecules, 4) help to explain the possible biological functions of aldehyde oxidase in vivo, and finally, 5) will resolve the continuing conundrum of the complementarity of biological functions of aldehyde oxidase and xanthine oxidase in vivo, since it has been shown on many occasions that they share some of their biological activities (Stanulovic, 1971a).

Knowledge of the conformation of the substrate-binding
site would also assist in the design of specific active site directed inhibitors with potential value in slowing the metabolic inactivation of pharmacologically active substrates for the enzyme.

8,8'-Dioxo-6,6'-azopurine (DOAP) is not adequately soluble in physiological buffers which seriously limits the investigation of its in vivo biological properties. 6,6'-Azopurine disodium is very soluble in these buffers, and hence can be administered at any desired dose. The aldehyde oxidase in liver would then convert it into 8,8'-dioxo-6,6'-azopurine, therefore, the investigation of its biological properties in vivo can be satisfactorily carried out.

The metabolic role of aldehyde oxidase is still under investigation. Three possible physiological functions for this enzyme have been postulated. The first is that aldehyde oxidase may be serving a protective function as a non-specific drug-metabolizing enzyme. It has been shown, for example, that the 7-hydroxylation and subsequent biologic inactivation of the anti-neoplastic agent methotrexate by aldehyde oxidase in rabbit proceeds so rapidly that, for all practical purposes, a toxic level of this compound for this species cannot be obtained (Redetzki et al., 1966; Johns and Loo, 1967).
A second possibility is that the nonphysiologic heterocyclic compounds which serve as substrates that can be hydroxylated by this enzyme bear a structural resemblance to an as yet unidentified physiologic nonaldehydic heterocyclic substrate for this enzyme (Johns, 1967). 6,6'-Azopurine disodium salt (NaAP), unique in its chemical structure, could serve this purpose if its presence in the body is demonstrated. Administration of an inhibitor of aldehyde oxidase at an adequate dosage should result in an increased urinary excretion, the hypothetical substrate, or NaAP. A third role postulated for aldehyde oxidase is the oxidation of aromatic aldehydes arising from dietary sources, or occurring in the course of intermediary metabolism (Johns, 1967).

As mentioned earlier, in depth study of the interactions between oxoazopurines and azopurines and human liver aldehyde oxidase will certainly help establish the metabolic function of human liver aldehyde oxidase.

The biochemical lesion in most cases of gout has not been elucidated, however, the major biochemical feature of gout is an elevated level of urate in the serum. Inhibition of xanthine oxidase by agents like allopurinol is being used as one of the therapeutic measures to decrease the serum urate levels. 8,8'-Dioxo-6,6'-azopurine (DOAP) is a
very potent xanthine oxidase inhibitor in vitro as well as in vivo. Thus, DOAP has a great potential of being developed as a therapeutic xanthine oxidase inhibitor in the treatment of gout.
CHAPTER VI
SUMMARY
CHAPTER VI

SUMMARY

1. 8,8'-DIOXO-6,6'-AZOPURINE MONOHYDRATE (DOAP)

A. Synthesis:
   i) Adenine dissolved in sulfuric acid was reacted with potassium bromide, potassium permanganate, and hydrogen peroxide. A bright-orange microcrystalline precipitate was isolated.
   ii) From the enzymatic oxidation of 6,6'-azo-purine disodium salt (NaAP) by rabbit liver aldehyde oxidase.

B. Chemical properties:
   i) Empirical formulae:
      at room temp.: \( \text{C}_{10}\text{H}_6\text{N}_10\text{O}_2\cdot\text{H}_2\text{O} \)
      at 105°C/1 hr/N\(_2\): \( \text{C}_{10}\text{H}_6\text{N}_10\text{O}_2 \)
   ii) Partially reduced to a hydrazo compound 8,8'-dioxo-6,6'-hydrazopurine monohydrate (DOHP).
   iii) Complete reductive cleavage resulted in 8-hydroxyadenine.

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iv) Air oxidation in alkali gave an azoxy compound, 8,8'-dioxo-6,6'-azoxypurine hexahydrate trisodium salt (NaDOAXP).

C. Physical properties:

i) Bright-orange microcrystals.

ii) Decomposition point $>300^\circ$C.

iii) uV characteristics:

- abs. EtOH: 230 (17.1 X $10^3$), 302 (5.6 X $10^3$), 442 (11.5 X $10^3$), and 490 (7.4 X $10^3$).
- 0.1 N KOH: 220 (25.2 X $10^3$), 245 (20.7 X $10^3$), and 513 (16.3 X $10^3$).
- 0.05 M potassium phosphate: 215 (15.8 X $10^3$), 242 (14.3 X $10^3$), 345 (4.8 X $10^3$), and 455 (9.0 X $10^3$).

iv) IR:

$C=O$ : 1760 cm$^{-1}$

$-N=H-$ : 1590 cm$^{-1}$

v) $R_f$ values:

- abs. EtOH: Pyridine: Dist. H$_2$O
  
  67 : 20 : 13 = 0.168

- n-Propanol: Conc. NH$_3$: Dist. H$_2$O
  
  60 : 30 : 10 = 0.267

- abs. EtOH: 0.5 M Ammonium acetate
  
  5 : 2 = 0.297
vi) nmr, δ ppm:
\[ C_2^\text{2-}H = 8.96, \text{amide } N_9^\text{2-}H = 12.22, \]
\[ \text{imidazole } N_7^\text{2-}H = 12.84. \]

D. Biochemical and Pharmacological properties:

i) A potent inhibitor of xanthine oxidase in vivo.

ii) Reduced to a hydrazo derivative, possibly by liver azoreductase system in vivo, and was excreted as such in rat urine.

iii) Inhibited rabbit liver aldehyde oxidase, to a lesser extent, in vitro.

iv) Was synthesized from the oxidation of 6,6'-azopurine disodium salt (NaAP) by rabbit liver aldehyde oxidase.

2. 8,8'-DIOXO-6,6'-HYDRAZOPURINE MONOHYDRATE (DOHP)

A. Synthesis:

i) From a partial reduction of 8,8'-dioxo-6,6'-azopurine monohydrate (DOAP by SnCl\(_2\)/HCl.

B. Chemical properties:

i) Empirical formulae:

\[ \text{at room temp.: } C_{10}H_8N_{10}O_2.H_2O \]
\[ \text{at } 100^\circ\text{C/1 hr/N}_2: C_{10}H_8N_{10}O_2. \]
ii) Slowly oxidized in alcoholic solution to 8,8'-dioxo-6,6'-azopurine (DOAP).

iii) Air oxidation in alkali gave an azoxy compound, 8,8'-dioxo-6,6'-azoxypurine hexahydrate trisodium salt (NaDOAXP).

C. Physical properties:

i) Tan-yellow precipitate.

ii) Decomposition point >300°C.

iii) Uv characteristics:

abs. EtOH: 280 (22.2 X 10³).
0.1 N HCl: 210 (34.5 X 10³), 287 (17.8 X 10³).

iv) Ir:

C=O : 1750 cm⁻¹

v) Rf values:

abs. EtOH: Pyridine: Dist. H₂O

67 : 20 : 13 = 0.194

n-Propanol: Conc. NH₃: Dist. H₂O

60 : 30 : 10 = 0.278

abs. EtOH: 0.5 M Ammonium acetate

5 : 2 = 351

vi) nmr, 6 ppm:

D. Biological properties:
   i) Excreted in rat urine as a metabolite of 8,8'-dioxo-6,6'-azopurine (DOAP).

3. 8,8'-DIOXO-6,6'-AZOXYPURINE HEXAHYDRATE TRISODIUM SALT (NaDOAXP)

A. Synthesis:
   i) From air oxidation of DOAP in alkali.

B. Chemical properties:
   i) Empirical formulae:
      at room temp.: \( \text{C}_{10}\text{H}_{3}\text{N}_{10}\text{O}_{3}\text{Na}_{3}\cdot6\text{H}_{2}\text{O} \)
      at 165°C/1 hr/N: \( \text{C}_{10}\text{H}_{3}\text{N}_{10}\text{O}_{3}\text{Na}_{3} \)
   ii) Reduced to 8,8'-dioxo-6,6'-azopurine (DOAP) instantly in acidic solution, and slowly in aqueous solution.

C. Physical properties:
   i) Cherry-red precipitate.
   ii) Decomposition point >300°C.
   iii) Uv characteristics:
      abs. EtOH: 245 (8.4 X 10^3), 367 (2.7 X 10^3), 416 (4.0 X 10^3), and 490 (5.5 X 10^3).
      0.1 N KOH: 220 (30.4 X 10^3), 245 (22.4 X 10^3), and 513 (18.8 X 10^3).
   iv) Ir:
-N=N- : 1540 cm\(^{-1}\)

v) \(R_f\) values:
   abs. EtOH: Pyridine: Dist. H\(_2\)O
   \(67 : 20 : 13\) = 0.164

   n-Propanol: Conc. NH\(_3\): Dist. H\(_2\)O
   \(60 : 30 : 10\) = 0.286

vi) nmr, \(\delta\) ppm:
   \(C_2-H = 8.56,\) imidazole \(N_7-H = 11.78.\)

4. \(6,6'\)-AZOPURINE HEMIHYDRATE DISODIUM SALT (NaAP)

A. Synthesis: (Giner-Sorolla, 1970)
   i) Air oxidation of \(6,6'\)-bisadenine in alkali.

B. Chemical properties: (Giner-Sorolla, 1970)
   i) Empirical Formula: \(C_{10}H_{14}N_{10}Na_2 \cdot \frac{1}{2} H_2O.\)
   ii) Aqueous solution decomposed when treated with dilute acid.

C. Physical properties:
   i) Red needles.
   ii) Melting point > 350°C.
   iii) Uv characteristics:
      abs. EtOH: 216 (21.7 \(\times\) 10\(^3\)), 416 (9.9 \(\times\) 10\(^3\)).
      0.1 N KOH: 220 (14.6 \(\times\) 10\(^3\)), 232 (15.6 \(\times\) 10\(^3\)), and 407 (9.5 \(\times\) 10\(^3\)).
0.05 M potassium phosphate: 212 (17.8 X 10^3), 392 (8.7 X 10^3).

iv) Ir:
\[ -N=N- : 1530 \text{ cm}^{-1} \]

v) \( R_f \) values:
- abs. EtOH: Pyridine: Dist. H₂O
  67 : 20 : 13 = 0.248
- abs. EtOH: 0.5 M Ammonium acetate
  5 : 2 = 0.773

vi) nmr, 6 ppm:
\[ C_2-H = 8.82, C_8-H = 8.38. \]

D. Biological properties:

i) Potent inhibition of rabbit liver aldehyde oxidase in vitro, causing 50% inhibition at a final concentration \((I)_{50}\) of 2.6 X 10^{-6} M, and with an inhibitor constant \((K_I)\) of 3.33 X 10^{-6} M at pH 7.8.

ii) Acted as substrate for rabbit liver aldehyde oxidase and was oxidized to 8,8'-dioxo-6,6'-azopurine (DOAP).

5. 6,6'-BISADENINE MONOHYDRATE (HP)

A. Synthesis: (Giner-Sorolla, 1970)

i) 6-Chloropurine in 50% aqueous ethanol and
6-hydrazinopurine in water were refluxed at 70°C for 6 hr.

B. Chemical properties: (Giner-Sorolla, 1970)

i) Empirical Formula: $\text{C}_{10}\text{H}_8\text{N}_{10}\cdot\text{H}_2\text{O}$.

ii) Alkaline solution turned red due to air oxidation (transformation into the azo derivative).

iii) Uv characteristics:

- abs. EtOH: 211 (6.1 x $10^3$), 272 (4.3 x $10^3$), and 345 (3.2 x $10^3$).
- 0.1 N HCl: 210 (24.3 x $10^3$), 274 (22.6 x $10^3$), and 338 (7.2 x $10^3$).

iv) $R_f$ values:

- abs. EtOH: Pyridine: Dist. H$_2$O
  - 67 : 20 : 13 = 0.236
- n-Propanol: Conc. NH$_3$: Dist. H$_2$O
  - 60 : 30 : 10 = 0.495

v) nmr, δ ppm:

$\text{C}_2$-H = 8.52, $\text{C}_8$-H = 8.52, imidazole N$_7$-H = 13.42, and hydrazo N-H = 10.04.

6. 6,6'-AZOXYPURINE DIHYDRATE DISODIUM SALT (NaAXP)

A. Synthesis: (Giner-Sorolla, 1970)

i) 6-Hydroxylamine was suspended in concen-
treated ammonium hydroxide for 5 days and precipitate was treated with sodium hydroxide.

B. Chemical properties: (Giner-Sorolla, 1970)

i) Empirical formula: $\text{C}_{10}\text{H}_4\text{N}_{10}\text{Na}_2\text{O}_2 \cdot \text{H}_2\text{O}$.

ii) Aqueous solution decomposed when treated with dilute acid.

C. Physical properties:

i) Red needles.

ii) Melting point $>350^\circ\text{C}$.

iii) UV characteristics:

abs. EtOH: $216 (21.5 \times 10^3), 416 (9.6 \times 10^3)$.

0.1 N KOH: $220 (15.7 \times 10^3), 232 (16.0 \times 10^3)$, and $407 (9.9 \times 10^3)$.

iv) IR:

$-\text{N=N-} : 1530 \text{ cm}^{-1}$

v) $R_f$ values:

abs. EtOH: Pyridine: Dist. H$_2$O

$67 : 20 : 13 = 0.248$

abs. EtOH: 0.5 M Ammonium acetate

$5: 2 = 0.773$

vi) nmr, $\delta$ ppm:

$\text{C}_2-\text{H} = 9.08, \text{C}_8-\text{H} = 8.64$. 
CHAPTER VII

FIGURES
Figure 1

Chemical structure of a parent member of a new class of compounds, i.e. oxoazopurines. 8,8'-dioxo-6,6'-azo-purine monohydrate (DOAP) was synthesized by reacting adenine dissolved in H$_2$SO$_4$ with KBr, KMnO$_4$ and H$_2$O$_2$. 
8,8'-DIOXO-6,6'-AZOPURINE MONOHYDRATE

(DOAP)

Figure 1
Figure 2

Keto-enol tautomerism of 8,8'-dioxo-6,6'-azopurine (DOAP)
KETO-ENOL TAUTOMERISM OF 8,8'-DIOXO-6,6'-AZOPURINE (DOAP)

Figure 2
Chemical structure of a hydrazo derivative of 8,8'-dioxo-6,6'-azopurine monohydrate (DOAP). It was synthesized by partial reduction of DOAP using SnCl₂/HCl at 70°C, and has the structure of 8,8'-dioxo-6,6'-hydrazopurine monohydrate (DOHP).
8,8'-DIOXO-6,6'-HYDRAZOPURINE MONOHYDRATE
(DOHP)

Figure 3
Chemical structure of sodium salt of an azoxy derivative of 8,8'-dioxo-6,6'-azopurine monohydrate (DOAP). It was synthesized by air oxidation of DOAP dissolved in 0.005 N NaOH and also of that of DOHP dissolved in 0.5 N NaOH and has the structure of 8,8'-dioxo-6,6'-azoxypurine hexahydrate trisodium salt (NaDOAXP).
8,8'-DIOXO-6,6'-AZOXYPURINE HEXAHYDRATE TRISODIUM SALT

(NaDOAXP)
The oxidation of 8,8'-dioxo-6,6'-hydrazopurine (DOHP) to 8,8'-dioxo-6,6'-azopurine (DOAP) was followed by monitoring the decrease in the optical density at 282 nm of a 10 µg/ml solution of DOHP in absolute ethanol at room temperature. No significant change in the optical density at 282 nm ($\lambda_{max}$ of DOHP) was found up to 30 min. After 30 min, the optical density at 282 nm started to decrease while that at 442 nm ($\lambda_{max}$ of DOAP) started to increase. After standing at room temperature for 48 hr the optical density at 282 nm completely disappeared and that at 442 nm reached a peak, indicating that DOHP was converted to DOAP.
OXIDATION OF 8,8'-DIOXO-6,6'-HYDRAZOPURINE (DOHP) TO 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) IN ABSOLUTE ETHANOL

λ max (nm)

DOAP (azo) 230, 302, 442, (490)
DOHP (hydr) 280, 442, (490)

Figure 5
Figure 6

Chemical structure of 6,6'-azopurine hemihydrate disodium salt (NaAP). Source: Giner-Sorolla, 1970.
6,6'-AZOPURINE HEMIHYDRATE DISODIUM SALT

(NaAP)

Figure 6
Figure 7

Chemical structure of 6,6'-bisadenine monohydrate (HP). Source: Giner-Sorolla, 1970.
6,6'-BISADENINE MONOHYDRATE
(6,6'-HYDRAZOPURINE MONOHYDRATE)
(HP)

Figure 7
Figure 8

Chemical structure of 6,6'-azoxy purine dihydrate disodium salt (NaAXP). Source: Giner-Sorolla, 1970.
6,6'-AZOXYPURINE DIHYDRATE DISODIUM SALT
(NaAXP)

Figure 8
Figure 9

Separation of authentic samples of purine derivatives on a 40 x 0.9 cm glass column packed with AG 50W-X8 (H⁺ form), 200-400 mesh resin. The column was eluted with a gradient system having a reservoir containing 2000 ml of 5 N HCl and a mixing chamber with 500 ml of distilled water. The running pressure was adjusted to give a constant flow rate of 1 tube containing 3 ml of the eluent per 5 min (0.6 psi). A total of 350 tubes were collected on an automatic fraction collector. Optical densities of these tubes were read at 208, 215, 250, 260, 280, and 305 nm, using a Beckman DB-G recording spectrophotometer.

Abbreviations used: 2,8-DOAd= 2,8-Dioxoadenine, 8-0Ad= 8-Oxoadenine, 2-0Ad= 2-Oxoadenine, Ad= Adenine, and DOHP= 8,8'-Dioxo-6,6'-hydrazopurine monohydrate.
Figure 9
Reductive cleavage of 25 mg of 8,8'-dioxo-6,6'-azo-purine (DOAP) dissolved in 150 ml of 1 N NaOH was carried out by reacting it with 1.0 gm of sodium hydrosulfite (Na₂S₂O₄). The reaction mixture was refluxed at 80°C for 1 hr. A 4 ml aliquote of the resulting yellow solution was neutralized to pH 7.0 by adding 1 N HCl, adjusted to equivalent of 0.5 N HCl and was applied to a 21.0 X 0.9 cm glass column packed with AG 50W-X8, 200-400 mesh, H⁺ form resin. The column was eluted with a gradient of HCl equipped with a reservoir containing 2000 ml of 5 N HCl and a mixing chamber containing 500 ml of distilled water. The running pressure was adjusted to a constant flow rate of 1 tube containing 3 ml of the eluent per 5 min (0.6 psi). A total 350 tubes were collected on an automatic fraction collector. The optical densities of each tube was determined at 208, 215, 250, 260, 280, and 350 nm, using a Beckman DB-G spectrophotometer.
SODIUM HYDROSULFITE/NaOH REDUCTIVE CLEAVAGE OF 8,8'-DIOXO-8,8'-AZOPURINE

Figure 10
Figure 11

Uv spectra of authentic sample of oxoadenine compounds in 1 N HCl.
UV SPECTRA IN 1 N HCl

AUTHENTIC SAMPLES OF OXOADENINE COMPOUNDS

max. abs., nm

---

- 2-OXOADENINE 283
- 8-OXOADENINE 280 (267)
- 2,8-DIOXOADENINE 305 (232)

WAVELENGTH, nm

Figure 11
Figure 12

UV spectra of AG 50W-X8 chromatographic fraction no. 127 obtained from \( \text{Na}_2\text{S}_2\text{O}_4/\text{NaOH} \) reductive cleavage of 8,8'-dioxo-6,6'-azopurine (DOAP).
Figure 12

AG 50W-X8 CHROMATOGRAPHIC FRACTION NO. 127 OBTAINED FROM Na2S2O4/NaOH REDUCTIVE CLEAVAGE OF 6,6'-DIOXO-6,6'-AZOPURINE

max. abs., nm
280 (267)
Figure 13

UV spectra of authentic sample of 8-oxoadenine in 1 N NaOH and dimethylsulfoxide (DMSO).
Figure 13

UV SPECTRA IN 1 N NaOH AND DIMETHYLSULFOXIDE (DMSO)

AUTHENTIC SAMPLES OF 8-OXOADENINE

max abs., nm

1 N NaOH 224, 280
DMSO 272
Figure 14

Uv spectra of 8-oxoadenine obtained from Na$_2$S$_2$O$_4$/NaOH reductive cleavage of 8,8'-dioxo-6,6'-azopurine and isolated from a AG 50W-X8 chromatographic column in 1 N NaOH and dimethylsulfoxide (DMSO).
8-OXOADENINE obtained from
Na$_2$S$_2$O$_4$/NaOH reductive cleavage
of 8,8'-dioxo-6,6'-azopurine and
isolated from a AG 50W-X8
chromatographic column

max. abs., nm

- - - 1N NaOH 224, 280
- - - DMSO 272

*Tube numbers 123-130 which were
dried under a nitrogen stream at
40°C yielding a yellow solid

Figure 14
Interconversion of oxoazopurines and the structure of the product of the reductive cleavage of 8,8'-dioxo-6,6'-azopurine (DOAP).
Figure 15
Figure 16

Nuclear magnetic resonance spectrum of 8,8'-dioxo-6,6'-azopurine monohydrate (DOAP).
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF
8,8'-DIOXO-6,6'-AZOPURINE MONOHYDRATE (DOAP)

Nucleus: $^1$H  
Instrument: Varian A-60A  
Frequency: 60  
Reference: TMS  
Solvent: DMSO-d$_6$  
Temp. ($^\circ$C): probe  
Sweep rate (Hz/sec): 4  
Conc. (mg/ml): 20  
Formula: C$_{10}$H$_6$N$_{10}$O$_2$.H$_2$O

Assignments:

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM (δ) relative to TMS</th>
<th>int. area</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.90</td>
<td>22</td>
<td>1</td>
<td>2 C$_2$-H protons</td>
</tr>
<tr>
<td>b</td>
<td>12.26</td>
<td>20</td>
<td>1</td>
<td>2 amide N$_9$-H protons</td>
</tr>
<tr>
<td>c</td>
<td>12.72</td>
<td>21</td>
<td>1</td>
<td>2 imidazole N$_7$-H protons</td>
</tr>
</tbody>
</table>

Figure 16
Figure 17

Nuclear magnetic resonance spectrum of 8,8′-dioxo-6,6′-azopurine monohydrate (DOAP): D₂O exchange.
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF
8,8'-DIOXO-6,6'-AZOPURINE MONOHYDRATE (DOAP)
(+ 25 µl D₂O)

| Nucleus: ¹H | Instrument: Varian A-60A | Frequency: 60 |
| Reference: TMS | Solvent: DMSO-d₆ | Conc. (mg/ml): 20 |

Temp. (°C): probe Sweep rate (Hz/sec): 4

Formula: C₁₀H₆N₁₀O₂·H₂O

<table>
<thead>
<tr>
<th>Peak PPM (δ) relative to TMS</th>
<th>int. area ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a 8.90</td>
<td></td>
<td>2 C₂-H protons</td>
</tr>
</tbody>
</table>

Figure 17
Figure 18

Nuclear magnetic resonance spectrum of 8,8'-dioxo-6,6'-hydrazopurine monohydrate (DOHP).
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF 8,8'-DIOXO-6,6'-HYDRAZOPURINE MONOHYDRATE (DOHP)

Nucleus: $^1$H  
Instrument: Varian A-60A  
Frequency: 60

Reference: TMS  
Solvent: DMSO-d$_6$  
Conc. (mg/ml): 20

Temp. ($^\circ$C):  
Sweep rate (Hz/sec): 4  
Probe:  
Formula: C$_{16}$H$_8$N$_{10}$O$_2$·H$_2$O

Assignments:

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM ($\delta$) relative to TMS</th>
<th>Int. area in mm.</th>
<th>Integer ratio</th>
<th>Type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.34</td>
<td>41</td>
<td>1</td>
<td>2 C$_2$-H protons</td>
</tr>
<tr>
<td>b</td>
<td>8.92</td>
<td>42</td>
<td>1</td>
<td>2 hydrazo H-N-N-H protons</td>
</tr>
<tr>
<td>c</td>
<td>10.42</td>
<td>40</td>
<td>1</td>
<td>2 amide N$_2$-H protons</td>
</tr>
<tr>
<td>d</td>
<td>11.88</td>
<td>39</td>
<td>1</td>
<td>2 imidazole N$_7$-H protons</td>
</tr>
</tbody>
</table>

Figure 18
Figure 19

Nuclear magnetic resonance spectrum of 8,8'-dioxo-6,6'-hydrazopurine monohydrate (DOHP). D$_2$O exchange.
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF
8,8'-DIOXO-6,6'-HYDRAZOPURINE MONOHYDRATE (DOHP)

(+ 25 µl D2O)

Nucleus: 1H  Instrument: Varian A-60A  Frequency: 60
Reference: TMS  Solvent: DMSO-d6  Conc. (mg/ml): 20
Temp. (°C): probe sweep rate (Hz/sec): 4

Formula: C10H8N10O2·H2O

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM (δ) relative to TMS</th>
<th>int. area</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.34</td>
<td>-</td>
<td>-</td>
<td>2 C2-H protons</td>
</tr>
</tbody>
</table>

Figure 19
Figure 20

Nuclear magnetic resonance spectrum of 8,8'-dioxo-6,6'-azoxypurine hexahydrate trisodium salt (NaDOAXP).
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF
8,8'-DIOXO-6,6'-AZOXPURINE HEXAHYDRATE TRISODIUM SALT (NaDOAXP)

Nucleus: \(^{1}\text{H}\)  
Instrument: Varian A-60A  
Reference: TMS  
Solvent: DMSO-\(d_6\)  
Temp. (\(^{\circ}\)C): probe  
Sweep rate (Hz/sec): 4  
Frequency: 60  
Conc. (mg/ml): 20  
Formula: C\(_{10}\)H\(_{10}\)N\(_{10}\)O\(_3\)Na\(_3\),6\(\text{H}_2\text{O}\)

Assignments:

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM ((\delta)) relative to TMS</th>
<th>int. area in mm.</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.56</td>
<td>35.5</td>
<td>2</td>
<td>2 (\text{C}_2)-H protons</td>
</tr>
<tr>
<td>b</td>
<td>11.78</td>
<td>17.5</td>
<td>1</td>
<td>1 imidazole</td>
</tr>
</tbody>
</table>

Figure 20
Figure 21

Nuclear magnetic resonance spectrum of 8,8'-dioxo-6,6'-azoxyprine hexahydrate trisodium salt (NaDOAXP): D$_2$O exchange.
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF
8,8'-DIOXO-6,6'-AZOXYPURINE HEXAHYDRATE TRISODIUM SALT (NaDOAXP)
(+ 25 µl D₂O)

Nucleus: \(^1\)H  
Instrument: Varian A-60A  
Frequency: 60  
Reference: TMS  
Solvent: DMSO-d₆  
Temp. (°C): 4  
Probe type:  
Sweep rate (Hz/sec): 4

Conc. (mg/ml): 20  
Formula: C\(_{10}\)H\(_{10}\)N\(_{10}\)O\(_3\)Na\(_3\)·6H\(_2\)O

Assignments:

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM (δ) relative to TMS</th>
<th>int. area</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.56</td>
<td>-</td>
<td>2</td>
<td>CH₂dSOCd₃</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H₂O</td>
</tr>
</tbody>
</table>

Figure 21
Figure 22

Nuclear magnetic resonance spectrum of 6,6'-azopurine hemihydrate disodium salt (NaAP).
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF
6,6'-AZOPURINE HEMIHYDRATE DISODIUM SALT (NaAP)

Nucleus: $^1$H  
Instrument: Varian A60-A  
Frequency (MHz): 60

Reference: TMS  
Solvent: DMSO-d$_6$  
Conc. (mg/ml): 20

Temp. ($^\circ$C), probe  
Sweep rate (Hz/sec): 4  
Formula: C$_{10}$H$_4$N$_{10}$Na$_2$.tH$_2$O

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM (6) relative to TMS</th>
<th>int. area in mm.</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.42</td>
<td>18</td>
<td>1</td>
<td>2 C$_8$-H protons</td>
</tr>
<tr>
<td>b</td>
<td>8.84</td>
<td>18</td>
<td>1</td>
<td>2 C$_2$-H protons</td>
</tr>
</tbody>
</table>

![Figure 22](image-url)
Figure 23

Nuclear magnetic resonance spectrum of 6,6’-azopurine hemihydrate disodium salt (NaAP): D₂O exchange.
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF
6,6'-AZOPURINE HEMIHYDRATE DISODIUM SALT (NaAP)

(+ 25 µl D₂O)

Nucleus: \(^1\)H  
Instrument: Varian A-60A  
Frequency (MHz): 60

Reference: TMS  
Solvent: DMSO-d₆  
Conc. (mg/ml): 20

Temp. (°C): probe Sweep rate (Hz/sec): 4

Formula: C₁₀H₄N₁₀Na₂·H₂O

### Assignments:

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM (δ) relative to TMS</th>
<th>int. area (in mm.)</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.32</td>
<td>19</td>
<td>1</td>
<td>2 C₈-H protons</td>
</tr>
<tr>
<td>b</td>
<td>8.76</td>
<td>19</td>
<td>1</td>
<td>2 C₂-H protons</td>
</tr>
</tbody>
</table>

Figure 23
Figure 24

Nuclear magnetic resonance spectrum of 6,6'-bisadine nine monohydrate (HP).
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF
6,6'-BISADENINE MONOHYDRATE (HP)

<table>
<thead>
<tr>
<th>Nucleus: (^1H)</th>
<th>Instrument: Varian A60-A</th>
<th>Frequency (MHz): 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference: TMS</td>
<td>Solvent: DMSO-d(_6)</td>
<td>Conc. (mg/ml): 20</td>
</tr>
<tr>
<td>Temp. (°C): probe</td>
<td>Sweep rate (Hz/sec):</td>
<td>Formula: C(_{10})H(<em>8)N(</em>{10}).H(_2)O</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assignments:</th>
<th>PPM (δ) relative to TMS</th>
<th>int. area in mm.</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.52</td>
<td>63</td>
<td>2</td>
<td>2 C(_2)-H and 2 C(_8)-H protons</td>
</tr>
<tr>
<td>b</td>
<td>10.04</td>
<td>31</td>
<td>1</td>
<td>2 hydrazo H-N-H protons</td>
</tr>
<tr>
<td>c</td>
<td>13.42</td>
<td>26</td>
<td>1</td>
<td>2 imidazole N(_7)-H protons</td>
</tr>
</tbody>
</table>

Figure 24
Figure 25

Nuclear magnetic resonance spectrum of 6,6'-azoxy-purine dihydrate disodium salt (NaAXP).
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF

6,6'-AZOXYPURINE DIHYDRATE DISODIUM SALT

(NaAXP)

Nucleus: $^1$H  
Instrument: Varian A60-A  
Frequency (MHz): 60

Reference: TMS  
Solvent: DMSO-d$_6$  
Conc. (mg/ml): 20

Temp. (°C): probe
Sweep rate (Hz/sec): 4

Formula: C$_{10}$H$_{10}$N$_{10}$O$_7$Na$_2$.2H$_2$O

Assignments:

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM (δ) relative to TMS</th>
<th>Int. area in mm</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.64</td>
<td>32</td>
<td>1</td>
<td>2 C$_6$-H protons</td>
</tr>
<tr>
<td>b</td>
<td>9.04</td>
<td>29</td>
<td>1</td>
<td>2 C$_2$-H protons</td>
</tr>
</tbody>
</table>

Figure 25
Figure 26

Nuclear magnetic resonance spectrum of adenine (6-aminopurine).
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF

ADENINE

Nucleus: $^1$H
Reference: TMS
Temp. ($^\circ$C): probe

Instruments: Varian A60-A
Solvent: DMSO-d$_6$
Sweep rate (Hz/sec): 4

Conc. (mg/ml): 20
Formula: C$_5$H$_5$N$_5$.

**Assignments:**

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM ($\delta$) relative to TMS</th>
<th>int. area in mm.</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>7.36</td>
<td>60</td>
<td>2</td>
<td>2 amino NH$_2$ protons</td>
</tr>
<tr>
<td>b</td>
<td>8.44</td>
<td>59</td>
<td>2</td>
<td>1 C$_2$-H and 1 C$_9$-H proton</td>
</tr>
<tr>
<td>c</td>
<td>13.28</td>
<td>29</td>
<td>1</td>
<td>1 imidazole N$_7$-H proton</td>
</tr>
</tbody>
</table>

**Figure 26**
Figure 27

Nuclear magnetic resonance spectrum of hypoxanthine (6-oxopurine).
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF HYPOXANTHINE

Nucleus: \(^1\)H
Instrument: Varian A60-A
Reference: TMS
Solvent: DMSO-\(d_6\)
Temp. (°C), probe
Sweep rate (Hz/sec): 4

Frequency (MHz): 60
Conc. (mg/ml): 20
Formula: \(C_5H_4N_4O\)

Assignments:

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM (δ) relative to TMS</th>
<th>int. area in mm.</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.28</td>
<td>40</td>
<td>1</td>
<td>1 C₆-H proton</td>
</tr>
<tr>
<td>b</td>
<td>8.43</td>
<td>41</td>
<td>1</td>
<td>1 C₇-H proton</td>
</tr>
<tr>
<td>c</td>
<td>12.88</td>
<td>35</td>
<td>1</td>
<td>1 amide N₁-H proton</td>
</tr>
<tr>
<td>d</td>
<td>13.76</td>
<td>39</td>
<td>1</td>
<td>1 imidazole N₇-H proton</td>
</tr>
</tbody>
</table>

Figure 27
Figure 28

Nuclear magnetic resonance spectrum of xanthine (2,6-dioxopurine).
NUCLEAR MAGNETIC RESONANCE SPECTRUM OF XANTHINE

Nucleus: $^1$H  
Instrument: Varian A60-A  
Frequency (MHz): 60

Reference: TMS  
Solvent: DMSO-d$_6$  
Conc. (mg/ml): 20

Temp. (°C): probe sweep rate (Hz/sec): 4

Frequency (MHz): 60

Cone. (mg/ml): 20

Formula: $C_9H_4N_4O_2$

<table>
<thead>
<tr>
<th>Peak</th>
<th>PPM (δ) relative to TMS</th>
<th>int. area in mm.</th>
<th>integer ratio</th>
<th>type of protons present</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>8.26</td>
<td>38.5</td>
<td>1</td>
<td>1 C$_6$-H proton</td>
</tr>
<tr>
<td>b</td>
<td>11.20</td>
<td>41.0</td>
<td>1</td>
<td>1 amide N$_1$-H proton</td>
</tr>
<tr>
<td>c</td>
<td>11.92</td>
<td>37.5</td>
<td>1</td>
<td>1 amide N$_3$-H proton</td>
</tr>
<tr>
<td>d</td>
<td>13.76</td>
<td>39.0</td>
<td>1</td>
<td>1 imidazole N$_7$-H proton</td>
</tr>
</tbody>
</table>

Figure 28
Figure 29

UV spectra of 8,8'-dioxo-6,6'-hydrazopurine (DOHP), 8,8'-dioxo-6,6'-azopurine (DOAP), and 8,8'-dioxo-6,6'-azoxypurine trisodium salt (NaDOAXP) in absolute ethanol obtained at appropriate concentrations on a Beckman DB-G recording spectrophotometer.
UV SPECTRA OF 8,8'-DIOXO-6,6'-HYDRAZOPURINE (DOHP),
8,8'-DIOXO-6,6'-AZOPURINE (DOAP), AND 8,8'-DIOXO-
6,6'-AZOXYPURINE TRISODIUM SALT (NaDOAXP) IN abs. EtOH

Figure 29
Figure 30

Uv spectra of 6,6'-bisadenine (HP), 6,6'-azopurine disodium salt (NaAP) and 6,6'-azoxypurine disodium salt (NaAXP) in absolute ethanol obtained at appropriate concentrations on a Beckman DB-G recording spectrophotometer.
UV SPECTRA OF 6,6'-BISADENINE (HP), 6,6'-AZOPURINE DISODIUM SALT (NaAP), AND 6,6'-AZOPYRINE DISODIUM SALT (NaAXP) IN ABS. ETH.

Figure 30
Figure 31

Uv spectra of 8,8'-dioxo-6,6'-hydrazopurine (DOHP), 8,8'-dioxo-6,6'-azopurine (DOAP), and 8,8'-dioxo-6,6'-azoxypurine trisodium salt (NaDOAXP) in 0.1 N KOH obtained at appropriate concentrations on a Beckman DB-G recording spectrophotometer.
UV SPECTRA OF 8,8'-DIOXO-6,6'-HYDRAZOPURINE (DOHP) 8,8'-DIOXO-6,6'-AZOPURINE (DOAP), AND 8,8'-DIOXO-6,6'-AZOXYPURINE TRISODIUM SALT (NaDOAXP) IN 0.1 N KOH

![UV Spectra Graph](Image)

**Figure 31**
Figure 32

Uv spectra of 6,6'-bisadenine (HP), 6,6'-azopurine disodium salt (NaAP) and 6,6'-azoxypurine disodium salt (NaAXP) in 0.1 N KOH obtained at appropriate concentrations on a Beckman DB-G recording spectrophotometer.
UV SPECTRA OF 6,6'-BISADENINE (HP), 6,6'-AZOPURINE DISODIUM SALT (NaAP), AND 6,6'-AZOXYPURINE DISODIUM SALT (NaAXP) IN 0.1 N KOH

Figure 32
Figure 33

Uv spectra of 8,8'-dioxo-6,6'-hydrazopurine (DOHP) and 6,6'-bisadenine (HP) in 0.1 N HCl obtained at appropriate concentrations on a Beckman DB-G recording spectrophotometer.
UV SPECTRA OF 5,5'-DIOXO-5,5'-HYDROZOPURINE (DOHP) AND
5,5'-BISADENINE (HP) IN 0.1 N HCl

Figure 33
Figure 34

Uv spectra of 8,8'-dioxo-6,6'-azopurine (DOAP) and 6,6'-azopurine disodium salt (NaAP) in 0.05 M potassium phosphate, pH 7.8 obtained at appropriate concentrations on a Beckman DB-G recording spectrophotometer.
UV SPECTRA OF 8,8'-DIOXO-8,8'-AZOPURINE (DOAP) AND 8,8'-AZOPURINE DISODIUM SALT (NaAP) IN 0.05 M POTASSIUM PHOSPHATE, pH 7.8

- 25 µg/ml DOAP
- 455 nm
- (345) nm
- 242 nm
- 215 nm
- 25 µg/ml NaAP
- 392 nm
- 212 nm

Figure 34
Figure 35

Uv spectra of $8.0 \times 10^{-5}$ M 8,8'-dioxo-6,6'-azopurine (DOAP) in 0.05 M potassium phosphate at pHs 6.2, 6.6, 7.0, 7.4, 7.8 and 8.2 were obtained on a Beckman DB-G recording spectrophotometer.
Figure 35
Figure 36

UV spectra of 8,8'-dioxo-6,6'-azopurine (DOAP) in 0.05 M potassium phosphate containing 0.005% EDTA, pH 7.8 obtained at appropriate concentration on a Beckman DB-G recording spectrophotometer.
UV SPECTRUM OF 8,8'-DIOXO-8,8'-AZOPURINE IN 0.05M POTASSIUM PHOSPHATE CONTAINING 0.005% EDTA, pH 7.8

max. abs., nm
455
(242)
242
215

Figure 36
Effect of pH on stability of 8,8'-dioxo-6,6'-azo-purine (DOAP) in 0.05 M potassium phosphate buffer at 25°C. Optical density at 455 nm of 8.0 × 10^{-5} M DOAP in 0.05 M potassium phosphate was monitored against the buffer for 10 min using a chart speed of 1 inch/min.
EFFECT OF pH ON ITALITY OF OAP IN O. OOS ISOIUIIAl. I'HOIPHATI IUFFER, 25°C

Figure 37

150 µl of 500 µg/ml DOP in 0.005M NaOH added to 2.85 ml of 0.05M Potassium Phosphate Buffer for a final conc. of 8 x 10^-5M DOAP.
Effect of pH on stability of 8,8'-dioxo-6,6'-azo-purine (DOAP) in 0.05 M potassium phosphate buffer at 25°C. Optical density at 455 nm of $8.0 \times 10^{-5}$ M DOAP in 0.05 M potassium phosphate was monitored against the buffer for 60 min using a chart speed 0.1 inch/min.
EFFECT OF pH ON STABILITY OF DOAP IN
0.05M POTASSIUM PHOSPHATE BUFFER, 25°C

pH 8.2

150 μl of 500 μg/ml DOAP in 0.005M NaOH added to 2.85 ml of 0.05M Potassium Phosphate Buffer for a final conc. of 8 x 10⁻⁷ M DOAP

pH 7.8

pH 7.4

pH 6.2

pH 6.0

pH 6.6

min. after addition of DOAP

Figure 38
Figure 39

Uv spectra of $8.0 \times 10^{-5}$ M $6,6'$-azopurine disodium salt (NaAP) in 0.05 M potassium phosphate at pHs 6.2, 6.6, 7.0, 7.4, 7.8, and 8.2 were obtained on a Beckman DB-G recording spectrophotometer.
Figure 39
Figure 40

Effect of pH on stability of 6,6'-azopurine disodium salt (NaAP) in 0.05 M potassium phosphate buffer at 25°C. Optical density at 390 nm of $8.0 \times 10^{-5}$ M NaAP in 0.05 M potassium phosphate was monitored against the buffer for 10 min using a chart speed of 1 inch/min.
EFFECT OF pH ON STABILITY OF NaAP IN 0.05M POTASSIUM PHOSPHATE BUFFER, 25°C.

Figure 40
Figure 41

Effect of pH on stability of 6,6'-azopurine disodium salt (NaAP) in 0.05 M potassium phosphate buffer at 25°C. Optical density at 390 nm of 8.0 \times 10^{-5} \text{ M} \text{NaAP} \text{in} \text{0.05 M potassium phosphate} \text{ was monitored against the buffer for 60 min using a chart speed of 0.1 inch/min.}
Figure 41
A$_{300}$ method used to investigate the in vitro inhibition of rabbit liver aldehyde oxidase by 6,6'-azopurine disodium salt (NaAP). Each experimental cuvette contained 2.25 ml of 0.05 M potassium phosphate buffer containing 0.005% EDTA, pH 7.8, 0.25 ml of 0.06 M solution of N$_1$-methylnicotinamide chloride, and 0.25 ml of 2.5 mg/ml solution of NaAP in 0.05 M potassium phosphate buffer pH 7.8. After a 5 min equilibration period at 25°C, 0.25 ml of 5.04 mg protein/ml rabbit liver aldehyde oxidase was added to the cuvette. The cuvette contents were rapidly mixed, and A$_{300}$ nm was recorded with time against a reference cell from which the substrate had been omitted.
INHIBITION OF RABBIT LIVER ALDEHYDE OXIDASE BY 6,6'-AZOPURINE DISODIUM SALT (NaAP)

(ΔA₃₀₀ METHOD)

0.05 M POTASSIUM PHOSPHATE BUFFER, pH 7.8, 25°C

250 µl OF ALDEHYDE OXIDASE (5.04 mg PROTEIN/ml)

Figure 42
Oxidation of $5.0 \times 10^{-3}$ M $N^1$-methylnicotinamide chloride by rabbit liver aldehyde oxidase. Each experimental cuvette contained 3 ml of reaction mixture containing an appropriate volume of 0.05 M potassium phosphate buffer containing 0.005% EDTA, pH 7.8, and 0.25 ml of 0.06 M solution of $N^1$-methylnicotinamide chloride. After a 5 min equilibration period at $25^\circ$C, 25, 50, 100, 150, 200, and 250 µl of 5.04 mg protein/ml rabbit liver aldehyde oxidase was added to the cuvette and its contents were mixed rapidly. Optical density of this solution at 300 nm was recorded against a reference cell from which the substrate had been omitted.
OXIDATION OF $5.0 \times 10^{-3}$ M $N^1$-METHYLNICOTINAMIDE CHLORIDE BY

RABBIT LIVER ALDEHYDE OXIDASE

Figure 43
Determination of optimal substrate and enzyme concentration for the oxidation of N\(^1\)-methylnicotinamide chloride by the rabbit liver aldehyde oxidase. A procedure same as that described for Figure 43 was used.
Figure 44

RABBIT LIVER ALDEHYDE OXIDASE (AO)

250 μl of AO
(5.04 mg PROTEIN/ml)

100 μl of AO
(5.04 mg PROTEIN/ml)

ΔO.D./min at 300 nm

10^{-4} M N-METHYLNICOTINAMIDE CHLORIDE
Figure 45

Inhibition of rabbit liver aldehyde oxidase by 6,6'-azopurine disodium salt (NaAP) at 25°C. Concentration of NaAP causing a 50% inhibition of rabbit liver aldehyde oxidase in vitro, i.e. (I)_{50} at 7.8. Each experimental cuvette contained an appropriate volume of 0.05 M potassium phosphate buffer containing 0.005% EDTA, pH 7.8, 0.25 ml of 0.06 M solution of N1-methylnicotinamide chloride, and an appropriate volume of 2.5 mg/ml solution of NaAP in 0.05 M potassium phosphate buffer, pH 7.8. After a 5 min equilibration period 0.25 ml of 5.04 mg protein/ml rabbit liver aldehyde oxidase was added to the cuvette. The cuvette contents were rapidly mixed, and A_{300} nm was recorded against a reference cell from which the substrate had been omitted.
RABBIT LIVER ALDEHYDE OXIDASE

(pH 7.8)

\[ (1)_{50} = 2.6 \times 10^{-6} \text{ M} \]

Figure 45
Inhibition of rabbit liver aldehyde oxidase by 6,6'-azopurine disodium salt (NaAP) at 25°C. Concentration of NaAP causing a 50% inhibition of rabbit liver aldehyde oxidase in vitro, i.e. (I)$_{50}$ at pH 8.2. The $\Delta A_{300}$ nm method described for Figure 45 was used.
RABBIT LIVER ALDEHYDE OXIDASE

(pH 8.2)

% INHIBITION OF ALDEHYDE OXIDASE

($\Delta A_{300}$ method)

$(l)_50 = 3.4 \times 10^{-6} M$

$X 10^{-5} \text{M 6,6'-AZOPURINE DISODIUM SALT}$

Figure 46
Inhibition of rabbit liver aldehyde oxidase by menadione at 25°C: Concentration of menadione causing a 50% inhibition of the enzyme in vitro, (I)_{50} at pH 7.8. The A_{300} nm method described for Figure 45 was employed substituting menadione for NaAP.
RABBIT LIVER ALDEHYDE OXIDASE
(pH 7.8)

(1)_50 = 1.1 \times 10^{-7} M

\% INHIBITION OF ALDEHYDE OXIDASE

\( \Delta A_{300} \) method

X \times 10^{-7} M MENADIONE SODIUM BISULFITE SALT

Figure 47
In vitro inhibition of rabbit liver aldehyde oxidase by 8,8'-dioxo-6,6'-azopurine (DOAP) at pH 8.2. The ΔA₃₀₀ nm method described for Figure 45 was employed using DOAP instead of NaAP.
RABBIT LIVER ALDEHYDE OXIDASE
(pH 8.2)

% INHIBITION OF ALDEHYDE OXIDASE

(ΔA300 method)

X 10^{-5} M 8,8'-DIOXO-6,6'-AZOPURINE (DOAP)

Figure 48
Lineweaver-Burk plot of the \textit{in vitro} inhibition of rabbit liver aldehyde oxidase by 6,6'-'azopurine disodium salt (NaAP) at pH 7.8. The $\Delta A_{300}$ nm method described for Figure 42 was used.
RABBIT LIVER ALDEHYDE OXIDASE

250 μl OF ENZYME (5.04 mg PROTEIN/ml)

pH 7.8

$\frac{1}{[S]} \cdot 10^{-2}$

(S = N-METHYLNICOTINAMIDE CHLORIDE)

Figure 49
Figure 50

Dixon plot of the \textit{in vitro} inhibition of rabbit liver aldehyde oxidase by 6,6'-azopurine disodium salt (NaAP) at pH 7.8. The $\Delta A_{300}$ nm method described for Figure 42 was used.
RABBIT LIVER ALDEHYDE OXIDASE

250 µl OF ENZYME (5.04 mg PROTEIN/ml)

pH 7.8

\[
\frac{1}{v} (\Delta A_{300}/\text{min}) \times 10^7
\]

\[
[1] M \times 10^{-5} \quad \text{(I = 6,6'-AZOPURINE DISODIUM SALT)}
\]

Figure 50
Figure 51

Lineweaver-Burk plot of the *in vitro* inhibition of rabbit liver aldehyde oxidase by menadione at pH 7.8. The \( \Delta A_{300} \) nm method described for Figure 45 was used.
RABBIT LIVER ALDEHYDE OXIDASE

250 µl OF ENZYME (5.04 mg PROTEIN/mI)

pH 7.8

\[ \frac{1}{(S)} M^{-1} \times 10^{-2} \quad (S = N^1-METHYLNICOTINAMIDE CHLORIDE) \]

Figure 51
Enzymatic synthesis of 8,8'-dioxo-6,6'-azopurine (DOAP) from the oxidation of 6,6'-azopurine disodium salt (NaAP) by rabbit liver aldehyde oxidase. In a 25-ml glass tube, 2.5 mg NaAP dissolved in 10 ml of dist. H₂O, 7.5 ml of 0.05 M potassium phosphate buffer containing 0.005% EDTA, pH 7.8, 2.5 ml of 5.04 mg protein/ml rabbit liver aldehyde oxidase were incubated at 25°C for 15 min. A brown precipitate was obtained from the reaction mixture, by adding 6.16 gm of solid ammonium sulfate (NH₄)₂SO₄ for a 50% saturation and centrifugation at 8000 rpm for 20 min at 4°C. The brown precipitate was washed twice by dissolving in 25 ml of dist. H₂O acidified with 2 ml of 5 N HCl, and centrifugation at 8000 rpm for 20 min at 4°C. At the end, a bright-orange microcrystalline precipitate having uv characteristics and Rₚ values exactly the same as those of authentic sample of DOAP was obtained.
ENZYMATIC SYNTHESIS OF 8,8'-DIOXO-6,6'-AZOPURINE (DOAP)

RABBIT LIVER ALDEHYDE OXIDASE

0.05 M POTASSIUM PHOSPHATE, pH 7.5, 25°C

6,6'-AZOPURINE DISODIUM SALTS → 8,8'-DIOXO-6,6'-AZOPURINE

Figure 52
Uv spectra of the reaction mixture containing 250 μl of 5.04 mg protein/ml rabbit liver aldehyde oxidase, 250 μl of 0.25 mg/ml 6,6'-azopurine disodium salt (NaAP), and 2.5 ml of 0.05 M potassium phosphate buffer containing 0.005% EDTA, pH 7.8 at 0 min (before adding enzyme), 15 min and 360 min after adding the enzyme.
ENZYMATIC SYNTHESIS OF 8,8'-DIOXO-8,8'-AZOPURINE
FROM 8,8'-AZOPURINE DISODIUM SALT (NeAP)
(experimental system)

Minutes after adding 250 μl of
5.04 mg/ml Rabbit Liver Aldehyde Oxidase
suspension to a cuvette containing
250 μl of 0.250 mg/ml NeAP in dist. H₂O
+ 2.50 μl of 0.05 M Potassium phosphate,
pH 7.6 containing 0.005% EDTA

---

Figure 53
Figu.re 54

Uv spectra of the reaction mixture containing 250 µl of 0.25 mg/ml 6,6'-azopurine disodium salt (NaAP) in dist. H2O and 2.50 ml of 0.05 M potassium phosphate containing 0.005% EDTA, pH 7.8 at 0 min, 15 min, and 260 min, showing that NaAP under these conditions did not change up to 360 min.
ENZYMATIC SYNTHESIS OF 8,8'-DIOXO-6,6'-AZOPURINE FROM 6,6'-AZOPURINE DISODIUM SALT (NaAP) (control system)

Minutes after adding 250 µl of 0.250 mg/ml NaAP in dist. H2O to 2.50 ml of 0.05 M Potassium phosphate, pH 7.8 containing 0.005% EDTA

0 min
--- 15 min
* * * * * * * * * * * 360 min

Figure 54
Figure 55

Uv spectra of enzymatically synthesized 8,8'-dioxo-6,6'-azopurine (DOAP), chemically synthesized DOAP, and 6,6'-azopurine disodium salt (NaAP) dissolved in absolute ethanol.
ENZYMATIC SYNTHESIS OF 8,8'-DIOXO-8,8'-AZOPURINE (DOAP) FROM 8,8'-AZOPURINE DISODIUM SALT (NaAP)

---

**Figure 55**
Figure 56

Calibration curve for 8,8'-dioxo-6,6'-azopurine (DOAP) in 0.1 N KOH.
CALIBRATION CURVE FOR 8,8'-DIOXO-6,6'-AZOPURINE DISSOLVED IN 0.1 N KOH

Figure 56
Urinary excretion of 8,8'-dioxo-6,6'-azopurine (DOAP) after a single subcutaneous injection of 25 mg/kg in an adult male rat. The urine of the rat was collected on an automatic fraction collector for every 2 hr. It was acidified by adding a drop of 5 N HCl to change the pH of the urine to 2.0 and centrifuged at 600 X g for 10 min to obtain an orange precipitate. The orange precipitate was washed 3 times with dist. H₂O and dissolved in 3.0 ml of 0.1 N KOH. The optical density of this solution was read at 513 nm and the amount of DOAP was calculated from the calibration curve.
2.59

**Figure 57**

**URINARY EXCRETION OF 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) AFTER A SINGLE SUBCUTANEOUS INJECTION OF 25 mg/kg IN AN ADULT MALE RAT**
Effect of a single subcutaneous injection of 25 mg/kg 8,8’-dioxo-6,6’-azopurine (DOAP) dissolved in 0.01 N NaOH on urine output of an adult male rat. After the injection, the rat was placed in a Holdge metabolic cage, and the urine was collected on an automatic fraction collector for every 2 hr for a period of 24 hr.
EFFECT OF A SINGLE SUBCUTANEOUS INJECTION OF 25 mg/kg
8,8'-DIOXO-6,6'-AZOPURINE (DOAP) DISSOLVED IN 0.01 N NaOH
ON URINE OUTPUT OF AN ADULT MALE RAT

Figure 58
Effect of a single subcutaneous injection of 25 mg/kg 8,8'-dioxo-6,6'-azopurine (DOAP) dissolved in 0.01 N NaOH on urine output of an adult male rat. After the injection, the rat was placed in a Holdge metabolic cage, and the urine was collected on an automatic fraction collector for every 2 hr for a period of 7 days.
EFFECT OF A SINGLE SUBCUTANEOUS INJECTION OF 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) DISSOLVED IN 0.01 N NaOH ON URINE OUTPUT OF AN ADULT MALE RAT

Figure 59
Effect of 25 mg/kg 8,8'-dioxo-6,6'-azopurine (DOAP) on urinary excretion of xanthine and hypoxanthine in an adult male rat. To a silica cuvette of 1 cm pathlength, 500 μl of the urine, collected as described in Chapter III, Section J (a), and 1500 μl of M/15 glycine buffer, pH 9.3, 25 μl of 0.4 units/ml uricase was added and the reaction mixture was incubated at room temperature for 15 min. To this solution, 200 μl of 1.5 N NaOH was added and after a 15 min interval, 200 μl of 1.6 N HCl, 300 μl of 0.6 M glycylglycine buffer pH 8.2, and 25 μl of 6.9 units/ml bovine milk xanthine oxidase was added. After an incubation period of 1 hr, 200 μl of 0.6 N NaOH, and 500 μl of 0.7 M glycine buffer, pH 9.4 was added and optical density of this solution was read at 292 nm against a blank which was prepared by using 25 μl of dist. H₂O instead of 6.9 units/ml bovine milk xanthine oxidase.

Then, 25 μl of 0.4 units/ml uricase was added to the reaction mixture while 25 μl of dist. H₂O was added to the blank. After incubating it at room temperature for 1 hr, the optical density of the reaction mixture was read at 292 nm vs blank. The amount of uric acid was calculated from the ΔA₂₉₂ nm as described in Chapter III, Section J (a).
EFFECT OF 25 mg/kg 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) ON URINARY EXCRETION OF XANTHINE AND HYPOXANTHINE IN AN ADULT MALE RAT

Figure 60
Figure 61

Effect of 25 mg/kg 8,8'-dioxo-6,6'-azopurine (DOAP) on uric acid excretion in an adult male rat. 1.0 ml of the urine from the rat was diluted to 10 ml by adding 9.0 ml of dist. H₂O. To 0.5 ml of this diluted urine, 1.0 ml of 0.7 glycine buffer, pH 9.4 and 5.7 ml of dist. H₂O was added. Optical density of this solution was read at 292 nm against dist. H₂O. Then, 10 μl of 0.4 units/ml uricase was added to it and the optical density of the final reaction mixture was read at 292 nm against dist. H₂O. The change in optical density at 292 nm before and after adding the uricase was obtained and the amount of uric acid was calculated from a calibration curve.
EFFECT OF 25 mg/kg 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) ON URINARY URIC ACID EXCRETION IN AN ADULT MALE RAT

Figure 61
Calibration curve for uric acid analysis in adult male rat urine. Change in optical density at 292 nm produced by degrading 5, 10, 20, 30, 40, and 50 μg of uric acid by 10 μl of 0.4 units/ml uricase was obtained employing the same procedure as described for Figure 61.
CALIBRATION CURVE FOR URIC ACID ANALYSIS

IN ADULT MALE RAT URINE

\[ \Delta \text{O.D.} \text{ at 292 nm} \]

\[ \mu g \text{ of URIC ACID} / 7.2 \text{ ml FINAL REACTION VOLUME} \]

Figure 62
Effect of 25 mg/kg 8,8'-dioxo-6,6'-azopurine (DOAP) on urinary excretion of creatinine in an adult male rat. In a 50-ml Erlenmyer flask containing 1.0 ml of the rat urine, 8.0 ml of 1/12 N H₂SO₄ and 1.0 ml of 10% sodium tungstate was added, the contents were mixed by gentle rotation and centrifuged at 600 X g for 10 min. 5.0 ml of the supernatant was added to a tube marked "test" and 5.0 ml of dist. H₂O to a tube marked "blank". 2.5 ml of freshly prepared alkaline picrate reagent was added to both tubes and they were incubated in a waterbath at 25°C for 1 hr. Optical density of the reaction mixture was read vs blank at 520 nm and the amount of creatinine in the urine was calculated from a calibration curve.
EFFECT OF 25 mg/kg 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) ON URINARY EXCRETION OF CREATININE IN AN ADULT MALE RAT

Figure 63
Figure 64

Calibration curve for creatinine analysis in adult male rat urine. 10, 20, 30, 40, 60, 80, and 100 μg of creatinine from a stock solution were subjected to the same procedure as described for Figure 63 and the optical densities at 520 nm were recorded.
CALIBRATION CURVE FOR CREATININE ANALYSIS IN ADULT MALE RAT URINE

Figure 64
Figure 65

Uv spectra of an authentic sample of 8,8'-dioxo-6,6'-hydrazopurine (DOHP) in 1 N HCl.
UV SPECTRUM OF AUTHENTIC SAMPLE OF
8,8'-DIOXO-6,6'-HYDRAZOPURINE (DOHP),
10 μg/ml in 1 N HCl
max. abs., nm
298

Figure 65
Figure 66

Uv spectra of AG 50W-X8 chromatographic fraction no. 196 obtained from urine of a rat subcutaneously injected with 25 mg/kg 8,8'-dioxo-6,6'-azopurine (DOAP) dissolved in 0.01 N NaOH.
AG 50W-X8 CHROMATOGRAPHIC FRACTION NO. 195 OBTAINED FROM URINE OF A RAT SUBCUTANEOUSLY INJECTED WITH 25 mg/kg 8,8'-DIOXO-6,6'-AZOPURINE DISSOLVED IN 0.01 N NaOH

max. abs., nm

290

Figure 66
CHAPTER VIII

CHARTS AND TABLES
Chart 1. SYNTHESIS OF OXOAZOPURINES

(6 mg/ml in 1 N H$_2$SO$_4$, total vol. 2500 ml)

Adenine

KBr (120 ml of 15 mg/ml soln. in dist. H$_2$O)

1 N KMnO$_4$ (800 ml)

30% H$_2$O$_2$ (50 ml added very slowly)

Each added with one hour inter-val while continuously stirring the reaction mixture.

BRIGHT ORANGE SUSPENSION

16 hr at 4°C

BRIGHT ORANGE SUSPENSION

600 X g/15 min

BRIGHT ORANGE PRECIPITATE

wash with 4 N H$_2$SO$_4$, dist. H$_2$O, acetone, dry at 75°C/16 hr

BRIGHT ORANGE MICROCRYSTALS

(DOAP)

SnCl$_2$/HCl

75°C/20 min

0.005 N NaOH

60 hr at 4°C

TAN YELLOW PPT

CHERRY RED PPT

(DOHP) 0.5 N NaOH

bubble air for 2 hr

(NaDOAXP)
Chart 2. **$A_{300}$ METHOD FOR ALDEHYDE OXIDASE ACTIVITY**

### a. CONTROL

<table>
<thead>
<tr>
<th>SAMPLE CELL</th>
<th>REFERENCE CELL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2.50 ml of 0.05 M potassium phosphate buffer, pH 7.8</td>
<td>1'. 2.75 ml of 0.05 M potassium phosphate buffer, pH 7.8</td>
</tr>
<tr>
<td>(equilibrate at 25°C for 5 min using chart speed of 0.5 in/min)</td>
<td></td>
</tr>
<tr>
<td>2. 0.25 ml of 0.06 M N$^{1}$-methylnicotinamide chloride</td>
<td></td>
</tr>
<tr>
<td>(equilibrate at 25°C for 5 min using chart speed of 0.5 in/min)</td>
<td></td>
</tr>
<tr>
<td>3. 0.25 ml of 5.04 mg protein/ml aldehyde oxidase suspension</td>
<td>3'. 0.25 ml of 5.04 mg protein/ml aldehyde oxidase suspension</td>
</tr>
<tr>
<td>(record $A_{300}$ using chart speed of 2 in/min)</td>
<td></td>
</tr>
</tbody>
</table>

### b. TEST

<table>
<thead>
<tr>
<th>SAMPLE CELL</th>
<th>REFERENCE CELL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2.25 ml of 0.05 M potassium phosphate buffer, pH 7.8</td>
<td>1'. 2.50 ml of 0.05 M potassium phosphate buffer, pH 7.8</td>
</tr>
<tr>
<td>(equilibrate at 25°C for 5 min using chart speed of 0.5 in/min)</td>
<td></td>
</tr>
<tr>
<td>2. 0.25 ml of 0.06 M N$^{1}$-methylnicotinamide chloride</td>
<td></td>
</tr>
<tr>
<td>(equilibrate at 25°C for 5 min using chart speed of 0.5 in/min)</td>
<td></td>
</tr>
<tr>
<td>3. 0.25 ml of 2.5 mg/ml NaAP in 0.05 M potassium phosphate buffer, pH 7.8</td>
<td>3'. 0.25 ml of 2.5 mg/ml NaAP in 0.05 M potassium phosphate buffer, pH 7.8</td>
</tr>
<tr>
<td>(equilibrate at 25°C for 5 min using chart speed of 0.5 in/min)</td>
<td></td>
</tr>
<tr>
<td>4. 0.25 ml of 5.04 mg protein/ml aldehyde oxidase suspension</td>
<td>4'. 0.25 ml of 5.04 mg protein/ml aldehyde oxidase suspension</td>
</tr>
<tr>
<td>(record $A_{300}$ using chart speed of 2 in/min)</td>
<td></td>
</tr>
</tbody>
</table>

The 0.05 M potassium phosphate buffer, pH 7.8 used in all the steps contained 0.005% EDTA.
Chart 3

ENZYMATIC SYNTHESIS OF
8,8'-DIOXO-6,6'-AZOPURINE MONOHYDRATE (DOAP)
FROM 6,6'-AZOPURINE DISODIUM SALT (NaAP)

(control)

2.5 mg of NaAP in 10 ml of dist. H₂O

+ 10 ml of 0.05 M potassium phosphate containing 0.005% EDTA, pH 7.8

Incubate at room temp. for 15 min

+ 6.26 gm of solid (NH₄)₂SO₄ (50% saturation)

8000 rmp/20 min at 4°C.

Yellow ppt

Dissolve in 25 ml of dist. H₂O and add 2 ml of 5 N HCl

8000 rmp/20 min at 4°C.

Yellow ppt

Dissolve in 25 ml of dist. H₂O and add 2 ml of 5 N HCl

8000 rmp/20 min at 4°C.
ENZYMATIC SYNTHESIS OF
8,8'-DIOXO-6,6'-AZOPURINE MONOHYDRATE (DOAP)
FROM 6,6'-AZOPURINE DISODIUM SALT (NaAP)

(experimental system)

2.5 mg of NaAP in 10 ml of dist. H₂O

+ 7.5 ml of 0.05 M potassium phosphate containing 0.005% EDTA, pH 7.8

+ 2.5 ml of 5.04 mg protein/ml of rabbit liver aldehyde oxidase

Incubate at room temp. for 15 min

+ 6.26 gm of solid (NH₄)_₂SO₄ (50% saturation)

8000 rmp/20 min at 4°C.

Brown ppt

dissolve in 25 ml of dist. H₂O and add 2 ml of 5 N HCl

8000 rmp/20 min at 4°C.

Orange-Brown ppt

dissolve in 25 ml of dist. H₂O and add 2 ml of 5 N HCl

8000 rmp/20 min at 4°C.

Bright-Orange ppt
### Table 1. ELEMENTAL ANALYSIS DATA FOR DOAP

**I. At Room Temperature (C_{10}H_{6}N_{10}O_{2}.H_{2}O)**

<table>
<thead>
<tr>
<th>Element</th>
<th>Anal. Calcd. %</th>
<th>Found %</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{10}</td>
<td>37.98</td>
<td>37.93</td>
<td>-0.05%</td>
</tr>
<tr>
<td>H_{6}</td>
<td>2.55</td>
<td>2.56</td>
<td>+0.01%</td>
</tr>
<tr>
<td>N_{10}</td>
<td>44.29</td>
<td>44.50</td>
<td>+0.21%</td>
</tr>
<tr>
<td>O_{3}</td>
<td>15.18</td>
<td>14.90</td>
<td>-0.28%</td>
</tr>
</tbody>
</table>

**II. Following Drying At 105° For 1 Hour Under Nitrogen (C_{10}H_{6}N_{10}O_{2})**

<table>
<thead>
<tr>
<th>Element</th>
<th>Anal. Calcd. %</th>
<th>Found %</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{10}</td>
<td>40.23</td>
<td>39.87</td>
<td>-0.41%</td>
</tr>
<tr>
<td>H_{6}</td>
<td>2.03</td>
<td>2.28</td>
<td>+0.25%</td>
</tr>
<tr>
<td>N_{10}</td>
<td>46.97</td>
<td>46.53</td>
<td>-0.44%</td>
</tr>
<tr>
<td>O_{2}</td>
<td>10.73</td>
<td>10.70</td>
<td>-0.03%</td>
</tr>
</tbody>
</table>
Table 2. DETERMINATION OF THE EMPIRICAL FORMULA OF DOAP

<table>
<thead>
<tr>
<th>I. At Room Temperature</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 38.25% ( \div ) 12 = 3.19 ( \div ) 0.93 = 3.43 x 3 = 10.3</td>
<td></td>
</tr>
<tr>
<td>H = 2.58% ( \div ) 1 = 2.58 ( \div ) 0.93 = 2.77 x 3 = 8.3</td>
<td></td>
</tr>
<tr>
<td>N = 44.44% ( \div ) 14 = 3.17 ( \div ) 0.93 = 3.41 x 3 = 10.2 or ( C_{10}H_8N_10O_3 )</td>
<td></td>
</tr>
<tr>
<td>O = 14.80% ( \div ) 16 = 0.93 ( \div ) 0.93 = 1.00 x 3 = 3.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Following Drying At 100° C For 1 Hour Under Nitrogen</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C = 40.02% ( \div ) 12 = 3.34 ( \div ) 0.66 = 5.06 x 2 = 10.1</td>
<td></td>
</tr>
<tr>
<td>H = 2.14% ( \div ) 1 = 2.14 ( \div ) 0.66 = 3.24 x 2 = 6.4 or ( C_{10}H_6N_10O_2 )</td>
<td></td>
</tr>
<tr>
<td>N = 46.20% ( \div ) 14 = 3.30 ( \div ) 0.66 = 5.00 x 2 = 10.0</td>
<td></td>
</tr>
<tr>
<td>O = 10.60% ( \div ) 16 = 0.66 ( \div ) 0.66 = 1.00 x 2 = 2.0</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. DETERMINATION OF WATER OF CRYSTALLIZATION OF
DOAP AT 100° C FOR 1 HOUR UNDER NITROGEN

\[ C_{10}H_6N_{10}O_2 \cdot H_2O \]

<table>
<thead>
<tr>
<th>Anal. Calcd. %</th>
<th>Sample Weight Loss Found %</th>
<th>Vaporized Water* Found %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.69</td>
<td>5.26</td>
<td>5.41</td>
</tr>
</tbody>
</table>

*Absorbed in magnesium perchlorate and the increase in weight of the drying agent measured.
### Table 4. ELEMENTAL ANALYSIS DATA FOR DOHP

#### I. At Room Temperature \((C_{10}H_{8}N_{10}O_{2}.H_{2}O)\)

<table>
<thead>
<tr>
<th></th>
<th>Anal. Calcd. %</th>
<th>Found %</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(_{10})</td>
<td>37.76</td>
<td>37.89</td>
<td>+0.13%</td>
</tr>
<tr>
<td>H(_{10})</td>
<td>3.14</td>
<td>2.89</td>
<td>-0.25%</td>
</tr>
<tr>
<td>N(_{10})</td>
<td>44.02</td>
<td>43.90</td>
<td>-0.12%</td>
</tr>
<tr>
<td>O(_{3})</td>
<td>15.08</td>
<td>15.31</td>
<td>+0.23%</td>
</tr>
</tbody>
</table>

#### II. Following Drying At 100° C For 1 Hour Under Nitrogen \((C_{10}H_{8}N_{10}O_{2})\)

<table>
<thead>
<tr>
<th></th>
<th>Anal. Calcd. %</th>
<th>Found %</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(_{10})</td>
<td>40.02</td>
<td>39.82</td>
<td>-0.10%</td>
</tr>
<tr>
<td>H(_{8})</td>
<td>2.66</td>
<td>2.41</td>
<td>-0.25%</td>
</tr>
<tr>
<td>N(_{10})</td>
<td>46.65</td>
<td>46.36</td>
<td>-0.29%</td>
</tr>
<tr>
<td>O(_{2})</td>
<td>10.66</td>
<td>10.58</td>
<td>-0.08%</td>
</tr>
</tbody>
</table>
### Table 5. DETERMINATION OF THE EMPIRICAL FORMULA OF DOHP

<table>
<thead>
<tr>
<th></th>
<th>At Room Temperature (C\textsubscript{10}H\textsubscript{10}N\textsubscript{10}O\textsubscript{3})</th>
<th></th>
<th>Following Drying At 100\degree C For 1 Hour Under Nitrogen (C\textsubscript{10}H\textsubscript{8}N\textsubscript{10}O\textsubscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C = 37.89% : 12 = 3.16 : 0.96 = 3.29 x 3 = 9.87</td>
<td></td>
<td>C = 39.82% : 12 = 3.32 : 0.66 = 5.03 x 2 = 10.06</td>
</tr>
<tr>
<td></td>
<td>H = 2.89% : 1 = 2.89 : 0.96 = 3.01 x 3 = 9.03</td>
<td></td>
<td>H = 2.41% : 1 = 2.41 : 0.66 = 3.65 x 2 = 7.30</td>
</tr>
<tr>
<td></td>
<td>N = 43.90% : 14 = 3.14 : 0.96 = 3.27 x 3 = 9.81</td>
<td></td>
<td>N = 46.36% : 14 = 3.31 : 0.66 = 5.02 x 2 = 10.04</td>
</tr>
<tr>
<td></td>
<td>O = 15.31% : 16 = 0.96 : 0.96 = 1 x 3 = 3.00</td>
<td></td>
<td>O = 10.58% : 16 = 0.66 : 0.66 = 1 x 2 = 2.00</td>
</tr>
</tbody>
</table>
Table 6. ELEMENTAL ANALYSIS DATA FOR NaDOAXP

I. At Room Temperature (C₁₀H₃N₁₀O₃Na₃.6H₂O)

<table>
<thead>
<tr>
<th></th>
<th>Anal. Calcd. %</th>
<th>Found %</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>24.59</td>
<td>24.99</td>
<td>+0.40%</td>
</tr>
<tr>
<td>H</td>
<td>3.08</td>
<td>3.07</td>
<td>+0.01%</td>
</tr>
<tr>
<td>N</td>
<td>28.68</td>
<td>28.64</td>
<td>-0.04%</td>
</tr>
<tr>
<td>O</td>
<td>29.50</td>
<td>28.69</td>
<td>-0.82%</td>
</tr>
<tr>
<td>Na</td>
<td>14.13</td>
<td>14.39</td>
<td>+0.26%</td>
</tr>
</tbody>
</table>

II. Following Drying At 165°C For 1 Hour Under Nitrogen (C₁₀H₃N₁₀O₃Na₃)

<table>
<thead>
<tr>
<th></th>
<th>Anal. Calcd. %</th>
<th>Found %</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>31.59</td>
<td>31.67</td>
<td>+0.08%</td>
</tr>
<tr>
<td>H</td>
<td>0.80</td>
<td>1.22</td>
<td>+0.42%</td>
</tr>
<tr>
<td>N</td>
<td>36.84</td>
<td>36.65</td>
<td>-0.19%</td>
</tr>
<tr>
<td>O</td>
<td>12.63</td>
<td>12.62</td>
<td>-0.01%</td>
</tr>
<tr>
<td>Na</td>
<td>18.15</td>
<td>18.37</td>
<td>+0.22%</td>
</tr>
</tbody>
</table>
Table 7. DETERMINATION OF THE EMPIRICAL FORMULA OF NaDOAXP

I. At Room Temperature \((C_{10}H_{3}N_{10}O_{3}Na_{3}.6H_{2}O)\)

<table>
<thead>
<tr>
<th>Element</th>
<th>%oretical</th>
<th>%oretical</th>
<th>Calculated</th>
<th>Experimental</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>24.99%</td>
<td>12</td>
<td>2.08</td>
<td>0.63</td>
<td>3.30 x 3</td>
</tr>
<tr>
<td>H</td>
<td>3.07%</td>
<td>1</td>
<td>3.07</td>
<td>0.63</td>
<td>4.87 x 3</td>
</tr>
<tr>
<td>N</td>
<td>28.64%</td>
<td>14</td>
<td>2.05</td>
<td>0.63</td>
<td>3.25 x 3</td>
</tr>
<tr>
<td>O</td>
<td>28.69%</td>
<td>16</td>
<td>1.80</td>
<td>0.63</td>
<td>2.86 x 3</td>
</tr>
<tr>
<td>Na</td>
<td>14.39%</td>
<td>23</td>
<td>0.63</td>
<td>0.63</td>
<td>1.00 x 3</td>
</tr>
</tbody>
</table>

II. Following Drying At 165°C For 1 Hour Under Nitrogen \((C_{10}H_{3}N_{10}O_{3}Na_{3})\)

<table>
<thead>
<tr>
<th>Element</th>
<th>%etheoretical</th>
<th>%oretical</th>
<th>Calculated</th>
<th>Experimental</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>31.67%</td>
<td>12</td>
<td>2.64</td>
<td>0.79</td>
<td>3.34 x 3</td>
</tr>
<tr>
<td>H</td>
<td>1.22%</td>
<td>1</td>
<td>1.22</td>
<td>0.79</td>
<td>1.54 x 3</td>
</tr>
<tr>
<td>N</td>
<td>36.65%</td>
<td>14</td>
<td>2.62</td>
<td>0.79</td>
<td>3.32 x 3</td>
</tr>
<tr>
<td>O</td>
<td>12.62%</td>
<td>16</td>
<td>0.79</td>
<td>0.79</td>
<td>1.00 x 3</td>
</tr>
<tr>
<td>Na</td>
<td>18.37%</td>
<td>23</td>
<td>0.80</td>
<td>0.79</td>
<td>1.01 x 3</td>
</tr>
</tbody>
</table>
Table 8. OXIDATION OF 8,8'-DIOXO-6,6'-HYDRAZOPURINE (DOHP) IN abs. EtOH

<table>
<thead>
<tr>
<th>Time after addition of abs. EtOH</th>
<th>Color of 10 μg/ml soln.</th>
<th>O.D. at 282 nm</th>
<th>O.D. at 442 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 min.</td>
<td>yellow</td>
<td>0.700</td>
<td>0.000</td>
</tr>
<tr>
<td>17 min.</td>
<td>yellow</td>
<td>0.685</td>
<td>0.000</td>
</tr>
<tr>
<td>30 min.</td>
<td>yellow</td>
<td>0.675</td>
<td>0.010</td>
</tr>
<tr>
<td>43 min.</td>
<td>yellow</td>
<td>0.660</td>
<td>0.015</td>
</tr>
<tr>
<td>56 min.</td>
<td>yellow</td>
<td>0.645</td>
<td>0.020</td>
</tr>
<tr>
<td>69 min.</td>
<td>yellow</td>
<td>0.625</td>
<td>0.030</td>
</tr>
<tr>
<td>82 min.</td>
<td>yellow</td>
<td>0.625</td>
<td>0.035</td>
</tr>
<tr>
<td>95 min.</td>
<td>yellow</td>
<td>0.600</td>
<td>0.045</td>
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<tr>
<td>108 min.</td>
<td>yellow</td>
<td>0.575</td>
<td>0.060</td>
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<tr>
<td>121 min.</td>
<td>yellow</td>
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<td>0.070</td>
</tr>
<tr>
<td>151 min.</td>
<td>golden-yellow</td>
<td>0.560</td>
<td>0.070</td>
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<tr>
<td>181 min.</td>
<td>golden-yellow</td>
<td>0.535</td>
<td>0.085</td>
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<tr>
<td>241 min.</td>
<td>golden-yellow</td>
<td>0.485</td>
<td>0.110</td>
</tr>
<tr>
<td>361 min.</td>
<td>golden-yellow</td>
<td>0.400</td>
<td>0.150</td>
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<tr>
<td>601 min.</td>
<td>golden-yellow</td>
<td>0.285</td>
<td>0.225</td>
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<tr>
<td>1441 min.</td>
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<td>1921 min.</td>
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<td>0.000</td>
<td>0.330</td>
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<td>2881 min.</td>
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<td>0.000</td>
<td>0.330</td>
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<tr>
<td>4291 min.</td>
<td>golden-yellow</td>
<td>0.000</td>
<td>0.330</td>
</tr>
<tr>
<td>Compound (Mol. wt)</td>
<td>Absorption Maxima and Molar Ext. Coe. X 10^3</td>
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</tr>
<tr>
<td>-------------------</td>
<td>-------------------------------------------</td>
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<tr>
<td></td>
<td>abs. EtOH</td>
<td>0.1 N KOH</td>
<td>0.1 N HCl</td>
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<tr>
<td>DOAP (311.2)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>230 (17.1)</td>
<td>220 (25.2)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>302 (5.6)</td>
<td>245 (20.7)</td>
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<td>442 (11.5)</td>
<td>513 (16.3)</td>
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</tr>
<tr>
<td></td>
<td>490 (7.4)</td>
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<td></td>
</tr>
<tr>
<td>DOHP (318.26)</td>
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<td>280 (22.2)</td>
<td>-</td>
<td>210 (34.5)</td>
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<tr>
<td>NaDOAXP (488.26)</td>
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<td></td>
<td>245 (8.4)</td>
<td>220 (30.4)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>367 (2.7)</td>
<td>245 (22.4)</td>
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<tr>
<td></td>
<td>416 (4.0)</td>
<td>513 (18.8)</td>
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<td></td>
<td>490 (5.5)</td>
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Table 10. ULTRAVIOLET CHARACTERISTICS OF AZOPURINES

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorption Maxima and Molar Ext. Coe. X 10^3</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>NaAP (319.2)</td>
<td>216 (21.7)</td>
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<td>416 (9.9)</td>
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<td>HP (286.28)</td>
<td>211 (6.1)</td>
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<td>272 (4.3)</td>
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<td>345 (3.2)</td>
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<tr>
<td>NaAXP (362.0)</td>
<td>216 (21.5)</td>
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<tr>
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<td>416 (9.6)</td>
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Table 11. $R_f$ VALUES OF OXOAZOPURINES

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<thead>
<tr>
<th>Solvent System</th>
<th>DOAP</th>
<th>DOHP</th>
<th>NaDOAXP</th>
<th>NaAP</th>
<th>HP</th>
<th>NaAXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>abs. EtOH : Pyridine : Dist. H$_2$O</td>
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<tr>
<td>67 : 20 : 13</td>
<td>0.17</td>
<td>0.19</td>
<td>0.16</td>
<td>0.25</td>
<td>0.24</td>
<td>0.25</td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
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<tr>
<td>n-Propanol : Conc. NH$_3$ : Dist. H$_2$O</td>
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<td></td>
</tr>
<tr>
<td>60 : 30 : 10</td>
<td>0.27</td>
<td>0.28</td>
<td>0.29</td>
<td>-</td>
<td>0.50</td>
<td>-</td>
</tr>
<tr>
<td>pH 12.0</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>abs. EtOH : 0.5 M Ammonium Acetate</td>
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<td></td>
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<tr>
<td>5 : 2</td>
<td>0.30</td>
<td>0.35</td>
<td>-</td>
<td>0.77</td>
<td>-</td>
<td>0.77</td>
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<tr>
<td>pH 7.40</td>
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<tr>
<td>Compound</td>
<td>$C_2$-H</td>
<td>$C_8$-H</td>
<td>amide $N_9$-H</td>
<td>imidazole $N_7$-H</td>
<td>hydrazo $N$-H</td>
<td>amino $NH_2$</td>
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<tr>
<td>------------------------------------------</td>
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<td>---------</td>
<td>--------------</td>
<td>------------------</td>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>8,8'-Dioxo-6,6'-azopurine (DOAP)</td>
<td>8.96</td>
<td>-</td>
<td>12.22</td>
<td>12.84</td>
<td>-</td>
<td>-</td>
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<tr>
<td>8,8'-Dioxo-6,6'-Hydrazopurine (DOHP)</td>
<td>8.34</td>
<td>-</td>
<td>10.42</td>
<td>11.88</td>
<td>8.92</td>
<td>-</td>
</tr>
<tr>
<td>8,8'-Dioxo-6,6'-Azoxypurine (NaDOAXP)</td>
<td>8.56</td>
<td>-</td>
<td>-</td>
<td>11.78</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6,6'-Azopurine Disodium salt (NaAP)</td>
<td>8.82</td>
<td>8.38</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6,6'-Bisadenine (HP)</td>
<td>8.52</td>
<td>8.52</td>
<td>-</td>
<td>13.42</td>
<td>10.04</td>
<td>-</td>
</tr>
<tr>
<td>6,6'-Azoxypurine Disodium salt (NaAXP)</td>
<td>9.08</td>
<td>8.64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Adenine (6-aminopurine)</td>
<td>8.44</td>
<td>8.44</td>
<td>-</td>
<td>13.28</td>
<td>-</td>
<td>7.36</td>
</tr>
<tr>
<td>Hypoxanthine (6-oxopurine)</td>
<td>8.43</td>
<td>8.28</td>
<td>12.88*</td>
<td>13.76</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xanthine (2,6-dioxopurine)</td>
<td>-</td>
<td>8.26</td>
<td>11.20*</td>
<td>13.76</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

* amide $N_1$-H proton,  # amide $N_3$-H proton
Table 13. INFRARED FREQUENCIES OF AZOPURINES AND OXOAZOPURINES

<table>
<thead>
<tr>
<th>Assignment</th>
<th>DOAP</th>
<th>NaAP</th>
<th>DOHP</th>
<th>HP</th>
<th>NaDOAXP</th>
<th>NaAXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\nu$NH(_\text{free})</td>
<td>3410m</td>
<td>3500sm</td>
<td>3200bm</td>
<td>3480m</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\nu$NH(_\text{bonded})</td>
<td>3600-</td>
<td>3450-</td>
<td>3500-</td>
<td>3700-</td>
<td>3600-</td>
<td>2200b</td>
</tr>
<tr>
<td>NH(_\text{max})</td>
<td>2780</td>
<td>3000</td>
<td>2980</td>
<td>3010</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2660</td>
<td>2920</td>
<td>2840</td>
<td>2810</td>
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<td>2570</td>
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</tr>
<tr>
<td>$\nu$CH</td>
<td>3090m</td>
<td>3100m</td>
<td>3050m</td>
<td>3040m</td>
<td>3030m</td>
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</tr>
<tr>
<td></td>
<td>3040m</td>
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<td>3050m</td>
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</tr>
<tr>
<td>$\nu$C=O</td>
<td>1760bs</td>
<td>1750bs</td>
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<td>$\delta$NH</td>
<td>1630wsh</td>
<td>1655s</td>
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<tr>
<td>N=N-</td>
<td>1590s</td>
<td>1535m</td>
<td>1540m</td>
<td>1530bm</td>
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<tr>
<td>$\nu$C=C, enol</td>
<td>1640s</td>
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(Continued on next page)
Table 13. INFRARED FREQUENCIES OF AZOPURINES AND OXOAZOPURINES (contd.)

<table>
<thead>
<tr>
<th>Assignment</th>
<th>DOAP</th>
<th>NaAP</th>
<th>DOHP</th>
<th>HP</th>
<th>NaDOAXP</th>
<th>NaAXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>C=C/C=N ring modes</td>
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<tr>
<td>1590bm</td>
<td>1590m</td>
<td>1605s</td>
<td>1600s</td>
<td>1605w</td>
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<td></td>
</tr>
<tr>
<td>1565w</td>
<td>1570w</td>
<td>1580sh</td>
<td>1540w</td>
<td>1545m</td>
<td>1550m</td>
<td></td>
</tr>
<tr>
<td>1460w</td>
<td>1540w</td>
<td>1530w</td>
<td>1440m</td>
<td>1450m</td>
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<td>1400w</td>
<td>1410w</td>
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<td>1435m</td>
<td>1420m</td>
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<td>1300w</td>
<td>1335m</td>
<td>1320m</td>
<td>1320w</td>
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<tr>
<td>1295w</td>
<td>1270s</td>
<td>1280m</td>
<td>1270w*</td>
<td>1270w*</td>
<td>1290*</td>
<td></td>
</tr>
<tr>
<td>1250m</td>
<td>1250w</td>
<td>1260m</td>
<td>1230m</td>
<td>1230m</td>
<td>1225w</td>
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<td>1200bw</td>
<td>1230m</td>
<td>1170m</td>
<td>1160w</td>
<td>1150w</td>
<td>1175w</td>
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<td>1085w</td>
<td>1100w</td>
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<td>1000w</td>
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<td>1040w</td>
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<td>925wsh</td>
<td>930w</td>
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<td>880w</td>
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<td>870w</td>
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<td>900-910wd</td>
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<td>820w</td>
<td>835w</td>
<td>815w</td>
<td>820w</td>
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<td>760w</td>
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<td>700bw</td>
<td>135w</td>
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<td>615wsh</td>
<td>620w</td>
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</table>

Abbreviations: s=strong, m=medium, w=weak, sh=shoulder, b=broad, d=doublet

* This band probably includes azoxy N-O stretch
<table>
<thead>
<tr>
<th>Assignments</th>
<th>Adenine</th>
<th>2-Oxoadenine Sulfate</th>
<th>2,8-Dioxo adenine</th>
<th>Hypoxanthine</th>
<th>Xanthine</th>
<th>Uric acid</th>
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</thead>
<tbody>
<tr>
<td>NH (free)</td>
<td>3300m</td>
<td>3240w</td>
<td>3350s</td>
<td>3240w</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3250s</td>
<td></td>
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</tr>
<tr>
<td>NH (bonded)</td>
<td>3130m</td>
<td>3000b</td>
<td>3030s</td>
<td>3200b</td>
<td>3200b</td>
<td>3300b</td>
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<tr>
<td>NH(_2) (scissors)</td>
<td>1720s</td>
<td>1660s</td>
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<td>NH (deformation)</td>
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<td>1725w</td>
<td>1710w*</td>
<td>1720w</td>
<td>1715w</td>
<td>1720w</td>
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<td>N(\rightarrow) O</td>
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<td>NH(_3) + Stretch</td>
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<td>C=O</td>
<td>1690s(^\Theta)(C(_2))</td>
<td>1710(^#)(C(_2))</td>
<td>1660(^#)(C(_6))</td>
<td>1660(^#)(C(_6))</td>
<td>1600bs(^#)(C(_6))</td>
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<td>1750s(^#)(C(_8))</td>
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<td>1700s(^#)(C(_2))</td>
<td>1680bs(^#)(C(_2))</td>
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<td>2960bs</td>
<td>3030bs</td>
<td>3000bs</td>
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</tr>
<tr>
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<td>1590s</td>
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<td>1570m</td>
<td>1650m</td>
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(Continued on next page)
Table 14. INFRARED FREQUENCIES OF OXOPURINES (Continued)

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<th>2-Oxoadenine Sulfate</th>
<th>2,8-Dioxo adenine</th>
<th>Hypoxanthine</th>
<th>Xanthine</th>
<th>Uric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>=C=C/C-N</td>
<td>1550w</td>
<td>1450s</td>
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<td>1445m</td>
<td>1455m</td>
<td>1340m</td>
</tr>
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<td>1495s</td>
<td>1320m</td>
<td>1450m</td>
<td>1410m</td>
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<td>1300m</td>
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<td>1405m</td>
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<td>1350m</td>
<td>1370m</td>
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<td>1200m</td>
<td>1340m</td>
<td>1305m</td>
<td>1325m</td>
<td>1300m</td>
</tr>
<tr>
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<td>1160m</td>
<td>1210w</td>
<td>1265m</td>
<td>1250m</td>
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</tr>
<tr>
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<td>1240m</td>
<td>1120m</td>
<td>1160w</td>
<td>1205m</td>
<td>1195m</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1145m</td>
<td>1070s</td>
<td>1090m</td>
<td>1140m</td>
<td>1145s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1110m</td>
<td>965m</td>
<td>1000w</td>
<td>1120m</td>
<td>1110m</td>
<td>1110m</td>
</tr>
<tr>
<td></td>
<td>920m</td>
<td>890w</td>
<td>950m</td>
<td>950m</td>
<td>1020m</td>
<td>1020m</td>
</tr>
<tr>
<td></td>
<td>890m</td>
<td>820w</td>
<td>900m</td>
<td>880m</td>
<td>950m</td>
<td>980m</td>
</tr>
<tr>
<td></td>
<td>860w</td>
<td>790w</td>
<td>850m</td>
<td>840sh</td>
<td>880w</td>
<td>860m</td>
</tr>
<tr>
<td></td>
<td>830w</td>
<td>710w</td>
<td>770m</td>
<td>780m</td>
<td>830m</td>
<td></td>
</tr>
<tr>
<td></td>
<td>770m</td>
<td></td>
<td>730m</td>
<td>715m</td>
<td>750m</td>
<td>765m</td>
</tr>
<tr>
<td></td>
<td>700w</td>
<td></td>
<td></td>
<td></td>
<td>710m</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations:  s= strong,  m= medium,  w= weak,  sh= shoulder,  b= broad,  d= doublet

* Overlapping by C₂ amide C=O

# plus amide stretch

@ plus NH₃⁺
<table>
<thead>
<tr>
<th>Peak tube No.</th>
<th>abs. max. in HCl, nm</th>
<th>Compound</th>
<th>abs. max. of authentic compd., nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>225, 282</td>
<td>Uric Acid</td>
<td>230, 282</td>
</tr>
<tr>
<td>67</td>
<td>208, 228, 259</td>
<td>Xanthine</td>
<td>207, 227, 259</td>
</tr>
<tr>
<td>87</td>
<td>207, 247</td>
<td>Hypoxanthine</td>
<td>207, 247</td>
</tr>
<tr>
<td>103</td>
<td>215</td>
<td>Creatinine</td>
<td>215</td>
</tr>
<tr>
<td>120</td>
<td>208, 232, 306</td>
<td>2,8-DOA</td>
<td>208, 232, 305</td>
</tr>
<tr>
<td>137</td>
<td>211, 267, 280</td>
<td>8-OA</td>
<td>212, 267, 280</td>
</tr>
<tr>
<td>180</td>
<td>208, 282</td>
<td>2-OA</td>
<td>208, 283</td>
</tr>
<tr>
<td>224</td>
<td>208, 260</td>
<td>Adenine</td>
<td>207, 262</td>
</tr>
<tr>
<td>284</td>
<td>210, 297</td>
<td>DOHP</td>
<td>210, 297</td>
</tr>
</tbody>
</table>
Table 16. RATS USED FOR IN VIVO INHIBITION OF XANTHINE OXIDASE AND METABOLISM OF 8,8'-DIOXO-6,6'-AZOPURINE (DOAP)

<table>
<thead>
<tr>
<th>Rat</th>
<th>Age in days</th>
<th>Weight in gm.</th>
<th>mg of DOAP dissolved in ml of 0.01 N NaOH for a final dose of 25 mg/kg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>110</td>
<td>380</td>
<td>9.500 mg DOAP/6.3 ml 0.01 N NaOH</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>210</td>
<td>5.250 mg DOAP/3.5 ml 0.01 N NaOH</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>228</td>
<td>5.700 mg DOAP/3.8 ml 0.01 N NaOH</td>
</tr>
<tr>
<td>4</td>
<td>63</td>
<td>235</td>
<td>5.875 mg DOAP/3.9 ml 0.01 N NaOH</td>
</tr>
</tbody>
</table>

*The concentration of DOAP in 0.01 N NaOH was 1.5 mg/ml.
Table 17. ANALYSIS OF DOAP IN RAT URINE: CALIBRATION CURVE FOR 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) IN 0.1 N KOH

<table>
<thead>
<tr>
<th>Conc. of DOAP in 0.1 N KOH (μg/ml.)</th>
<th>Optical Density at 513 nm.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>2.5</td>
<td>0.135</td>
</tr>
<tr>
<td>5.0</td>
<td>0.275</td>
</tr>
<tr>
<td>10.0</td>
<td>0.560</td>
</tr>
<tr>
<td>15.0</td>
<td>0.710</td>
</tr>
<tr>
<td>20.0</td>
<td>1.140</td>
</tr>
<tr>
<td>22.0</td>
<td>1.100</td>
</tr>
</tbody>
</table>
Table 18. EXCRETION OF 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) AFTER A SINGLE SUBCUTANEOUS INJECTION OF 25 mg/kg in RAT URINE

<table>
<thead>
<tr>
<th>Hours after the injection of DOAP</th>
<th>O.D. at 513 nm#</th>
<th>Mg/ml of DOAP</th>
<th>g of DOAP excreted in 2 hour urine sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.225 ± 0.0014</td>
<td>4.5</td>
<td>13.5</td>
</tr>
<tr>
<td>4</td>
<td>0.990 ± 0.0013</td>
<td>20.0</td>
<td>60.0</td>
</tr>
<tr>
<td>6</td>
<td>1.130 ± 0.0016</td>
<td>20.8</td>
<td>62.4</td>
</tr>
<tr>
<td>8</td>
<td>0.880 ± 0.0017</td>
<td>17.7</td>
<td>53.1</td>
</tr>
<tr>
<td>10</td>
<td>0.560 ± 0.0013</td>
<td>11.3</td>
<td>39.9</td>
</tr>
<tr>
<td>12</td>
<td>0.670 ± 0.0012</td>
<td>13.5</td>
<td>40.5</td>
</tr>
<tr>
<td>14</td>
<td>0.315 ± 0.0011</td>
<td>6.3</td>
<td>18.9</td>
</tr>
<tr>
<td>16</td>
<td>0.410 ± 0.0010</td>
<td>8.2</td>
<td>24.6</td>
</tr>
<tr>
<td>18</td>
<td>0.510 ± 0.0010</td>
<td>10.3</td>
<td>30.9</td>
</tr>
<tr>
<td>20</td>
<td>0.390 ± 0.0011</td>
<td>7.9</td>
<td>23.7</td>
</tr>
<tr>
<td>22</td>
<td>0.225 ± 0.0016</td>
<td>4.5</td>
<td>13.5</td>
</tr>
<tr>
<td>24</td>
<td>0.045 ± 0.0014</td>
<td>0.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

\#An average of three experiments is listed with standard deviations.
Table 19. EFFECT OF A SINGLE SUBCUTANEOUS INJECTION OF 25 mg/kg 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) DISSOLVED IN 0.01 N NaOH ON URINE OUTPUT OF AN ADULT MALE RAT

<table>
<thead>
<tr>
<th>Hours after the injection of DOAP</th>
<th>24 hr. before 0.01 N NaOH</th>
<th>Urine volume, ml</th>
<th>24 hr. after 0.1 N NaOH</th>
<th>24 Hr. after DOAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.85</td>
<td>1.50</td>
<td>3.25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.20</td>
<td>2.55</td>
<td>4.55</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.30</td>
<td>2.30</td>
<td>5.30</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2.50</td>
<td>2.50</td>
<td>6.80</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.80</td>
<td>1.80</td>
<td>8.50</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2.50</td>
<td>2.50</td>
<td>8.90</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>3.25</td>
<td>3.25</td>
<td>9.60</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>5.45</td>
<td>5.25</td>
<td>10.80</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>4.60</td>
<td>3.10</td>
<td>9.50</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.10</td>
<td>1.00</td>
<td>9.00</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>0.75</td>
<td>0.85</td>
<td>7.20</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.70</td>
<td>0.70</td>
<td>7.00</td>
<td></td>
</tr>
<tr>
<td>ml. of std. soln.* of uric acid</td>
<td>µg of uric acid</td>
<td>ml. of glycine buffer# added</td>
<td>ml. of dist. H₂O added</td>
<td>O.D. at 292 nm@</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------</td>
<td>-------------------------------</td>
<td>------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>0.1</td>
<td>5</td>
<td>1.0</td>
<td>6.1</td>
<td>0.050</td>
</tr>
<tr>
<td>0.2</td>
<td>10</td>
<td>1.0</td>
<td>6.0</td>
<td>0.080</td>
</tr>
<tr>
<td>0.4</td>
<td>20</td>
<td>1.0</td>
<td>5.8</td>
<td>0.165</td>
</tr>
<tr>
<td>0.6</td>
<td>30</td>
<td>1.0</td>
<td>5.6</td>
<td>0.235</td>
</tr>
<tr>
<td>0.8</td>
<td>40</td>
<td>1.0</td>
<td>5.4</td>
<td>0.335</td>
</tr>
<tr>
<td>1.0</td>
<td>50</td>
<td>1.0</td>
<td>5.2</td>
<td>0.418</td>
</tr>
</tbody>
</table>

*Standard solution of uric acid was purchased from Sigma Chemical Co., St. Louis, Mo., containing 0.05 mg of uric acid/ml.

#0.7 M glycine buffer, pH 9.4.

@ Average of three experiments.

10 µl of 0.4 units/ml of Uricase was used in all experiments.
### Table 21. EFFECT OF 25 mg/kg 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) ON URIC ACID EXCRETION IN AN ADULT MALE RAT URINE

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine vol., ml.</th>
<th>O.D. at 292 nm of 0.5 ml. of urine sample*</th>
<th>O.D. at 292 nm of 1.0 ml. of urine sample</th>
<th>Uric Acid µg/ml of urine</th>
<th>Uric Acid mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.0</td>
<td>0.0300 ± 0.0014</td>
<td>0.060</td>
<td>7.0</td>
<td>0.70</td>
</tr>
<tr>
<td>2</td>
<td>29.4</td>
<td>0.0375 ± 0.0015</td>
<td>0.075</td>
<td>8.5</td>
<td>0.85</td>
</tr>
<tr>
<td>3</td>
<td>32.0</td>
<td>0.0325 ± 0.0013</td>
<td>0.065</td>
<td>7.5</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* a single subcutaneous injection of 0.01 N NaOH

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine vol., ml.</th>
<th>O.D. at 292 nm of 0.5 ml. of urine sample*</th>
<th>O.D. at 292 nm of 1.0 ml. of urine sample</th>
<th>Uric Acid µg/ml of urine</th>
<th>Uric Acid mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.8</td>
<td>0.0225 ± 0.0011</td>
<td>0.055</td>
<td>7.0</td>
<td>0.70</td>
</tr>
<tr>
<td>2</td>
<td>26.7</td>
<td>0.0375 ± 0.0010</td>
<td>0.075</td>
<td>8.5</td>
<td>0.85</td>
</tr>
<tr>
<td>3</td>
<td>27.3</td>
<td>0.0300 ± 0.0013</td>
<td>0.060</td>
<td>7.0</td>
<td>0.70</td>
</tr>
</tbody>
</table>

* a single subcutaneous injection of 25 mg/kg DOAP dissolved in 0.01 N NaOH

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine vol., ml.</th>
<th>O.D. at 292 nm of 0.5 ml. of urine sample*</th>
<th>O.D. at 292 nm of 1.0 ml. of urine sample</th>
<th>Uric Acid µg/ml of urine</th>
<th>Uric Acid mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90.4</td>
<td>0.0100 ± 0.0010</td>
<td>0.020</td>
<td>2.5</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>31.0</td>
<td>0.0300 ± 0.0014</td>
<td>0.060</td>
<td>7.0</td>
<td>0.70</td>
</tr>
<tr>
<td>3</td>
<td>42.0</td>
<td>0.0200 ± 0.0012</td>
<td>0.040</td>
<td>5.0</td>
<td>0.50</td>
</tr>
<tr>
<td>4</td>
<td>35.6</td>
<td>0.0175 ± 0.0010</td>
<td>0.035</td>
<td>4.5</td>
<td>0.45</td>
</tr>
<tr>
<td>5</td>
<td>31.2</td>
<td>0.0225 ± 0.0010</td>
<td>0.055</td>
<td>7.0</td>
<td>0.70</td>
</tr>
<tr>
<td>6</td>
<td>30.8</td>
<td>0.0300 ± 0.0011</td>
<td>0.060</td>
<td>7.0</td>
<td>0.70</td>
</tr>
<tr>
<td>7</td>
<td>29.3</td>
<td>0.0250 ± 0.0013</td>
<td>0.050</td>
<td>6.0</td>
<td>0.60</td>
</tr>
<tr>
<td>8</td>
<td>30.1</td>
<td>0.0375 ± 0.0013</td>
<td>0.075</td>
<td>8.5</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* an average of three experiments is listed with Standard Deviations
Table 22. EFFECT OF 25 mg/kg 8,8'-DIOXO-6,6'-AZOPURINE (DOAP) ON XANTHINE
AND HYPOXANTHINE EXCRETION IN AN ADULT MALE RAT URINE

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine vol., ml.</th>
<th>O.D. 292 nm*</th>
<th>Uric Acid µg/ml</th>
<th>mg% of urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.0</td>
<td>0.018 ± 0.0014</td>
<td>1.76</td>
<td>0.18</td>
</tr>
<tr>
<td>2</td>
<td>29.4</td>
<td>0.021 ± 0.0016</td>
<td>2.05</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td>32.0</td>
<td>0.028 ± 0.0013</td>
<td>2.74</td>
<td>0.27</td>
</tr>
</tbody>
</table>

a single subcutaneous injection of 0.01 N NaOH

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine vol., ml.</th>
<th>O.D. 292 nm*</th>
<th>Uric Acid µg/ml</th>
<th>mg% of urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.8</td>
<td>0.021 ± 0.0014</td>
<td>2.05</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>26.7</td>
<td>0.023 ± 0.0016</td>
<td>2.25</td>
<td>0.23</td>
</tr>
<tr>
<td>3</td>
<td>27.3</td>
<td>0.020 ± 0.0013</td>
<td>1.95</td>
<td>0.19</td>
</tr>
</tbody>
</table>

a single subcutaneous injection of 25 mg/kg DOAP dissolved in 0.01 N NaOH

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine vol., ml.</th>
<th>O.D. 292 nm*</th>
<th>Uric Acid µg/ml</th>
<th>mg% of urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90.4</td>
<td>0.069 ± 0.0013</td>
<td>6.74</td>
<td>0.67</td>
</tr>
<tr>
<td>2</td>
<td>31.0</td>
<td>0.101 ± 0.0016</td>
<td>9.87</td>
<td>0.99</td>
</tr>
<tr>
<td>3</td>
<td>42.0</td>
<td>0.032 ± 0.0014</td>
<td>3.13</td>
<td>0.31</td>
</tr>
<tr>
<td>4</td>
<td>35.6</td>
<td>0.029 ± 0.0015</td>
<td>2.83</td>
<td>0.28</td>
</tr>
<tr>
<td>5</td>
<td>31.2</td>
<td>0.030 ± 0.0011</td>
<td>2.93</td>
<td>0.29</td>
</tr>
<tr>
<td>6</td>
<td>30.8</td>
<td>0.021 ± 0.0016</td>
<td>2.05</td>
<td>0.20</td>
</tr>
<tr>
<td>7</td>
<td>29.3</td>
<td>0.020 ± 0.0013</td>
<td>1.95</td>
<td>0.19</td>
</tr>
<tr>
<td>8</td>
<td>30.1</td>
<td>0.021 ± 0.0012</td>
<td>2.05</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* an average of three experiments is listed with Standard Deviations.
Table 23. CALIBRATION CURVE FOR CREATININE ANALYSIS IN ADULT MALE RAT URINE.

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>ml of stock soln.</th>
<th>ml of dist. H&lt;sub&gt;2&lt;/sub&gt;O</th>
<th>O.D. at 520 nm vs blank</th>
<th>µg of creatinine per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
<td>9.9</td>
<td>0.040</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>0.20</td>
<td>9.8</td>
<td>0.060</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>0.40</td>
<td>9.6</td>
<td>0.135</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
<td>9.4</td>
<td>0.210</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>0.80</td>
<td>9.2</td>
<td>0.290</td>
<td>80</td>
</tr>
<tr>
<td>7</td>
<td>1.00</td>
<td>9.0</td>
<td>0.365</td>
<td>100</td>
</tr>
</tbody>
</table>

* The stock soln. of creatinine was 1.0 mg/ml in 0.1 N HCl.
Table 24. EFFECT OF 25 mg/kg 8,8' -DIOXO-6,6'-AZOPURINE (DOAP) ON CREATININE EXCRETION IN AN ADULT MALE RAT URINE

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine vol., ml.</th>
<th>$A_{520nm}^*$</th>
<th>Creatinine $\mu g/ml$</th>
<th>Creatinine mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.8</td>
<td>0.200 ± 0.0014</td>
<td>55.0</td>
<td>5.50</td>
</tr>
<tr>
<td>2</td>
<td>25.2</td>
<td>0.210 ± 0.0016</td>
<td>58.0</td>
<td>5.80</td>
</tr>
<tr>
<td>3</td>
<td>28.2</td>
<td>0.215 ± 0.0013</td>
<td>60.0</td>
<td>6.00</td>
</tr>
</tbody>
</table>

a single subcutaneous injection of 0.01 N NaOH

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine vol., ml.</th>
<th>$A_{520nm}^*$</th>
<th>Creatinine $\mu g/ml$</th>
<th>Creatinine mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.0</td>
<td>0.230 ± 0.0013</td>
<td>63.5</td>
<td>6.35</td>
</tr>
<tr>
<td>2</td>
<td>22.8</td>
<td>0.210 ± 0.0011</td>
<td>58.0</td>
<td>5.80</td>
</tr>
<tr>
<td>3</td>
<td>26.7</td>
<td>0.190 ± 0.0012</td>
<td>52.5</td>
<td>5.25</td>
</tr>
</tbody>
</table>

a single subcutaneous injection of 25 mg/kg DOAP dissolved in 0.01 N NaOH

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine vol., ml.</th>
<th>$A_{520nm}^*$</th>
<th>Creatinine $\mu g/ml$</th>
<th>Creatinine mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96.8</td>
<td>0.050 ± 0.0010</td>
<td>13.5</td>
<td>1.35</td>
</tr>
<tr>
<td>2</td>
<td>42.9</td>
<td>0.135 ± 0.0014</td>
<td>37.0</td>
<td>3.70</td>
</tr>
<tr>
<td>3</td>
<td>31.6</td>
<td>0.175 ± 0.0013</td>
<td>48.0</td>
<td>4.80</td>
</tr>
<tr>
<td>4</td>
<td>45.6</td>
<td>0.120 ± 0.0014</td>
<td>33.0</td>
<td>3.30</td>
</tr>
<tr>
<td>5</td>
<td>21.3</td>
<td>0.230 ± 0.0010</td>
<td>63.5</td>
<td>6.35</td>
</tr>
<tr>
<td>6</td>
<td>28.2</td>
<td>0.210 ± 0.0013</td>
<td>58.0</td>
<td>5.80</td>
</tr>
<tr>
<td>7</td>
<td>26.3</td>
<td>0.230 ± 0.0016</td>
<td>63.5</td>
<td>6.35</td>
</tr>
<tr>
<td>8</td>
<td>25.0</td>
<td>0.220 ± 0.0010</td>
<td>60.5</td>
<td>6.05</td>
</tr>
</tbody>
</table>

* an average of three experiments is listed with Standard Deviations.
### Table 25. DETERMINATION OF PROTEIN OF RABBIT LIVER ALDEHYDE OXIDASE.

<table>
<thead>
<tr>
<th>Dilution of the stock soln.</th>
<th>$A_{215}$</th>
<th>$A_{225}$</th>
<th>$\Delta$ O.D. ($A_{215} - A_{225}$)</th>
<th>$\mu$g protein/ml in dil. soln. (O.D. X 144)</th>
<th>mg protein/ml of the stock soln.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 250</td>
<td>0.860</td>
<td>0.790</td>
<td>0.070</td>
<td>10.08</td>
<td>2.52</td>
</tr>
<tr>
<td>1 : 500</td>
<td>0.550</td>
<td>0.490</td>
<td>0.060</td>
<td>3.64</td>
<td>4.32</td>
</tr>
<tr>
<td>1 : 1000</td>
<td>0.385</td>
<td>0.350</td>
<td>0.035</td>
<td>5.04</td>
<td>5.04</td>
</tr>
<tr>
<td>1 : 2000</td>
<td>0.280</td>
<td>0.260</td>
<td>0.020</td>
<td>2.88</td>
<td>5.76</td>
</tr>
</tbody>
</table>

* $\mu$g protein/ml in diluted stock solution X dilution factor ÷ 1000.

Average of 5.04 mg protein/ml in the final stock solution of rabbit liver aldehyde oxidase.
Table 26. DETERMINATION OF OPTIMAL SUBSTRATE CONCENTRATION FOR RABBIT LIVER ALDEHYDE OXIDASE.

<table>
<thead>
<tr>
<th>Substrate conc. *</th>
<th>Amt of aldehyde oxidase #, µl</th>
<th>Initial velocity O.D./min @</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.33 X 10⁻⁴</td>
<td>100</td>
<td>0.010</td>
</tr>
<tr>
<td>8.33 X 10⁻⁴</td>
<td>100</td>
<td>0.020</td>
</tr>
<tr>
<td>1.66 X 10⁻³</td>
<td>100</td>
<td>0.030</td>
</tr>
<tr>
<td>3.33 X 10⁻³</td>
<td>100</td>
<td>0.035</td>
</tr>
<tr>
<td>5.00 X 10⁻³</td>
<td>100</td>
<td>0.025</td>
</tr>
<tr>
<td>8.33 X 10⁻³</td>
<td>100</td>
<td>0.200</td>
</tr>
<tr>
<td>1.66 X 10⁻²</td>
<td>100</td>
<td>0.200</td>
</tr>
</tbody>
</table>

* N¹-methylnicotinamide chloride

# 5.04 mg protein/ml rabbit liver aldehyde oxidase.

@ Change in optical density/min at 300 nm.
Table 27. DETERMINATION OF OPTIMAL SUBSTRATE CONCENTRATION FOR RABBIT LIVER ALDEHYDE OXIDASE.

<table>
<thead>
<tr>
<th>Substrate Conc. M</th>
<th>Amt of aldehyde oxidase μl</th>
<th>Initial velocity O.D./min</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.33 x 10⁻⁴</td>
<td>250</td>
<td>0.025</td>
</tr>
<tr>
<td>1.66 x 10⁻³</td>
<td>250</td>
<td>0.055</td>
</tr>
<tr>
<td>3.33 x 10⁻³</td>
<td>250</td>
<td>0.060</td>
</tr>
<tr>
<td>5.00 x 10⁻³</td>
<td>250</td>
<td>0.060</td>
</tr>
<tr>
<td>8.33 x 10⁻³</td>
<td>250</td>
<td>0.060</td>
</tr>
<tr>
<td>1.66 x 10⁻²</td>
<td>250</td>
<td>0.055</td>
</tr>
</tbody>
</table>

* N¹-methylnicotinamide chloride

# 5.04 mg protein/ml rabbit liver aldehyde oxidase

@ Change in optical density/min at 300 nm.
Table 28. INHIBITION OF RABBIT LIVER ALDEHYDE OXIDASE BY 6,6'-AZOPURINE DISODIUM SALT (NaAP) AT 25°C: (I)$_{50}$AT pH 7.8

<table>
<thead>
<tr>
<th>Concentration of NaAP ul of stock soln*</th>
<th>FBC#, M</th>
<th>A$_{300}$/min</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.085</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1.3 X 10$^{-6}$</td>
<td>0.055</td>
<td>35.29</td>
</tr>
<tr>
<td>10</td>
<td>2.6 X 10$^{-6}$</td>
<td>0.045</td>
<td>47.05</td>
</tr>
<tr>
<td>25</td>
<td>6.5 X 10$^{-6}$</td>
<td>0.025</td>
<td>70.58</td>
</tr>
<tr>
<td>50</td>
<td>1.3 X 10$^{-5}$</td>
<td>0.010</td>
<td>88.23</td>
</tr>
<tr>
<td>100</td>
<td>2.6 X 10$^{-5}$</td>
<td>0.005</td>
<td>94.11</td>
</tr>
<tr>
<td>125</td>
<td>3.3 X 10$^{-5}$</td>
<td>0.000</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.085</td>
<td>-</td>
</tr>
</tbody>
</table>

5.0 X 10$^{-3}$ M N$^1$-methylnicotinamide chloride was used as substrate.

* Stock solution of NaAP was prepared by dissolving 2.5 mg of NaAP in 10 ml of dist. H$_2$O

# Final bath concentration
Table 29. INHIBITION OF RABBIT LIVER ALDEHYDE OXIDASE BY 6,6'-AZOPURINE DISODIUM SALT (NaAP) AT 25°C: (I)50 AT pH 8.2

<table>
<thead>
<tr>
<th>Concentration of NaAP ul of stock soln*</th>
<th>FBC#, M</th>
<th>A300/min</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.085</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1.3 X 10^-6</td>
<td>0.060</td>
<td>29.41</td>
</tr>
<tr>
<td>10</td>
<td>2.6 X 10^-6</td>
<td>0.055</td>
<td>35.29</td>
</tr>
<tr>
<td>25</td>
<td>6.4 X 10^-6</td>
<td>0.030</td>
<td>64.71</td>
</tr>
<tr>
<td>.50</td>
<td>1.3 X 10^-5</td>
<td>0.010</td>
<td>88.23</td>
</tr>
<tr>
<td>100</td>
<td>2.6 X 10^-5</td>
<td>0.005</td>
<td>94.12</td>
</tr>
<tr>
<td>125</td>
<td>3.3 X 10^-5</td>
<td>0.000</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.085</td>
<td>-</td>
</tr>
</tbody>
</table>

5.0 X 10^-3 M N1-methyl nicotinamide chloride was used as substrate

* Stock solution of NaAP was prepared by dissolving 2.5 mg of NaAP in 10 ml of dist. H2O

# Final bath concentration
<table>
<thead>
<tr>
<th>Concentration of Menadione ul of stock soln.*</th>
<th>FBC# M</th>
<th>A&lt;sub&gt;300&lt;/sub&gt;/min</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.080</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>5.3 X 10^-8</td>
<td>0.047</td>
<td>41.88</td>
</tr>
<tr>
<td>10</td>
<td>1.0 X 10^-7</td>
<td>0.043</td>
<td>46.88</td>
</tr>
<tr>
<td>25</td>
<td>2.6 X 10^-7</td>
<td>0.027</td>
<td>66.25</td>
</tr>
<tr>
<td>50</td>
<td>5.3 X 10^-7</td>
<td>0.010</td>
<td>87.50</td>
</tr>
<tr>
<td>100</td>
<td>1.0 X 10^-6</td>
<td>0.000</td>
<td>-</td>
</tr>
</tbody>
</table>

5.0 X 10^-3 M N<sup>1</sup>-methylnicotinamide chloride was used as substrate

* Stock solution of Menadione was prepared by dissolving 2 mg of Menadione Sodium Bisulfite into 200 mls of dist. H<sub>2</sub>O

# Final bath concentration
Table 31. INHIBITION OF RABBIT LIVER ALDEHYDE OXIDASE BY 8,8'-DIOXO-6,6'-AZO-PURINE (DOAP) AT 25°C: (I)50 AT pH 8.2

<table>
<thead>
<tr>
<th>Concentration of DOAP u1 of stock soln*</th>
<th>FBC#, M</th>
<th>A300/min</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>0.085</td>
<td>-</td>
</tr>
<tr>
<td>100 u1 of 0.005 N NaOH</td>
<td>-</td>
<td>0.085</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>5.3 X 10^{-6}</td>
<td>0.080</td>
<td>5.88</td>
</tr>
<tr>
<td>25</td>
<td>1.3 X 10^{-5}</td>
<td>0.075</td>
<td>11.76</td>
</tr>
<tr>
<td>50</td>
<td>2.6 X 10^{-5}</td>
<td>0.068</td>
<td>20.58</td>
</tr>
<tr>
<td>100</td>
<td>5.3 X 10^{-5}</td>
<td>0.065</td>
<td>23.53</td>
</tr>
<tr>
<td>250</td>
<td>1.3 X 10^{-4}</td>
<td>0.065</td>
<td>23.53</td>
</tr>
<tr>
<td>500 u1</td>
<td>2.6 X 10^{-4}</td>
<td>0.065</td>
<td>23.53</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>0.085</td>
<td>-</td>
</tr>
</tbody>
</table>

5.0 X 10^{-3} M N1-methylnicotinamide chloride was used as substrate

* Stock solution of DOAP was prepared by dissolving 2 mg of DOAP in 4.0 ml of 0.005 N NaOH

# Final bath concentration
Table 32. INHIBITION OF RABBIT LIVER ALDEHYDE OXIDASE BY 6,6'·AZOPURINE DISODIUM SALT (NaAP) AT pH 7.8, 25°C: LINEWEAVER-BURK PLOT

<table>
<thead>
<tr>
<th>Substrate (N¹-methylnicotinamide chloride) Concentration $\frac{1}{M} \times 10^{-2}$</th>
<th>$\frac{1}{V} \times 10^{-1}$ at Inhibitor (NaAP) Concentration</th>
<th>2.6 $\times 10^{-6}$ M</th>
<th>6.5 $\times 10^{-6}$ M</th>
<th>1.3 $\times 10^{-5}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (No inhibition)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2.50</td>
<td>3.64</td>
<td>5.00</td>
<td>6.67</td>
</tr>
<tr>
<td>10</td>
<td>2.00</td>
<td>2.22</td>
<td>3.33</td>
<td>4.00</td>
</tr>
<tr>
<td>4</td>
<td>1.82</td>
<td>1.82</td>
<td>1.90</td>
<td>2.00</td>
</tr>
<tr>
<td>2</td>
<td>1.67</td>
<td>1.54</td>
<td>1.67</td>
<td>1.74</td>
</tr>
<tr>
<td>1</td>
<td>1.54</td>
<td>1.43</td>
<td>1.54</td>
<td>1.54</td>
</tr>
</tbody>
</table>
Table 33. INHIBITION OF RABBIT LIVER ALDEHYDE OXIDASE BY 6,6'-AZOPURINE DISODIUM SALT (NaAP) AT pH 7.8, 25°C: DIXON PLOT

<table>
<thead>
<tr>
<th>Substrate (N^1-methylnicotinamide chloride) Conc., M</th>
<th>( \frac{1}{V} ) ( \times 10^{-1} ) at Inhibitor (NaAP) Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 ( \times 10^{-4} )</td>
<td>2.6 ( \times 10^{-6} ) M</td>
</tr>
<tr>
<td>2.5 ( \times 10^{-3} )</td>
<td>2.42</td>
</tr>
</tbody>
</table>
Table 34.  INHIBITION OF RABBIT LIVER ALDEHYDE OXIDASE BY MENADIONE

AT pH 7.8, 25°C: LINEWEAVER-BURK PLOT

<table>
<thead>
<tr>
<th>Substrate Concentration (N\textsuperscript{1}-methylnicotinamide chloride)</th>
<th>( \frac{1}{V} ) Concentration</th>
<th>1 X 10\textsuperscript{-1} M at Inhibitor (Menadione)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ( \text{M} \times 10^{-2} )</td>
<td>Control (No inhibition)</td>
<td>1.0 X 10\textsuperscript{-7} M</td>
</tr>
<tr>
<td>20</td>
<td>2.50</td>
<td>3.33</td>
</tr>
<tr>
<td>10</td>
<td>2.00</td>
<td>2.86</td>
</tr>
<tr>
<td>4</td>
<td>1.81</td>
<td>2.50</td>
</tr>
<tr>
<td>2</td>
<td>1.66</td>
<td>2.35</td>
</tr>
<tr>
<td>1</td>
<td>1.54</td>
<td>2.22</td>
</tr>
</tbody>
</table>
Table 35. IDENTIFICATION OF THE PRODUCT OF ENZYMATIC OXIDATION
OF 6,6'-AZOPURINE DISODIUM SALT (NaAP) BY RABBIT
LIVER ALDEHYDE OXIDASE

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>DOAP</th>
<th>ppt. 1</th>
<th>R_f values</th>
<th>ppt. 2</th>
<th>ppt. 3</th>
<th>NaAP</th>
<th>ppt. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>abs. EtOH : Pyridine : Dist. H_2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67 : 20 : 13</td>
<td>0.165</td>
<td>0.162</td>
<td>0.165</td>
<td>0.165</td>
<td>0.256</td>
<td>0.256</td>
<td></td>
</tr>
<tr>
<td>n-Propanol : Conc. NH_3 : Dist. H_2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 : 30 : 10</td>
<td>0.357</td>
<td>0.363</td>
<td>0.370</td>
<td>0.370</td>
<td>0.593</td>
<td>0.585</td>
<td></td>
</tr>
<tr>
<td>abs. EtOH : 0.5 M Ammonium Acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:2</td>
<td>0.085</td>
<td>0.091</td>
<td>0.085</td>
<td>0.093</td>
<td>0.399</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Precipitates 1, 2, and 3 were the final orange precipitates obtained from the brown-orange DOAP-protein complex, precipitated by using 50% ammonium sulfate saturation, incubation in boiling water-bath for 10 min, and by adding 5 N HCl respectively. Precipitate 4 was obtained from the control system (Ref. Chart 3)
CHAPTER IX

BIBLIOGRAPHY
CHAPTER IX

BIBLIOGRAPHY


The dissertation submitted by Arun L. Jadhav has been read and approved by the following Committee:

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Jan. 5, 1977
Date

Joseph R. Davis, M.D., Ph.D.
Director's Signature