Effect of Anti-Arthritic Drugs on Sulfhydryl Reactivity of Rat Serum (36438)

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Subnormal levels of serum sulfhydryl groups have been observed in various connective tissue diseases including rheumatoid arthritis (1–3). Lorber *et al.* (1) postulated that impaired sulfhydryl group reactivity in rheumatoid arthritis may be due to accelerated formation of disulfide bonds which could result in macroglobulin formation, protein denaturation and autoimmunity.

In support of this hypothesis, many drugs which are beneficial in the treatment of rheumatoid arthritis alter sulfhydryl group reactivity and inhibit protein denaturation. Clinically used gold salts have been reported to inhibit protein denaturation (4) as well as lysosomal enzyme activity (5, 6) by presumably reacting with sulfhydryl groups. Oronsky *et al.* (7) have shown that sulfhydryl binding agents are capable of suppressing the inflammatory process and that the antiinflammatory activity of these agents and certain nonsteroidal antiinflammatory drugs is, at least partly, related to their effects on sulfhydryl groups. Nonsteroidal antiinflammatory drugs also protect proteins against denaturation (8–11) and Gerber *et al.* (12) have shown that these drugs exhibit a high degree of specificity in accelerating a sulfydryl–disulfide interchange reaction between human sera or bovine serum albumin and an aromatic disulfide. Recently, Swingle *et al.* (13) reported a significant correlation between the ability of nonsteroidal antiinflammatory drugs to accelerate this interchange reaction and their protective effect on guinea pig uv erythema.

However, Butler *et al.* (14) reported that antiinflammatory agents produced little or no acceleration of the sulfhydryl–disulfide interchange reaction in rat serum.

The above clinical and experimental observations strongly suggest that biochemical abnormalities may play an important role in the pathogenesis of rheumatoid arthritis and underscore the need for further research to elucidate the pathological and pharmacological significance of sulfhydryl group reactivity.

Since the laboratory rat is frequently used to evaluate potential antiarthritic agents, we investigated the utility of rat serum to measure alterations of sulfhydryl group reactivity produced by antiarthritic drugs.

The specific objectives of the present investigation were (1) to determine if the sulfhydryl group reactivity of clinically used gold salts could be measured by a sulfhydryl–disulfide interchange reaction between rat serum sulfhydryl groups and dithiobisnitrobenzoic acid and (2) to reevaluate the effects of nonsteroidal antiinflammatory drugs on the sulfhydryl–disulfide interchange reaction in rat serum.

Methods and Materials. Serum was obtained from male Wistar (Purina) rats weighing approximately 200–400 g. The rats were anesthetized with ether inhalation, and blood samples were obtained by cardiac puncture.

For *in vitro* studies, drugs dissolved in 1 ml 0.1 *M* phosphate buffer pH 7.4 were incubated 30–60 min with equal volumes of fresh, pooled undiluted or diluted rat serum. Following incubation, 0.5 ml dithiobisnitrobenzoic acid (DNBA) was added to a final concentration of 65 μM. The resulting interchange reaction between DNBA and serum sulfhydryl groups releases a colored product thionitrobenzoic acid (14) which was measured spectrophotometrically at 440 mμ. Drug concentrations are expressed as final concentration after addition of DNBA.

TABLE I. Effect of *N*-ethylmaleimide on the SH-SS Interchange Reaction in Rat Serum.

Drug	Concentration (μg/ml)[a]	Δ OD (2'-1') $\times 10^{-3}$ Mean[b] (Range)	% Inhibition
N-Ethylmaleimide	12	5 (3–8)	95
	9	41 (36–45)	60
	6	69 (57–76)	33
	3	89 (89–89)	13
Control	0	102 (92–113)	—

[a] Incubated with rat serum for 30 min.

[b] Absorbance change between 1 and 2 min post DNBA mean of four samples per dose level and six samples in control group.

In studies of drug-induced acceleration of the interchange reaction, 100% transmission was established with drug-serum reference samples lacking DNBA.

For oral administration, drugs were prepared as homogenized suspensions in aqueous 0.5% gum tragacanth and administered in a volume of 10 ml/kg body wt by incubation with a blunted 20-gauge needle. Gold sodium thiomalate, prepared in 0.5% benzyl alcohol, was administered im in a volume of 0.5 ml/kg body wt. Following administration of drugs and appropriate control vehicles, individual sera were obtained and assessed for reactivity with DNBA as described above.

All data were statistically analyzed for significant differences between drug and control (vehicle) samples using the Student's *t* test.

Results. Effects of gold salts in vitro. In order to establish the sensitivity of our test procedure in detecting SH binding agents, the effect of various concentrations of *N*-ethylmaleimide, a known sulfhydryl reagent, on the interchange reaction between rat serum sulfhydryl groups and DNBA was determined. As shown in Table I, the inhibition of the interchange reaction produced by this reagent was dose related. The concentration of *N*-ethylmaleimide which produced a 50% inhibition of the interchange reaction in rat serum was calculated to be 7.3 μg/ml.

Figure 1 shows the effects of various concentrations of gold sodium thiomalate on the interchange reaction in rat serum. As shown, gold sodium thiomalate produced a dose related inhibition of the interchange reaction rate.

Utilizing absorbance change from 1 to 2 min to quantitate reaction rates, gold sodium thiomalate was found to inhibit the interchange reaction by 50% at an estimated concentration of 13 μg gold/ml. In a similar experiment, gold thioglucose inhibited the reaction by 50% at an estimated concentra-

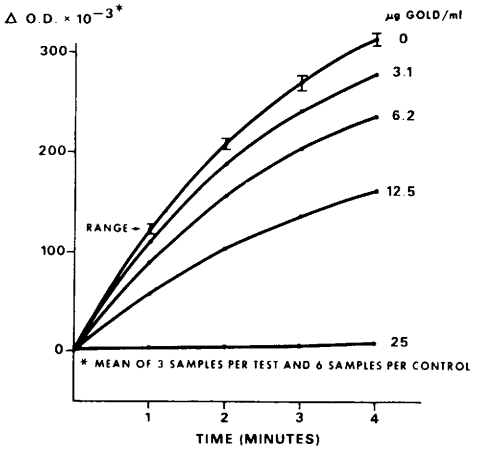

FIG. 1. Effect of gold sodium thiomalate on SH–SS interchange reaction in rat serum. Drug solutions were incubated for 60 min with equal volumes of undiluted rat serum.

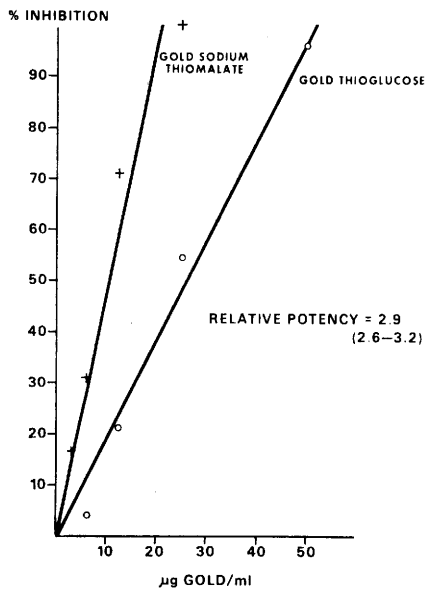

FIG. 2. Relative potency of gold thioglucose and gold sodium thiomalate on inhibiting the SH–SS interchange reaction in rat serum. Relative potency determined by slope ratio assay utilizing optical density (△ OD 2′–1′ post DNBA) data. Values are based on the mean of four samples per dose level and seven samples in the control group.

tion of 27 μg gold/ml. To confirm the apparent difference in potency of gold sodium thiomalate and gold thioglucose on inhibiting the interchange reaction, their relative potency in this procedure was determined.

As shown in Fig. 2, inhibition of the sulfhydryl–disulfide interchange reaction rate produced by both gold sodium thiomalate and gold thioglucose was dose dependent. However, gold sodium thiomalate was found to be 2.9 (2.6–3.2) times more potent than gold thioglucose in inhibiting the interchange reaction rate.

Effect of nonsteroidal antiinflammatory drugs (NAIFD) in vitro. The following experiments were designed to evaluate the effects of nonsteroidal antiinflammatory drugs (NAIFD) on the interchange reaction. Figure 3 illustrates the effect of serum concentration on the sensitivity of the procedure in detecting the activity of NAIFD. The upper portion of Fig. 3 shows results obtained following incubation of equal volumes of phenylbutazone, indomethacin and acetylsalicylic acid with 1 ml rat serum. As shown, only acetylsalicylic acid significantly accelerated the reaction. The results illustrated in the lower portion of Fig. 3 were obtained following incubation of the drugs with 0.5 ml rat serum diluted with equal volumes of phosphate buffer.

Using diluted rat serum, all three nonsteroidal antiinflammatory drugs were found to significantly accelerate the sulfhydryl–disulfide interchange reaction. It should be noted that by 7 min after addition of DNBA, the changes in optical density (OD) of drug samples were equivalent to the control indicating the drugs did not alter the maximum extent of the reaction. It is also evident that drug-induced acceleration occurs within 1 min after addition of DNBA and then the reaction rate rapidly plateaus. Figure 4 further illustrates alterations of the reaction rate by antiinflammatory drugs in comparison to ethacrynic acid, a known sulfhydryl reagent. It is apparent from the results shown in Fig. 4 that change in absorbance from 1 to 2 min, previously used to

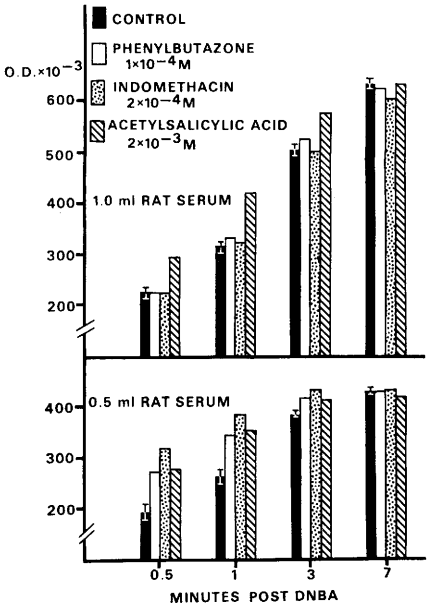

FIG. 3. Effect of serum concentration on the acceleration of the SH–SS interchange reaction by nonsteroidal antiinflammatory drugs. Values represent the mean of three samples per group. I = Control Range.

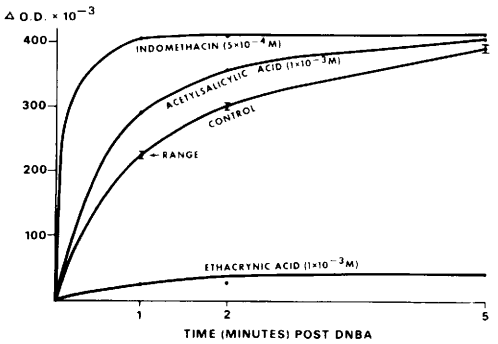

FIG. 4. Effect of indomethacin and acetylsalicylic acid on the SH–SS interchange reaction in rat serum. Each point represents mean value of three samples.

assess inhibition of the reaction rate, is not an appropriate index of drug-induced acceleration. We, therefore, used per cent increase above control absorbance at 1 minute to quantitate drug-induced acceleration of the interchange reaction.

The effect of various antiarthritic drugs on the interchange reaction *in vitro* are summarized in Table II. Nonsteroidal antiinflammatory drugs, phenylbutazone, indomethacin and acetylsalicylic acid produced significant acceleration at $10^{-3}M$ and $10^{-4}M$, although acetylsalicylic acid was only marginally effective at $10^{-4}M$. Prednisolone was ineffective at $10^{-4}M$ but significantly accelerated the reaction at $10^{-3}M$. Chloroquine diphosphate produced a slight inhibition of the reaction at $10^{-4}M$ and slight but inconsistent effects at $10^{-3}M$.

Dose responses and relative potencies of nonsteroidal antiinflammatory drugs on accelerating the interchange reaction *in vitro* are illustrated in Fig. 5. On a molar basis, phenylbutazone was calculated to be 1.5 times more potent than indomethacin and 22 times more potent than acetylsalicylic acid.

In vivo *administration of antiarthritic drugs*. Since results obtained *in vitro* are often misleading due to the absorption, distribution and metabolism characteristics of drugs *in vivo*, the effects of antiarthritic drugs on the interchange reaction were determined following their *in vivo* administration to male Wistar rats. The results are summarized in Table III.

Sera from rats orally treated with phenylbutazone, indomethacin, and acetylsalicylic acid exhibited an accelerated reaction with DNBA compared to sera obtained from vehicle treated rats, whereas prednisolone or chloroquine diphosphate administered at comparable doses were ineffective. In contrast, sera obtained from rats treated with im injections on gold sodium thiomalate exhibited a marked decrease in reactivity with DNBA.

Phenylbutazone and acetylsalicylic acid were administered orally to rats at four equivalent dose levels in order to determine their relative *in vivo* potency on altering sulfhydryl group reactivity in rat serum. Sera obtained from rats orally treated with phenylbutazone and acetylsalicylic acid exhibited significant increased reactivity with DNBA following doses as low as 25 and 50 mg/kg, respectively (Table IV). Their relative *in vivo* potency in this experiment was calculated to be 1.6 (1.1–2.3).

TABLE II. Effect of Antiarthritic Drugs on the SH–SS Interchange Reaction in Rat Serum.

	Percent difference from control OD (1 min)[a]	
Drug	$1 \times 10^{-4}M$	$1 \times 10^{-3}M$
Phenylbutazone	+39[b]	+85[b]
	+33[b]	+98[b]
	+32[b]	
Indomethacin	+15[b]	+89[b]
	+21[b]	+94[b]
Acetylsalicylic acid	+7[b]	+22[b]
	+3[c]	+31[b]
		+24[b]
		+30[b]
		+15[b]
Prednisolone	+2[c]	+26[b]
	0	+35[b]
Chloroquine diphosphate	−16[b]	−10[c]
	−2[c]	+13[b]
	−7[b]	−2[c]

[a] Based on mean values of three samples per group.

[b] Indicates significant difference from control: $p \leq .01$.

[c] Not significant: $p > .05$.

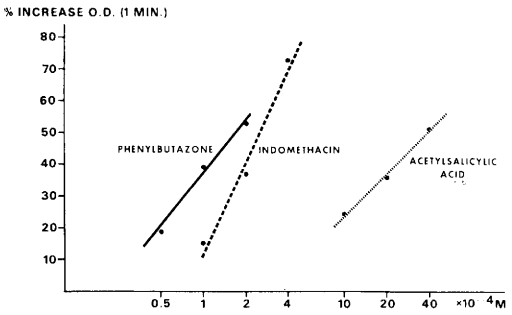

FIG. 5. Relative potency of phenylbutazone, indomethacin and acetylsalicylic acid on accelerating the SH–SS interchange reaction in rat serum. Each point represents mean value of three samples.

Discussion. Although the mode of action of gold salts in the treatment of rheumatoid arthritis is not known, the therapeutic activity of these heavy metal mercaptides is believed to be due to their reactivity with sulfhydryl groups resulting in inhibition of lysosomal enzyme activity (5, 6) and/or protein denaturation (4). In the present investigation, the sulfhydryl group reactivity of gold sodium thiomalate and gold thioglucose, was measured directly by a simple sulfhydryl–disulfide interchange reaction between rat serum sulfhydryl groups and dithiobisnitrobenzoic acid.

The clinically used gold salts were found to produce a potent, dose related inhibition of the interchange reaction rate. These results suggest that the interchange reaction utilizing rat serum sulfhydryl groups is a useful method to determine sulfhydryl group reactivity of potential antiarthritic agents following their *in vitro* or *in vivo* administration.

It is noteworthy that two agents reported to produce remissions in the clinical manifestation of rheumatoid arthritis, gold sodium thiomalate and chloroquine, produced an inhibition of sulfhydryl interchange reaction rather than acceleration as seen with NAIFD which have only a reported symptomatic relief in rheumatoid arthritis.

Nonsteroidal antiinflammatory agents have been reported to increase the sulfhydryl group reactivity of human sera or bovine serum albumin (12) but not rat serum (14). Under our test conditions, nonsteroidal antiinflammatory drugs (phenylbutazone, indomethacin, and acetylsalicylic acid) significantly accelerated the sulfhydryl group reactivity of rat serum following their *in vitro* or *in vivo* administration.

The discrepancy between our results and those of Butler *et al.* (14) may be due to differences in experimental protocol including: serum concentration, utilization of drug-serum reference samples, and methods of assessing drug-induced acceleration of reaction rates. Our results indicate that increased pharmacological sensitivity is obtained using diluted rather than undiluted rat serum in the interchange reaction. The reason for this enhanced sensitivity is presently under investigation but is thought to be due to increased drug-sulfhydryl group ratio and/or decrease in reaction rates.

Butler *et al.* (14) reported increased optical density of rat serum containing phenylbutazone which was believed to be due to turbidity of the drug sample. In order to control alterations of optical density by the test drugs, we utilized drug-serum reference sam-

TABLE III. Effect of Antiarthritic Drugs on the SH–SS Interchange Reaction following *In Vivo* Administration to Rats.

Drug	Dose mg/kg PO (3-hr pretreat)	Percent difference from control OD[a] (1 min)
	100	+69[b]
Phenylbutazone		+64[b]
	50	+58[b]
Indomethacin	100	+42[b]
	20	+7[c]
Acetylsalicylic	200	+59[b]
Acid	100	+57[b]
Prednisolone	100	+2[c]
Chloroquine diphosphate	100	+11[c]
	(I.M.–18 hr)	(ΔOD 2–1 min)
Gold sodium thiomalate	40	−79[b]
		−86[b]

[a] Based on mean values of five rats per group.

[b] Indicates significant difference from vehicle treated (control) rats: $p \leq .01$.

[c] Not significantly different from control group: $p > .05$.

TABLE IV. Relative Potency of Phenylbutazone and Acetylsalicylic Acid on Accelerating the SH–SS Interchange Reaction following Oral Administration to Rats.

Drug	Dose mg/kg PO (3 hr pretreat)	Percent difference[a] from control OD (1 min)
Phenylbutazone	200	+78[b]
	100	+58[b]
	50	+42[b]
	25	+23[b]
Acetylsalicylic acid	200	+59[b]
	100	+45[b]
	50	+47[b]
	25	+ 8[c]

[a] Based on mean values of six rats per group.

[b] Indicates significant difference from vehicle treated (control) rats: $p \leq .01$.

[c] Not significant: $p > .05$.

ples to establish 100% transmission. The negative results following *in vivo* administration of antiinflammatory drugs to normal rats reported by Butler *et al.* (14) were based on optical density changes from 1- to 2-min post DNBA. The results of our investigation have shown that this method of evaluation is not appropriate for the assessment of drug-induced acceleration of the interchange reaction in rat serum.

The utilization of rat serum to evaluate alterations in sulfhydryl group reactivity following *in vivo* administration of antiarthritic drugs may provide a procedure which will (1) more accurately reflect drug-protein interactions *in vivo*, (2) result in better correlations between biochemical effects and antiinflammatory potency and (3) eliminate variables due to drug absorption and metabolism occurring *in vivo*.

Summary and Conclusions. The effect of antiarthritic drugs on a sulfhydryl–disulfide interchange reaction between rat serum sulfhydryl groups and dithiobisnitrobenzoic acid was determined. Gold sodium thiomalate and gold thioglucose produced potent, dose dependent inhibition of the interchange reaction. Thus, the interchange reaction utilizing rat serum appears to be a sensitive and quantitative procedure to measure the sulfhydryl group reactivity of potential antiarthritic agents.

Nonsteroidal antiinflammatory drugs (phenylbutazone, indomethacin and acetylsalicylic acid) administered *in vitro* or *in vivo,* accelerated the interchange reaction in diluted rat serum. The mechanism by which these agents produce this effect and its relevance to their therapeutic activity is not known at the present time. In contrast, the *in vivo* administration of Prednisolone or chloroquine diphosphate did not significantly alter sulfhydryl group reactivity in rat serum.

The results of our investigation suggest that the sulfhydryl–disulfide interchange reaction between rat serum sulfhydryl groups and dithiobisnitrobenzoic acid is a useful procedure in establishing the profile of potential antiarthritic agents.

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