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Trypanothione is the primary target for arsenical drugs against African trypanosomes

(chemotherapy)

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ABSTRACT **The trypanosomatid metabolite N^1,N^8-bis-(glutathionyl)spermidine (trypanothione) has been demonstrated to form a stable adduct with the aromatic arsenical drug melarsen oxide [*p*-(4,6-diamino-*s*-triazinyl-2-yl)aminophenyl arsenoxide]. The stability constant of the melarsen–trypanothione adduct (Mel T) has been determined to be 1.05×10^7 M^{-1}. When bloodstream *Trypanosoma brucei* are incubated with either melarsen oxide or the 2,3-dimercaptopropanol adduct of melarsen oxide (melarsoprol), Mel T is the only arsenical derivative detectable in acid-soluble extracts of the cells. Trypanothione may therefore be regarded as a primary target for aromatic arsenical derivatives against African trypanosomes. The selective toxic action of these compounds might arise through sequestration of intracellular trypanothione in the form of Mel T, or Mel T itself may be toxic within the cell. The latter possibility is illustrated by the finding that Mel T is an inhibitor of trypanothione reductase from *T. brucei* (K_i = 9.0 μM)—an enzyme that is central to the regulation of the thiol/disulfide redox balance in the parasite and absent from the host.**

The development of antimicrobial arsenical drugs stems from the pioneering studies on chemotherapy conducted by Paul Ehrlich and his colleagues in the early part of this century. In most clinical situations, the use of such compounds has, for some time, been superseded by the development of more selective agents. However, in the absence of suitable alternatives, the melaminyl aromatic arsenicals remain the drugs of choice for the treatment of late-stage African trypanosomiasis (sleeping sickness) (1). The bloodstream form of the African trypanosome is extremely sensitive to trivalent aromatic arsenicals. On exposure to the drug melarsen oxide [*p*-(4,6-diamino-*s*-triazinyl-2-yl)aminophenyl arsenoxide], these organisms rapidly lose their motility and cell lysis soon follows. A number of theories have been put forward to account for these effects and for some years the prevailing view has been that the extreme toxicity of trivalent arsenicals toward African trypanosomes may be attributed to inhibition of parasite pyruvate kinase (2). As this organism lacks both functional oxidative phosphorylation and tricarboxylic acid cycle and depends entirely on glycolysis for ATP generation (3), it was believed to be uniquely sensitive to such an effect. However, more recent studies indicate that inhibition of glycolysis is secondary to the lytic effect of melarsen oxide and is not the result of primary inhibition of pyruvate kinase (4). Thus, despite >80 years of research, the mode of action of trypanocidal arsenicals remains completely unknown.

As early as 1909, Ehrlich himself proposed that trivalent arsenicals might be active by virtue of their ability to react with cellular sulfhydryl groups essential to parasite survival. The discovery that trypanosomatids contain the dithiol metabolite N^1,N^8-bis(glutathionyl)spermidine (trypanothione) (5) prompted us to investigate the interaction of this compound with aromatic arsenicals *in vitro* and within intact *Trypanosoma brucei*. We now report that the dithiol form of trypanothione [dihydrotrypanothione, Try(SH)$_2$] forms a stable adduct with melarsen oxide *in vitro* and *in vivo* and that this compound is an effective inhibitor of trypanothione disulfide reductase, an enzyme unique to trypanosomatids.

MATERIALS AND METHODS

All reagents were of the highest purity available. Melarsen oxide and the adduct of melarsen oxide with 2,3-dimercaptopropanol (Mel B) were obtained from E. Friedheim (Rockefeller University); the adduct of melarsen oxide with 2,3-dimercaptosuccinate (Mel W; "trimelarsen", lot no. 209-1) was from Specia (Paris). Trypanothione was chemically synthesized as described (6).

HPLC Analysis. Thiols and arsenical derivatives were separated by ion-paired reverse-phase HPLC on a Hewlett–Packard HP1090 fitted with a Beckman–Altex C_{18} column. Compounds were detected by UV absorbance or by fluorescence following postcolumn derivatization with fluorescamine. Elution systems and methods have been described (7).

Mass Spectrometric and Amino Acid Analysis. Amino acid analysis of the adduct of melarsen oxide with dihydrotrypanothione (Mel T) was performed as described (5). Mel T was analyzed by fission fragment ionization mass spectrometry by Brian T. Chait (Rockefeller University).

Spectroscopic Studies. Digitalized spectral data were obtained by using a microprocessor-controlled Hewlett–Packard 8450A UV/Vis spectrophotometer. Reactions of melarsen oxide (40 μM) with varying concentrations of thiol were carried out in 10 mM phosphate buffer (pH 7.0) containing 150 mM KCl and 1 mM EDTA in a total volume of 1 ml. Spectra were measured in the range 204–340 nm and stored in memory standards. Difference spectra were calculated at each thiol concentration by subtracting spectra of melarsen oxide (standard M) and thiol (standard T_n) alone from the spectra of the mixture (standard A_n), such that the difference spectrum = standard A_n − (standard M + standard T_n). Difference spectra obtained show isosbestic points at 253 and 277 nm and minimum and maximum absorbances at 299 and 267 nm, respectively. These values were found to be identical for all thiols and dithiols tested. Thus, formation of thioarsenite adducts was measured from the difference in absorbance at 299 minus 267 nm (ΔOD). Maximum ΔOD corresponds to

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Abbreviations: Try(S)$_2$, trypanothione disulfide; Try(SH)$_2$, dihydrotrypanothione; melarsen oxide, *p*-(4,6-diamino-*s*-triazinyl-2-yl)aminophenyl arsenoxide; Mel B, Mel W, and Mel T, adducts of melarsen oxide with 2,3-dimercaptopropanol, 2,3-dimercaptosuccinate, and dihydrotrypanothione, respectively; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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complete conversion of melarsen oxide. Extinction coefficients of Mel T and the melarsen glutathione complex were determined to be 20.1 and 20.3 $mmol^{-1} \cdot cm^{-1}$, respectively. Association constants (K_a) were determined by adaptation of the method developed by Zahler and Cleland (8). Stock solutions of the dithioarsenite complexes were incubated in 0.1 M Hepes buffer containing 1 mM EDTA (pH 8.0) at 26°C (total volume, 1 ml) in a concentration range of 200–500 μM, 200–1000 μM, and 10–50 μM for Mel B, Mel W, and Mel T, respectively. After 3 min, 0.01 ml of a 1-mM solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in buffer was added and the increase in absorbance at 412 nm was measured with time. Initial absorbance was then determined by extrapolation to zero time after subtracting initial absorbance due to DTNB.

Exposure of *T. brucei* to Arsenical Drugs. Bloodstream trypomastigote forms of *T. brucei* (EATRO 427, clone 118) were grown in 300- to 400-g rats as described (7). Purified trypanosomes were resuspended (2×10^6 ml^{-1}) in 60 mM sodium phosphate buffer (pH 8.0) containing 44 mM NaCl, 50 mM glucose, and bovine serum albumin (1 mg/ml). Aliquots (10 ml) were incubated at 25°C for 5 min before the addition of melarsen oxide (10 μM) or Mel B (50 or 75 μM). At intervals, 5 ml of 10 mM glutathione was added to terminate uptake of the drug and the cells were immediately pelleted by centrifugation. The trypanosomes were resuspended and washed once in 1.5 ml of 60 mM sodium phosphate buffer, pH 8.0/44 mM NaCl/50 mM glucose and then centrifuged. The resulting pellet was extracted with 1.0 ml of cold 5% trichloroacetic acid containing 0.01 M HCl. Following centrifugation, the supernatant was extracted with ethyl acetate (5 × 2 vol), dried under reduced pressure, and redissolved in 20% (vol/vol) propylene glycol for HPLC analysis.

Trypanothione Reductase Assays. Trypanothione disulfide [$Try(S)_2$] reductase from *Crithidia fasciculata* was purified as described (9); partially purified enzyme (6.6 units/ml) from *T. brucei* was obtained by modification of this procedure. Activity was measured spectrophotometrically at 340 nm at 25°C in 0.1 M Hepes buffer (pH 7.8) containing 1 mM EDTA and 0.15 mM NADPH. Enzyme was preincubated for 5 min with Mel T before addition of $Try(S)_2$.

RESULTS

Formation of Trypanothione–Melarsen Oxide Adducts (Mel T). The reaction between $Try(SH)_2$ and melarsen oxide to form a 25-atom macrocycle was readily demonstrated by HPLC analysis. Following exposure to melarsen oxide, $Try(SH)_2$ was completely converted to an equimolar mixture of two dithioarsane adducts (Fig. 1). Amino acid and mass spectrometric analyses [$(M + H)^+C_{36}H_{56}N_{15}O_{10}S_2As$ found 998.5, calculated 998.4; (M + Na) found 1020.4, calculated 1020.5] on the isolated adduct were consistent with the structure given in Fig. 2. For convenience, and in concert with previously coined abbreviations (Mel B, Mel W, etc.), the trypanothione–melarsen oxide adduct will henceforth be abbreviated Mel T. As illustrated in Fig. 2, the two products of the reaction between $Try(SH)_2$ and melarsen oxide represent a diastereoisomeric mixture of dithioarsane derivatives. Trivalent arsenic has approximately tetrahedral geometry; therefore two diastereoisomeric cyclic dithioarsane will be generated when the sulfhydryl functions are nonequivalent, as is the case if neighboring groups R_1 and R_2 are not identical. In Mel T asymmetry of the cysteine sulfur atoms is caused by the N^4 nitrogen in the spermidine moiety. This interpretation was supported by HPLC analysis of Mel B (R_1 = OH, R_2 = CH_2OH), for which two forms could be resolved, and Mel W (R_1 = R_2 = COOH), which was homogeneous. Similarly, when melarsen oxide was allowed to react with an analogue of $Try(SH)_2$ in which the spermidine moiety had been replaced by diaminooctane (10), only one dithioarsane product was formed.

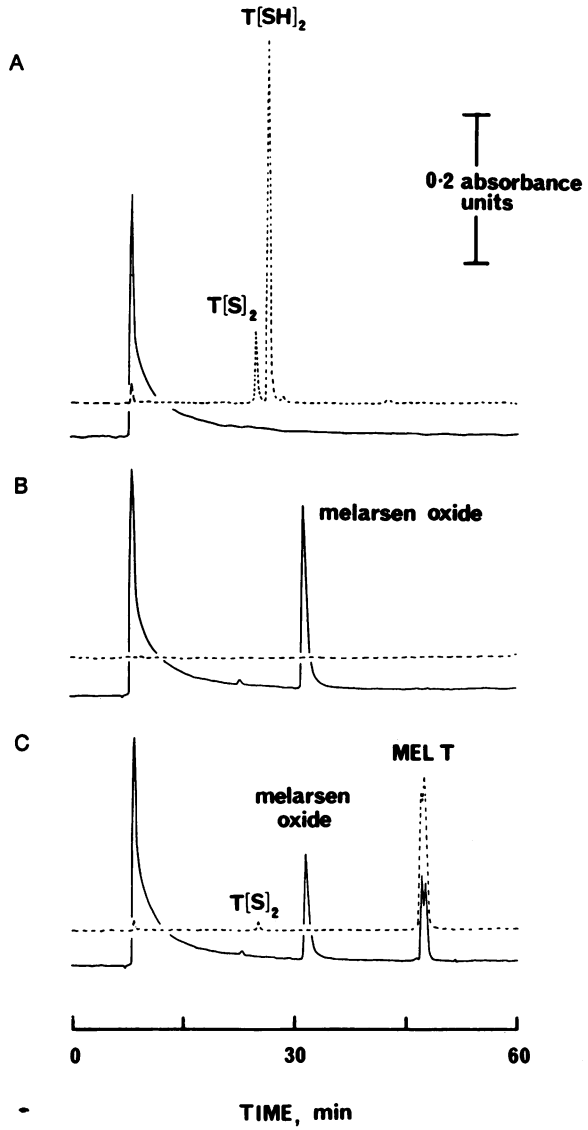

FIG. 1. Formation of a stable complex between $Try(SH)_2$ and melarsen oxide, measured by HPLC. (*A*) $Try(SH)_2$. (*B*) Melarsen oxide. (*C*) $Try(SH)_2$ incubated with melarsen oxide. The gradient applied was 0–60% solvent B over 60 min. $Try(SH)_2$, $Try(S)_2$, and the arsenical derivative [Mel T] were detected by fluorescence (dashed lines) following reaction with fluorescamine; melarsen oxide and Mel T were detected by absorbance (solid lines) at 280 nm.

Affinity of Trypanothione and Other Thiols for Melarsen Oxide. The reaction of melarsen oxide with $T(SH)_2$ and other thiols was followed spectrophotometrically by monitoring the conversion of melarsen oxide (λ_{max} = 272 nm) to the corresponding dithio–melarsen adducts (λ_{max} = 283 nm). By calculating difference spectra, as described in the previous section, the amount of adduct formed with increasing thiol concentration could be determined. Fig. 3 shows the results of these titrations and serves to illustrate the marked difference in affinity between monothiols and dithiols for melarsen oxide. In the case of $Try(SH)_2$ and the other dithiols, ΔOD increases linearly until the formation of the 1:1 molar complex is complete. In contrast, the monothiol species GSH reached a maximum ΔOD on addition of 10 mM (not shown), >125 times that of $Try(SH)_2$. Cysteine reacted similarly; in this case ΔOD_{max} is in excess of 10 mM and could not be determined. The actual association constant K_a for $T(SH)_2$ with melarsen oxide was determined by a method developed

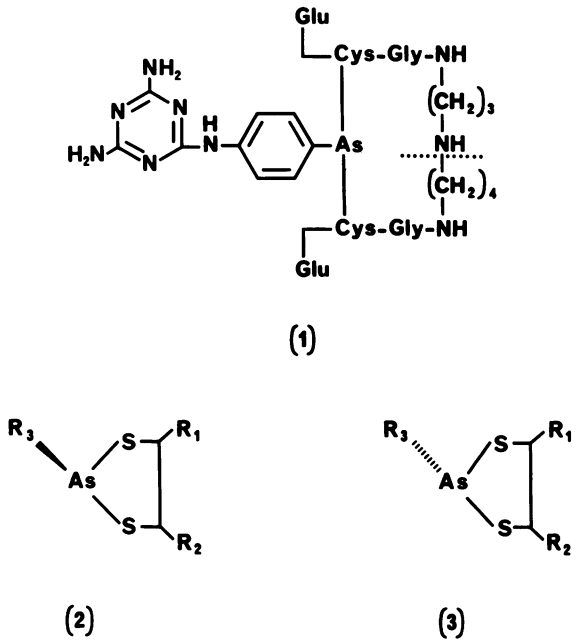

FIG. 2. Structure of the melarsen oxide–Try(SH)$_2$ derivative (Mel T). The dotted line in **1** indicates the plane of asymmetry in the peptide. Structures **2** and **3** illustrate how the approximately tetrahedral geometry of the arsenic atom in melarsen oxide (R_3 = melaminophenyl moiety) results in the formation of two diastereoisomeric forms.

by Zahler and Cleland (8). Mel T (initial concentration [A]) was dissolved in buffer and allowed to come to equilibrium [Mel T $\rightleftharpoons$ Try(SH)$_2$ + melarsen oxide] and free thiol was measured by the addition of DTNB. As DTNB reacts instantaneously with thiol but only slowly with Mel T, it was possible to determine the initial Try(SH)$_2$ concentration ([T]) by extrapolation to zero time. The association constant K_a was then calculated from the equation $K_a = [A] - [T]/[T]^2$. A plot of $[T]^2$ versus [A] gave a straight line with slope $1/K_a$ (Fig. 4) from which a mean K_a of $1.21 \times 10^7\ M^{-1}$ ($n = 10$) was obtained. Similar experiments with Mel B and Mel W gave association constants of 7.93×10^{10} ($n = 8$) and 4.50×10^{10} ($n = 15$), respectively. By using these values of K_a, it was possible to calculate the expected ΔOD values in the spectrophotometric titration experiments. The calculated plot for

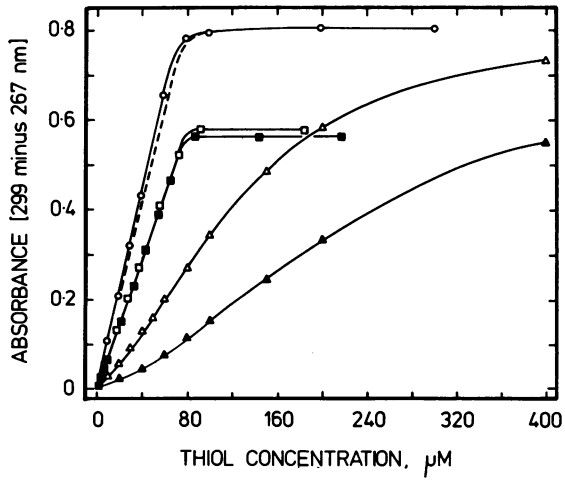

FIG. 3. Titration curves showing the formation of melaminophenyl–thioarsenite complexes with mono- and dithiols. Values are expressed as the concentration of thiol groups. ○, Try(SH)$_2$; □, Mel W; ■, Mel B; △, glutathione; ▲, cysteine. The dashed line is calculated from the value for K_a for Mel T.

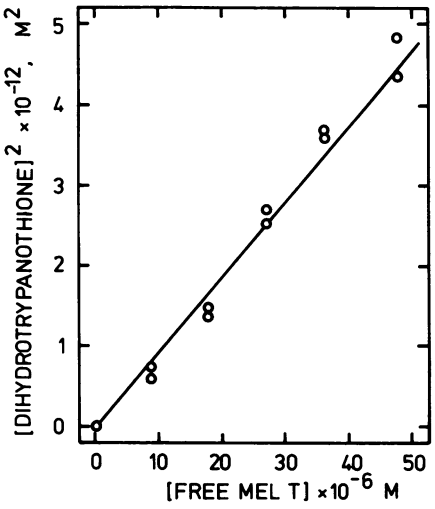

FIG. 4. Determination of K_a for Mel T.

Mel T (broken line, Fig. 3) fits well with the experimentally determined data and those for Mel B and Mel W exactly superimpose, thus confirming the validity of the method.

Formation of Mel T in *T. brucei*. These experiments were complicated by the extreme sensitivity of trypanosomes to melarsen oxide. Nevertheless, as illustrated in Fig. 5, it was possible to demonstrate the ability of melarsen oxide to react with intracellular Try(SH)$_2$ in intact bloodstream forms of *T. brucei*. Following exposure to melarsen oxide, Mel T was

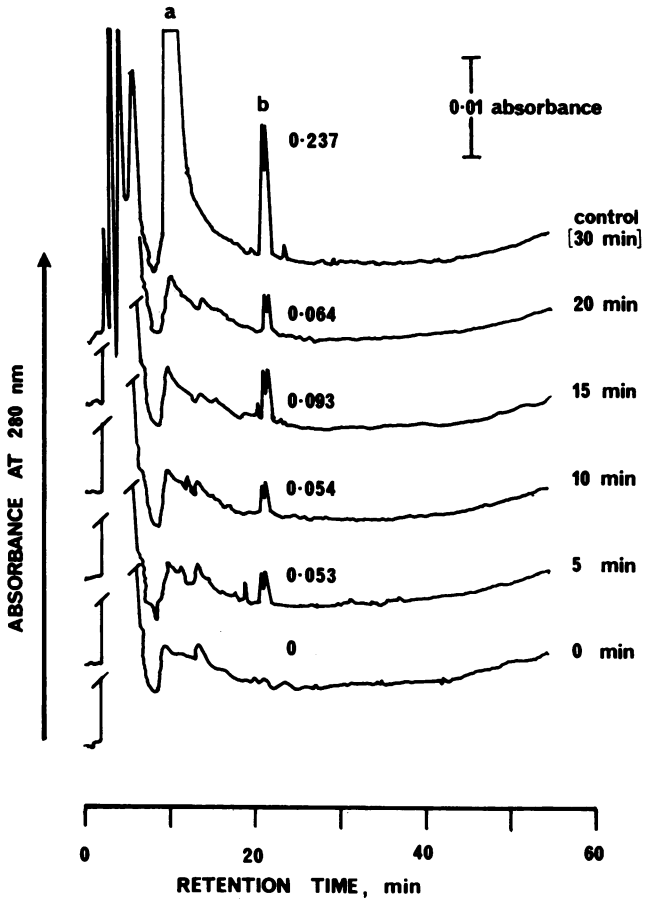

FIG. 5. Formation of Mel T in intact bloodstream forms of *T. brucei* exposed to melarsen oxide. Separation of the concentrated extracts by HPLC is described in the text. Peak a is due to melarsen oxide added to the 30-min sample after extraction with acid. Peak b is Mel T. The amount of Mel T formed is given in nmol per 10^8 cells.

clearly discernible in the concentrated cell extracts after 5 min; it then increased to a maximum of 0.1 nmol per 10^8 cells after 15 min and decreased thereafter. No free melarsen oxide could be detected in the extracts. Although microscopic examination 30 min after addition of drug showed that ≈50% of the cells had become swollen and nonmotile, it is likely that the bulk of the intracellular $Try(SH)_2$ remained intact. This is supported by the recovery of unreacted $Try(SH)_2$ on addition of excess melarsen oxide to the concentrated extract (Fig. 5, 30-min control). In the same experiment intracellular GSH and $Try(SH)_2$ concentrations were determined to be 2.57 ± 0.02 and 0.91 ± 0.09 nmol per 10^8 cells [0.43 mM and 0.16 mM, assuming 10^8 cells have a volume of 5.8 μl (11)], respectively. Thus the intracellular concentration of Mel T reached 17 μM, accounting for ≈10% of the intracellular $Try(SH)_2$, before cell lysis commenced. Mel T was also formed when intact bloodstream forms of *T. brucei* were exposed to Mel B. Following a 30-min exposure to 50 and 75 μM Mel B, 0.7% and 2.6%, respectively, of the intracellular $Try(SH)_2$ had been converted to Mel T (not shown). Neither Mel B nor free melarsen oxide could be detected in the cell extracts, suggesting that exchange between 2,3-dimercaptopropanol and $Try(SH)_2$ was occurring within the cell.

Inhibition of $Try(S)_2$ Reductase by Mel T. Mel T was shown to be an effective inhibitor of $Try(S)_2$ reductase purified from either *C. fasciculata* or *T. brucei*. Both enzymes were inhibited competitively with respect to $Try(S)_2$ with K_i values of 36 μM and 9 μM for *C. fasciculata* and *T. brucei*, respectively (Fig. 6 *A* and *B*). In the same series of experiments the K_m for $Try(S)_2$ for the *T. brucei* enzyme was determined to be 58 μM, similar to the reported value of 53 μM for the enzyme from *C. fasciculata* (9).

DISCUSSION

The glutathione–spermidine conjugate trypanothione is the principal intracellular thiol of *T. brucei* and other trypanosomatids, accounting for >68% of the intracellular glutathione (12). Unlike mammalian cells, trypanosomes do not possess classical glutathione reductase or glutathione peroxidase activities. Instead, these vital enzymes have been replaced by isofunctional trypanothione-dependent counterparts (9, 13). Trivalent arsenical derivatives are known to form stable complexes with enzymes, coenzymes, or other compounds that contain two or more proximal thiol groups (14). For this reason we investigated the possibility that trypanothione and/or the trypanothione-dependent enzymes might be involved in the trypanocidal action of arsenical drugs. The data presented here indicate that trivalent melaminyl arsenical drugs become concentrated within bloodstream forms of *T. brucei* in the form of a macrocyclic dithioarsane adduct with trypanothione. This derivative, Mel T, can be isolated and is considerably more stable than the corresponding adduct that might be formed by the other major thiol species of the cell, glutathione and cysteine. In light of this information, it is necessary to reevaluate currently held theories that relate to the selective toxic effects of aromatic arsenical drugs on African trypanosomes. Two aspects of the problem may be considered separately—namely, the mode of entry of the drug and its site of action.

Trypanosomes are thought to concentrate melarsen oxide against a concentration gradient (15), but it is not known whether this occurs by simple diffusion or by a membrane-mediated process. In either event, the present experiments suggest that melarsen oxide will complex with trypanothione to form Mel T on entry to the cell and, assuming that Mel T will not readily diffuse across cell membrane, drug exit will be prevented. The importance of this effect is illustrated by the fact that Mel T is also formed *in vivo* when the organisms are exposed to the dimercaptopropanol adduct of melarsen

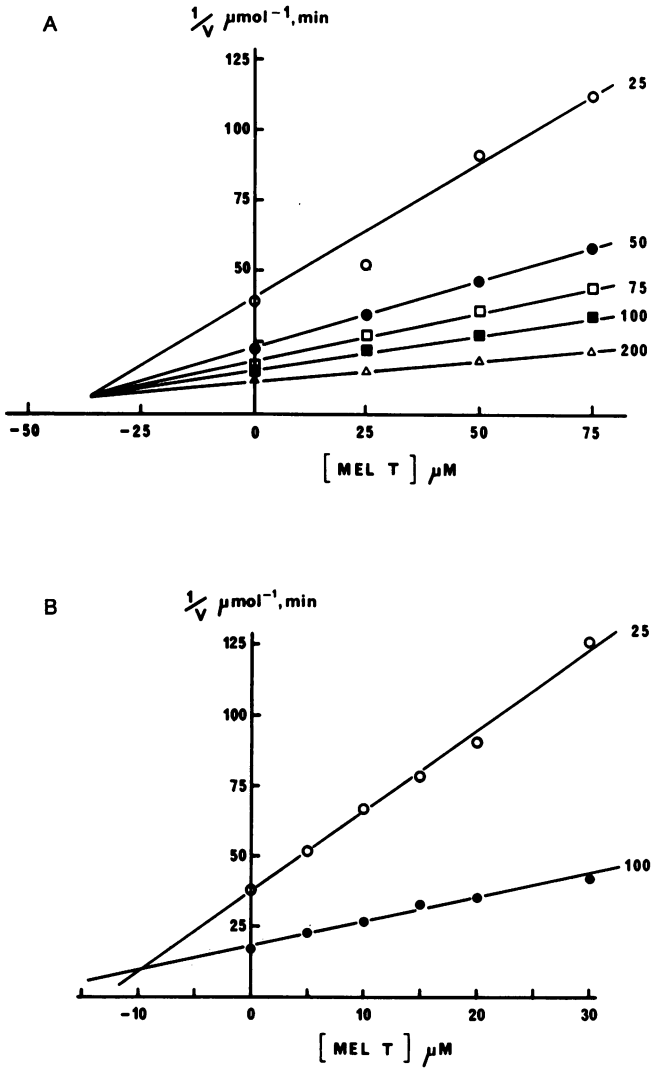

FIG. 6. Kinetics of inhibition of trypanothione reductase by Mel T. (*A*) Pure *C. fasciculata* enzyme. (*B*) Partially purified *T. brucei* enzyme. The values on the lines refer to the concentrations of $Try(SH)_2$ in μM used in the assays.

oxide, Mel B. Although the latter compound has a stability constant 3 orders of magnitude greater than the trypanothione adduct, dithiol exchange occurs because Mel B, on initial entry into the cell, encounters the large pool of trypanothione and Mel T is formed with release of dimercaptopropanol. As dimercaptopropanol is freely permeable to cell membranes it will exit the cell down a diffusion gradient, therefore facilitating further formation of Mel T. Trypanothione therefore represents an abundant and reasonably high-affinity target for both arsenoxide and dithioarsane derivatives.

Having formed within the cell, the Mel T complex might act to "steer" the arsenical drug toward its ultimate cellular target. Alternatively, the complex might itself be directly toxic. The latter effect is illustrated by the ability of Mel T to inhibit $Try(S)_2$ reductase activity. In the present experiments, a mean intracellular concentration of 17 μM Mel T could be achieved before cell lysis was observed. This concentration is 2-fold higher than the K_i for Mel T against the *T. brucei* enzyme and would have caused considerable inhibition of this activity.

In conclusion, the present experiments suggest that the toxicity of aromatic arsenicals toward African trypanosomes may be mediated by the presence of trypanothione. This hypothesis is supported by recent studies that have revealed

a pronounced synergism between the trypanocidal activity of aromatic arsenical drugs and the ornithine decarboxylase inhibitor difluoromethylornithine (DFMO) (16, 17). DFMO selectively blocks the biosynthesis of spermidine and hence trypanothione in trypanosomes (7). Thus, DFMO-induced depletion of trypanothione and its concomitant sequestration by melaminyl arsenical drugs offers an explanation of the synergism between these apparently unrelated compounds.

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1. Apted, F. I. C. (1970) in *The African Trypanosomiases*, ed. Mulligan, H. W. (George Allen & Unwin, London), pp. 684–710.
2. Flynn, I. W. & Bowman, I. R. R. (1974) *Comp. Biochem. Physiol. B* **48**, 261–273.
3. Fairlamb, A. H. & Opperdoes, F. R. (1986) in *Carbohydrate Metabolism in Cultured Cells*, ed. Morgan, M. J. (Plenum, New York), pp. 183–224.
4. Van Schaftingen, E., Opperdoes, F. R. & Hers, H.-G. (1987) *Eur. J. Biochem.* **166**, 653–661.
5. Fairlamb, A. H., Blackburn, P., Ulrich, P., Chait, B. T. & Cerami, A. (1985) *Science* **227**, 1485–1487.
6. Henderson, G. B., Ulrich, P., Fairlamb, A. H. & Cerami, A. (1986) *J. Chem. Soc. Chem. Commun.*, 593–594.
7. Fairlamb, A. H., Henderson, G. B., Bacchi, C. J. & Cerami, A. (1987) *Mol. Biochem. Parasitol.* **24**, 185–191.
8. Zahler, W. L. & Cleland, W. W. (1968) *J. Biol. Chem.* **243**, 716–719.
9. Shames, S. L., Fairlamb, A. H., Cerami, A. & Walsh, C. T. (1986) *Biochemistry* **25**, 3519–3526.
10. Henderson, G. B., Fairlamb, A. H., Ulrich, P. & Cerami, A. (1987) *Biochemistry* **26**, 3023–3027.
11. Opperdoes, F. R., Baudhuin, P., Coppens, I., De Roe, C., Edwards, S. W., Weijers, P. J. & Misset, O. (1984) *J. Cell Biol.* **98**, 1178–1184.
12. Keithly, J. S. & Fairlamb, A. H. (1988) in *The First Centenary (1885–1985) New Strategies for Control*, NATO–ASI Series, ed. Hart, D. T. (Plenum, New York), Vol. 163, pp. 729–737.
13. Henderson, G. B., Fairlamb, A. H. & Cerami, A. (1987) *Mol. Biochem. Parasitol.* **24**, 39–45.
14. Johnstone, R. M. (1963) in *Metabolic Inhibitors*, eds. Hochster, R. M. & Quastel, J. H. (Academic, New York), Vol. 2, pp. 99–117.
15. Albert, A. (1973) in *Selective Toxicity* (Chapman & Hall, London), pp. 130–172.
16. McCann, P. P., Bacchi, C. J., Nathan, H. C. & Sjoerdsma, A. (1983) in *Mechanisms of Drug Action*, eds. Singer, T. P. & Ondarza, R. N. (Academic, New York), pp. 159–173.
17. Jennings, F. W. (1988) *Trans. R. Soc. Trop. Med. Hyg.* **82**, 572–573.