Studies on the Mechanism of Action of Salicylates VII: Effect of a Few Anti-inflammatory Agents on Uridine-5′-diphosphoglucose Dehydrogenase

K. H. LEE and MICHAEL R. SPENCER

Abstract □ Phenylbutazone, oxyphenbutazone, indomethacin, flufenamic acid, and mefenamic acid, like salicylic acid, inhibit the oxidation of uridine-5′-diphosphoglucose (UDPG) competitively with nicotinamide adenosine dinucleotide (NAD) and noncompetitively with UDPG. The *Km* and *Ki* values of these inhibitory agents are presented.

Keyphrases □ Salicylates—action mechanism □ Uridine-5′-diphosphoglucose dehydrogenase—anti-inflammatory agents effect □ UV spectrophotometry—analysis

It has been known for some time that salicylic acid inhibits acid mucopolysaccharide synthesis (1). However, its action on molecular level has not been studied until recently. Bollet, in 1961, reported that salicylic acid and phenylbutazone inhibit glucosamine-6-phosphate synthesis which is one of the first-step reactions in mucopolysaccharide synthesis (2). The enzyme involved is L-glutamine: D-fructose-6-phosphate aminotransferase (EC 2.6.1.16). Bollet's work was confirmed by Schönhöfer in 1966 (3). Later, Kalbhen *et al.* using fibroblast tissue culture, demonstrated that salicylic acid and a few other antiphlogistic drugs inhibited the incorporation of glucosamine-1-^{14}C into mucopolysaccharide (4). Their findings suggest that these drugs also inhibit mucopolysaccharide formation at a site or sites other than glucosamine synthesis. In a previous communication, the authors have shown that salicylic acid competitively inhibits UDP-glucuronyl-transferase (2.4.1.17) reaction (5). The authors also reported that salicylic acid most effectively inhibits uridine-5′-diphosphoglucose (UDPG) dehydrogenation reaction competitively with nicotinamide-adenosine-dinucleotide (NAD) and noncompetitively with UDPG (5). The enzyme involved is UDPG-dehydrogenase (1.1.1.22). The *Km* and *Ki* values for competitive inhibition are 1×10^{-4} *M* and 2×10^{-3} *M*, respectively. The lowest concentration of NAD used in the study was 2×10^{-4} *M* and the activity of the enzyme in the presence of 1.25×10^{-3} *M* of salicylic acid was about 74% of the control. In tissues, the concentration of NAD is in the neighborhood of 10^{-7} *M*. The authors believe this is the main and most sensitive site on which salicylic acid acts. It is important to know whether this is the common site of action of other structurally unrelated anti-inflammatory agents. For this purpose, the effect of a few commonly used anti-inflammatory agents, namely, phenylbutazone, oxyphenbutazone, indomethacin, flufenamic acid, and mefenamic acid, on UDPG-dehydrogenase were studied and the results are reported in the present paper.

EXPERIMENTAL

Materials and Chemicals—UDPG (sodium salt Sigma grade), UDPG-dehydrogenase, type III; and NAD, grade III (Sigma Chemical Company, St. Louis, Mo.) were used. Phenylbutazone and oxyphenbutazone (supplied by Dr. T. A. Terzakis of Geigy Pharmaceuticals, Division of Geigy Chemical Corp., Ardsley, N. Y.); mefenamic acid and flufenamic acid (supplied by C. V. Winder of the Research Laboratories of Parke, Davis and Co., Ann Arbor, Mich.);

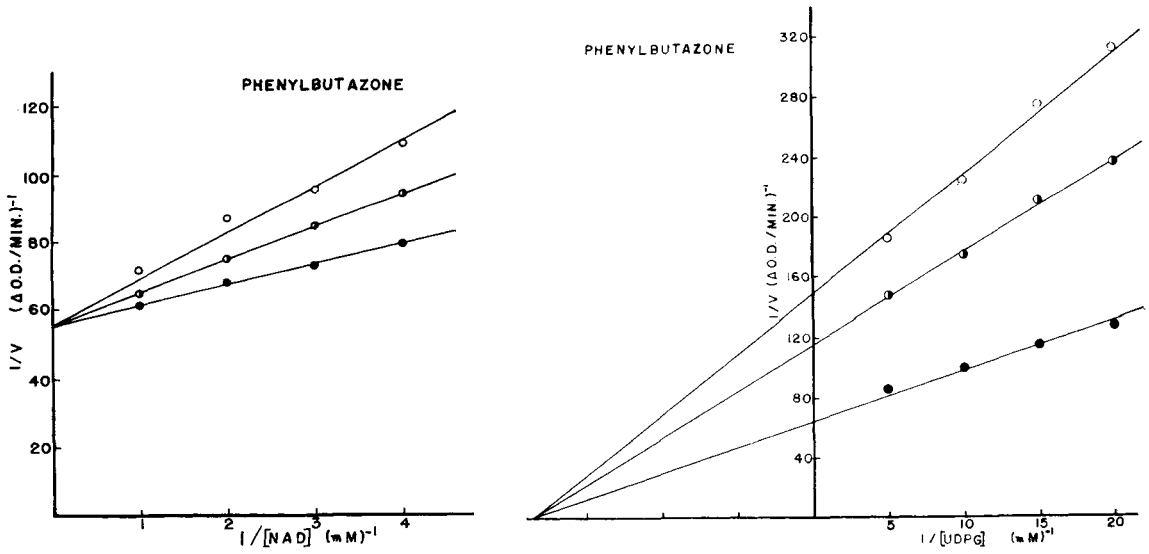

Figure 1—*Inhibition of UDPG-dehydrogenase by phenylbutazone. Left: competitive with NAD. Right: noncompetitive with UDPG. Key:* ●, *control;* ◑, 2×10^{-3} M *phenylbutazone;* ○, 3×10^{-3} M *phenylbutazone.*

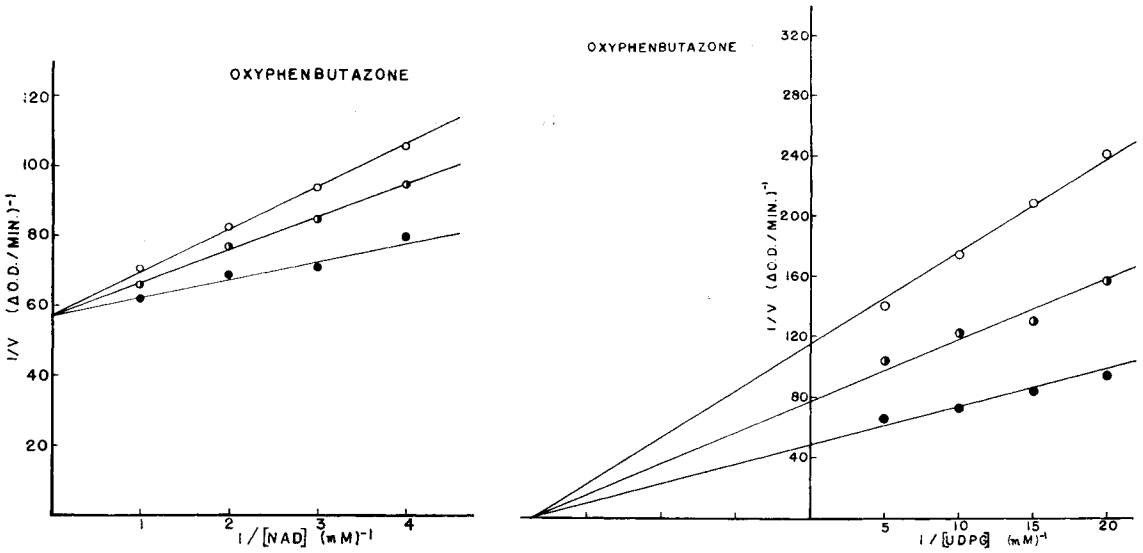

Figure 2—*Inhibition of UDPG-dehydrogenase by oxyphenbutazone. Left: competitive with NAD. Right: noncompetitive with UDPG. Key:* ●, *control;* ◑, *2* × 10^{-3} M *oxyphenbutazone;* ○, *3* × 10^{-3} M *oxyphenbutazone.*

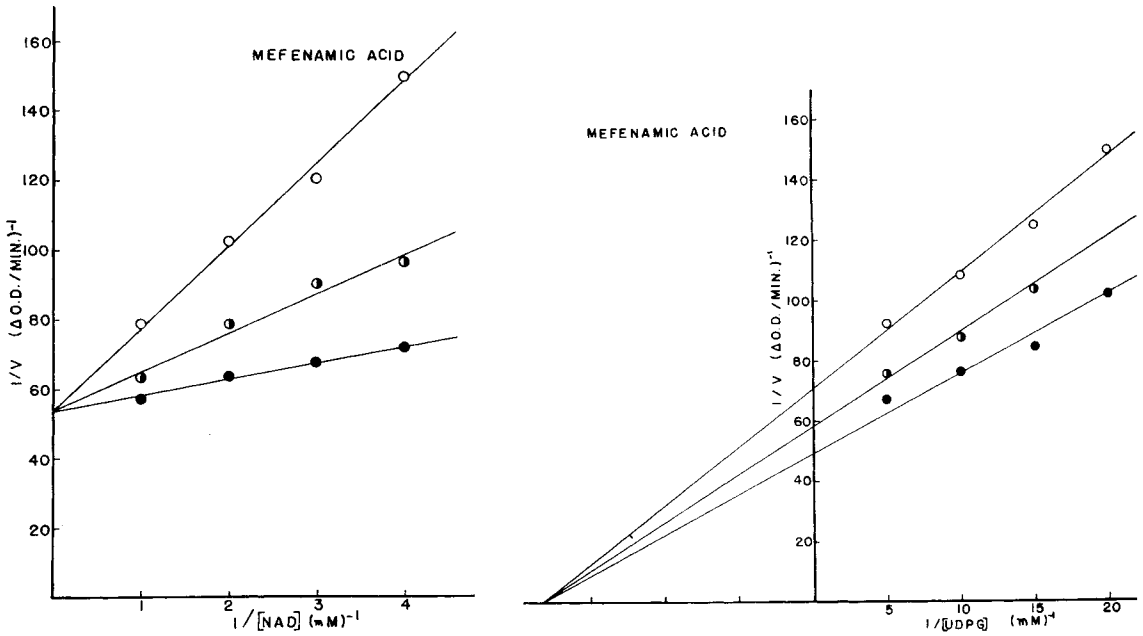

Figure 3—*Inhibition of UDPG-dehydrogenase by mefenamic acid. Left: competitive with NAD. Right: noncompetitive with UDPG. Key:* ●, *control;* ◑, *2* × 10^{-4} M *mefenamic acid;* ○, *4* × 10^{-4} M *mefenamic acid.*

and indomethacin (supplied by Dr. W. B. Gall of the Research Laboratories of Merck, Sharp & Dohme, Div. of Merck and Co., Inc., Rahway, N. J.) were also used.

UDPG-dehydrogenase Assay—The method described by Strominger *et al.* was followed with some necessary modifications to adapt to the quantities of reactants and reaction mixtures and the spectrophotometer used (6). The activity of the enzyme was estimated from the initial rate of reduction of NAD recorded by a recording spectrophotometer (Beckman DB) at 340 mμ in silica cells of 1.0 cm. light path at room temperature after the addition of the enzyme. The reaction mixture contained, in addition to enzyme, 0.1 μ*M* UDPG and 0.5 μ*M* NAD in a total volume of 4.0 ml. of 0.1 *M* glycine

Table I—The *Ki* Values of Anti-inflammatory Agents as Inhibitors of UDPG-Dehydrogenase

Drugs	Competitive with NAD		Noncompetitive with UDPG	
	Km, M	*Ki, M*	*Km, M*	*Ki, M*
Phenylbutazone	1.08×10^{-4}	2.37×10^{-3}	5.51×10^{-5}	2.47×10^{-3}
Oxyphenbutazone	1.03×10^{-4}	2.71×10^{-3}	5.31×10^{-5}	2.60×10^{-3}
Mefenamic acid	1.05×10^{-4}	1.35×10^{-4}	5.62×10^{-5}	9.47×10^{-4}
Flufenamic acid	1.03×10^{-4}	1.88×10^{-4}	5.57×10^{-5}	7.16×10^{-4}
Indomethacin	1.11×10^{-4}	1.56×10^{-4}	5.02×10^{-5}	2.64×10^{-4}
Salicylate	0.96×10^{-4}	2.10×10^{-3}	5.40×10^{-5}	8.70×10^{-3}

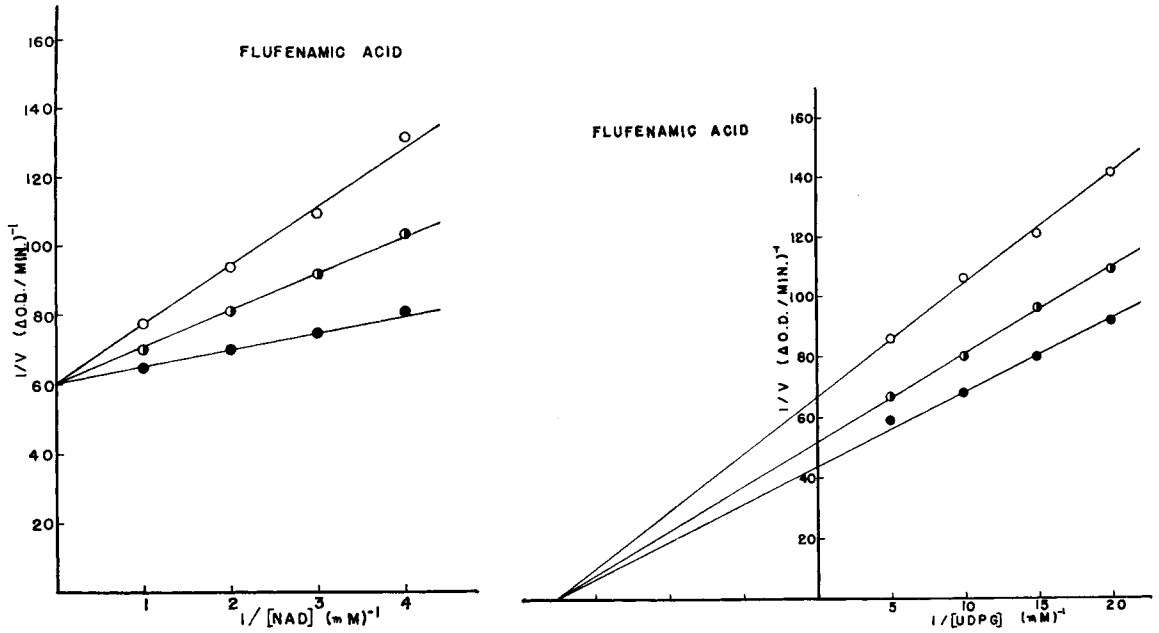

Figure 4—*Inhibition of UDPG-dehydrogenase by flufenamic acid. Left: competitive with NAD. Right: noncompetitive with UDPG. Key:* ● *control;* ◑, *2* × 10^{-4} M *flufenamic acid;* ○, *4* × 10^{-4} M *flufenamic acid.*

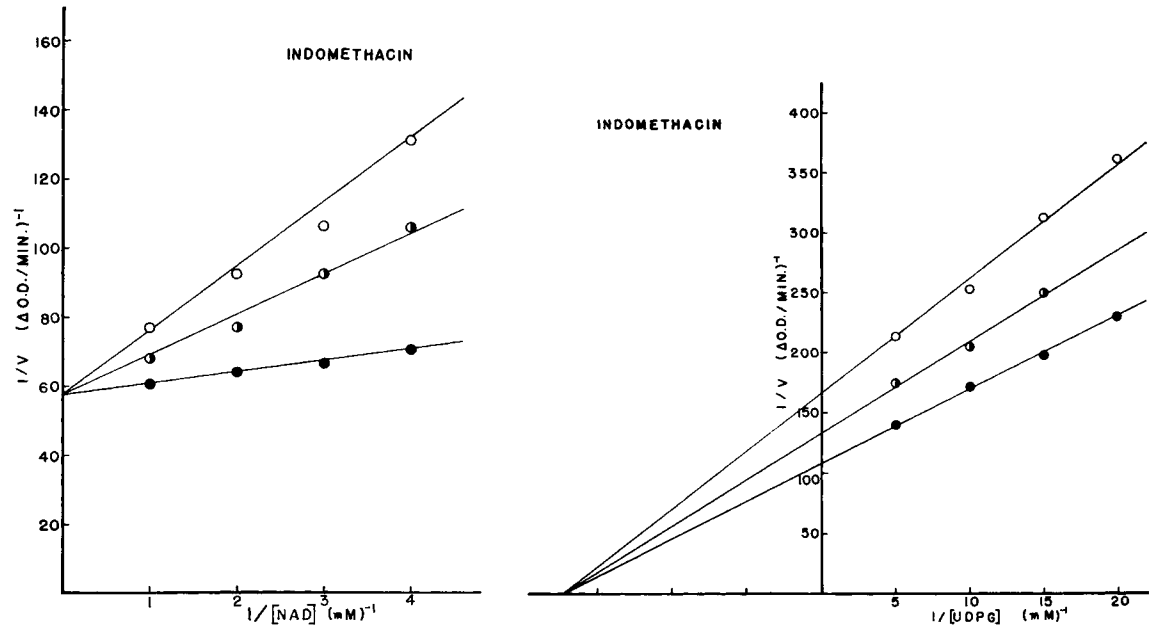

Figure 5—*Inhibition of UDPG-dehydrogenase by indomethacin. Left: competitive with NAD. Key:* ●, *control;* ◑, *2* × 10^{-4} M *indomethacin;* ○, *5* × 10^{-4} M *indomethacin. Right: noncompetitive with UDPG. Key:* ●, *control;* ◑, *0.5* × 10^{-4} M; ○, *1* × 10^{-4} M.

buffer, pH 8.7. A blank without UDPG was used. For the studies on mechanism of action, graded concentrations of either NAD or UDPG were used. Mefenamic acid, flufenamic acid, and indomethacin were dissolved in 95% ethanol. One-tenth milliliter of each drug solution was added to the reaction mixture. One-tenth milliliter of 95% alcohol was added to each of the controls. Phenylbutazone and oxyphenbutazone were dissolved in glycine buffer.

RESULTS AND DISCUSSION

The Lineweaver-Burk plots of the inhibition of a few anti-inflammatory agents on UDPG-dehydrogenase are shown in Figs. 1–5 (7). It is obvious that all of the five useful drugs studied behave essentially the same way toward UDPG-dehydrogenase as salicylic acid. They inhibit UDPG-dehydrogenase competitively with NAD and noncompetitively with UDPG. Phenylbutazone and oxyphenbutazone exert almost identical effect on the enzyme. The therapeutic potency of these two drugs are about the same. Flufenamic acid, mefenamic acid, and indomethacin are more active than phenylbutazone, oxyphenbutazone, or salicylic acid.

The calculated *Km* and *Ki* values of these drugs as competitive inhibitors with NAD and as noncompetitive inhibitors with UDPG are listed in Table I. The *Km* and *Ki* values of salicylic acid are also included for comparison. For competitive inhibition, the *Ki* values, in all cases, are very close to that of the *Km* which indicates that these drugs are very potent inhibitors of UDPG-dehydrogenase. These drugs are slightly less effective as noncompetitors of UDPG. All of the drugs studied are more potent than salicylic acid in inhibiting UDPG-dehydrogenase.

These anti-inflammatory agents studied also retard wound healing (8). The results presented in this paper support the idea that these anti-inflammatory agents retard would healing primarily by a mechanism which involves mucopolysaccharide synthesis (5).

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Rate Assay for Estimation of Thiol Affinity of Sulfhydryl-Reactive Agents: Estimation of SH-Reactivity

R. T. LOUIS-FERDINAND and G. C. FULLER

Abstract □ An interaction between cysteine SH groups and β-nitrostyrene was suggested from alterations in the ultraviolet spectrum of β-nitrostyrene following the addition of cysteine, but not serine. In the presence of β-nitrostyrene or phenylmercuric chloride, the rate of color development resulting from the reaction of cysteine and DTNB was reduced; increasing the concentration of β-nitrostyrene resulted in increased interference in the color development. Excess DTNB did not mitigate the influence of β-nitrostyrene on color development; however, excess cysteine was found to overcome the effects of the β-nitrostyrene on the development of color. The implication of this interference on single point SH content determinations is discussed. Application of inhibition of the cysteine DTNB reaction to estimate SH-reactivity of thiol-reactive agents is described.

Keyphrases □ Thiol affinity—sulfhydryl-reactive agents □ Sulfhydryl-thiol affinity—rate assay □ Cysteine–β-nitrostyrene interaction □ UV spectrophotometry—reaction monitoring

The inactivation of biochemical systems following exposure to sulfhydryl (SH)-reactive compounds and the reversal of the inhibition by thiols has been described earlier (1, 2). *In vitro* estimation of the relative affinities of SH-reactive agents for SH groups in such biological systems would be facilitated by the availability of a suitable model capable of reflecting variable SH-reactivity within a given series of compounds. In this investigation the ability of a series of SH-reactive agents to interfere with the rate of a disulfide-thiol reaction was examined, and employed to compare the SH-reactivity of certain mercurial and nitroolefinic derivatives.

EXPERIMENTAL

Reagents used were analytical grade or equivalent. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was used (Aldrich Chemical Co., Milwaukee, Wis.). Deionized water was used for all solutions. The β-nitrostyrene derivatives were prepared by the Department of Pharmaceutical Chemistry, University of Rhode Island.

The UV spectra of 2-ml. aliquots of β-nitrostyrene solutions (0.1 μmole/ml. of 0.1 *M* phosphate buffer pH 7.3) following the addition of 0.1 μmole/ml. solutions of cysteine or serine (0.1–0.2 ml.), were recorded using a spectrophotometer (Beckman DBG) and an accessory linear-log 25.4 cm. (10-in.) recorder. Reaction cells were maintained in a 30° thermostat-controlled spectrophotometer chamber during incubation and measurement periods.

Sulfhydryl reactivity was determined by following the rate of the color-forming reaction between cysteine and DTNB (3) in the presence and in the absence of the SH-reactive agent. The incubation media contained 7 ml. cysteine (0.25 m*M*), 10 ml. ethylenediaminetetraacetate (EDTA) (0.5 *M*) adjusted to pH 7.3 with potassium hydroxide, 5 ml. deionized water, and 6 ml. 0.1 *M* phosphate buffer (pH 7.3). Following the addition of ethanolic solutions of β-nitrostyrene or aqueous solutions of mercurials (0.05–0.15 ml.), the mixtures were incubated at 30° for 20 min. Twenty-five microliters of DTNB (4 mg./ml.) (prepared fresh daily in 0.1 *M* phosphate buffer pH 7.3) were pipeted into the sample cell to start color development. The rate of absorbance increase at 412 mμ was estimated from straight line portions of the recordings, which were commenced 5 sec. after the addition of DTNB.

RESULTS

Effect of Cysteine on the Ultraviolet Spectrum of β-Nitrostyrene—The effect of the addition of cysteine solutions on the UV spectrum of β-nitrostyrene is shown in Fig. 1. The alterations observed include a decrease in the absorption at 300 mμ, an increase in the absorption at 250 mμ, and a progressive shift in the absorption maximum at 230 mμ to shorter wavelengths. Isosbestic points in the family of spectra can be seen near 250 and 263 mμ. The result of similar additions of serine to β-nitrostyrene solutions led to no qualitative spectral changes. The quantitative alterations seen with serine were no greater than those to be expected from dilution of β-nitrostyrene solutions. These data are suggestive of an interaction between β-nitrostyrene and SH groups of cysteine.

Sulfhydryl Assay—Development of colored reaction product was followed by measuring absorption at 412 mμ, after the addition of DTNB to a solution of cysteine. Enhanced cysteine and product