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DRUG RESISTANCE IN TRYPANOSOMES; EFFECTS OF METABOLIC INHIBITORS, *p*H AND OXIDATION-REDUCTION POTENTIAL ON NORMAL AND RESISTANT *TRYPANOSOMA RHODESIENSE*

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A wide variety of metabolic inhibitors tested *in vitro* for trypanocidal activity on normal and drug-resistant strains of *Trypanosoma rhodesiense* showed no relation between acquired drug resistance and changes in specific enzymatic function. Oxidation-reduction potential is an important factor in trypanocidal action but is not obviously related to the development of resistance. The dependence on *p*H of the trypanocidal action of ionizing drugs against both normal and resistant trypanosomes supports the postulate that the development of resistance involves physical changes in cell structures associated with the uptake of drug.

The development of acquired drug resistance in pathogenic trypanosomes is likely to involve at least two changes in the parasite, (i) a modification in the protein surface structure associated with drug uptake, and (ii) a change in metabolism whereby drug-sensitive enzyme reactions are modified, by-passed or eliminated; (i) and (ii) may obviously be related.

Attempts to elucidate mechanism (i) by cross-resistance analysis and selective interference experiments have been described (Williamson and Rollo, 1959; Williamson, 1959); the ionic basis of this behaviour has now been investigated by measuring the effect of *p*H on the trypanocidal action *in vitro* of representative anionic, cationic and feebly ionized drugs on normal and resistant parasites.

Little evidence exists for mechanism (ii) although changes in dehydrogenase activity of a melarsen-resistant *Trypanosoma rhodesiense* have been described (Williamson, 1953a). With the advent of a stilbamidine-fast strain (Fulton and Grant, 1955) which is resistant to a very wide variety of trypanocides (Williamson and Rollo, 1959) and would therefore be expected to show metabolic changes most obviously, a preliminary examination was made of its response to a number of metabolic inhibitors (Williamson, 1953b). Some differences, of a fairly low order, from the normal response were detected in the groups of phosphorase and dehydrogenase inhibitors; the differential action of some of these compounds has now been studied further by examination of the individual enzymatic processes likely to be involved.

As pathogenic African trypanosomes appear to rely on dehydrogenase rather than metalloporphyrin oxidase systems, and as some kinds of trypanocidal drug action are known to be affected by oxidation-reduction potential (von Jancsó and von Jancsó, 1936; Brownlee, Goss, Goodwin, Woodbine and Walls, 1950), this factor has been examined in detail electrometrically and by the use of redox indicators. Marked differences occurred in the reaction of normal and resistant strains, but although oxidation-reduction potential was apparently related to trypanocidal activity it was not obviously connected with the development of resistance.

METHODS

Trypanosome Strains.—The strains of *T. rhodesiense* and the tests of trypanocidal activity *in vivo* were as described (Williamson and Rollo, 1959).

Metabolic Inhibitor Experiments.—(i) Twenty-four different "specific" enzyme inhibitors were tested *in vitro* under standardized conditions against the normal and stilbamidine-resistant strains of *T. rhodesiense*. The "trypanocidal titre" of each compound was determined by the technique of Yorke

and Murgatroyd (1930), as described by Williamson and Rollo (1959), using a standard incubation period of 4 hr. at 37° (and, in some instances, also of 24 hr.). A mixture of equal parts by volume of fresh sterile horse serum and an aqueous solution containing 0.85% NaCl and 0.2% glucose was used as a supporting medium. The titre, for comparative purposes, has been expressed here as the reciprocal of the lowest molar concentration which lysed or immobilized at least 90% of parasites. Fourfold serial dilutions were made throughout and, where differences in reaction from the normal were detected in the resistant strain, experiments were repeated using serial dilutions with a factor of 2. A difference of one dilution-level can be considered significant within any one experiment, where the same inhibitor solution was titrated simultaneously against equivalent numbers of parasites of both strains in dilution series of four to six tubes. The short incubation period was chosen in order that inhibitor activity on trypanosome motility and viability could be roughly compared with that known to be exerted on other cellular or cell-free systems. The "resistance factor" is given by the ratio of the lowest trypanocidal concentrations for the resistant and normal strain respectively.

(ii) For experiments on phosphate metabolism, suspensions of normal and resistant trypanosome strains were prepared from infected blood obtained by cardiac puncture of heavily infected rats. The parasites were washed and suspended in a glucose-Ringer-phosphate saline containing 50% sterile inactivated horse serum, and the concentration was measured in a counting-chamber. The composition of the saline was as follows: 150 mM-NaCl, 11 mM-glucose, 0.2 mM-$MgCl_2$, 0.2 mM-K_2SO_4, 2.67 mM Sörensen phosphate buffer *p*H 7.4. Volumes of 3 ml. in capped sterile centrifuge tubes were incubated at 37° in a constant temperature water-bath. After centrifugation, 25% trichloroacetic acid was added to the parasite-free supernatant medium (1 vol. to 2 or 3 vol. of medium). After centrifuging again, 1.0 ml. samples of the protein-free supernatant were analysed for inorganic phosphate by the method of Gomori (1942); total phosphate was obtained by digestion according to Moraczewski and Kelsey (1948). Acid-soluble phosphate in trypanosome deposits was determined by disrupting the cells with 0.45 ml. of acetone followed by 24 hr. shaking at room temperature with 5 ml. of 5% trichloroacetic acid.

(iii) For respiration experiments, O_2 uptake in air was measured in conventional Warburg flasks at a shaking rate of 120/min., using a total volume of 3.0 ml. of parasite suspension and 0.15 ml. of 10% KOH in the centre well. At the end of the incubation period (30 min. at 37°) the contents of the flasks were transferred by Pasteur pipette to centrifuge tubes and, after centrifugation, the parasite-free supernatant fluid was analysed for phosphate. Parasite homogenates were prepared according to McIlwain (1948) by grinding with "Microid" alumina; all operations, from blood collection onwards, were carried out as far as possible at 0° to 5°. 2,4-Dinitrophenol and NaF solutions were made up in the medium for suspending parasites.

(iv) For experiments on citrate metabolism, washed erythrocyte-free suspensions of normal and stilbamidine-resistant strains of *T. rhodesiense* were prepared by differential centrifugation of heparinized infected blood taken by cardiac puncture from heavily infected rats. Homogenates were prepared by the method of McIlwain (1948). The suspending medium was a solution of 1% (v/v) sterile inactivated horse serum in a buffered glucose saline consisting of 116 mM-NaCl, 8.9 mM-glucose, 197 μM-$MgCl_2$, 230 μM-K_2SO_4 and 13.3 mM Sörensen phosphate buffer *p*H 7.4, the whole medium containing 333 μM-sodium citrate. Aerobic incubates (3 ml.) were set up for a period of 3 hr. in capped sterile tubes at 37° in a constant temperature water-bath, and parasite concentrations were determined with a counting chamber. Solutions of sodium fluoroacetate were prepared freshly in the supporting medium.

Analyses of the citrate content of the medium were made by the method of Pucher, Sherman and Vickery (1936) as modified by Buffa and Peters (1950), and the results are expressed as μg. of anhydrous citric acid. All reagents were A.R. grade, and the light petroleum (67–70° boiling fraction; May & Baker) used in the extractions was redistilled and washed with acid (M.A.R. concentrated H_2SO_4) several times, followed by washing with water, drying and redistillation.

After incubation, 1.0 ml. of 25% trichloroacetic acid was added to each tube, and the tubes were put in ice for 15 min. The precipitate was filtered off, washed twice with 5% trichloroacetic acid, and the filtrate was then boiled for 1 min. with 0.3 ml. of 50% H_2SO_4; subsequent analytical steps were as described by Buffa and Peters (1950).

Oxidation-Reduction Potential Experiments.—(i) The trypanocidal titres of fourteen reversibly reducible dyes were determined as described above for the metabolic inhibitors, except that the medium consisted of a mixture of equal volumes of sterile inactivated horse serum and Sörensen phosphate-buffered saline (*p*H 7.4); the buffer contained 0.2% glucose.

(ii) Electrometric determinations were made on erythrocyte-free trypanosome suspensions prepared by differential centrifugation of blood taken by cardiac puncture of heavily infected rats. The parasites were washed and finally suspended in a medium consisting of a mixture of equal volumes of M/15 Sörensen phosphate buffer *p*H 7.4 and an aqueous solution of 0.85% NaCl and 0.2% glucose; the whole contained 1% (v/v) sterile inactivated horse serum. Parasite concentration was checked with a counting chamber and the suspensions were incubated in 4½ × 1 in. rimless tubes in a constant-temperature water-bath at 37°. Three tubes were set up simultaneously, each containing about 15 ml. of suspension at a parasite concentration of approximately 10^7/ml. and each fitted with a bung carrying a platinum electrode, an

agar-KCl bridge, gas inlet and outlet tubes, and a glass stirring rod. A source of O_2-free N_2 was connected through a water-bubbler to the gas inlet, and the free ends of the salt bridges, together with a saturated calomel electrode, dipped into a vessel containing saturated KCl solution. A Cambridge *p*H meter used as a millivoltmeter, with a wandering crocodile clip connection to the platinum electrodes, measured the potential in each incubation tube as required. At the end of the incubation period, the *p*H of the suspensions was checked with the glass electrode, and a drop was examined microscopically for parasite motility.

Effect of p*H on Trypanocidal Action* in Vitro.—The trypanocidal titre of the test compounds after 4 hr. incubation at 37° was measured as described above, but in media of controlled *p*H. The drugs were dissolved in an unbuffered medium consisting of a mixture of 1 part by volume of sterile inactivated horse serum and 9 parts by volume of glucose saline (0.85% NaCl and 0.2% glucose). Serial dilutions were made in a similar serum medium consisting of 1 part by volume of horse serum and 9 parts by volume of a 50/50 (v/v) mixture of glucose saline and M/15 Sörensen phosphate buffer. Before use, the *p*H of the resultant buffered serum-saline mixtures was checked with the glass electrode.

TABLE I

TRYPANOCIDAL ACTIVITY OF ENZYME INHIBITORS ON NORMAL AND STILBAMIDINE-FAST *T. RHODESIENSE IN VITRO* AT 37°

Incubation period, 4 hr., except for the numerals in parentheses. In these experiments, incubation was for 24 hr.

Site of Inhibition	Inhibitor	Trypanocidal Titre (M^{-1})	Resistance Factor
Metallo-oxidases	Cyanide	32	1
	Azide	128	1
	2,2'-Dipyridyl	512	1
	Hydroxylamine	320	1
Metal ion co-factors	Dithizone	5,120	1
	Oxine	786,000	1
	Fluoride	32	1
Dehydrogenases	Urethane	12	(2)
	Pentobarbitone	32	(0·25)
	Thiopentone	128	(1–0·25)
Thiol enzymes	Iodoacetate	32,800	1
	$HgCl_2$	2,050	1
	Arsenite	32,800	1
	Tetrathionate	128	1
Phosphorases	Alloxan	64	(4)
	1,2-Naphthaquinone-4-sulphonate	8,190	1
	Ninhydrin	2,050	2
	Pyrophosphate	32	1
	Phlorrhizin	32	1
	2,4-Dinitrophenol	128	2
	Methylene blue	131,000	4
Cholinesterase	Eserine	2,050	1
Amine oxidase	Ephedrine	512	1
Pyruvate utilization	Malonate	6	1
	Fluoroacetate	5	(0·5)
—	Pentamidine	237,000	16
	Melarsen oxide	30,000,000	64

RESULTS

Table I shows the activity *in vitro* of 24 different "specific" enzyme inhibitors against normal and stilbamidine-fast strains of *T. rhodesiense*. The inhibitors are grouped as far as possible according to their specific reactivities. Some differential action against the resistant strain is apparent in the dehydrogenase and phosphorase inhibitor groups, and also with fluoroacetate. The relatively high trypanocidal activity of oxine suggested that this might be reversed by cobaltous ions in the same way as its antibacterial action (Albert, Rubbo, Goldacre and Balfour, 1947); Table II shows that this specific reversal of trypanocidal action does occur.

TABLE II

REVERSAL BY Co^{2+} OF THE TRYPANOCIDAL ACTION OF 8-HYDROXYQUINOLINE (OXINE) ON *T. RHODESIENSE IN VITRO* AT 37°

Incubation period, 4 hr.

Metal Ion	Trypanosome Numbers (as % Control)	
	Metal Ion (M/393,000)	Metal Ion (M/393,000) + Oxine (M/786,000)
Ca^{2+}	147	5·6
Mg^{2+}	128	<1
Mn^{2+}	120	<1
Cd^{2+}	0	0
Fe^{2+}	92	<1
Co^{2+}	114	117
Zn^{2+}	139	<1
Ni^{2+}	100	0
Cu^{2+}	128	0
Medium only	100	
Oxine M/786,000	<1	<1

Experiments on phosphate metabolism are recorded in Tables III, IV and V. All suspensions (concentration: 10^8 to 10^9 trypanosomes/3 ml.), with negligible variants, produced an increase in the inorganic phosphate of the medium, which originally contained about 6 μmole of orthophosphate. Neither the addition of NaF nor an increase in the Mg^{2+} content of the medium to 0.02 M appreciably inhibited this shedding of

TABLE III

LOSS OF INORGANIC PHOSPHORUS FROM STATIC TRYPANOSOME SUSPENSIONS AT 37°; LACK OF EFFECT OF NaF AND 2,4-DINITROPHENOL

+ Indicates an increase and − a decrease in phosphorus content of the medium compared with the control (parasite-free medium or suspension at the start of incubation period).

Inhibitor Added	Change in Medium Inorganic P Content (μmole/10^9 Trypanosomes/hr.)		
	Mean	No. of Expts.	Range
None	+0·55	11	(−0·39 to +1·6)
NaF (3 to 10 mM)	+0·86	6	(−0·5 to +1·5)
2,4-Dinitrophenol (0·75 to 2·5 mM)	+0·87	4	(0 to +2·0)

TABLE IV

CHANGES IN TRICHLOROACETIC ACID-SOLUBLE PHOSPHORUS OF TRYPANOSOMES IN STATIC SUSPENSIONS AT 37° IN RELATION TO CHANGES IN PHOSPHORUS CONTENT OF MEDIUM

No. of Parasites/ 3 ml. ($\times 10^{-6}$)	Change in P Content of Medium (μmole/10^9/hr.)			Trichloroacetic Acid-soluble P in Trypanosomes (μmole/10^9)					
				At 0 hr.			Change after 1 hr.		
	Inorganic	Organic	Total	Inorganic	Organic	Total	Inorganic	Organic	Total
900	+0·84	+3·3	+4·14	2·1	2·7	4·8	−0·65	−2·7	−3·35
984	+1·16	+1·05	+2·21	1·34	2·07	3·4	−0·99	−0·52	−1.51

phosphate by the trypanosomes; 2,4-dinitrophenol was similarly without effect on the normal or the resistant strain (Table III).

Two experiments, in which the trichloroacetic acid-soluble (organic) phosphate of the trypanosome deposits was determined before and after incubation, indicated that the increase in the phosphate content of the medium is caused largely, if not entirely, by irreversible breakdown and loss of the labile intracellular organic phosphate in the trypanosomes (Table IV). The conditions of preparation of the suspension, and of abnormal cultural environment and population density, are most probably responsible for this loss.

Static suspensions of trypanosomes were used in the experiments detailed in Tables III and IV. Table V shows the results of experiments with

TABLE V

LOSS OF INORGANIC PHOSPHORUS FROM SHAKEN TRYPANOSOME SUSPENSIONS AT 37°; LACK OF EFFECT OF 2,4-DINITROPHENOL

N indicates normal strain; and R, stilbamidine-fast strain. O_2 uptakes are the means of five experiments, except that marked with an asterisk, which is the mean of three experiments.

2,4-Dinitrophenol Conc. (μM)	Strain	No. of Expts.	Increase in Medium Inorganic P (μmole/10^9 Trypanosomes/hr.)	O_2 Uptake (μmole/ 10^9 Trypanosomes/hr.)
0	N	10	+1·55 (−3·49 to +4·97)	50·0
	R	6	+1·74 (−1·94 ,, +5·68)	49·4
47	N	5	+0·26 (−3·49 to +3·04)	51·1
	R	6	+2·26 (0 ,, +3·68)	47·8
188	N	6	+1·94 (0 to +6·66)	40·5*
	R	7	+1·81 (−1·94 ,, +4·06)	49·7
750	N	6	+2·46 (0 to +3·42)	53·3
	R	7	+2·13 (−3·87 ,, +4·06)	49·8
3,000	N	5	+1·16 (0 to +3·81)	50·8
	R	5	+0·39 (−1·94 ,, +2·0)	40·6

shaken suspensions. In these experiments with the Warburg respirometer in which O_2 uptake was also measured, the shedding of inorganic phosphate into the medium by the shaken suspensions (concentration: 10^8 to 10^9 trypanosomes/3 ml.) was about twice as great as that observed with static suspensions. The presence of 2,4-dinitrophenol (47 to 3,000 μM) had no noticeable effect on this loss, and there was no detectable difference between normal and resistant strains. In several experiments the changes in absolute amounts of phosphate were small and at the lower limits of precision of the analytical method used; this accounts for the variability of the results and the apparent phosphate uptake in some instances.

The effect of 2,4-dinitrophenol on O_2 uptake is shown in Table VI. The suspension concentration in these ten experiments ranged from 170 to

TABLE VI

EFFECT OF 2,4-DINITROPHENOL ON THE O_2 UPTAKE IN AIR OF SUSPENSIONS OF NORMAL AND STILBAMIDINE-RESISTANT STRAINS OF *T. RHODESIENSE* AT 37°

N indicates a normal strain, and R a stilbamidine-resistant strain.

2,4-Dinitrophenol Conc. (μM)	Strain	$qO_2/10^9$ Parasites in Expt. No.					Mean Change (%) from Control qO_2
		1	2	3	4	5	
	N	1,555	1,540	1,162	746	590	
	R	1,648	1,295	907	916	762	
47	N	1,860	1,386	1,172	700	604	+1·4
	R	1,678	1,114	893	913	751	−3·1
188	N			1,332	771	615	+7·5
	R	1,832	1,243	891	894	704	−0·8
750	N	1,860	1,395	1,424	669	628	+5·8
	R	1,730	1,264	916	932	740	+0·5
3,000	N	1,850	1,332	1,176	795	542	+1·1
	R	1,265	1,074	846	777	628	−15·9
Parasite concentration/ 3 ml.($\times 10^{-6}$)	N	170	221	427	646	902	
	R	272	510	629	629	765	

902×10^6 trypanosomes/3 ml., and an inverse relationship was observed between respiratory rate and parasite concentration. In a series of 26 experiments in which the parasite concentration varied from 64×10^6/3 ml. to $1{,}248 \times 10^6$/3 ml. the corresponding respiratory rate range ($qO_2/10^9$ trypanosomes) was 2,196 to 311, which is similar to that observed by von Brand and Tobie (1948). The respiratory rate throughout is based on the uptake observed during the first 30 min. of the experimental incubation period.

There was some stimulation of O_2 uptake by 2,4-dinitrophenol at the lower drug concentrations and at the lowest parasite concentrations, especially on the normal strain. The O_2 uptake of the resistant strain tended rather to be inhibited; this inhibition was most marked, by comparison with the normal strain, at a concentration of 3 mM 2,4-dinitrophenol. The difference in response (17%) is significant; using Student's t test, the significance level is 0.02 to 0.05.

Two experiments with homogenates were made in the Warburg respirometer. Each flask contained 3.0 ml. of a dilution of homogenate in the standard medium equivalent to 350×10^6 and 560×10^6 trypanosomes respectively in the two series. In both, microscopic examination of the homogenates showed almost complete cell disruption. Over a 30 min. incubation period, the O_2 uptake of these homogenates was negligible. The inorganic phosphate content of the medium was determined after protein precipitation by tipping in 1.0 ml. of 25% (v/v) trichloroacetic acid from the side-arm of the reaction flask at varying intervals. No phosphate uptake was demonstrable at incubation periods of 15 min and 30 min. in the presence or absence of 3 mM 2,4-dinitrophenol.

Table VII, which gives the results of experiments on citrate metabolism, shows clearly that there is no aerobic utilization of citrate, and no accumulation of citrate in the presence of fluoroacetate, either in whole suspensions or in cell-free preparations of normal or stilbamidine-resistant strains of *T. rhodesiense*.

Table VIII shows the trypanocidal titre of fourteen reversibly-reducible substances including twelve "redox indicators." The values quoted for the oxidation-reduction potential of the half-reduced substances (E'_o) are for *p*H 7.0 and temperature 30°. In the case of janus green, the final reduction stage is well defined, corresponding to $E'_o = -0.255$ V, but an intermediate blue-red change corresponding to $E'_o = -0.035$ V is quoted by von Jancsó and von Jancsó (1936). Electrometric titration with hydrosulphite under N_2 has shown a red-blue change in the neighbourhood of 0 V followed by a sharp end-point at the -0.25 V level; there are thus no grounds for supposing, as Page and Robinson (1943) have done, that the B.D.H. product used by them (and in the present work) differs essentially from the dye used by von Jancsó and von Jancsó (1936). The E'_o value used here is -0.035 V.

From these results, trypanocidal activity is obviously not a simple function of oxidation-reduction potential, but, with the exception of Bindschedler's green, the eight most highly active compounds have E'_o values in the region $+0.15$ V to -0.15 V.

TABLE VII

EFFECT OF FLUOROACETATE ON CITRATE METABOLISM OF NORMAL AND STILBAMIDINE-RESISTANT *T. RHODESIENSE*

Incubate	Citric Acid after 3 hr. at 37° (μg.)			
	Normal Strain		Resistant Strain	
	Free Parasites	Homogenate	Free Parasites	Homogenate
Medium	192	192	192	192
" +parasites	201	183	183	192
" + +M/120" fluoroacetate	183	178	183	183
Parasites/3 ml. ($\times 10^{-6}$)	32	21	32	90

TABLE VIII

TRYPANOCIDAL ACTIVITY *IN VITRO* AT 37° OF REVERSIBLY-REDUCIBLE TEST COMPOUNDS AGAINST NORMAL AND STILBAMIDINE-RESISTANT *T. RHODESIENSE* (*p*H: 7·4)

An asterisk indicates drugs obtained from British Drug Houses Ltd., redox indicator. †, British Drug Houses Ltd., standard stain. ‡, G. T. Gurr Ltd. §, Braid and Co. Ltd.

Test Substance	E'_o (V)	No. of Expts.	Trypanocidal Titre (M^{-1}) at		Resistance Factor	
			4 hr.	24 hr.	4 hr.	24 hr.
Ferricyanide	+0·43	2	<1,000	2,000–8,000	1	1
Alloxan	+0·406	2	32–64	128	1	4
*Bindschedler's green (Zn double salt) [C.I. No. 819 (1st Edn.); 49405 (2nd Edn.)]	+0·224	2	64,000	64,000–256,000	1	1
*o-Cresol-2,6-dichlorophenol-indophenol	+0·181	1	8,000–32,000	32,000	1	
*Toluylene blue (C.I. No. 820; 49410)	+0·115	1	8,000	32,000	1	1
‡Thionine (C.I. No. 920; 52000)	+0·063	1	32,000	512,000	4	4
†Brilliant cresyl blue (C.I. No. 877; 51010)	+0·047	3	32,000	512,000	4	1
§Toluidine blue (C.I. No. 925; 52040)	+0·011	2	32,000	128,000	1	1
†Methylene blue (C.I. No. 922; 52015)	+0·011	4	128,000	2,000,000	4	4
†Janus green (C.I. No. 133; 11045)	−0·035	3	512,000	33,000,000	1	1
†Nile blue (C.I. No. 913; 51180)	−0·122	4	32,000	512,000	1	
†Indigo carmine (C.I. No. 1180; 73015)	−0·125	4	8,000	32,000	4–16	16
*Phenosafranine (C.I. No. 840; 50200)	−0·252	4	2,000	128,000	4	1
‡Neutral red (C.I. No. 825; 50040)	−0·325	2	2,000	32,000	1	1

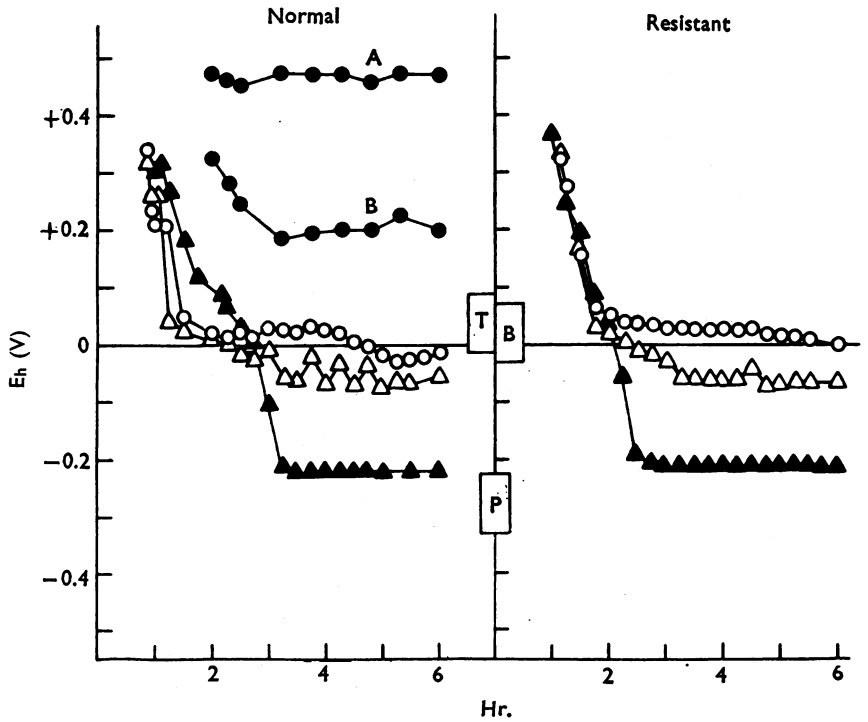

FIG. 1.—Effect of redox dyes on the oxidation-reduction potential of suspensions of normal and stilbamidine-resistant *T. rhodesiense* at 37° and *p*H 7·4 in O_2-free N_2. ● A, medium only; ● B, suspension only; O, thionine (T, M/8,500); Δ, brilliant cresyl blue (B, M/133,000); ▲, phenosafranine (P, M/8,300). Blocks on central ordinate show 2 to 98% reduction zone for T, B, and P.

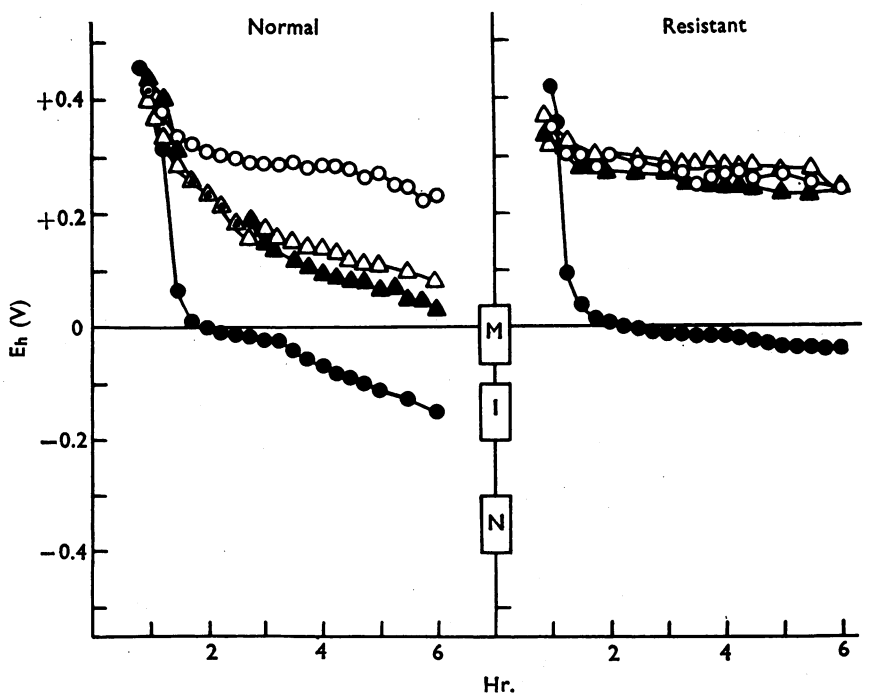

FIG. 2.—Effect of redox dyes on the oxidation-reduction potential of suspensions of normal and stilbamidine-resistant *T. rhodesiense* at 37° and *p*H 7.4 in O_2-free N_2. O, neutral red (N, M/8,300); Δ, indigo carmine (I, M/8,300); ▲, indigo carmine (M/33,000); ●, methylene blue (M, M/8,300). Blocks on central ordinate show 2 to 98% reduction zone for N, I, and M.

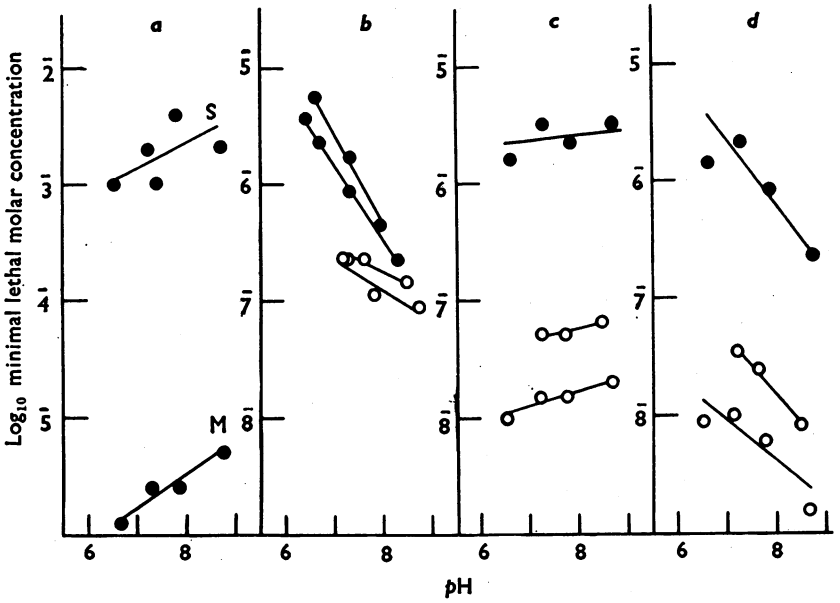

FIG. 3.—Effect of *p*H on the trypanocidal action of (*a*) oxophenarsine (M) and suramin (S), (*b*) pentamidine, (*c*) melarsen oxide, and (*d*) melarsen oxide as cation, against *T. rhodesiense in vitro* at 37°. Incubation time, 4 hr. O, Normal strain; ●, strain resistant to drug under test.

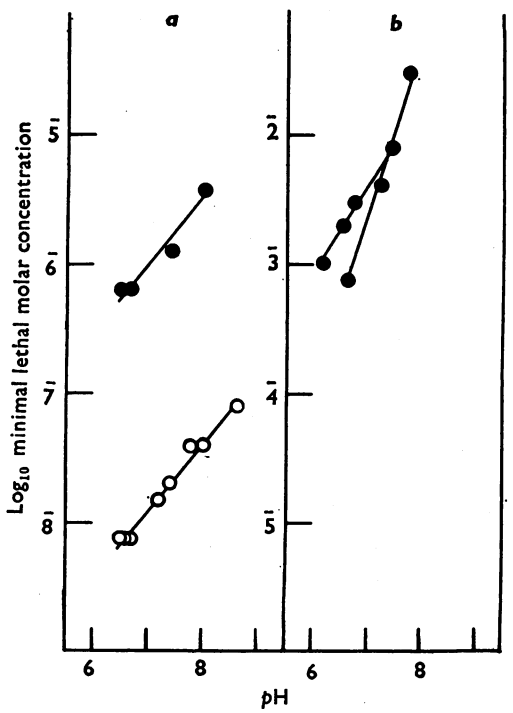

FIG. 4.—Effect of *p*H on the trypanocidal action of (*a*) butarsen and (*b*) indigo carmine, against *T. rhodesiense in vitro* at 37°. Incubation time, 4 hr. O, Normal strain; ●, strain resistant to drug under test.

In the graphs of results of electrometric measurements in trypanosome suspensions (Figs. 1 and 2), the ordinate represents the measured oxidation-reduction potential (E_h, referred to the normal H_2 electrode) at 37° and *p*H 7.4, and the abscissa represents time of incubation in hr., zero being the time of withdrawal of infected blood for the preparation of the suspension. Blocks on a central ordinate indicate the 2 to 98% reduction range of potential of the indicators used at *p*H 7.4 and 37° ($E'_o \pm 0.051$ V); the indicators were those to which the stilbamidine-resistant strain showed some resistance *in vitro*.

A few initial experiments with aerobic suspensions showed variable and erratic potentials, but anaerobically the potentials were stable and reproducible. The medium alone showed a steady level of about +0.47 V, and a trypanosome suspension without added dyes showed a fall of about 0.25 V from this level in the first 2 to 3 hr. of incubation followed by a slower decline to a level of about +0.2 V after 6 hr. This probably reflects the initial rapid O_2 uptake of the parasites followed by progressive decline and the increasing liberation of reducing substances by dying cells (Fig. 1).

The dependence on *p*H of the trypanocidal action *in vitro* of pentamidine, melarsen oxide, butarsen and indigo carmine is shown in Figs. 3 and 4.

The trypanocidal action *in vitro* of oxophenarsine and suramin was less dependent on *p*H (Fig. 3*a*) as might be expected from the lack of actively ionizing substituents in oxophenarsine and the relative inactivity *in vitro* of suramin, the mode of action of which is unlikely to be similar to that of the other drugs in the group tested.

Discussion

Few of the metabolic inhibitors listed in Table I can be considered specific for one given enzyme reaction, but several share a common chemical property, such as the ability to combine with or inactivate thiol groups, or to form chelate complexes with metal ions, which can reasonably be taken to underlie any observed inhibitory or lethal effect. The details of metabolism in *T. rhodesiense* have been established as yet only fragmentarily, and a full interpretation of inhibitor action can be made only by methods involving chemical determination of intermediate substances formed in the interrupted metabolic chain. Nevertheless, specific inhibitors showing trypanocidal activity in high dilution may be valuable indicators of the presence of a given enzyme reaction; conversely, any striking lack of lethal effect in low dilution may indicate the absence of a particular enzyme, though here the interpretation can be complicated by the cell-permeability factor.

The insensitivity of the respiration of pathogenic African trypanosomes to cyanide is well known (von Brand and Johnson, 1947; von Brand and Tobie, 1948) and is taken to indicate the absence of metalloporphyrin oxidases; this is reflected in the results here. Cyanide definitely stimulates motility, possibly by stimulation of anaerobic glycolysis or, as von Brand and Tobie (1948) have indicated, by keto-fixation (for example, cyanhydrin formation with pyruvic acid, the main end-product of glycolysis). The somewhat greater inhibition with azide may be connected with its ability also to inhibit phosphorases.

2,2′-Dipyridyl chelates strongly at blood *p*H specifically with Fe^{2+} ions, and markedly inhibits the anaerobic glycolysis of *T. lewisi* (Ryley, 1951). It has a less marked but appreciable action on *T. rhodesiense*; hydroxylamine has the same order of activity.

Several divalent metal ions in trace amounts are specific co-factors for enzyme reactions, for example, Mg^{2+} for enolase, Fe^{2+} and Cu^{2+} for the oxidation of ascorbic acid and glutathione respectively. At *p*H 7.3 dithizone combines specifically with Zn^{2+}, Co^{2+}, Pb^{2+}, Cu^{2+} and Cd^{2+} ions, and oxine with Mn^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+} ions (Albert and Gledhill, 1947). Fluoride inactivates the enzyme enolase by combination with Mg^{2+} ions. Both dithizone and oxine have a marked trypanocidal action, especially the latter, the trypanocidal properties of which were observed by Liese (1928). Since the antibacterial action of oxine is due to the formation of toxic complexes with Fe and Cu^{2+} ions (Albert *et al.*, 1947; Rubbo, Albert and Gibson, 1950; Albert, Gibson and Rubbo, 1953) and is reversible by Co^{2+} ions, the possibility that a similar mechanism might operate in its trypanocidal activity was investigated. Of nine divalent metal ions tested (see Table II) only Co^{2+} reversed the trypanocidal action of oxine (at the concentration used, Cd^{2+} ions were toxic). The protective action of Co^{2+} on the metal-catalysed oxidation of a group vital to metabolism, as indicated by Rubbo *et al.* (1950), may thus operate in *T. rhodesiense*; this is not improbable in view of the major importance of glycolysis to the parasites requiring the participation of thiols such as glutathione which is easily oxidized in the presence of metal ions (Ryley, 1953). The value of M/786,000 for oxine activity was unchanged by further incubation up to 24 hr. and seems to indicate that the trypanosomes are more sensitive than Gram-positive bacteria to oxine, if M/100,000 is regarded as the characteristic antibacterial concentration. This may reflect the relatively greater dependence of pathogenic trypanosomes on intact thiol groups if the action of oxine involves metal-catalysed thiol oxidations.

Central nervous system depressant drugs are relatively specific dehydrogenase inhibitors, and *T. rhodesiense* is known to possess dehydrogenase systems. Urethane and pentobarbitone (Nembutal) have relatively little effect, though the sulphur analogue of the latter, thiopentone, shows appreciable activity. However, it is possible that the effect is developed over the longer incubation period (24 hr.), when the resistant strain shows a resistance to urethane and a sensitivity to pentobarbitone and thiopentone, compared with the normal strain. This is not unexpected in view of the differences in dehydrogenase substrate activation already demonstrated between normal and melarsen-resistant strains of *T. rhodesiense* (Williamson, 1953a).

Inhibitors of thiol enzymes act by alkylation, mercaptide formation or oxidation, and their high degree of trypanocidal activity reflects the well-known importance of functional thiol groups in *T. rhodesiense* metabolism; tetrathionate, which inhibits by oxidation, is not, however, markedly

active. Some overlap with other inhibitor types occurs in this group, as iodoacetate is a specific inhibitor of triosephosphate dehydrogenase, and probably alloxan and naphthaquinonesulphonate, and possibly phlorrhizin, which are phosphorase inhibitors, owe this action to thiol inactivation.

The term " phosphorase " is the generic term coined by Dixon (1951) to include enzymes " which put on or remove or transfer phosphoric acid groups." The participation of phosphate in *T. rhodesiense* metabolism is likely by analogy with other pathogenic trypanosomes in which a phosphorylative glycolytic process has been shown, and by implication from the results in Tables III, IV and V.

1,2-Naphthaquinone-4-sulphonate and ninhydrin show marked relative trypanocidal activity and are presumed to act on thiol enzymes of the phosphokinase type. The presence of this type of enzyme in *T. evansi* and its inhibition by arsenoxide has been shown by Marshall (1948). Pyrophosphate and phlorrhizin are relatively inactive, but alloxan and 2,4-dinitrophenol show appreciable activity.

The main interest in this group is the differential action of four inhibitors on the resistant strain. The latter shows resistance to methylene blue, 2,4-dinitrophenol, ninhydrin and alloxan. 2,4-Dinitrophenol and methylene blue interfere with oxidative phosphorylation, and this property is shared by several other dyestuffs and some amidine and guanidine compounds, including trypanocidal derivatives. Alteration of drug-sensitive systems such as these might be expected to occur in the resistant trypanosome strain, but the results in Tables III, IV, V and VI show that phosphate uptake is not demonstrable under the experimental conditions here, and that there is no differential action of 2,4-dinitrophenol on the resistant strain.

Eserine is a specific inhibitor of cholinesterase and, since acetylcholine has been demonstrated in *T. rhodesiense* (Bülbring, Lourie and Pardoe, 1949), some eserine inhibition might be expected. The activity here is not marked (2,050 compared with approximately 10^6 on cholinesterase).

Ephedrine is a specific inhibitor of amine oxidase, which is inhibited by diamidines. Protein metabolism in *T. rhodesiense* has not been extensively investigated and is probably small *in vitro*, although amino-acid utilization occurs (Williamson and Rollo, 1952). Ephedrine inhibition here is marked but not outstanding.

Malonate and fluoroacetate are inhibitors of component reactions of the Krebs cycle involved in the utilization of pyruvate. Since pyruvate is the main end product of *T. rhodesiense* glycolysis, utilization is probably negligible, though it may occur to a small degree (Ryley, 1956) and is likely in *T. evansi* (Marshall, 1948). Malonate, a specific inhibitor of succinic dehydrogenase, has no significant inhibitory activity. Fluoroacetate, which inhibits the aconitase reaction and promotes citrate accumulation, is likewise relatively inactive, though some activity is developed over the longer incubation period. A differential action is, however, shown against the resistant strain which develops some sensitivity to the inhibitor. Guanidine compounds, including the trypanocidal Synthalin B, can cause citrate accumulation in animals and tissue preparations (Buffa, Peters and Wakelin, 1951), and it has been found that the melarsen-resistant strain develops marked citrate dehydrogenase activity not shown by the normal strain (Williamson, 1953a). Nevertheless, Table VII shows that there is no significant citrate utilization or accumulation in *T. rhodesiense* suspensions and no differential effect of fluoroacetate on the stilbamidine-fast strain. Ryley (1956) in experiments on *T. rhodesiense* showed that citrate was produced by this trypanosome in serum-free media.

A number of trypanocides such as phenylarsenoxides, acriflavine, stilbamidine and several dyestuffs have been shown to act as mitotic poisons. For this reason, and also because one of the more recently-developed radiomimetic compounds, tretamine (triethylene melamine; used in the treatment of leukaemia and Hodgkin's disease), is based on the melamine structure which appears to be involved in the action of the diamidine and melamine arsenical trypanocides, a number of radiomimetic compounds were tested for trypanocidal action, with the aim of relating further specific metabolic inhibitions to resistance development.

Tretamine, as noted earlier, has no trypanocidal activity. Two other active compounds of this sort, busulphan [Myleran; 1:4-di(methanesulphonoxy)butane], used in the treatment of myeloid leukaemia, and 8-azaguanine (carcinostatic for certain mouse tumours, and assumed to be a guanine antagonist), were tested, but showed no trypanocidal activity. Colchicine, the antimitotic action of which, like arsenicals, appears to be confined to suppression of spindle-formation, is similarly inactive as a trypanocide. All tests were made in living mice, as these compounds are primarily active against dividing cells.

So far as drug resistance is concerned, the above results with metabolic inhibitors would seem to

indicate that, in the stilbamidine-fast strain, the development of a high degree of resistance to a wide variety of trypanocides is not generally associated with comparably gross changes in intermediary metabolism. This conclusion accords with the inferences of Williamson and Rollo (1959) and of Williamson (1959) that resistance involves a steric modification of specific structures associated with the preliminary reversible adsorption stage of trypanocidal drug action.

A further aspect of drug-resistance capable of experimental investigation emerged from the work with enzyme inhibitors. Many of the latter act by irreversibly oxidizing or reducing susceptible cell components operating in a narrow range of oxidation-reduction potential. Oxidation-reduction changes in resistant cells were therefore investigated in two ways: (a) by measurement of the overall trypanocidal activity of twelve different oxidation-reduction indicators covering a potential range of +0.43 V to −0.34 V, and (b) by direct electrometric measurement of the oxidation-reduction potentials of O_2-free suspensions of parasites in the presence and absence of selected indicators.

Table VIII shows the results of investigation (a). The twelve indicator dyes used, with the exception of indophenol and indigo carmine, have superficially similar structures. They are either derivatives of diamino-substituted phenazines, oxazines or thiazines, or of indamine (Bindschedler's green and toluylene blue), but these similarities are not sufficiently close to isolate oxidation-reduction potential as a determinant activity factor. Using a more varied range of dyes, Page and Robinson (1943) showed a complete absence of correlation between oxidation-reduction potential and antibacterial activity against *Staphylococcus aureus*. Inspection of the antibacterial titres recorded by these workers for ten dyes used in the present study reveals, however, that the trypanocidal is much greater than the antibacterial activity, and that the most active antibacterial dyes have E'_0 values between +0.05 V and −0.12 V, which is within the range recorded above for maximal trypanocidal activity (Table VIII). Although, in this restricted series of dyes, lethality *in vitro* for *T. rhodesiense* and *S. aureus* appears to be related to oxidation-reduction potential, a number of examples may be cited to show that high trypanocidal activity is not necessarily associated with the possession of an E'_0 value in the region of 0 V: (i) the aminoacridine, acriflavine, which has a trypanocidal titre (M^{-1}) of 6.6×10^7 (Yorke and Murgatroyd, 1930), has a strongly negative E'_0 value of about −0.4 V (Breyer, Buchanan and Duewell, 1944); (ii) a number of trypanocidal phenylarsonic acids examined polarographically show strongly negative half-wave potentials of −1.26 V to −1.47 V against the normal calomel electrode (Breyer, 1939); and (iii) trypanocidal aromatic diamidines, in which the aromatic nuclei are joined by a saturated chain and are substituted by groups which in themselves do not possess an oxidation-reduction potential, are not reduced between +0.2 V and −1.9 V against the saturated calomel electrode at the dropping mercury electrode (H. Campbell, personal communication).

The stilbamidine-resistant strain is resistant *in vitro* to the phenothiazine dyes—thionine and methylene blue, to the phenoxazine dye—brilliant cresyl blue, to the phenazine dye—phenosafranine, and to the sulphonated indigo dye—indigo carmine; resistance to alloxan has already been noted above. The cross-resistance to these dyes is not related to their oxidation-reduction potential, and in fact recapitulates in another context the early observations of Ehrlich, Gonder, Werbitzki and others (summarized by Morgenroth, 1914) on the cross-resistance between certain aromatic arsenicals and dyes derived from the pyronine, acridine, selenopyronine, phenoxazine, phenothiazine and phenoselenazine series, in trypanosome strains made resistant to one or other of the two groups. The tricyclic anthracene-type nucleus, with one or two heteroatoms in the meso positions, characterizes dyes of the type listed above to which resistance is shown, but is present only in an incomplete form in the indophenol and indamine dyes, to which no resistance is shown here. Nor is the strain resistant to two other structurally divergent dyes, janus green, in which an aminophenazonium salt similar to phenosafranine is connected by an azo-linkage to dimethylaniline, and nile blue, which is a naphthophenoxazine. No resistance is shown to the phenothiazine dye toluidine blue or to the phenazine dye neutral red, both of which are closely related to methylene blue and to brilliant cresyl blue. The relatively high degree of cross-resistance to indigo carmine is of particular interest, since this is the only acid-substituted dye in the group tested and the possession of substituents able to ionize as anions at blood *p*H has generally been correlated with activity against resistant strains of the type used here. Some elucidation of this result is afforded by the electrometric experiments discussed below.

Some other biological reactions of a number of dyes used here may be considered. Several are well-known respiratory accelerants for a wide variety of cells and tissues, and, in fact, von Jancsó and von Jancsó (1936) suggested that the redox dyes which interfered with trypanocidal action did so by acting as accessory electron carriers for oxidations poisoned by the drug. Dickens (1935) found that phenosafranine was a highly potent inhibitor of the Pasteur effect in normal and tumour tissues, and Dickens (1936a, 1936b, 1939) showed similar activity in a number of related dyes. The ability of these dyes to raise aerobic glycolysis towards anaerobic levels was not correlated with respiratory acceleration, but there was a tendency for the more electropositive dyes to be inactive or to increase the Pasteur effect. Of particular interest is the inclusion of the trypanocides acriflavine, Synthalin B and undecane diamidine, and of "Styryl 430" in the group of highly active inhibitors. More recently, and more specifically, this inhibitory capacity has been correlated with depression of aerobic phosphorylation in cell-free tissue preparations (Judah and Williams-Ashman, 1951) and in rat tissues *in vivo* and *in vitro* (Dianzani and Scuro, 1956). A possibly related effect, where degradation of cozymase is inhibited, is restricted among the dyes tested to basic, quaternary dyes of the phenosafranine type (McIlwain, 1950; McIlwain and Grinyer, 1950). A comparison of these various dye reactions is presented in Table IX. The experimental materials are diverse: Dickens worked with rat kidney and brain slices, and rat and mouse tumour tissue, McIlwain (1950) with mammalian brain and spinal cord tissue extracts and guinea-pig brain slices, and Judah and Williams-Ashman (1951) with cell-free rabbit and rat kidney and liver particulate fractions. As the latter workers have emphasized, results on cell-free and intact cell preparations cannot be strictly compared since, in addition to the complex of molecular properties determining dye access to whole cells, species and tissue specificity factors are involved. No obvious parallelism with trypanocidal activity or cross-resistance behaviour can be discerned, but the association of Pasteur effect inhibition and trypanocidal activity is clearly marked with acriflavine, "Styryl 430," the trypanocidal aliphatic diamidines and diguanidines, and with dyes such as janus green. Trypanosomes of the *T. brucei* group have a high rate of aerobic glycolysis; further increase, with increased acid production, might be toxic, as Dickens (1939) has suggested. It may be significant, also, that the structural requirements postulated for inhibitory activity in the Pasteur reaction (Dickens, 1936b) apply to most of the few compounds (phenanthridine, cinnoline and quinaldine quaternary salts) found to be active against the arsenic-refractory *T. congolense,* where the aerobic glycolysis rate, though marked, is not so high as in the *T. brucei* group (von Brand, 1951).

TABLE IX

SOME BIOLOGICAL REACTIONS OF DYES AND TRYPANOCIDES

Columns marked with an asterisk indicate values obtained from Dickens (1936a, 1936b, and 1939); †, McIlwain (1950), McIlwain and Grinyer (1950); ‡ Judah and Williams-Ashman (1951). § Refers to guinea-pig brain cortex slices (McIlwain and Grinyer, 1950).

Test Compound	Respiratory Acceleration*	Release of Aerobic Glycolysis*	Cozymase Degradation†	Depression of Aerobic Phosphorylation‡
Ferricyanide	−	−		
Bindschedler's green	−	−		
Toluylene blue		−		±
Thionine	++	−		+++
Brilliant cresyl blue	++	−	±	+++
Methylene blue	+		−	+++
Janus green	−	(+§)	+++	+++
Nile blue	−			
Phenosafranine	−	+++	+++	+++
Neutral red	−	−(+§)	+	±
Acriflavine	−	+++		
Undecane diamidine	−	+++	−	+++
"Styryl 430"		+++	−	

No aerobic reduction of twelve of the fourteen redox indicators (all those tested except alloxan and methylene blue) was observed at 37° with trypanosome preparations under the following conditions: (i) M/2,500 dyes in a mixture of equal amounts of horse serum and glucose-saline with heat-killed normal, living normal and stilbamidine-resistant *T. rhodesiense* at a concentration of about 10^7/ml. incubated up to 20 hr.; (ii) M/8,000 dyes in phosphate-buffered glucose-saline mixture (*p*H 7.4) containing water-lysed normal and stilbamidine-resistant *T. rhodesiense* (approximately 10^7/ml.) incubated up to 96 hr.

Anaerobic incubation at 37° in evacuated Thunberg tubes of (i) M/4,500 dyes in phosphate-buffered glucose-saline (*p*H 7.4) containing saponin-lysed normal and stilbamidine-resistant *T. rhodesiense* (1.4×10^6/ml.) showed no reduction up to 17.5 hr., and of (ii) dye concentrations equivalent to the minimum lethal concentration *in vitro* (at a parasite concentration of approximately 1×10^6/ml.), in phosphate-buffered glucose-saline (*p*H 7.4) containing living normal and stilbamidine-resistant *T. rhodesiense* (4.2×10^6/ml.) incubated up to 18 hr., showed some reduction of *o*-cresol-2,6-dichlorophenol-indophenol, brilliant cresyl blue and toluidine blue. In several instances, the minimum lethal concentrations *in*

vitro were below visible coloration levels, and electrometric measurements were undertaken to avoid this and other defects of the colorimetric method.

Both normal and resistant trypanosome suspensions took up potentials with thionine (M/8,500) and brilliant cresyl blue (M/33,000) characteristic of the 2 to 98% reduction range of these dyes (Fig. 4). With phenosafranine (M/8,300), both strains took up a steady potential (−0.210 to −0.225 V) characteristic of minimal dye reduction, and may represent the lower limit of reducing capacity of the suspensions under these conditions, since neutral red (M/8,300) with a more negative E′₀ value is not reduced by either strain (Fig. 2). This lower limit of reducing capacity falls within the redox potential range of flavins and flavoproteins (Dixon, 1951) and may reflect the dependence of pathogenic African trypanosomes on this type of oxidase rather than on metalloporphyrin systems, as noted earlier.

Methylene blue (M/8,300) showed a differential action: with the normal strain the 2 to 98% reduction zone was passed through rapidly, and the suspension potential continued to become more negative, reaching a level of −0.165 V after 6.5 hr.; with the resistant strain, the suspension maintained a steady potential well within the 2 to 98% reduction zone (Fig. 2). As the dye concentration was near-toxic for the normal strain, the rapid fall in potential with this strain probably represented increased dye uptake followed by cell injury, death and release of reducing substances; with the resistant strain dye access was limited.

A differential action is more marked with the acid dye indigo carmine (indigo disulphonate) (Fig. 2). This dye was not reduced by either strain, but at concentrations of M/8,300 and M/33,000 a fall in potential of 0.3 to 0.4 V occurred with the normal but not with the resistant strain. The difference is not so marked at the less toxic concentration of M/133,000. Again dye access was probably involved; addition of saponin to the resistant strain suspension containing M/33,000 dye produced a rapid fall in potential of about 0.3 V, suggesting release of previously inaccessible reducing systems capable of reacting with the dye. Indigo carmine, unlike the other dyes tested here, is an acid ionized at *p*H 7.4 and it is probable that resistance to indigo carmine involves changes in the ionization of cationic receptors associated with dye uptake. Experiments on the effect of *p*H on trypanocidal action *in vitro* have shown that indigo carmine uptake by a resistant strain was *p*H dependent (Fig. 4).

Although oxidation-reduction potential was apparently related to trypanocidal activity, it was not in itself obviously related to the development of resistance, and the marked differences revealed with some indicators by the electrometric method can most satisfactorily be explained by alterations in dye transport across the cell wall. The electrometric method seems a particularly sensitive way of detecting such transport if the substances investigated are capable of reacting with indifferent electrodes.

The importance of ionization as a factor in the action of selectively toxic agents has been stressed by Albert (1951). Antibacterial diamidines and acridines are two drug series with trypanocidal members which have been investigated in this way. The dependence on *p*H of the antibacterial action of the former group has been noted by Bernheim (1943) and by Elson (1945); Fig. 3b shows that the trypanocidal action of pentamidine is similarly determined by *p*H. Since the pK_a of pentamidine is 11.4, the drug is 100% ionized over the *p*H range tested, and the observed increase in activity with increasing *p*H is most probably due to competition with H^+ for an anionic receptor group ionizing over this range, as has been suggested by Albert, Rubbo, Goldacre, Davey and Stone (1945) for the similar behaviour of antibacterial acridines. The reaction to pentamidine of the resistant strain is also *p*H-dependent over the same range as the normal strain. This parallelism implies that the same anionic receptor for pentamidine is functioning in both the normal and resistant strains, and that the reduced drug uptake by the resistant strains reflects a decrease in the relative proportion of these receptors. A change of this sort might produce a shift in the isoelectric point of the parasite protein, as envisaged by Schueler (1947), and it is hoped to investigate this point further by electrophoretic methods and by potentiometric titration of separated protein material.

Melarsen oxide (Fig. 3c) shows no marked dependence on *p*H, but recalculation of the minimum lethal concentrations of drug in terms of the cationic form present (the pK_a of the basic group in melarsen is 7.64) shows that the most active form of the drug is the cation, and that, as in pentamidine, these cations compete with H^+ for an anionic receptor (Fig. 3d). In view of the cross-resistance behaviour described earlier, and of the similarity of the *p*H-activity curves of pentamidine and of the cation of melarsen oxide, this anionic receptor may well be the same for both drug types, possibly the hydroxyl group of tyrosine or of nucleic acid purines and pyrimidines, as

suggested by Albert (1951) for antibacterial acidines.

The *p*H-activity curves of the anionic trypanocide butarsen (Fig. 4a) reveal a similar mechanism involving a cationic receptor ionizing in the *p*H 6 to 9 range for both the normal and resistant strains. Within this range, the butarsen molecule is present as an anion to the extent of 95 to 99.9%, and the observed increased activity with lowered *p*H is therefore less likely to be due to an increased proportion of the more freely cell-permeable non-ionized molecules, as Eagle (1945) suggests, than to a change in the extent of ionization of a cationic receptor. Two such possible receptors are the α-amino group of cystine and the imidazolyl group of histidine; the latter group is "generally responsible for most of the buffering power of proteins in the physiological *p*H range" and is the most important binding site for zinc and other ions in the case of serum albumin (Edsall, Felsenfeld, Goodman, and Gurd, 1954).

The trypanocidal action of indigo carmine is also *p*H-dependent (Fig. 4b). The available results refer only to a strain resistant to the dye, but as indigo carmine is fully ionized over the *p*H range tested, the variation in activity may reasonably be ascribed to the varying ionization of a cationic receptor.

These results lend further support to the thesis that acquired drug resistance in trypanosomes is a process which involves physical changes in specific cellular structures associated with preliminary drug uptake rather than alterations in drug-sensitive metabolic function.

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