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METABOLISM OF OLTIPRAZ AND GLUTATHIONE REDUCTASE INHIBITION

NICOLE MOREAU,* THIERRY MARTENS,*† MAURICE-BERNARD FLEURY† and JEAN-PIERRE LEROY‡

*Centre d'Etudes et de Recherches de Chimie Organique Appliquée (C.E.R.C.O.A.), 8 rue Henry Dunant Thiais 94320; †Laboratoire de Chimie Analytique, Faculté de Pharmacie, 4 avenue de l'Observatoire, 75270 Paris Cedex 06, France; and ‡Rhone-Poulenc-Santé, Centre de Recherche de Vitry, 13 quai Jules Guesde, 94407 Vitry-sur-Seine Cedex, France

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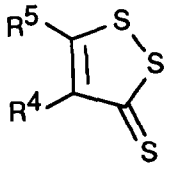
Abstract—A decrease in glutathione reductase (GR) activity was observed in *Schistosoma mansoni* isolated from oltipraz(OPZ)-treated mice. Yeast and *Schistosoma mansoni* GR-activity was inhibited by OPZ derivatives only. These OPZ-derivatives showed *in vitro* schistosomicidal activity. Using yeast GR and dithiolium salts of OPZ, time-dependent inactivation and gel chromatography experiments revealed irreversible inhibition dependent on the redox state of the enzyme. Binding of radiolabelled ([^{3}H]7-methyl-8-methylthio-pyrrolo[1,2-*a*]pyrazine disulphide **1b**) obtained from OPZ was observed using exclusion chromatography and equilibrium dialysis. These results indicate that GR can be considered as the target of schistosomicidal activity of OPZ. The lack of inhibitory activity of OPZ and dithiole-thione analogues, and the potent activity of the corresponding pyrrolo-pyrazine derivatives, is consistent with the hypothesis that OPZ is a pro-drug.

The 4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione oltipraz (OPZ) is a slowly acting drug, active against *Schistosoma mansoni* infections [1]. OPZ is metabolized in the host to pyrrolo[1,2-*a*]pyrazine derivatives [2]. In previous papers [3, 4], a mechanistic interpretation of this biochemical process was proposed and the transient species, unsymmetrical and symmetrical pyrrolo[1,2-*a*]pyrazine disulphides, were isolated by the use of nucleophiles or electrochemical reduction process. With OPZ analogues **2** and **3**, the same procedure led to indolizine disulphides. These disulphides exhibited a schistosomicidal activity *in vitro* when obtained from OPZ or active analog **3**, whereas they were inactive when the parent dithiole-thione **2** was inactive. Bueding *et al.* [5] have shown that the antischistosomal action of OPZ was concomitant with the lowering of the glutathione (GSH) level in worms and have suggested that this depletion would be damaging to the parasite. Moreover, OPZ is known to inhibit cysteine uptake by the parasites [6] but does not inhibit parasite glutathione biosynthesis [7].

1: R^5 = 2-pyrazinyl, $R^4 = CH_3$
2: R^5 = 2-pyridyl, $R^4 = CH_3$
3: R^5 = 2-pyridyl, $R^4 = CO_2C_2H_5$
4: R^5 = 5-pyrimidinyl, $R^4 = CH_3$



In this work, we turned our attention towards glutathione reductase and we hypothesized that the decrease in GSH level observed in *S. mansoni* after OPZ treatment might be partly due to a non-reversible loss of reductase activity caused by an OPZ transient metabolic species. So we measured the activity of GR in worms from OPZ-treated mice. Using both a yeast enzyme and the GR isolated from schistosomes, we looked for an inhibition of GR activity by OPZ and its derivatives.

MATERIALS AND METHODS

Reagents

NADPH, GSSG and yeast GR were products from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

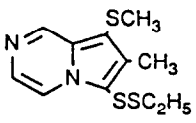
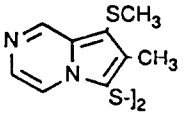
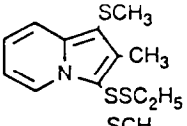
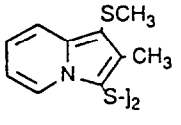
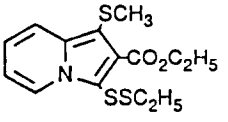
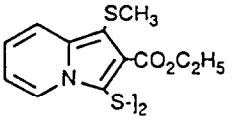
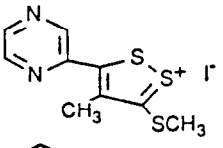
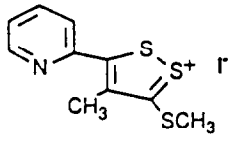
Organisms and extracts

Paired worms were picked up manually from the mesenteric and portal veins of CD_1 female mice (Charles River, St Aubin-les-Elboeuf, France) infected 2 months previously with 120 cercariae of a Brazilian strain of *S. mansoni*. The worm pairs were washed twice in physiological serum, suspended in 0.25 M sucrose and disrupted at 4° using a Thomas potter homogenizer. The homogenate was centrifuged and the supernatant used for enzymatic and inhibition assays. Centrifugation was carried out using an SW 41 rotor in a Beckman ultracentrifuge (40,000 rpm) for 1 hr at 4°.

Schistosomicidal activity in vitro

Schistosomes aseptically recovered from mice infected 2 months previously were placed in a survival medium (50% tyrode and 50% calf serum, plus 40 μg of streptomycin sulfate and 100 mUnits of penicillin sodium salt per mL) to which variable concentrations of the products to be assayed were added. Four pairs of worms were used for each concentration. Compounds to be assayed were dissolved in DMF/water (1:9 v/v) and one part of this solution was added to nine parts of survival medium. The concentration of the stock solution was chosen so that increasing concentrations from 3 to 100 μg/mL were used in the assay. After 72 hr at 37°, immobile worms were counted under a binocular lens. Immobile schistosomes

Table 1. Activity of OPZ and derivatives

Compounds		Inhibition of GR activity (%)* Yeast GR	*S. mansoni* GR	Schistosomicidal activity *in vitro*†
	**1**	0	0	1
	1a	40	50	0.3
	**1b**	60	100	0.3
	**2**	0	0	0
	2a	0	0	0
	**2b**	0	0	0
	**3**	0	0	0.1
	3a	50	50	0.5
	**3b**	60	50	0.1
	**4**	0	0	1
	1c	100	70	0.1
	**2c**	0	0	0

* The concentration of compound was 50 μM. Assays were carried out as described in Materials and Methods.

† The schistosomicidal activity *in vitro* was measured by using concentrations up to 100 μg/mL as described in Materials and Methods. Activities of the products were referred to OPZ activity.

were placed for 4 hr at 37° in a survival medium, devoid of test compound. In this restoration medium, immobile worms were considered to be dead.

OPZ derivatives

Unsymmetrical pyrrol[1,2,-*a*]pyrazine and indolizine disulphides were obtained by nucleophilic attack of dithiole-thiones [3], symmetrical disulphides by electrochemical reduction [4] and 4-methyl-3-methylthio-1,2-dithiolium salts by methylation of dithiole-thiones [8, 9]. [^{3}H]7-methyl-8-methylthio-pyrrolo[1,2-*a*]pyrazine disulphide **1b**: after electrolysis of OPZ (1 mM in DMF), an aliquot was methylated using 25 mCi of [^{3}H]ICH_3 (5000 Ci/mol). The [^{3}H]**1b** (1500 Ci/mol) was isolated, dissolved in ethanol (2 mL) and used as a stock solution kept at 4°.

Assay of GR activity

GR activity was measured at 37° according to Calberg and Mannervik [10]. In a 1 mL cuvette, 0.5 mL phosphate buffer (0.2 M, 2 mM EDTA, pH 7.0), 50 μL NADPH (2 mM, in Tris–HCl 10 mM, pH 7.0), 50 μL GSSG (20 mM in water) and a volume of deionized water were mixed giving a final total volume of 1 mL. The reaction was initiated by the addition of 50 μL of 5 μg/mL solution of yeast GR

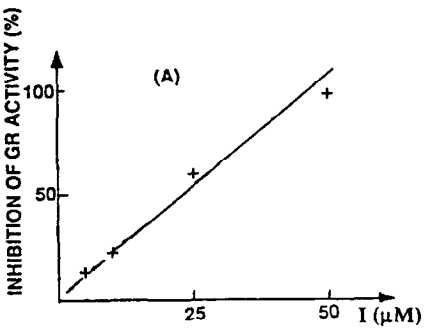


Fig. 1. Effect of inhibitor (I) **1b** on the activity of *S. mansoni* GR at fixed concentrations of I (5, 10, 25 and 50 μM).

in 0.05 M phosphate buffer, pH 7.0, containing 2 mM EDTA. The decrease in absorbance at 340 nm was monitored at 37°. One unit of activity was defined as the oxidation of 1 μmol NADPH/min. GR activity in treated worms: mice infected 6 to 7 weeks ago by *S. mansoni* were treated with 200 mg OPZ per kg body weight administered once by oral intubation. The GR activity was measured as above described using 50 μL of supernatant.

Inhibition procedure. In a 1 mL cuvette, 0.5 mL phosphate buffer at 37°, 50 μL NADPH, 10 μL of inhibitor solution (5 mM in ethanol), 50 μL enzyme solution or 50 μL of supernatant were added. After 10 min at 37°, 50 μL GSSG were added. The control assay was performed using the same procedure to prevent the error due to NADPH-induced denaturation of yeast enzyme activity [11]. Time-dependent inactivation of GR was carried out as described

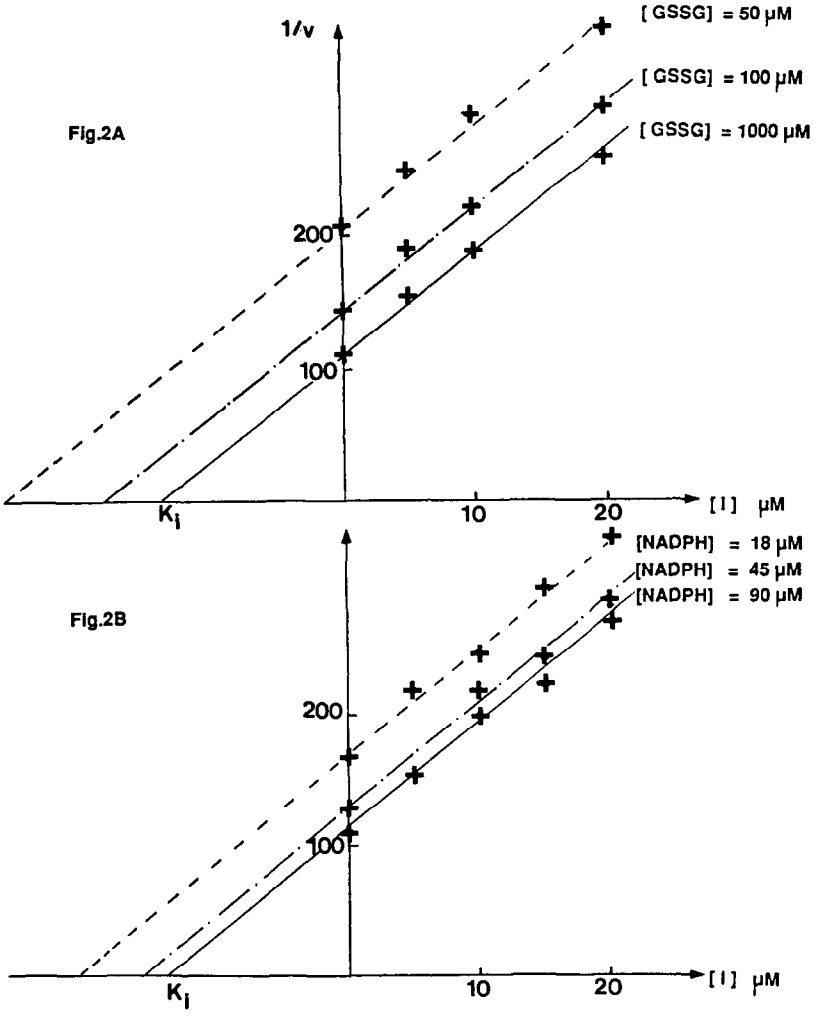


Fig. 2. Dixon plot for compound **1c**. Yeast enzyme was used and the concentrations of I(**1c**) were 0, 5, 10, 15 and 20 μM. The rate v is in μmol NADPH/min. (A) GSSG concentrations are 50, 100 and 1000 μM at fixed 100 μM NADPH concentration. The intercept with abscissa rises to K_i (1 + K_m/[S]). Assuming a K_m of 45 μM for GSSG (from 1/v versus 1/[S] plot), the calculated values were 20 and 27 μM for 100 and 50 μM respectively (found 19 and 26 μM). (B) NADPH concentrations are 18, 45 and 90 μM at fixed 1 mM GSSG concentration. The calculated intercepts with abscissa are 16 and 19 μM for 45 and 18 μM respectively (found 16 and 20 μM), assuming a K_m of 6.5 μM.

above with the following modifications: the temperature was 25° and the preincubation time of the enzyme plus NADPH varied from 0 to 10 min. Kinetic experiments were carried out at 25° without preincubation with NADPH.

Ultrogel AcA 202 gel filtration. The reaction mixture (similar to that described under the inhibition procedure but with an enzyme solution of 12 μg/mL and without GSSG), was loaded onto a (35 × 1 cm) AcA 202 Ultrogel column equilibrated with phosphate buffer. The elution was run out with the same buffer at 14 mL/hr, 700 μL fraction, and GR activity was assayed using 500 μL of each fraction. To check the effect of the pyrrolo[1,2-*a*]pyrazine disulphide **1b** or of the dithiolium salt **1c**, 10 μL of a 5 mM ethanolic solution and 200 μL of a 0.25 mM aqueous solution were added respectively before chromatography.

Binding assay using gel filtration

The same procedure as above was used but 10 μL of [^{3}H]**1b** stock solution was added to the non-labelled compound. The radioactivity of 500 μL of each fraction was measured by liquid scintillation in 5 mL of Beckman ready solvent solution HPb.

Equilibrium dialysis

Equilibrium dialysis was carried out with rotating cells of 500 μL total volume (Dianorm instruments, Munich, F.R.G.). Two compartments (250 μL each) were separated by a semi-permeable membrane (Visking, Union Carbide, U.S.A.). The reaction mixture, 150 μL phosphate buffer (0.2 M, pH 7.0) containing NADPH 1 mM, EDTA 2 mM, yeast enzyme 36 μg and 10 μL [^{3}H]**1b** stock solution, was incubated for 20 min at 37°. After addition of 50 μL of 2-methoxyethyl ether, the mixture was introduced into one compartment of the cell and 200 μL of phosphate buffer with 25% (v/v) of solvent into the other.

RESULTS AND DISCUSSION

The GR activity was assayed in schistosomes recovered from infected mice either not treated or treated 2 days before with 200 mg OPZ per kg body weight. Three assays, using 230, 230 and 325 untreated worm pair respectively, gave an average value of 1 ± 0.05 mUnits/worm pair (each value was the mean of three experiments). The assay on treated worms used 230 pairs and gave an activity of 0.5 ± 0.05 mUnits/worm pair (mean of three experiments). The results of the inhibition of GR and the *in vitro* schistosomicidal activities of the assayed products are shown in Table 1. Unsymmetrical **1a** and symmetrical **1b** pyrrolo[1,2-*a*]pyrazine disulphides prepared from OPZ were inhibitors of yeast and worm GR activity. The indolizine disulphides **3a** and **3b** obtained from active analogue **3** showed inhibitory properties, whereas indolizine compounds **2a** and **2b** obtained from inactive analogue **2** were devoid of effect on GR activity. Using the dithiolium salts **1c** and **2c**, we observed that only the schistosomicidal active salt **1c** obtained from OPZ was an inhibitor of yeast and worms GR activity. Furthermore, the drug concentration having an activity on the enzyme, and *in vitro* on the worms, are of the same order of magnitude.

These results are in agreement with the implication of GR in the schistosomicidal activity of OPZ: a 50% decrease in GR activity is observed in worms from treated mice, as compared with untreated controls; both yeast and schistosome enzymes are inhibited only by the products showing an *in vitro* schistosomicidal activity. Products inactive *in vitro* have no inhibitory effect on the enzymes, clearly showing that the inhibition observed is not an artefact, but is a specific effect. GR inhibition could be the cause of the death of the worms as a consequence of an alteration in the GSSG/GSH balance, which could initiate a cascade of events ultimately leading to various oxidative damages. This phenomenon could explain the GSH depletion observed in the worms of the OPZ-treated mice [5].

The inhibition was observed when the product was preincubated with enzyme (yeast and worm) and NADPH. **1b** showed a strong effect on the worm enzyme and a linear relationship between amount of inhibition and concentration (Fig. 1). The low water solubility of **1b** and analogues did not permit a detailed study of inhibition mechanism to be obtained. We therefore turned towards the dithiolium salt **1c**, a soluble derivative of OPZ, which showed a fair activity on the yeast enzyme. Figure 2 shows the results obtained for kinetic measurements without preincubation with NADPH. Dixon plots ($1/v$ versus [I] (**1c**)) for a range of GSSG and NADPH concentrations (Fig. 2A and B) are consistent with an uncompetitive inhibition with respect to two substrates, the K_i being 14 μM.

Also, Fig. 3 shows the time-dependent inactivation of the enzyme with varying concentrations of **1c** (5, 10 and 20 μM). The experiments were carried out at two concentrations of NADPH, 60 μM (Fig. 3A) and 100 μM (Fig. 3B). It can be underlined that (1) if the time-dependent experiments were carried out without NADPH, this cofactor being added lastly to initiate the reaction, there was no time-dependent inactivation; (2) the inhibition observed after preincubation with NADPH was reversible by addition of 1 mM GSH.

The rate constants for time-dependent inactivation of the enzyme calculated are shown in Table 2. The values of these constants (k_{obs}) suggest two observations: (1) when the NADPH concentration rose from 60 to 100 μM, k_{obs} varied in the same ratio; (2) when the inhibitor (**1c**) concentration was doubled (from 5 to 10 μM and then from 10 to 20 μM) the rate constants were not doubled as could be expected in the case of bimolecular inactivation. In contrast, the ratios of k_{obs} for different concentrations of **1c** are in agreement with the theoretical values calculated assuming the occurrence of an active-site-directed inactivation mechanism [12], according to the reaction scheme (where E is the reduced enzyme):

$$E + I \overset{K_i}{\leftrightarrows} E \cdots I \overset{k_{obs}}{\rightarrow} E - I.$$

Without preincubation with NADPH, only the first reversible part of the mechanism was observed; this is the case in the kinetic experiments.

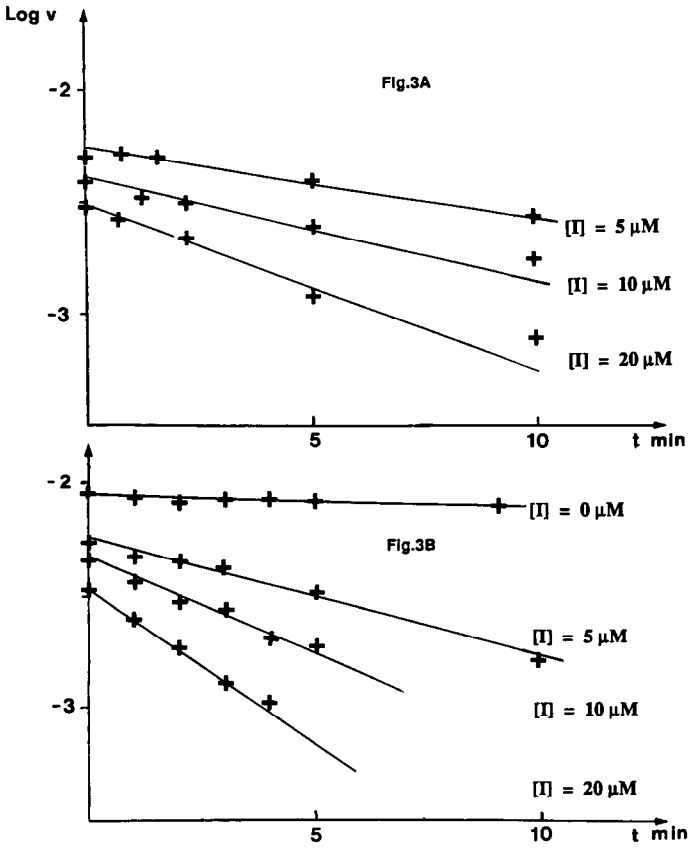


Fig. 3. Time-dependent inactivation. Yeast enzyme was used and the concentration of I (**1c**) were 5, 10 and 20 μM. (A) 60 μM NADPH; (B) 100 μM NADPH.

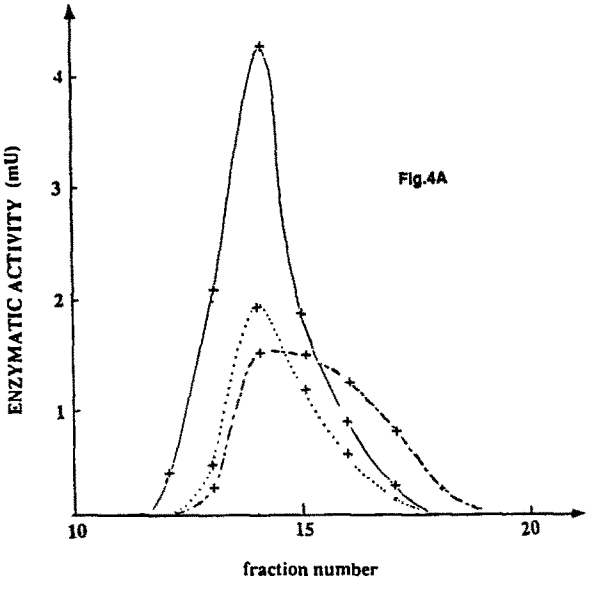
Chromatographic studies (Fig. 4) showed an irreversible loss (50%) of GR activity after incubation of the enzyme with inhibitors (**1b** and **1c**). Chromatography of yeast GR in the presence of labelled **1b** is shown in Fig. 4B. Some radioactivity was associated with the enzyme peak, provided that NADPH was included in the reaction mixture, indicating a binding of the inhibitor to the GR. Equilibrium dialysis assays confirm the binding of **1b** to GR, but no accurate estimation of the binding can be obtained, due to the low solubility of the product.

The catalytic behaviour of GR requires the reduction of the active site by NADPH [13, 14]. The requirement of NADPH for inhibition expression, the time-dependent inactivation of the enzyme and the observation of NADPH dependent binding of [^{3}H]**1b**, are in agreement with the formation of covalent enzyme–inhibitor complex dependent on the redox state of the enzyme. We could propose a mechanism implying the formation of a mixed disulphide between the reduced enzyme and the product according to Scheme 1. The recovery of GR-

Table 2. Rate constants of the time-dependent inactivation

	NADPH 100 μM			NADPH 60 μM		
I (μM)	5	10	20	5	10	20
k_{obs} (/min)	0.12	0.20	0.30	0.073	0.11	0.17
k_{obs} ratio	1.66	1.50		1.50	1.54	
Theoretical k_{obs} ratio	1.60	1.45		1.60	1.45	

k_{obs} are 2,3 the slope of the plot log v versus t (Fig. 3). The k_{obs} ratio are calculated for [I] (5 to 10 μM and 10 to 20 μM). The theoretical values of the ratio are calculated according to active-site-directed inactivation mechanism [12].



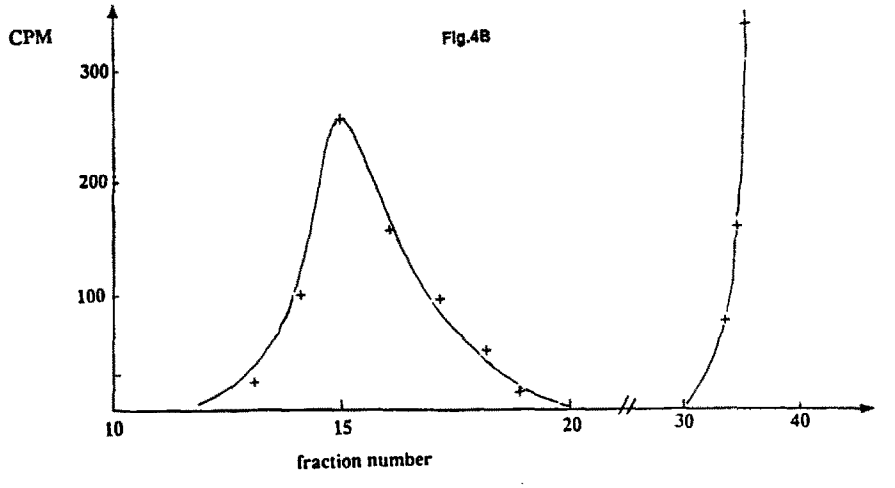
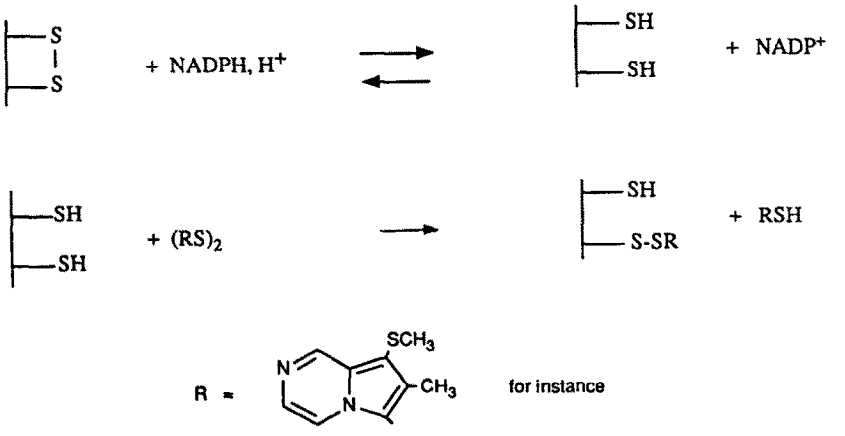


Fig. 4. Ultrogel AcA 202 gel filtration of yeast GR–inhibitor mixtures. (4A) Enzymatic activity without inhibitor (——), in the presence of 50 μM of **1b** (—·—), and of 50 μM of **1c** (...); (4B) Retention of [^{3}H] **1b** by the enzyme. Elution, enzyme assays and radioactivity measurements were performed as described in Materials and Methods.



Scheme 1.

activity after GSH addition confirms this hypothesis. The FAD domain of GR contains four cysteinyl residues [15]. Cys-58 and Cys-63, at the active site of the enzyme could be implicated in the inhibition by OPZ-derivatives, as represented in Scheme 1, but the two other cysteinyl residues could as well be responsible for the inactivation. Only radiolabelling experiments could show which residues are actually affected. Using compound **1c** we obtain reversible kinetics which allows us to determine the K_i value. This fact remains compatible with the above irreversible phenomenon provided a transient reversible complex can be formed between the reduced enzyme and **1c** before the covalent complex formation [12].

An important fact is the inactivity of OPZ and dithiole–thione analogues: neither OPZ nor analogues **3** and **4** are inhibitors of GR, whereas they are active on the schistosomes. Unsymmetrical and symmetrical pyrrolo-pyrazine **1a** and **1b** (indolizine **3a** and **3b**) disulphides, obtained from OPZ (analogue **3**), are inhibitors of GR, whilst the disulphides **2a** and **2b** derived from the inactive dithiole–thione **2** are devoid of inhibitory properties. These results strongly indicate that the disulphides are active *in vitro* on the worms and on the isolated enzyme provided that they derive from an active dithiole–thione. This fact confirms previous suggestions [3] that OPZ could be a pro-drug.

Since disulphides are inactive *in vivo*, it would be interesting to have an insight into the metabolic pathway of OPZ in schistosomes to see whether or not the worm is able to produce such metabolites.

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