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INHIBITION BY SULFASALAZINE OF LTC SYNTHETASE AND OF RAT LIVER GLUTATHIONE *S*-TRANSFERASES

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Abstract—Sulfasalazine inhibited the formation of sulfidopeptide leukotrienes in ionophore A23187-challenged rat basophil leukemia cells in a dose-dependent fashion (EC_{50} = 0.11 mM). This compound also inhibited the solubilized, particulate LTC synthetase of RBL cells (EC_{50} = approximately 0.4 mM in the presence of a standard substrate mixture). The inhibition of LTC synthetase was paralleled by the capacity of sulfasalazine to potently inhibit several subfractions of the cytosolic rat liver glutathione *S*-transferases. The kinetics of the inhibition of the glutathione *S*-transferases, with 2,4-dinitrochlorobenzene as the substrate, were consistent with competitive inhibition with respect to glutathione (K_i values 0.21 ± 0.05 to 0.46 ± 0.096 μM in three discrete fractions). Inhibition with respect to the chromophoric substrate was uncompetitive in two of the three fractions examined (K'_i values 0.61 ± 0.13 and 1.05 ± 0.14 μM) and non-competitive in the third (K_i = 0.72 μM). The inhibition of the LTC synthetase of RBL cells was also competitive with respect to glutathione (K_i = 120 μM). Both 5-aminosalicyclic acid and *N'*-2-pyridylsulfanilamide inhibitied the one glutathione *S*-transferase fraction which was examined, and *N'*-2-pyridylsulfanilamide also inhibited the LTC synthetase. However, the kinetics of the inhibition of the liver enzyme by these compounds were not consistent with a competitive mechanism relative to glutathione, and the K_i values were at least 100 times greater than the ones for sulfasalazine on the same enzyme.

Sulfasalazine has been used for many years for the management of inflammatory bowel disease. The mode of action of this compound has remained unclear although there have been suggestions that it acts by inhibiting folate uptake or folate-requiring enzymes [1–4], inhibits the destruction of prostaglandins via the prostaglandin 15-dehydrogenase [5, 6], inhibits prostaglandin formation [7, 8], inhibits neutrophil action [9] possibly by interfering with the binding of chemotactic peptides to their receptors on neutrophils [10], inhibits myeloperoxidase-catalyzed histamine release from mast cells [11], inhibits thromboxane formation in platelets [12], and inhibits DNA synthesis in leukocytes [13]. More recently, it has been reported that sulfasalazine can inhibit the soybean lipoxygenase [14] and, indeed, that it inhibits the formation of 5-lipoxygenase products in human polymorphonuclear neutrophils [15], in human colonic mucosa [16], and in isolated rat peritoneal cells [17]. The studies with human neutrophils [15], in particular, suggested that 5-aminosalicylic acid, which is one of the biological degradation products of sulfasalazine, may be a selective inhibitor of the formation of LTA_4 from 5-hydroperoxyeicosatetraenoic acid (5-HPETE) since the formation of 5,12-dihydroxyeicosatetraenoic acids was inhibited while the formation of 5-hydroxyeicosatetraenoic acid (5-HETE) was not.

Because of our ongoing studies with the LTC synthetase (LTA_4: glutathione *S*-transferase) of rat basophil leukemia (RBL) cells [18], we were interested in the possibility that sulfasalazine might inhibit this enzyme as well. The present paper describes studies which demonstrate that sulfasalazine is a potent competitive inhibitor for glutathione both in rat liver glutathione *S*-transferases and in the RBL LTC synthetase.

MATERIALS AND METHODS

Chemicals. Bromsulfophthalein, 2,4-dinitrochlorobenzene (DNCB), 3,4-dichloronitrobenzene (DCNB), and *trans*-4-phenyl-3-buten-2-one, all from the Aldrich Chemical Co., Milwaukee, WI, 5-aminosalicylic acid, ethacrynic acid, Δ^5-androstene-3,17-dione, hematin and glutathione, all from the Sigma Chemical Co., St. Louis, MO, 1,2-epoxy-3-(*p*-nitrophenoxy) propane (ENPP) and Triton X-100 from Eastman Kodak, Rochester, NY, epoxy-activated Sepharose 6B, chromatofocusing reagents and adsorbents from Pharmacia Fine Chemicals, Piscataway, NJ, the calcium ionophore, A23187, from Calbiochem-Behring, San Diego, CA, and triethyltin chloride and cumene hydroperoxide from ICN Life Sciences and K & K Pharmaceuticals Group, respectively, Plainview, NY, were purchased from the suppliers. Sulfasalazine was a gift from the Lederle Laboratories of American Cyanamide. *N'*-2-Pyridylsulfanilamide [19] and leukotriene A_4 (LTA_4 [20]) were prepared by Drs. Herman W. Smith and Douglas R. Morton, Jr., of these laboratories by published procedures. Glutathionyl sepharose was prepared by published procedures [21].

Isolation and characterization of rat liver glutathione S-transferases. The procedures employed for the isolation of the glutathione *S*-transferase frac-

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tions from rat liver cytosol have been described elsewhere [22]. Briefly, glutathione *S*-transferase activity in the 100,000 *g* supernatant fraction from rat liver homogenates was isolated by adsorption to and elution from glutathionyl sepharose and was then fractionated by serial chromatofocusing at increasing pH. The fractions which were eluted were characterized for their activity using both DCNB and DNCB [23], and the fractions which were used in the present studies defined clear-cut peaks of activity in which biologic activity with the two substrates and absorption in the ultraviolet at 280 nm appeared to coincide. The pooled fractions were further characterized with respect to their substrate specificity [24–26] and inhibitor profile when DNCB was used as the substrate [27]. In the latter studies, the uninhibited reaction was always measured in any given reaction tube before the addition of inhibitor, and inhibition was then expressed as a fraction of the uninhibited rate. To overcome errors in the quantitation of the results which arose from the variability in the uninhibited reaction, the rate of the uninhibited reaction was averaged across all comparable incubations and the inhibited rates were then calculated using the corrected uninhibited rate and the observed ratio of the inhibited to the uninhibited reaction. The activities of the fractions with different substrates were expressed as μmoles product formed·min^{-1}·(mg protein)$^{-1}$ based on the published extinction coefficients of the reaction products. The EC_{50} values (concentration causing 50% inhibition) for the inhibitors were computed from the relationship of the $\log_{10}$ of the inhibitor concentration to the percentage inhibition, using the method of least squares and a minimum of three inhibitor concentrations, all falling between 15 and 85% inhibition. Protein concentrations were determined by the method of Folin.

Kinetic studies and statistical treatment of the results. The evaluation of the kinetics of the inhibition of the glutathione *S*-transferases by sulfasalazine was carried out under conditions where one of the substrates (DNCB or glutathione) was present at saturating concentration and the concentration of the other substrate was varied around the apparent K_m. The results were normalized as described above. The data were analyzed by the method of Lineweaver and Burk, testing for the goodness of fit of the data under the assumption that there was either a common intercept on the Y axis (competitive model) or a common slope (uncompetitive model) using the F test of the general linear model procedure of SAS [28], and weighting the results by the sample size for any given point. Spuriously large residuals (observed minus predicted values) were eliminated by testing the significance of the appropriate indicator variables after adjusting for the slopes and intercepts. If the competitive model was consistent with the data, the common intercept gave an estimate of V_{max}, and a plot of the slopes of the Lineweaver–Burk plots against the associated sulfasalazine concentration allowed estimates to be made of K_m and K_i. Analogously, if the uncompetitive model was consistent with the data and the competitive model was not, K_i'/V_{max} could be estimated from the common slopes, and a plot of the intercepts (X = 0) against the inhibitor concentration could be used to solve for K_i'. The standard errors which are associated with these estimates can be estimated based on Fieller's formula [29]. In the one instance where this process did not result in a definitive answer, the Lineweaver–Burk plot was, in fact, consistent with non-competitive inhibition. Forcing the lines through a common intercept on the horizontal axis and plotting the intercepts on the vertical axis against the inhibitor concentration yielded estimates of K_i and V_{max}.

Tests with intact RBL cells and with solubilized LTC synthetase. RBL cells were cultured in Minimal Essential Medium (Eagle) which was supplemented with glutamine, streptomycin, penicillin and gentamycin as described [18]. For the measurement of the inhibition of leukotriene synthesis in intact cells, the harvested, washed cells were suspended at a concentration of 1.875×10^7 cells/ml in modified Tyrode's buffer (containing, in g/l: NaCl, 8.0; KCl, 0.20; NaH_2PO_4, 0.050; $NaHCO_3$, 1.0; $CaCl_2 \cdot 2H_2O$, 0.12; glucose 1.0; and gelatin, 1.0), which was 10 mM with respect to 4-(2-hydroxyethyl)-1-piperazine-ethanosulfonic acid (Hepes), pH 7.0, and were incubated at 30° for 20 min. The cells were then distributed (0.2 ml/tube) into 12 × 75 mm plastic culture tubes (Sarsted, Princeton, NJ) which contained 0.2 ml of the same buffer containing double strength inhibitor solutions. After 2.5 min of further incubation, 50 μl of a neutralized 0.1 M cysteine solution was added to each tube, and this was followed in another 2.5 min by 50 μl of a 120 μg/ml solution of the calcium ionophore. The stock solution from which the ionophore dilution was prepared contained 10 mg/ml of A23187 in dimethyl sulfoxide and was stored between uses at 0°. Incubation was continued for 20 min at which time the reaction was stopped by chilling the tubes in an ice bath and adding 2 ml per tube of absolute methanol to dissociate any bound leukotrienes and to precipitate the proteins. After standing (30 min, 0°) supernatant fractions were collected by centrifugation and were dried under vacuum at 45° in a Speed Vac Concentrator (Savant Instruments Inc., Hicksville, NY).

The LTC synthetase was solubilized from the high speed particulate fraction of RBL cell homogenates using 0.3% Triton X-100 as previously described [18]. The assay for LTC synthetase activity consisted of incubating the enzyme in the presence of 3 mM glutathione in 0.45 ml of 10 mM Hepes buffer which was 1 mM with respect to EDTA, pH 7.0, and adding 50 μl of a freshly prepared dilution of LTA_4 (100 μM concentration of the lithium salt of LTA_4 in the same buffer which also contained 10 mg/ml bovine serum albumin) to initiate the reaction. Incubations were stopped after 10 min as described above, and, after drying, the samples were processed by adsorption to and elution from Dowex 50 as described [18] before they were bioassayed for LTC on the guinea pig ileum using a synthetic LTC_4 reference standard. The identity of the generated biologic activity to leukotriene C_4 was established by the comparison of elution times on high pressure liquid chromatography using 10 μm reverse phase (C18) column (IBM No. SM3112943, Danbury, CT) eluted isocratically with water–methanol–tetrahydrofuran–

acetic acid (45:30:25:0.1), pH 5.5, at a flow rate of 1 ml/min.

The fit of the data from the bioassay to the kinetic curves was not as good as was that from the spectrophotometric determinations. Therefore, the results were further normalized before they were used for kinetic estimates. This was accomplished by relying on the fact that there was a range of inhibitor concentrations over which there was a linear relationship between the $\log_{10}$ of the inhibitor concentration and the amount of product formed. Using this relationship, values for the inhibited rate of reaction could be obtained by interpolation, using all the values on the curve rather than having to rely on the individual values with their larger associated errors. These interpolated values were then compared across substrate concentrations by the method of Lineweaver and Burk as described above to determine the nature of the inhibition which was observed.

RESULTS

Inhibition of leukotriene synthesis in RBL cells. The inhibition of leukotriene synthesis in ionophore-challenged RBL cells by sulfasalazine, 5-aminosalicylic acid and *N'*-2-pyridylsulfanilamide is described in Fig. 1. The EC_{50} values for these compounds were 0.11, 0.38 and 0.65 mM respectively. A similar plot for the effects of sulfasalazine on the generation of LTC_4 from LTA_4, using the solubilized LTC synthetase of RBL cells, is shown in Fig. 2. The EC_{50} for sulfasalazine (0.94 mM) was higher than that for the intact cells but this may be a reflection of the higher glutathione concentration (see below). The EC_{50} for *N'*-2-pyridylsulfanilamide was 0.83 mM while there was no significant inhibition of this reaction with 5-aminosalicylic acid up to a concentration of 20 mM (data not shown).

Characterization of rat liver glutathione S-transferase fractions. The elution profiles of the three fractions of cytosolic glutathione *S*-transferase from rat liver which were studied are shown in Fig. 3. The substrate specificity of these pooled fractions is summarized in Table 1. Note that all three fractions converted significant amounts of LTA_4 to LTC_4, and that there were differences in the relative activities of these fractions with respect to several of the substrates. The product(s) of the reaction of LTA_4 with glutathione in the presence of the liver enzyme fractions was characterized by comparing its elution time on reverse phase HPLC to those of synthetic leukotriene standards [22]. Although several peaks absorbing in the ultraviolet were detected, only one of these corresponded in elution time to a known leukotriene, and that was LTC_4. There was no evidence of any LTD_4 or LTE_4 in the preparations. Furthermore, the behavior of the products in the bioassay on the guinea pig ileum was consistent with the properties of LTC_4 (slow attainment of maximum contraction and difficulty in washing out the sample). The absence of LTD_4 and LTE_4 in the samples is consistent with the fact that the γ-glutamyl transpeptidase which would convert LTC to LTD is a particulate enzyme and the enzyme fractions we used were all derived from the high speed supernatant fraction of rat liver homogenates.

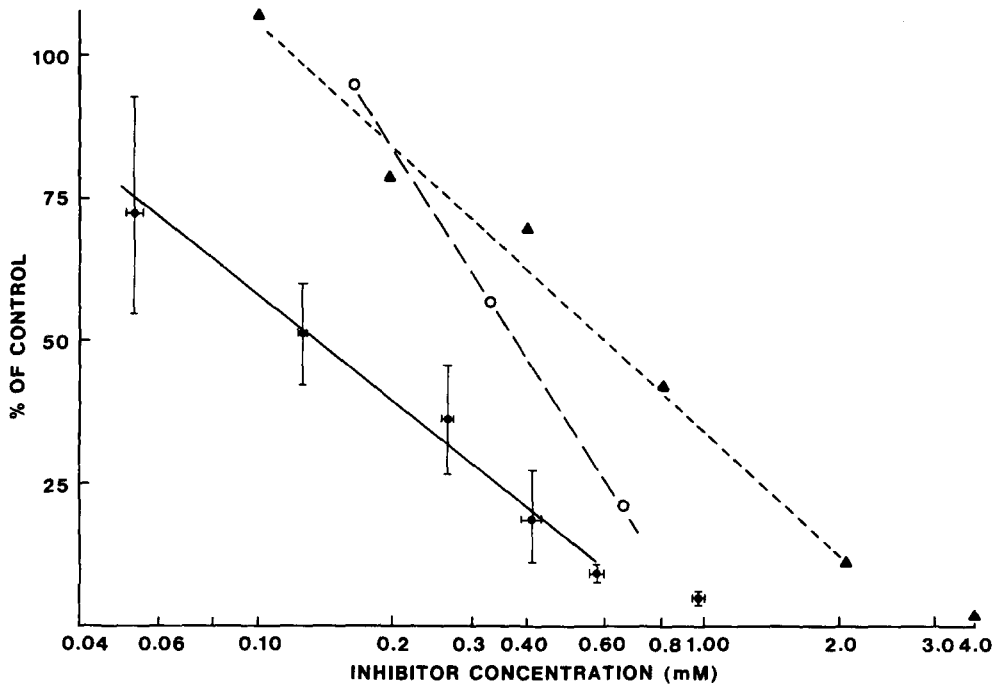

Fig. 1. Dose dependence of the inhibition of sulfidopeptide leukotriene production in A23187-challenged RBL cells by sulfalazine (●——●; average of four experiments), 5-aminosalicylic acid (○——○) and *N'*-2-pyridylsulfanilamide (▲---▲). Results are expressed as percent of control, where control production was (range of four experiments) from 400 to 600 pmoles (expressed as LTC)/7.5 × 10^6 cells in 1 ml of incubation medium in 20 min. See Materials and Methods for details.

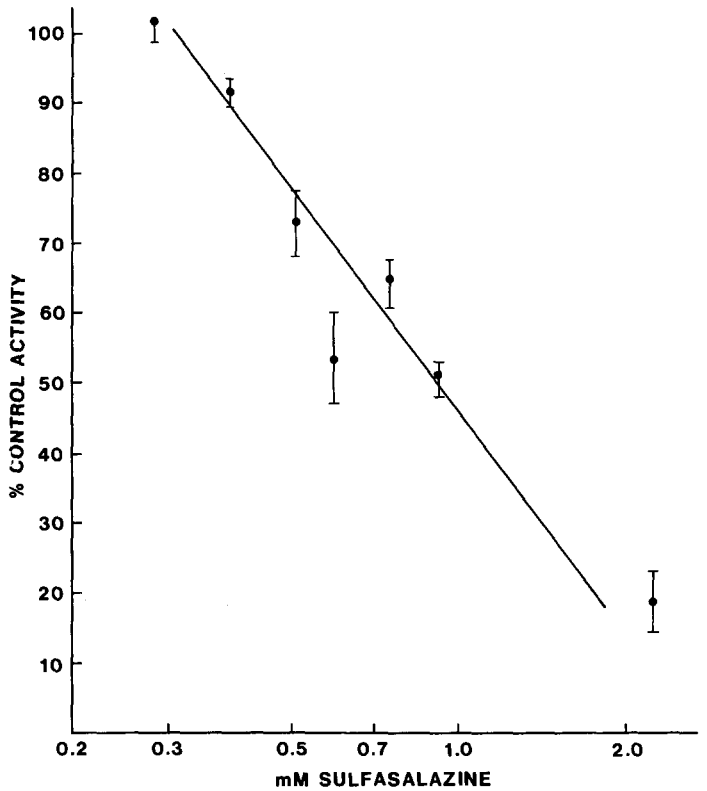

Fig. 2. Dose dependence of the inhibition of LTC production by solubilized RBL cell LTC synthetase by sulfasalazine. The enzyme preparation was the 100,000 *g* supernatant fraction from the incubation of the 100,000 *g* pellet from RBL homogenates with 0.3% Triton X-100 at 4° for 30 min. Incubations were in the presence of 0.3% Triton X-100, 3 mM glutathione, 10 μM LTA_4 lithium salt and 1 mg/ml bovine serum albumin in pH 7.0 buffer containing 1 mM EDTA for 10 min at 37°. Control LTC production (range of four experiments) was 58–132 pmoles/mg protein/min. See Materials and Methods for details.

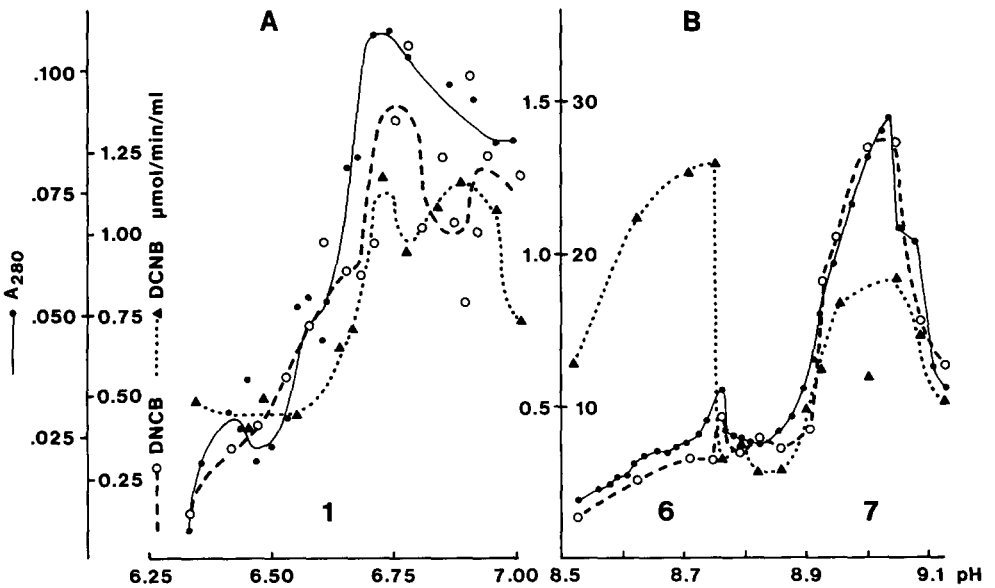

Fig. 3. Elution profiles on chromatofocusing separations of the affinity-purified rat liver glutathione *S*-transferase fractions that were employed. Key: solid circles and solid line, absorption at 280 nm; open circles and dashed line, enzyme activity with DNCB as substrate; closed triangles and short dashed line, activity with DCNB as substrate. Results are plotted against the pH of the fractions which was obtained by interpolation of the values determined for each fifth fraction. Note that the scales for panel A (fraction 1) are markedly different from the scales for panel B.

Table 1. Substrate specificity of rat liver glutathione *S*-transferase subfractions*

Substrate	Specific activities [μmoles·(mg protein)$^{-1}$·min^{-1}] Fraction number† 1	6	7
DNCB	6.39	4.74	12.6
DCNB	0.711	0.560	1.41
ENPP	0.198	0.302	0.468
Ethacrynic acid	0.152	0.0116	0.0331
Bromsulfophthalein	0.0685	0.140	0.235
trans-4-Phenyl-3-buten-2-one	0.0155	0.097	0.0344
Leukotriene A_4	0.0898‡	0.173‡	0.375‡
Cumene hydroperoxide§	1.24	0.092	0.809

* Results are based on least squares calculations using a minimum of three different enzyme concentrations.
† None of these fractions gave any reaction with Δ^5-androstene-3,12 dione.
‡ Activity is in nmoles·(mg protein)$^{-1}$·min^{-1}.
§ Glutathione peroxidase activity.

The susceptibility of the same enzyme fractions to inhibition by a series of known inhibitors of glutathione *S*-transferases, as well as by sulfasalazine and its breakdown products, is summarized in Table 2. Although there were some differences in the susceptibility of the different fractions to inhibition, these were not nearly as great as the differences in substrate specificity. Nor did these inhibition profiles shed any light on the identity of the isozymes of glutathione *S*-transferase in the fractions [27].

We were interested in determining if the generation of LTC_4 by the rat liver enzymes might also be susceptible to inhibition by sulfasalazine. As shown in Fig. 4, there was a large difference in the susceptibilities of the LTC-generating activities of the different fractions to inhibition by sulfasalazine. While no inhibition was seen with fraction 1 up to an inhibitor concentration of 0.3 mM, the activity of fraction 7 was inhibited by more than 50% even at 0.03 mM sulfasalazine. This range is much greater than the range of EC_{50} values for the same inhibitor when DNCB was used as the substrate.

Kinetics of the inhibition by sulfasalazine. A possible explanation for the differences in EC_{50} that were encountered when the activity of sulfasalazine was measured with different substrates might be in the nature of the inhibition. We therefore studied the kinetics of the inhibition of the glutathione *S*-transferases. Since the glutathione *S*-transferase reaction is a bimolecular reaction, we made the simplifying assumption that, by employing one substrate at a concentration which is well above the apparent K_m,

Table 2. Inhibitor profile of rat liver glutathione *S*-transferase subfractions*

Inhibitor	EC_{50} (μM) Fraction number 1	6	7
Bromsulfophthalein	22.5	29.2	27.1
Hematin	0.136	0.134	0.122
Triethyltin chloride	0.026	0.069	0.040
Sulfasalazine	1.56	1.06	0.95
5-Aminosalicylic acid	ND†	ND	59.9
N'-2-Pyridylsulfanilamide	ND	ND	115

* All assays were performed using DNCB (0.687 mM) as substrate. Results are based on least squares calculations from the relationship of $\log_{10}$ of dose of inhibitor against percent inhibition.
† Not determined.

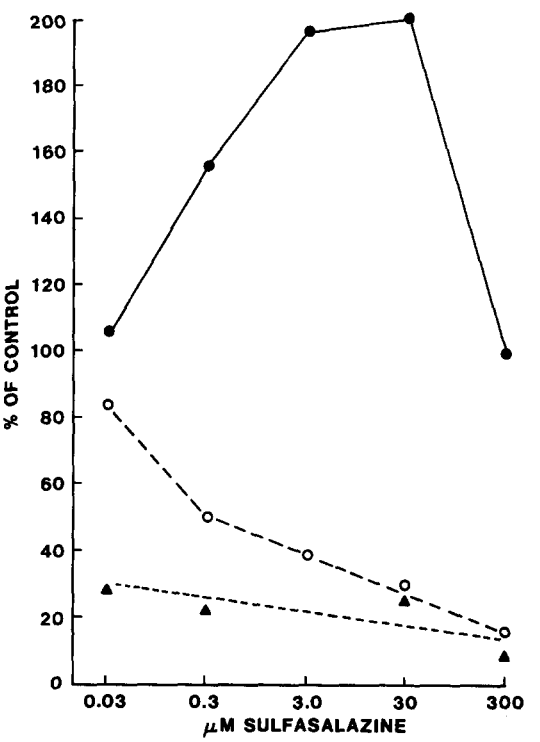

Fig. 4. Dose dependence of the inhibition of the generation of LTC by subfractions of rat liver glutathione *S*-transferases by sulfasalazine. Key: (●——●) fraction 1; (○——○) fraction 6; (▲---▲) fraction 7. Results are expressed as percent of control production. See Table 1 for the uninhibited production of LTC by these fractions.

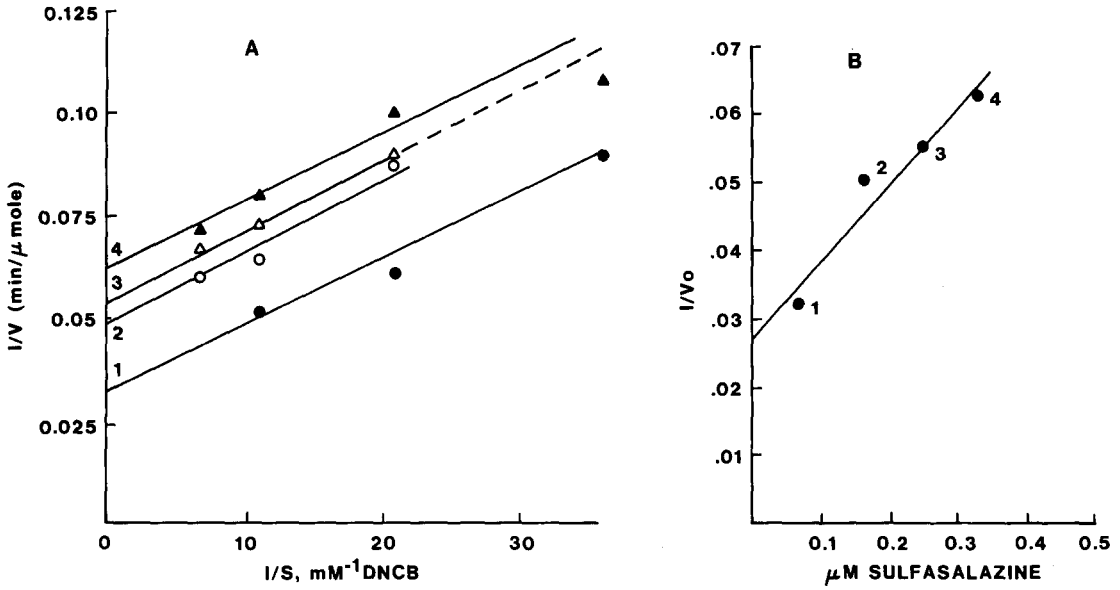

Fig. 5. Kinetics of the inhibition of fraction 1 of rat liver glutathione *S*-transferase by sulfasalazine, under conditions of a constant (3 mM) glutathione concentration and various DNCB concentrations. Panel A: Lineweaver–Burk plot; Line 1, 0.67 μM sulfasalazine; Line 2, 0.162 μM sulfasalazine; Line 3, 0.25 μM sulfasalazine; and Line 4, 0.33 μM sulfasalazine. Panel B: plot of the Y axis intercepts against the concentration of sulfasalazine.

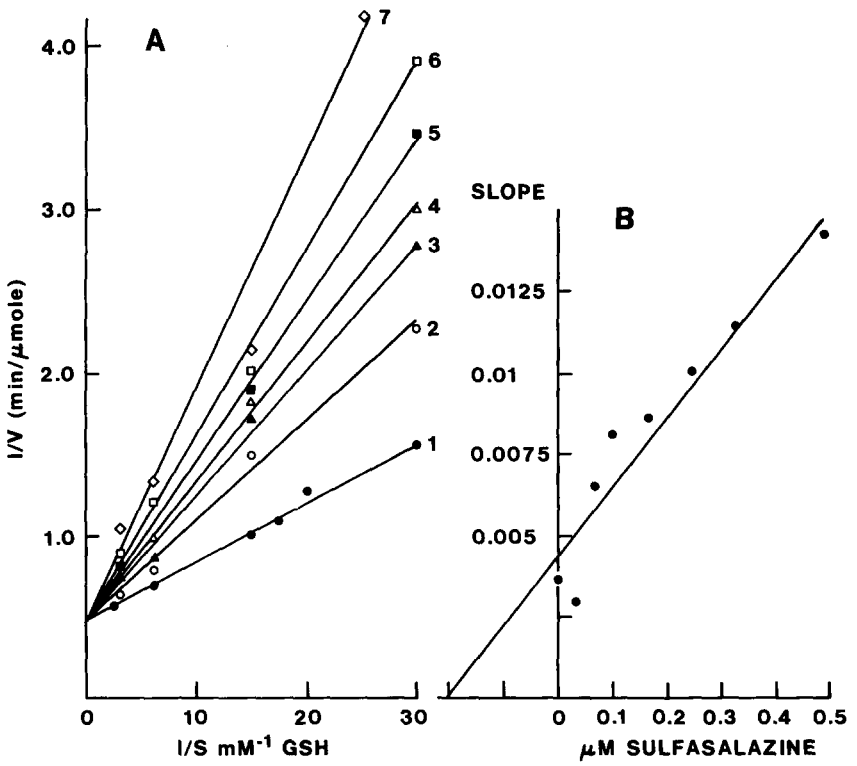

Fig. 6. Kinetics of the inhibition of fraction 1 of rat liver glutathione *S*-transferase by sulfasalazine, under conditions of a constant (0.69 mM) DNCB concentration and various glutathione concentrations. Panel A: Lineweaver–Burk plot; Line 1, no sulfasalazine; Line 2, 0.067 μM sulfasalazine; Line 3, 0.10 μM sulfasalazine; Line 4, 0.167 μM sulfasalazine; Line 5, 0.25 μM sulfasalazine; Line 6, 0.38 μM sulfasalazine; and Line 7, 0.50 μM sulfasalazine. Panel B: plot of the slopes of the double-reciprocal plots against the concentration of sulfasalazine.

Table 3. Summary of kinetics of inhibition of glutathione *S*-transferase fractions by sulfasalazine with dinitrochlorobenzene as substrate

Fraction no.	Variable substrate	Type of inhibition	V_{max} (μmoles/min)	K_m (mM)	K_i (μM)	K_i' (μM)
1	GSH	Comp.	19.8 ± 2.1	0.084 ± 0.013	0.214 ± 0.049	
	DNCB	Uncomp.	26.4 ± 2.0	0.022 ± 0.003		0.61 ± 0.13
6	GSH	Comp.	32.4 ± 2.2	0.166 ± 0.027	0.280 ± 0.072	
	DNCB	Non-Comp.*	0.68 ± 0.02	0.171	0.72†	
7	GSH	Comp.	60.6 ± 11.1	0.155 ± 0.031	0.464 ± 0.096	
	DNCB	Uncomp.	55.2 ± 4.6	0.083 ± 0.010		1.05 ± 0.14

* Derived from the relationship, slope of Lineweaver–Burk plot = $K_s[1 + i/K_i]/V$, where $1/K_s$ = intercept on the horizontal axis.

† Derived from a plot of intercepts on vertical axis against sulfasalazine concentration (K_i is the intercept on the horizontal axis).

and then varying the second substrate, conditions would approximate those of a unimolecular reaction. All three enzyme fractions were evaluated in detail. The results for fraction 1 are presented in Figs. 5 and 6 and are representative of the results which were obtained with the other fractions. Figure 5 is a plot of the kinetics of inhibition of fraction 1, with glutathione at 3 mM and the concentration of DNCB being varied. It is clear that the inhibition was consistent with an uncompetitive model (slopes of the Lineweaver–Burk plots were parallel). By contrast (Fig. 6), when DNCB was held at 0.69 mM and the concentration of glutathione was varied, the plot of the results was consistent with competitive inhibition. Similar studies with fractions 6 and 7 confirmed that the inhibition by sulfasalazine was competitive with respect to glutathione. In the case of fraction 6, the double-reciprocal plots intersect on the horizontal axis, suggesting that the inhibition may have been non-competitive with respect to DNCB. However, in the case of fraction 7, the inhibition with respect to DNCB was again found to be uncompetitive. The kinetic constants which could be computed from these plots are summarized in Table 3. It is clear that, although the K_m for each of the substrates varied somewhat from one fraction of

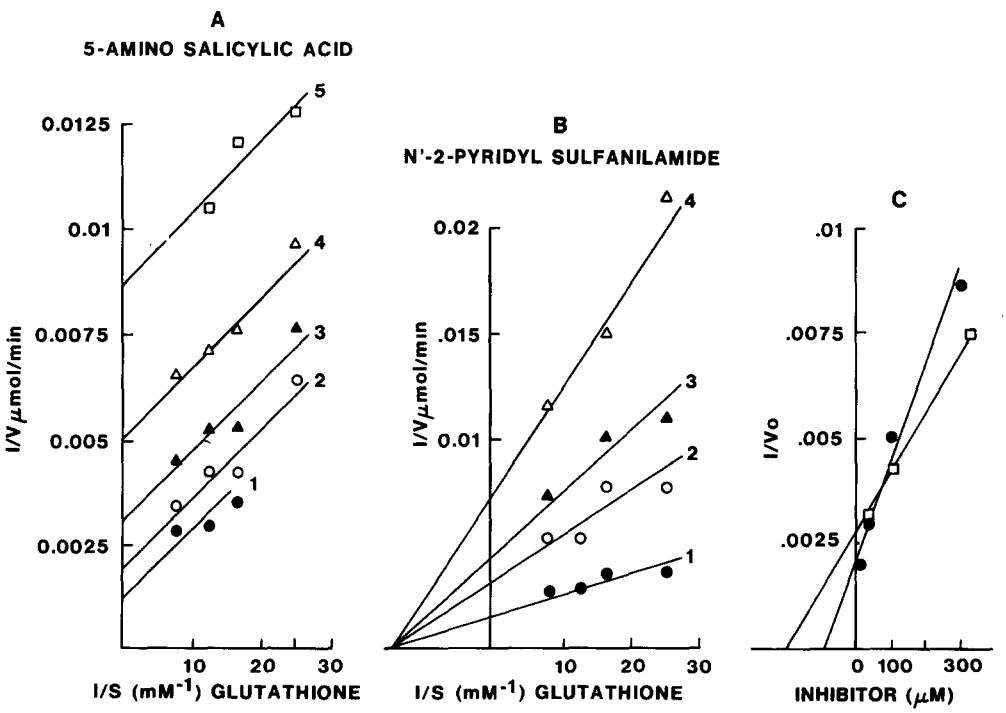

Fig. 7. Kinetics of the inhibition of fraction 7 of rat liver glutathione *S*-transferase by 5-aminosalicylic acid (panel A) and *N'*-2-pyridylsulfanilamide (panel B) under conditions of constant (0.69 mM) DNCB concentration and variable glutathione concentration. Panel C is a plot of the vertical intercepts from panels A and B against the concentration of sulfasalazine. Inhibitor concentrations: Panel A: Line 1, 0; Line 2, 10 μM sulfasalazine; Line 3, 30 μM sulfasalazine; Line 4, 100 μM sulfasalazine; and Line 5, 300 μM sulfasalazine. Panel B: Line 1, 0; Line 2, 33 μM; Line 3, 100 μM; and Line 4, 330 μM. Panel C: (●) 5-aminosalicylic acid, and (○) *N'*-2-pyridylsulfanilamide.

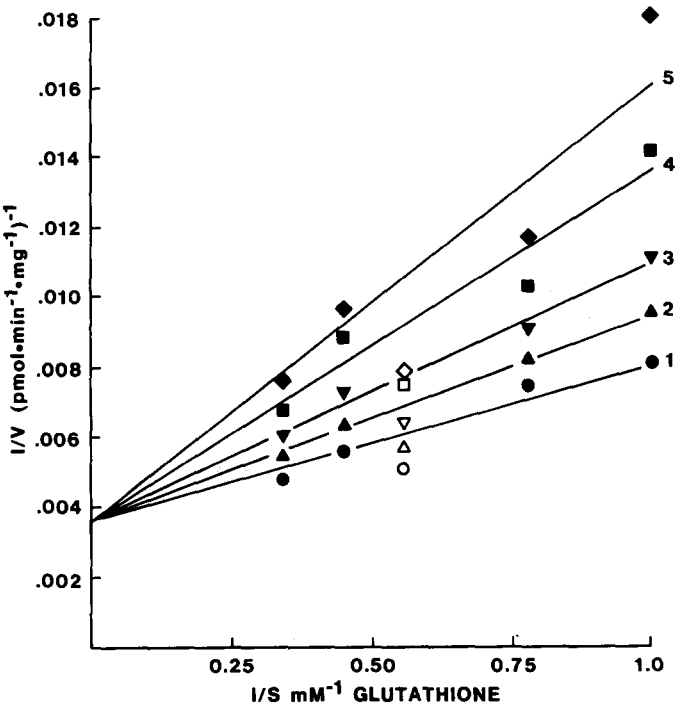

Fig. 8. Kinetics of the inhibition of solubilized RBL cell LTC synthetase by sulfasalazine under conditions of constant (10 μM) LTA_4 concentration and variable glutathione concentration. Results are shown as a Lineweaver–Burk plot of experimental points which were normalized as described in Materials and Methods. Key: Line 1, 0.40 μM; Line 2, 0.50 μM; Line 3, 0.60 μM; Line 4, 0.75 μM; and Line 5, 0.90 μM sulfasalazine. The open symbols were not included in the computations.

the enzyme to the next, the "saturating" concentrations that were chosen were, indeed, several multiples of the computed K_m values.

We next asked whether the inhibition of the liver glutathione *S*-transferases by either of the sulfasalazine breakdown products might follow similar kinetics to those which we had found for sulfasalazine itself. As shown in Fig. 7, this was not the case. The inhibition by 5-aminosalicylic acid was consistent with uncompetitive kinetics relative to glutathione while the inhibition by *N'*-2-pyridylsulfanilamide was consistent with non-competitive kinetics. K_i values were 0.1 and 0.2 mM respectively.

Finally, in view of the differences in the susceptibility of the liver fractions to inhibition when LTA_4 served as the substrate, we turned to the kinetics of inhibition of the RBL cell-derived LTC synthetase. As shown in Fig. 8, the kinetics that were observed were consistent with a competitive model in which sulfasalazine competes with glutathione on the enzyme. It should be stressed, however, that in contrast to the studies with the liver enzymes, the substrate concentration of the second substrate (LTA_4) was far from saturating in this case. Indeed, preliminary estimates of the K_m for LTA_4 by the LTC synthetase of RBL cells are around 0.06 mM (M. K. Bach and J. R. Brashler, unpublished observations), and it is not feasible to use sufficient amounts of this precious substrate to achieve conditions where the concentration of LTA is not limiting the reaction.

DISCUSSION

The results reported here confirm the reported observation that sulfasalazine is an inhibitor of the formation of products of the 5-lipoxygenase pathway of arachidonate metabolism. However, in addition to the possibility that this compound might inhibit the 5-lipoxygenase itself, and thereby account for the inhibition of the formation of 5-HETE and LTB_4 [15, 16], our results suggest that this compound may also inhibit the final step in the synthesis of the sulfidopeptide leukotrienes, the coupling of glutathione to LTA_4. The results presented here do not permit us to conclude unequivocably to what extent the possible inhibition of the 5-lipoxygenase of RBL cells by sulfasalazine contributed to the overall inhibitory activity. Clearly, the EC_{50} for the reaction in intact cells (Fig. 1) was lower than the EC_{50} for the isolated LTC synthetase (Fig. 2) or for the 5-lipoxygenase of neutrophils [15, 16]. The possibility that the 5-lipoxygenase of RBL cells is more sensitive to inhibition by this compound than is the enzyme in neutrophils cannot be ruled out; on the other hand, given the strong dependence of the inhibition on the glutathione concentration, it seems likely that some or all of the discrepancy in the EC_{50} between the intact cells and the isolated enzyme could be explained by differences in the effective glutathione concentrations.

It was not possible to relate the differences in substrate specificity of our isolated fractions (Table

1) to the described differences between the highly purified isozymes of this enzyme which have been described [24–26]. Unfortunately, none of our fractions reacted significantly with Δ^5-androstene-dione. However, comparison of substrate specificity ratios which could be formed to those published [30] suggests that fraction 1 may correspond to form AC which, according to Mannervik *et al.* elutes at the second lowest pH from chromatofocusing while fraction 7 may correspond to form A_2 which is the fourth peak to elute as the pH is decreased. There is no resemblance in these ratios between those we have observed for fraction 6 and those of any of the forms described. The inhibitor profiles (Table 2) were not instructive in efforts to compare our enzyme fractions to the purified subfractions which have been described in the literature since the EC_{50} values which we observed did not differ significantly from one fraction to the next while the very same inhibitors have been reported to be diagnostic in the identification of the purified subfractions [30].

The ability of sulfasalazine to inhibit the LTC synthetase was clearly not unique to this one form of glutathione *S*-transferase since, as we showed, the same compound could also inhibit the glutathione *S*-transferases in rat liver. As already noted, there was a marked difference in the ability of this compound to inhibit these enzymes when DNCB was the second substrate compared to its activity when LTA was the second substrate. It is not clear if the variable potency of sulfasalazine with the latter substrate can be explained entirely by the differences in K_m of the different enzyme fractions for LTA or whether part of this difference might be explained by the other enzymatic activities which these fractions may contain. For example, fraction 1, which was the least sensitive to inhibition by sulfasalazine, contained the highest glutathione peroxidase activity (Table 1). On the other hand, fraction 7, which was the most sensitive to sulfasalazine, had almost as much peroxidase activity associated with it as did fraction 1.

The kinetics of the inhibitory action of sulfasalazine are interesting. Most of the compounds that have been reported to inhibit the glutathione *S*-transferases are hydrophobic electrophylic molecules that compete with the chromogenic substrates which are ordinarily used to study these enzymes. Except for propylthiouracil [31], sulfasalazine is the only compound we know which displays kinetics which are competitive relative to glutathione. Neither of these molecules bears any obvious resemblance to the glutathione molecules which they apparently are capable of displacing on the enzyme. This is the more striking in the case of sulfasalazine where the breakdown products do not compete with glutathione (Fig. 7) even though the intact molecule does.

The marked susceptibility of the liver glutathione *S*-transferases to inhibition by sulfasalazine when DNCB was used as the second substrate may be surprising in view of the generally accepted low toxicity of sulfasalazine in clinical use. It has been reported that a significant fraction of the orally-administered sulfasalazine is taken up into the circulation unchanged and that 25% of the amount which is taken up is actually excreted in the bile unchanged [32]. Yet, with the exception of a few rare reports of sulfasalazine-induced hepatotoxicity [33, 34], sulfasalazine has not been known to have a hepatotoxic liability. It may be that the local glutathione concentration in the liver is so high, and the half-life of intact sulfasalazine in the liver so short that no permanent damage is done. In any event, the results presented here identify a new biochemical activity of this pluripotent molecule.

REFERENCES

1. J. L. Franklin and I. H. Rosenberg, *Gastroenterology* **64**, 517 (1973).
2. G. J. Dhar, J. Selhub and I. H. Rosenberg, *Gastroenterology* **70**, 878A (1976).
3. A. M. Reisenauer and C. H. Halsted, *Biochim. biophys. Acta* **659**, 62 (1981).
4. J. Selhub, G. J. Dhar and I. H. Rosenberg, *J. clin. Invest.* **61**, 221 (1978).
5. P. K. Moore and J. R. S. Hoult, *Biochem. Pharmac.* **31**, 969 (1982).
6. C. N. Berry, J. R. S. Hoult, S. H. Peers and H. Agback, *Biochem. Pharmac.* **32**, 2863 (1983).
7. J. R. S. Hoult and P. K. Moore, *Br. J. Pharmac.* **68**, 719 (1980).
8. C. J. Hawkey and M. Lo-Casto, *Gut* **24**, 213 (1983).
9. L. Molin and O. Stendahl, *Acta med. Scand.* **206**, 451 (1979).
10. W. F. Stenson, J. Mehta and I. Spilberg, *Biochem. Pharmac.* **33**, 407 (1984).
11. O. Stendahl, L. Molin and M. Lindroth, *Int. Archs Allergy appl. Immun.* **70**, 277 (1983).
12. W. F. Stenson and E. Lobs, *Biochem. Pharmac.* **32**, 2205 (1983).
13. A. T. M. M. Ali, G. S. Basran and J. Morley, *Lancet* i 506 (1982).
14. J. C. Sircar, C. F. Schwender and M. E. Carethers, *Biochem. Pharmac.* **32**, 170 (1983).
15. W. F. Stenson and E. Lobs, *J. clin. Invest.* **69**, 494 (1982).
16. P. Sharon and W. F. Stenson, *Gastroenterology* **86**, 453 (1984).
17. S. T. Nielsen, C. B. Buonato and L. Beninati, *J. Allergy clin. Immun.* **73**, 194 (1984).
18. M. K. Bach, J. R. Brashler and D. R. Morton, Jr., *Archs Biochem. Biophys.* **230**, 455 (1984).
19. R. Winterbottom, *J. Am. chem. Soc.* **62**, 160 (1940).
20. E. J. Corey, D. A. Clark, G. Goto, A. Marfat, C. Mioskowski, B. Samuelsson and S. Hammarstrom, *J. Am. chem. Soc.* **102**, 1436 (1980).
21. P. C. Simons and D. L. Vanderjagt, *Analyt. Biochem.* **82**, 334 (1977).
22. M. K. Bach and J. R. Brashler, *Prost. Leuk. Med.*, **17**, 125 (1985).
23. W. H. Habig and W. B. Jakoby, *Meth. Enzym.* **77**, 398 (1981).
24. B. Mannervik and H. Jensson, *J. biol. Chem.* **257**, 9909 (1982).
25. T. D. Boyer, W. C. Kenney and D. Zakim, *Biochem. Pharmac.* **32**, 1843 (1983).
26. J. D. Hayes and J. Chalmers, *Biochem. J.* **215**, 581 (1983).
27. S. Yalçin, H. Jensson and B. Mannervik, *Biochem. biophys. Res. Commun.* **114**, 829 (1983).
28. A. A. Ray (Ed.), *SAS User's Guide: Statistics*. SAS Institute, Cary, NC (1982).
29. D. J. Finney, *Statistical Methods in Bioassay*, p. 508. Macmillan, New York (1978).

30. B. Mannervik, C. Guthenberg, H. Jensson, M. Warholm and P. Alin, in *Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects* (Eds. A. Larsson, S. Orrenius, A. Holmgren and B. Mannervik), p. 75. Raven Press, New York (1984).
31. K. Kariya and T. Matsue, *Experientia* **39**, 896 (1983).
32. K. M. Das, J. R. Chowdhury, B. Zapp and J. W. Fara, *Gastroenterology* **77**, 280 (1979).
33. R. S. Kanner, F. J. Tedesco and M. H. Kalser, *Am. J. dig. Dis.* **23**, 956 (1978).
34. M. D. Smith, G. E. Gibson and R. Rowland, *Aust. N. Z. J. Med.* **12**, 76 (1982).