tuations in solvent strength can cause shifting of peaks and changes in peak height ratios. Resolution of theophylline and β-hydroxytheophylline can be optimized for each column by adjustments in the methanol concentration of ±2%.

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6,6′-Azopurine, a Potent *In Vitro* Inhibitor of Rabbit Liver Aldehyde Oxidase

Keyphrases □ 6,6′-Azopurine—inhibition of rabbit liver aldehyde oxidase, *in vitro*, prevention of azathioprine cytotoxicity □ Enzyme inhibitors—6,6′-azopurine, inhibition of rabbit liver aldehyde oxidase, *in vitro*, prevention of azathioprine cytotoxicity □ Azathioprine—prevention of cytotoxicity by 6,6′-azopurine

To the Editor:

Hepatic aldehyde oxidase (aldehyde:oxygen oxidoreductase, EC 1.2.3.1) oxidizes many purines at the C_8-position (1–3) and has been implicated in the metabolism of azathioprine (3), methotrexate and its dialkyl and dichloro derivatives (4), formycin B (5, 6), cyclophosphamide, and the *N*-alkylphenothiazines (7, 8). Inhibitors of aldehyde oxidase and xanthine oxidase, either endogenous or therapeutically administered, may change the conversion rate of azathioprine into active and inactive metabolites. This factor becomes significant for the development of treatment schedules for the most favorable balance between therapeutic effect and drug toxicity.

We wish to report the finding of such an inhibitor. During investigations of oxoazopurine pharmacology (9, 10), we observed that 6,6′-azopurine disodium salt (I) was a potent inhibitor of rabbit liver aldehyde oxidase and also acted as a substrate for this enzyme. The oxidation of I by the enzyme resulted in the formation of a known potent xanthine oxidase inhibitor, 8,8′-dioxo-6,6′-azopurine (II) (9).

Table I—Identification of the Product of Enzymic Oxidation of 6,6′-Azopurine Disodium Salt (I) by Rabbit Liver Aldehyde Oxidase

Solvent System	R_f Values[a] Chemically Synthesized II	Enzymatically Synthesized II	I
Ethanol–pyridine–water (67:20:13)	0.17	0.16	0.26
n-Propanol–ammonia (concentrated)–water (60:30:10)	0.36	0.37	0.59
Ethanol–0.5 *M* ammonium acetate (5:2)	0.09	0.09	0.4

[a] Chromatographic analyses were carried out using Whatman chromatography grade 1 paper. Results shown are averages of triplicate determinations.

A modified literature method (1) was used to obtain a partially purified aldehyde oxidase preparation from rabbit liver homogenates containing 5.04 mg of protein/ml. It was assayed using a spectrophotometric method based on measuring the rate of N^1-methylnicotinamide chloride oxidation at 300 nm (1, 6). The conversion of N^1-methylnicotinamide chloride to its 2-pyrone was measured by monitoring the increase in absorbance at 300 nm upon addition of the enzyme at ambient temperature to reaction mixtures containing, in 3 ml: substrate (0.5, 1, 2.5, 5, or 10 m*M*), desired concentration of I ranging from 2.6 to 13 μ*M* in 5 m*M* pH 7.8 potassium phosphate buffer, and 0.005% edetic acid. The double-reciprocal plot (Fig. 1) of the inhibition of rabbit liver aldehyde oxidase showed that I was a competitive inhibitor of this enzyme with a k_i of 3.3×10^{-6} *M*. The k_m for N^1-methylnicotinamide chloride was 318 μ*M*.

For the enzymatic synthesis of II from I, the following procedure was used. Compound I, 2.5 mg, was dissolved in 10 ml of water to obtain a clear yellow solution. To this solution were added 7.5 ml of 0.05 *M* potassium phosphate buffer containing 0.005% edetic acid at pH 7.8 and 2.5 ml of aldehyde oxidase suspension (5.04 mg of protein/ml). A control reaction was carried out using the same reactants except that 2.5 ml of the buffer was added in the last step instead of the aldehyde oxidase suspension. 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 g of solid

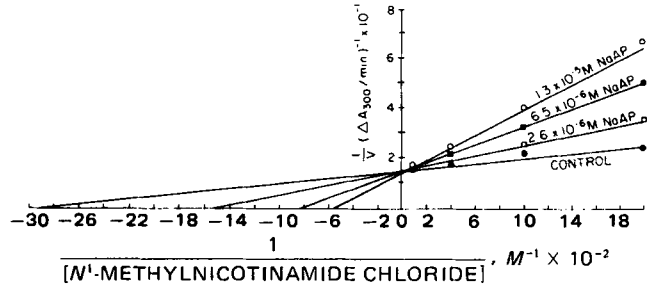

Figure 1—*Double-reciprocal plots of the* in vitro *inhibition of rabbit liver aldehyde oxidase by 6,6′-azopurine disodium salt (I, NaAP).*

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ammonium sulfate/liter of the reaction mixture. The solutions were centrifuged at 8000 rpm for 20 min at 4°. 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 water separately, and 2.0 ml of 5 *N* HCl was added to each solution. These solutions were centrifuged at 8000 rpm for 20 min at 4°, and the precipitates were treated according to the same procedure 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 as 8,8′-dioxo-6,6′-azopurine by comparing the R_f values in three different solvent systems (Table I) and the UV characteristics with authentic samples of II. The yellow precipitate was identified as I. Compound I was unchanged under the same conditions even after 6 hr in the absence of enzyme.

Compound I is the first example of a purine dimer that can react with rabbit liver aldehyde oxidase. This finding is significant 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 (3). Compound I can be considered as a sequential inhibitor of two enzymes involved in azathioprine metabolism. It should inhibit aldehyde oxidase and be converted to II, which is a potent inhibitor of another enzyme in the metabolic pathway, xanthine oxidase. Thus, investigation of the inhibition of mammalian aldehyde oxidase by I may provide such an agent.

Because of the significance of aldehyde oxidase in the metabolism of various biologically active *N*-heterocyclic compounds, I can be used in the investigation of the mechanism of the action of these compounds.

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Effect of Altered Plasma Protein Binding on Apparent Volume of Distribution

Keyphrases ◻ Volume of distribution—effect of altered plasma protein binding, pharmacokinetics ◻ Drug binding—plasma, volume of distribution, effect of altered plasma protein binding ◻ Plasma protein binding—effect of alterations on apparent drug volume of distribution ◻ Models, pharmacokinetic—apparent volume of distribution, effect of altered plasma protein binding

To the Editor:

Changes in the apparent volume of distribution occur with age, disease, and displacement (drug interaction) and in the presence of saturable binding. Relationships have been developed (1–6) to explain these changes based on alterations in plasma and/or tissue binding. Our discussion is restricted to measurement of drug concentrations in plasma. Gillette (1) showed that the volume of distribution, V, can be expressed by:

$$V = \alpha(V_f + XV_T) + (1 - \alpha)V_p \qquad \text{(Eq. 1)}$$

where α is the fraction unbound in plasma, V_f is the volume into which the unbound drug is distributed, X is the ratio of tissue drug concentration to unbound plasma drug concentration, V_T is the tissue volume, and V_p is the apparent volume of distribution of the plasma protein to which the drug binds. A simplified relationship, based on the physiological concepts of Gillette (1, 4–6), was proposed (2, 3) as follows:

$$V = V_P + (V_T)\left(\frac{\alpha}{\alpha_T}\right) \qquad \text{(Eq. 2)}$$

where V_P is the plasma volume, V_T is the volume outside plasma into which the drug distributes, and α and α_T are the fractions unbound in these two compartments.

The relationship of Eq. 2 does not take into account that plasma proteins are distributed throughout the extracellular fluids. When the binding to proteins in plasma is altered, similar changes are expected in the binding to these proteins located in other extracellular fluids. Furthermore, this relationship cannot distinguish between binding to these proteins and binding elsewhere in the body. This distinction is important for anticipating changes in the volume of distribution on altering drug binding and the converse.

The following derivation provides a method for making this distinction. From mass balance considerations:

$$A = A_P + A_E + A_R \qquad \text{(Eq. 3)}$$

where:

A = total amount of drug in the body
A_P = total amount of drug in plasma
A_E = total amount of drug in the extracellular fluid outside plasma

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