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ANTIPARASITIC NITROIMIDAZOLES—IV

SERUM PROTEIN BINDING AND MODE OF ACTION OF 2-(4′-CARBOXYSTYRYL)-5-NITRO-1-VINYLIMIDAZOLE

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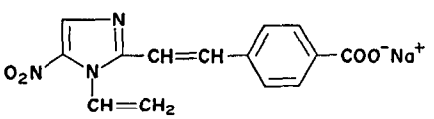
Abstract—The serum protein binding, uptake by parasites and mode of action of 2-(4′-carboxystyryl)-5-nitro-1-vinylimidazole (I) were investigated. Compound I was strongly bound to serum proteins in all species, especially the rat. The albumin of this species appeared to possess a single high-affinity site not present in mouse or dog albumin. Only unbound I was taken up by *Trichomonas vaginalis*. *In vitro*, a short exposure of *Trichomonas vaginalis* to levels of I above 5 μg/ml led to their subsequent death even after transfer to fresh medium. At low concentrations, I was degraded by the organisms which then recovered. Compound I reacted *in vitro* with thiol compounds and enzymes. It inactivated three glycolytic enzymes in intact *Trichomonas vaginalis* and *Trypanosoma rhodesiense* and caused a marked depletion of intracellular ATP before any effects on motility were apparent.

IN A search for new agents useful in the treatment of parasitic diseases, Ross *et al.*[1,2] synthesized a series of substituted 2-styryl-5-nitroimidazoles. Many of these showed good activity against *Trichomonas vaginalis*, *Entamoeba histolytica* and several strains of trypanosomes. One compound, the sodium salt of 2-(4′-carboxystyryl)-5-nitro-1-vinylimidazole (I) was the subject of studies on serum protein binding, uptake by parasites and mode of action, which are reported below.



SCHEME I.

MATERIALS AND METHODS

Chemicals. Compound I was prepared as described by Ross *et al.*[2] [^{14}C]I was prepared from [carbonyl-^{14}C]terephthalaldehyde, synthesized by the Radiochemical Centre, Amersham. Dioxan for scintillation counting was purchased from Union Carbide, Hythe, 2,5-diphenyloxazole (PPO) and 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) from Packard Instruments, Reading and Sephadex from Pharmacia G.B., London. Yeast extract L20, liver digest L27 and bacteriological

peptone L37 were obtained from Oxoid, London and horse serum No. 2 from Wellcome Reagents, Beckenham. Enzymes and cofactors were bought from the Boehringer Corp., London or the Sigma Chemical Co., London. All other chemicals were obtained from the British Drug Houses, Poole.

Experimental organisms. Trichomonas vaginalis (Poole strain) was subcultured every 2 days into the following medium at 37°: 250 ml 0·2 M-KH_2PO_4, 148 ml 0·2 M-NaOH, 602 ml water, 8 g NaCl, 1 g yeast extract, 5 g liver digest, 20 g glucose, 2 g cysteine HCl, 0·5 ml 0·1 %-methylene blue, 1 ml 0·5 %-Ca pantothenate and 10 g peptone. The medium was adjusted to pH 6·0–6·1, autoclaved and 100 ml sterile horse serum added. All cultures were used after 24 hr and were checked for motility, numbers and contamination.

Entamoeba histolytica (Rahman strain) was maintained at 37° in the following medium: 250 ml 0·2 M-KH_2PO_4, 0·2 M-NaOH, 553 ml water, 5 g NaCl, 1 g yeast extract and 5 g liver digest. The medium was adjusted to pH 7·0–7·1, autoclaved and 100 ml sterile horse serum and 30 ml 20 % rice starch suspension were added. For use, cultures were grown for 24 hr in the absence of starch, centrifuged at 45 *g* for 3 min, resuspended in isotonic NaCl and recentrifuged three times. This procedure markedly reduced the numbers of mixed gut bacteria which were also present in the culture as a nutrient.

Trypanosoma rhodesiense (Liverpool or EATRO 1276 strains) was maintained by passaging in mice. When required, four rats were infected. After 4 days, 5 ml of blood were taken by cardiac puncture from each rat and mixed with an equal volume of isotonic NaCl (buffered with phosphate to pH 7·1 and containing 1 mg/ml disodium diaminoethanetetra-acetate (EDTA) and 2 mg/ml glucose). After centrifuging for 2 min at 650 *g* the upper layer was discarded and the layer of trypanosomes immediately above the red blood cells collected and diluted in the buffered saline.

Assays. Compound I was assayed by its u.v. absorption at 375 nm in a Unicam SP500 spectrophotometer, or by radioactive counting using [^{14}C]I. Radioactivity was measured in a Packard Tri-Carb 3320 scintillation counter, using 1 ml of aqueous solutions or cell suspensions and 10 ml of Bray's solution[3] per vial. Counting efficiencies were corrected using internal standards.

The ATP content of *T. vaginalis* was assayed by the firefly luciferase method of Forrest and Walker.[4] One ml cell extract, 0·1 ml firefly tail supernatant and 2 ml 0·01 M-phosphate buffer (pH 7·4) were mixed in a scintillation counting vial which was placed in the Packard counter. Luminescence was measured from 0·25 to 0·45 min after mixing. Approximately 100,000 counts/0·2 min were obtained with a standard solution of 10 ng ATP/ml, the blank count being about 7500.

Pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40) was assayed by reduction of the pyruvate formed with lactate dehydrogenase (L-lactate–NAD oxidoreductase, EC 1.1.1.27) and measuring the concurrent oxidation of $NADH_2$ at 340 nm in a Unicam SP800 spectrophotometer. The following were mixed in a 1 cm cell at 25°: 2.57 ml 0·1 M-Tris(hydroxymethyl)methylamine buffer (pH 7·6), 0·15 ml 5 mg/ml-phosphoenolpyruvate (cyclohexyl-ammonium salt, in 0·5 M-$MgSO_4$ and 2 M-KCl), 0·2 ml 10 mg/ml-ADP (sodium salt), 0·05 ml 10 mg/ml-$NADH_2$ (sodium salt), 0·02 ml 2 mg/ml–lactate dehydrogenase and 0·01 ml homogenate.

Glyceraldehyde phosphate dehydrogenase (D-glyceraldehyde-3-phosphate–NAD oxidoreductase, EC 1.2.1.12) and phosphoglycerate kinase (ATP-3-phospho-D-

glycerate 1-phosphotransferase, EC 2.7.2.3) were estimated in a coupled assay. Each enzyme was assayed by adding a large excess of the other and following the oxidation of $NADH_2$ as above. The following were mixed in a 1 cm cell at 25°: 2·17 ml 0·1 M-Tris(hydroxymethyl)methylamine buffer (pH 7·6), 0·2 ml 0·024 M-K_2H PO_4, 0·15 ml 8·4 mg/ml-NaF, 0·05 ml 10 mg/ml-disodium diaminoethanetetra-acetate (EDTA) in buffer, 0·05 ml 10 mg/ml-$NADH_2$ (sodium salt), 0·2 ml 10 mg/ml-ATP (sodium salt), 0·1 ml 50 mg/ml-3-phosphoglycerate (cyclohexylammonium salt), 0·02 ml homogenate and 0·02 ml 4 mg/ml-phosphoglycerate kinase or 5 mg/ml-glyceraldehyde-phosphate dehydrogenase.

Measurement of serum protein binding of compound I. Blood was taken from the hearts of hamsters, rats and mice, an ear vein of rabbits and an arm vein of humans. It was allowed to clot for 1 hr at room temperature and serum collected after centrifuging at 3000 *g* for 5 min. The horse "serum" used was the commercial product used in the growth media, and was in fact heat-treated recalcified plasma.

Binding was measured by equilibrium dialysis or, in some experiments, by the Sephadex method of Hummel and Dreyer.[5] In the first method, 1·5 ml fresh serum (diluted with buffer if necessary) was placed in a dialysis bag made from 15 cm of 6·5 mm dia. Visking tubing, previously boiled for 5 min in 0·1% acetic acid and washed. The bag was inserted in a tube containing 3·5 ml 0·02 M-phosphate buffer (pH 7·4) containing 0·15 M-NaCl and I (usually 20 μg/ml). After incubation for 24 hr at 37° the concentrations of I inside and outside the bag were assayed.

In the alternative method, a 20 × 1 cm^2 column of Sephadex G-25, jacketed to 37°, was equilibrated with 5 μg/ml compound I in a Ringer–phosphate buffer. Three ml of serum were applied and elution continued with compound I in buffer. Fractions were assayed for I and for protein (by u.v. absorption at 280 nm, correcting for the absorption of I at this wavelength). The protein peak carried with it a peak of bound compound I, which was in equilibrium with the original (free) concentration of 5 μg/ml. The binding of compound I and its standard error were calculated from the ratio of free to bound I and the protein concentration in the fractions collected.

The albumin content of the sera used was measured by the method of Ness, Dickerson and Pastewka.[6]

RESULTS

Protein binding: effect on uptake by T. vaginalis. The structure of compound I, containing lipophilic and anionic groups, suggested that it would be strongly bound to serum proteins. This was found to be the case in all the species tested (except the horse), though there were significant differences between species (Table 1). As mentioned earlier, the horse serum was a heat-treated commercial product, used because of its inclusion in growth media. This result may therefore not reflect true protein binding in the horse. Similar results were obtained using the dialysis and Sephadex methods, indicating that both gave an accurate result.

In another experiment the binding was measured at several concentrations of I with fresh serum from each of three species (Table 2). In the rat the percentage of bound I fell as its free concentration was increased (to levels much higher than required therapeutically but in the range obtained in toxicology experiments). Results using mouse or dog serum were more variable, but no similar reduction in binding at high levels was evident.

TABLE 1. BINDING OF COMPOUND I IN SERUM

Method (species)	Free I concn (μg/ml)	Bound/free ratio	Bound/total (%)
Dialysis:			
Rat	6·9 ± 0·5	28·4 ± 3·5	96·6 ± 0·4
Rabbit	8·5 ± 0·4	15·7 ± 1·9	94·0 ± 0·7
Hamster	8·2 ± 0·2	14·9 ± 2·5	93·7 ± 1·0
Mouse	9·3 ± 0·3	8·5 ± 1·4	89·5 ± 1·5
Dog	8·1 ± 0·1	5·4 ± 0·2	84·5 ± 0·5
Horse	0·27 to 1·34	0·75 ± 0·08	43·0 ± 2·5
Human (M.E.T.)	8·4 ± 0·2	15·9 ± 0·9	94·1 ± 0·3
Human (T.J.W.)	8·6 ± 0·5	12·5 ± 1·1	92·6 ± 0·6
Sephadex:			
Rat	5·0	36·0 ± 2·7	97·3 ± 0·2
Mouse	5·0	11·3 ± 1·7	91·9 ± 1·1

Results are given as the mean ±S.E.M. of four results (dialysis method) or five results (Sephadex method). In dialysis the serum was at a concentration of 10 per cent; the results have been corrected to 100 per cent serum. The horse serum result is however based on two results at each of two I concentrations, using 50 per cent serum. See the text for comments on this result.

TABLE 2. SERUM BINDING OF COMPOUND I AT VARIOUS CONCENTRATIONS

	I concn (μg/ml)			Bound/total (%)	
Species	Free	Total	Ratio*	Observed	Calculated
Rat	0·565	38·1	0·216	98·5	98·8
	0·834	63·5	0·361	98·7	98·5
	5·40	124	0·677	95·6	95·5
	8·50	155	0·840	94·5	94·4
	14·7	191	1·014	92·3	93·0
	25·0	304	1·597	91·8	91·8
	36·4	406	2·125	91·0	91·0
	52·4	544	2·824	90·4	90·3
Mouse	1·46	10·2	0·079	85·7	89·8
	11·7	122	0·997	90·4	89·7
	22·7	221	1·796	89·7	89·5
	35·7	448	3·745	92·0	89·4
	190·0	739	5·890	87·8	88·7
	142·0	1120	8·899	87·3	88·0
Dog	8·13	52·3	0·357	84·5	86·8
	30·7	118	0·788	82·5	86·6
	50·1	372	2·606	86·5	86·3
	65·0	640	4·650	89·9	86·1

* Ratio of molecules bound I/molecules albumin.

The results are means of duplicates, carried out by dialysis. The sera were used at a concentration of 10 per cent; the results have been corrected to 100 per cent serum.

For each concentration the number of I molecules bound per albumin molecule was calculated, the concentrations of albumin in the sera used being: rat, 38 mg/ml; mouse, 24 mg/ml; dog, 27 mg/ml. The data were applied to a Scatchard plot[7] and equations of the type used by Karush[8] fitted. The mouse and dog results indicated the presence of a large number of identical weak binding sites on each albumin molecule. Assuming this number to be 55 (the number of ϵ-amino groups[9]) the association constant (K) of each site was: mouse, 449 M^{-1}; dog, 229 M^{-1}. The calculated percentages bound (Table 2) are based on these values. For the rat serum, a good fit to the experimental points was obtained if the albumin molecules possessed 55 weak binding sites ($K = 263\ M^{-1}$) and, on average, 0·4 strong binding site ($K = 6 \times 10^5\ M^{-1}$).

Since both *in vitro* and *in vivo* tests of antiparasitic activity are conducted in the presence of blood proteins, experiments were carried out to confirm that only the free compound is available for activity. A culture of *T. vaginalis* was centrifuged and portions resuspended in the following media, containing [^{14}C]I at 1 or 5 μg/ml: (a) control medium, containing only salts, glucose and cysteine at the levels given in Materials and Methods section, (b) control medium with 10 per cent fresh rat serum, (c) control medium with 10 per cent horse serum. After 10–60 min the cultures were centrifuged, washed in medium (a) free of I, and their radioactivity measured and expressed as I taken up. The motility of the trichomonads remained normal during the incubation except that after 60 min in 5 μg/ml I it was markedly reduced.

Figure 1 shows that rat serum strongly inhibited the uptake of I at both concentrations, but horse serum had little effect. As the control uptake was maximal after 30–60 min, the inhibition was most marked at short time intervals. The mean uptake in the first 30 min, expressed as a percentage of the control uptake (± S.E.M. on eight

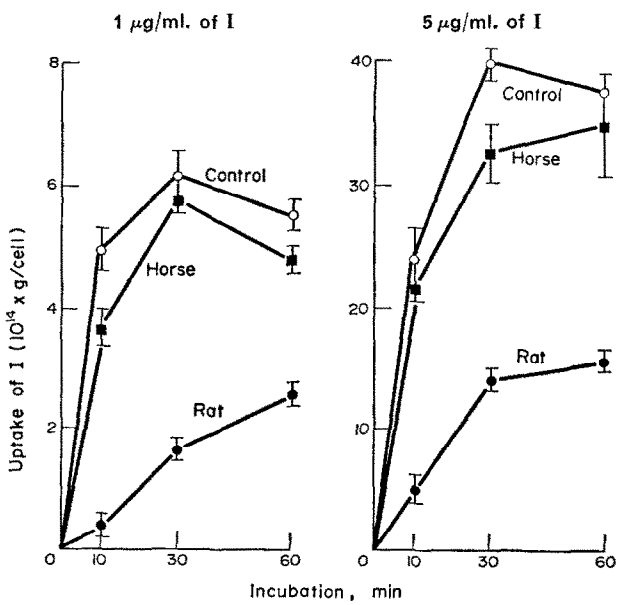


FIG. 1. The effect of serum on the uptake of compound I by *T. vaginalis*. *T. vaginalis* were incubated in [^{14}C]I (1 or 5 μg/ml) in protein-free medium (controls) or media containing 10 per cent rat or horse serum. Their content of I is given as the mean ±S.E.M. of eight (control) or four replicates. Note the difference of scale at the two concentrations of I.

replicates) was, horse serum: 90·5 ± 4·1 per cent; rat serum, 24·6 ± 2·3 per cent. The percentage of compound I unbound in 10 per cent serum was calculated from the data in Table 1 to be: horse serum, 93·0 ± 0·5 per cent; rat serum, 26·1 ± 3·1 per cent. Clearly *T. vaginalis* takes up only the free compound.

Uptake and elimination of compound I *by* T. vaginalis. [^{14}C]I was added to a growing culture of *T. vaginalis* to a concentration of 1 or 5 μg/ml. At intervals samples were removed and the motility of the organisms checked. Their numbers were measured with a Coulter counter, and their radioactivity determined and expressed as their content of I (although the chemical form of the radioactivity was not established). The results are shown in Fig. 2.

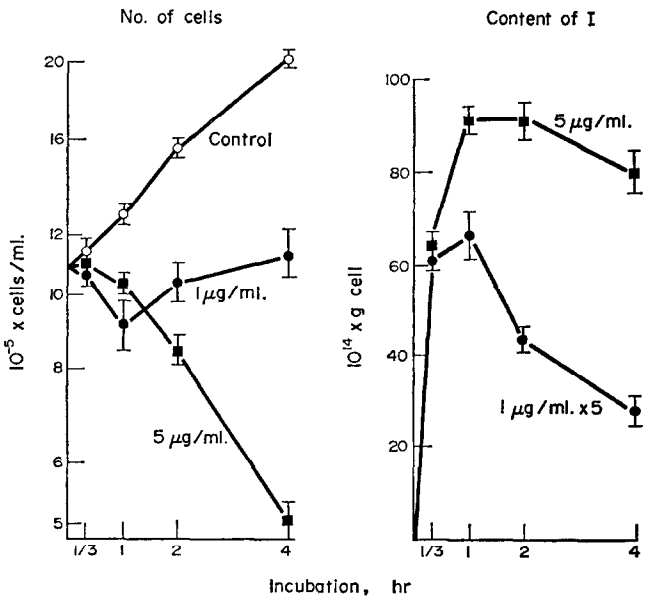


FIG. 2. The effect of compound I on *T. vaginalis in vitro*. *T. vaginalis* were incubated with [^{14}C]I (1 or 5 μg/ml). The numbers of cells and their content of I are given as the means ±S.E.M. of four replicates. The contents at 1 μg/ml have been multiplied by 5 before plotting.

As in the previous experiment (Fig. 1), the uptake of I was proportional to its concentration only for the first 20 min. (In both Figs. 1 and 2 the uptake at 1 μg/ml has been plotted at five-times the scale of that at 5 μg/ml, to facilitate comparison of the two concentrations.) The I content of organisms exposed to 5 μg/ml reached a maximum by 1 hr, when they were nearly immotile, and their numbers dropped rapidly thereafter. However organisms exposed to 1 μg/ml appeared to eliminate the compound. Their motility was depressed at 1 hr, but then returned to normal and they resumed dividing, though less actively than a control culture.

In another experiment (Table 3) the effect of short exposures of *T. vaginalis* to various concentrations of I was studied. Incubation for 20 min or 2 hr at low concentrations of I (0·25 or 1 μg/ml) caused a slight drop in the number of organisms, but on centrifuging briefly and resuspending in medium free of I, multiplication resumed.

TABLE 3. EFFECT OF SHORT EXPOSURES TO I ON *T. vaginalis*

First incubation		Number cells (% initial) 2nd incubation (no I):			I content (10^{14} × g/cell) 2nd incubation (no I):		
I concn (μg/ml)	Time	0	2 hr	16 hr	0	2 hr	16 hr
0.25	20 min	92	142	283	11·0	2·5	0·7
	2 hr	85	88	218	6·3	2·5	0·2
1·0	20 min	89	137	111	51·6	12·1	4·8
	2 hr	79	71	144	38·5	16·7	6·1
5·0	20 min	114	81	37	134	45	45
	2 hr	68	22	28	140	140	100
25·0	20 min	103	44	21	142	206	123
	2 hr	66	10	0	264	343	

T. vaginalis were incubated with [^{14}C]I for 20 min or 2 hr and then transferred to I-free medium. Results are the means of six replicates (initial incubation) or three replicates (recovery). Two experiments have been combined in which the initial numbers of cells/ml were 5·3 × 10^5 and 5·0 × 10^5.

The I content of the cells was dropping even before the end of the first incubation and it was eliminated rapidly on transfer to medium free of I. Little effect on motility was seen.

In contrast, exposure to high concentrations of I (5 or 20 μg/ml) for 20 min or 2 hr caused a rapid fall in the number of cells, which continued after transfer to fresh medium. Motility was markedly reduced or absent. The compound was only partly (at 5 μg/ml) or not at all (at 20 μg/ml) eliminated in the I-free medium. The I content per cell appeared to rise when the numbers dropped sharply, perhaps because of reuptake. However the radioactive material in the cells after 2 hr was not I but a metabolite, not extractable into ethyl acetate.

The apparent elimination of the compound by *T. vaginalis* after a few hours, even when it was still present in the medium at low concentration, was next investigated. It was found that at 1 μg/ml only 12 per cent of the radioactivity in the medium was in fact compound I after 4 hr. While carrying out this work it was found that the compound reacted with cysteine, present in the media (see later). However at 37° only about 10 per cent of the compound reacted with cysteine per hour, while when *T. vaginalis* was present the degradation was about five times faster. Some of the above experiments were repeated in cysteine-free medium, with essentially identical results.

To confirm that degradation of I by the trichomonads was the cause of the elimination of the drug from the cells, a culture was incubated with [^{14}C]I at 1 μg/ml for 4 hr. In the following hour its apparent I content dropped from 13·2 to 11·5 × 10^{-14} g/cell. However if the cells were transferred to fresh medium also containing [^{14}C]I at 1 μg/ml for 1 hr they took up the compound to a content of 22·7 × 10^{-14} g/cell. Conversely a new culture, resuspended in the medium from the 4 hr incubation, took up only 0·2 × 10^{-14} g/cell. Clearly the organisms had degraded the compound to a metabolite.

Effect of compound I *on nucleic acid and ATP synthesis in* T. vaginalis. Several antiparasitic drugs are known to act by interference with nucleic acid synthesis. The effect of compound I (5 μg/ml) on the incorporation of [^{14}C]adenine into the nucleic acids of *T. vaginalis* was therefore measured in some preliminary experiments. Five min after exposure to the compound (when the motility of the organisms was still normal) adenine incorporation into DNA and RNA was depressed by 55 and 36 per cent, respectively. The incorporation of tritiated thymine and uracil, respectively, into DNA and RNA, was however depressed less severely and it was therefore thought that the defect might reside not in nucleic acid synthesis but in the conversion of [^{14}C]adenine to [^{14}C]ATP. This would be expected to affect the incorporation of [^{14}C]adenine to a greater extent than the other bases.

Compound I was therefore added to a culture of *T. vaginalis* at 5 μg/ml. At intervals the number of cells was measured in a Coulter counter and aliquots were centrifuged, washed in isotonic NaCl and their ATP content determined. A marked drop in ATP content was observed in 5 min and was maximal in 10 min (Table 4), while the motility of the organisms was not reduced until after 20–30 min. Table 4 contains the combined data from several experiments. While the control ATP content was rather variable, the effect of the compound was consistent, as shown by the standard errors in the last column.

TABLE 4. EFFECT OF I ON THE ATP CONTENT OF *T. vaginalis*

Incubn. time (min)	No. results	ATP content 10^{15} × g/cell		Reduction (%)
		Control	With I	
5	3	407 ± 92	264 ± 44	33·7 ± 3·5
10	2	505 ± 102	103 ± 59	80·6 ± 7·3
15	4	403 ± 43	76 ± 15	81·6 ± 2·3
30	2	360 ± 208	78 ± 48	79·0 ± 1·1

The ATP content was measured at intervals after the addition of 5 μg/ml I to a culture of trichomonads. Results are means ±S.E.M. (i.e. half-range for two results).

The reaction of compound I *with thiol compounds and enzymes.* Compound I was found to react with thiol compounds in dilute aqueous solution, its u.v. absorption maxima at 303 and 376 nm being replaced by one at 330 nm. At 37° in 0·5 M phosphate buffer (pH 7·4) the pseudo-first-order rate constants were 2·49 M^{-1} min^{-1} with cysteine and 0·126 M^{-1} min^{-1} with glutathione. The products of the reaction have not been identified but are inactive against parasites. Qualitatively similar reactions occurred with other nitroimidazoles; the presence of the vinyl and styryl moieties was not necessary.

In view of the rapid inhibition of ATP synthesis in parasites by I, its effect was examined on the following glycolytic enzymes, known to be sensitive to thiol reagents: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase

(PGK) and pyruvate kinase (PK). The crystalline enzymes were inhibited by I in the range 1–30 μg/ml, GAPDH being the most sensitive. Pre-incubation of the enzymes and compound increased the inactivation to 100 per cent, indicating that the reaction was irreversible.

The levels of these enzymes were next assayed in trichomonads incubated in the presence of I, washed and homogenized in 0·1 M Tris(hydroxymethyl)methylamine buffer, pH 7.6. The homogenate was centrifuged and the supernatant used. The dilution effectively stopped any further inactivation. All three enzymes were rapidly inactivated (Table 5), PGK being the most sensitive. The time course of the inactiva-

TABLE 5. INACTIVATION OF GLYCOLYTIC ENZYMES BY COMPOUND I

Organism (strain)	Incubation: I μg/ml	Incubation: Time (min)	% Reduction in: GAPDH*	PGK	PK
Trichomonas vaginalis (Poole)	5	5	11, 23	64, 64	21, 22
	5	20	70, 72	75, 76	70, 73
Trypanosoma rhodesiense (Liverpool):	100	15	5, 18	90, 97	44, 44
(EATRO 1276):	200	15	17, 22	51, 72	31, 38

* See text for key.

The parasites were incubated as indicated above and their enzyme activity compared with that of a control culture incubated without the compound. Results are from two experiments.

tion was similar to that for the inhibition of ATP synthesis and preceded any loss of motility. Similar results were obtained using *Trypanosoma rhodesiense*, except that much higher concentrations of the compound had to be used. Using *Entamoebae histolytica* the levels of PK and PGK were too low for assay with the limited number of organisms available. No inhibition of GAPDH was found using I at 200 μg/ml for 15 min; since this enzyme was the least affected in the other parasites, this experiment was inconclusive.

After subcutaneous injection of 400 mg/kg of compound I (close to the LD_{50}) into four mice no changes in GAPDH, PGK or PK levels were detected in brain or liver at 15 min, when blood levels were maximal at about 0·5 mg/ml.

DISCUSSION

As anticipated from its structure, compound I was strongly bound to serum proteins. The binding varied between species, and it appeared that while the serum albumins of the rat, mouse and dog each bore a large number of weak binding sites, rat albumin also possessed 0·4 site/molecule of very high affinity. Fractional values for the number of high-affinity sites have been obtained by other workers (Davison and Smith)[10] and are probably due to heterogeneity of the albumin or to competition from other anions. Although this result is almost certainly an oversimplification, it does suggest that the particularly strong binding in rat serum is due to only one site per

molecule. Measurements of the uptake of the compound by *T. vaginalis* in the presence of rat or horse serum confirmed the usually-accepted hypothesis that only the free drug is available for antiparasitic action.

The effects of compound I on *T. vaginalis*, *in vitro*, were strongly concentration-dependent. At levels of 5 μg/ml and above, not only were the organisms rapidly killed, but they continued to die even after transfer to drug-free medium. Although they then appeared incapable of eliminating the drug, this was probably not responsible for their delayed death, for the radioactive content of the cells was not I but a metabolite. It is of course possible that the metabolite was toxic to the trichomonads, but perhaps more likely that at high levels sufficient irreversible damage was caused to the cells to produce their death later, even in the absence of drug. Such an effect obviously is of potential chemotherapeutic benefit.

At low concentrations of the compound (1 μg/ml and less) the organisms initially took it up, but then appeared to eliminate it and to recover even when it was still present in the medium. This effect was found to be due to the rapid degradation of all the compound in the medium to inactive products. This effect is clearly of great importance in the *in vitro* testing of antiparasitic activity of this and similar compounds, and could easily lead to an underestimate of potential *in vivo* activity, where the drug is being continuously absorbed and circulated.

This degradation was partly due to the reaction of the compound with the cysteine present in the medium. This effect was quantitatively unimportant in our short-term experiments, though it could play a part in tests of activity lasting for 24 hr. The major degradation was carried out by the trichomonads themselves, and resulted in unidentified but highly-polar metabolites. This study shows that in the chemotherapy of *T. vaginalis* with this and similar compounds it is necessary to achieve a high intracellular concentration, even for a short time, so as to damage the organisms sufficiently to lead to their ultimate death and to block the metabolism of the compound which would otherwise lead to the recovery of the trichomonads.

A marked inhibition of the incorporation of radioactive bases into nucleic acids occurred in *T. vaginalis* treated with I some time before any discernible effect on motility or numbers of cells. This was however a secondary effect, dependent on a block in the synthesis of the ATP required for the conversion of exogenous bases to nucleotides. The defect in ATP synthesis coincided with the irreversible inactivation of three glycolytic enzymes, probably by an attack on thiol groups since compound I reacted readily with these in model systems. The mode of action of the compound against *T. vaginalis* therefore appears to be inactivation of energy-producing enzymes. A similar inactivation of glycolytic enzymes occurred in two strains of *Trypanosoma rhodesiense*, suggesting a comparable mode of action.

A similar inactivation of glycolytic enzymes could not be detected in mice after injection of near-toxic doses of compound I. Since the crystalline enzymes from rabbit muscle were inactivated readily *in vitro*, it would appear that the compound does not readily enter mammalian tissues and that at least part of its specificity for parasites must depend on transport into the cell.

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