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DRUG RESISTANCE IN TRYPANOSOMES; CROSS-RESISTANCE ANALYSES

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Eight strains of *Trypanosoma rhodesiense*, made resistant respectively to atoxyl, butarsen, acriflavine, stilbamidine, Surfen C, suramin, and pontamine sky blue 5BX, have been examined for cross-resistance to representatives of nine structurally dissimilar groups of trypanocide. On the basis of their predominant ionic form at blood *p*H, these groups are considered in three main classes: (*a*) feebly ionized (neutral aromatic arsenicals), (*b*) ionized as cations (melaminyl arsenicals and antimonials, acridine derivatives, diguanidines and diamidines, 6-aminoquinoline and 6-aminocinnoline derivatives, phenanthridinium derivatives, triphenylmethane dyes), and (*c*) ionized as anions (carboxylated aromatic arsenicals and sulphonated naphthylamine derivatives). The results are discussed in relation to those of other workers and to possible modes of trypanocidal drug action. Cross-resistance behaviour is not wholly explicable on an ionic basis; the results suggest that stereospecific structural changes associated with initial drug uptake occur in resistant trypanosomes.

The novel structure and properties of the "melamine" class of arsenical and antimonial trypanocides introduced by Friedheim (Friedheim, 1939, 1940, 1944; Friedheim and Berman, 1946; Friedheim, Vogel, and Berman, 1947) led us to produce a strain of *Trypanosoma rhodesiense* resistant to melarsen (disodium *p*-melaminylphenylarsonate), a representative member of the new drug group. We found that this strain was unexpectedly, and almost totally, resistant also to trypanocidal diguanidines and diamidines (Rollo and Williamson, 1951). Subsequently we tested a wider variety of trypanocides against this and five other different resistant strains (Williamson and Rollo, 1952; Goodwin and Rollo, 1955), using these "therapeutic sieves," in Ehrlich's analogy, to sift out any common factors of activity not otherwise apparent among unrelated drug types. During this latter survey, we showed that a stilbamidine-resistant strain (Fulton and Grant, 1955) was cross-resistant to the "melamine" trypanocides, thus demonstrating a reciprocal resistance relationship between the "melamine" and diamidine classes of drug.

One possible mechanism for trypanocidal drug resistance can be based on the familiar assumption that there are at least two stages in drug action, a preliminary fixation and a subsequent lethal action, of which the former is modified in the resistant trypanosome so that the drug is no longer taken up (Clark, 1933; King and Strangeways, 1942). This is essentially the fifty-year-old *chemorezeptor* hypothesis of Ehrlich which, as Schnitzer and Grunberg (1957) indicate, "has not been replaced by either a more lucid or more comprehensive working hypothesis." We have attempted to elaborate this hypothesis with evidence from experiments on selective interference with trypanocidal action, the effect of *p*H and oxidation-reduction potential on trypanocidal action *in vitro*, the activity of selective enzyme inhibitors on normal and resistant trypanosomes *in vitro* and the physical and chemical properties of homogenates of normal and resistant trypanosomes. The present paper concerns the production and cross-resistance behaviour of a number of drug-fast strains, several of which have not hitherto been described.

METHODS

Trypanosome Strains

All the resistant strains described here are offshoots of a parent strain of *T. rhodesiense* (Yorke, Adams, and Murgatroyd, 1929) isolated from man in 1923 and subsequently maintained by blood passage in mice. Dr. J. D. Fulton, of the National Institute

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for Medical Research, very kindly provided us with the stilbamidine-resistant strain; the other resistant strains were obtained as follows:

Atoxyl (sodium p-aminophenylarsonate)-antimony potassium tartrate(tartar emetic)-fast.—This was developed 28 years ago by Yorke and Murgatroyd (1930); its resistance is still unchanged.

Suramin-fast.—This was developed in 1937 to 1938 by Dr. F. Hawking in the Liverpool School of Tropical Medicine and subsequently treated by Fulton and Yorke (1941). Its resistance was maintained unimpaired up to the most recent time of examination (1953 to 1954).

Melarsen-, butarsen-, and Surfen C-fast strains.—These were developed by us in the Liverpool School of Tropical Medicine (1948 to 1949) and re-established subsequently by one of us (J. W.) in the London School of Hygiene and Tropical Medicine and in the West African Institute for Trypanosomiasis Research Laboratories at Vom, Nigeria, where the acriflavine- and pontamine sky blue 5BX-fast strains were also developed.

All resistant strains were produced by intraperitoneal injection of a minimal effective dose of drug into infected mice or rats, followed by blood passage of the relapse strain and successive repetition of this procedure, increasing the dose where necessary as the strain developed resistance.

Drug Response Tests

These were made with batches of five or more mice of approximately 20 g. body weight, each given a single intraperitoneal drug injection (0.5 ml./20 g.) 48 hr. after intraperitoneal infection with approximately 20,000 trypanosomes; at this stage microscopic examination of blood showed one to ten parasites per field (objective 1/6 in., ocular ×10). Under these conditions, the terms "minimal effective dose" (ED90), "minimal curative dose" (CD90), and "maximum tolerated dose" (LD10), as used here and in the accompanying tables, are defined as:

ED90: That dose of drug which clears parasites from the peripheral blood for at least 24 hr. in at least 90% of infected mice, no trypanosomes being visible in thirty random microscope fields of a coverslip preparation of a drop of tail blood.

CD90: That dose which clears parasites from the peripheral blood for at least 28 days in at least 90% of infected mice.

LD10: That dose which kills not more than 10% of treated mice. Confidence limits of these doses for a 0.95 probability lie approximately in the range 70 to 150%, calculated by the method of Litchfield and Wilcoxon (1949).

Trypanocidal Action in Vitro

This was measured by the technique of Yorke and Murgatroyd (1930). Trypanosomes from infected rat blood were washed free of red cells and plasma and resuspended in glass-capped Kahn tubes at a concentration of 10^6/ml. in a sterile medium consisting of equal parts of rabbit serum and glucose-saline (0.85% NaCl, 0.2% glucose). Micro-pipettes for serial dilution were prepared and weight-calibrated as described by Fildes (1931).

Paper Chromatography of Acid Dyes

R_F values of dyes were obtained from ascending chromatograms on Whatman No. 50 paper, using a benzyl alcohol/ethanol/10N-H_2SO_4 mixture in proportions of 45/25/15 (v/v), and on Whatman No. 1 paper using an *n*-butanol/acetic acid/water mixture in proportions of 40/10/50 (v/v).

Origin of Trypanocides

Butarsen [γ-(*p*-arsenosophenyl)butyric acid monohydrate], tryparsamide (sodium *p*-carbamoylmethylaminophenylarsonate), pentamidine isethionate [1,5-di-(*p*-amidinophenoxy)pentane diisethionate], stilbamidine hydrochloride [di(*p*-amidino)stilbene dihydrochloride], and dimidium bromide (2,7-diamino-10-methyl-9-phenylphenanthridinium bromide) from May and Baker.

Homidium bromide (Ethidium bromide, 2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide) from Boots. Oxophenarsine hydrochloride (Mapharside, hemialcoholate of 3-amino-4-hydroxyphenylarsenoxide hydrochloride) and melarsen (disodium *p*-melaminylphenylarsonate) from Parke, Davis. Surfen [1,3-di(4-amino-2-methylquinol-6-yl)-urea hydrochloride] from Bayer Products. Surfen C [Congasin, di(4-amino-2-methylquinol-6-yl)melamine] from Bayer I.G. Farbenindustrie. Synthalin B (dodecamethylenebisguanidine dihydrochloride) as used by Lourie and Yorke (1937). Decane diamidine (decamethylenebisamidine dihydrochloride) and 3-acetamido-4-carboxyphenylarsenoxide from the late Dr. H. King, National Institute for Medical Research.

Quinapyramine [Antrycide, 4-amino-6-(2-amino-6 - methylpyrimidin - 4 - ylamino)quinaldine - 1:1′ - dimetho-sulphate], suramin {hexasodium salt of 3″, 3″-urylenebis[8-(3 - benzamido - 4 - methylbenzamido)-naphthalene-1,3,5,-trisulphonic acid]}, and para rosaniline [Colour Index No. 676 (1st Edn.); 42500 (2nd Edn.)] from Imperial Chemical Industries. Berenil [di(*p* - amidinophenyl) - N - 1,3, - triazene diaceturate trihydrate] from Farbwerke Hoechst. Cinnoline 528 [N^1N^3-di(4-aminocinnol-6-yl)guanidine dimethiodide] as used by Lourie, Morley, Simpson, and Walker (1951). Trypan blue (a) [C.I. No. 477 (1st Edn.); 23850 (2nd Edn.)], azovan blue [tetrasodium 4,4′-di(8-amino - 1 - hydroxy - 5,7 - disulphonaphth - 2 - ylazo)-3,3′-ditolyl], pontamine sky blue 5BX [C.I. No. 520 (1st Edn.); 24400 (2nd Edn.)], trypan red [C.I. No. 438 (1st Edn.); 22850 (2nd Edn.)], congo red [C.I. No. 370 (1st Edn.); 22120 (2nd Edn.)], benzopurpurin 4B [C.I. No. 448 (1st Edn.); 23500 (2nd Edn.)], azo blue [C.I. No. 463 (1st Edn.); 23680 (2nd Edn.)], vital red [C.I. No. 456 (1st Edn.); 23570 (2nd Edn.)], dianil blue 2R [C.I. No. 465 (1st Edn.); 23690 (2nd Edn.)], isamine blue [C.I. No. 710 (1st Edn.); 42700 (2nd Edn.)], and xylene cyanol FF [C.I. No. 715 (1st Edn.); 43535 (2nd Edn.)] from G. T. Gurr, Ltd.

Trypan blue (b), chlorazol fast pink BK [C.I. No. 353 (1st Edn.); 25380 (2nd Edn.)], acriflavine (mixture of 2,8-diaminoacridine dihydrochloride and 2,8-diamino-10-methylacridinium chloride), proflavine (2,8-diaminoacridine) sulphate, and aminacrine (5-amino-acridine) hydrochloride from British Drug Houses.

RESULTS

Table I shows the effective doses of active trypanocides in mice infected with the normal parent strain of *T. rhodesiense*. The following compounds were inactive at the LD10 level: aminacrine, azovan blue, pontamine sky blue 6BX, trypan red, congo red, benzopurpurin 4B, azo blue, vital red, dianil blue 2R, isamine blue, and xylene cyanol FF.

TABLE I

ACTIVITY OF TEST DRUGS AGAINST NORMAL *T. RHODESIENSE*

Intraperitoneal doses in mg./kg. of body weight. The activity of trypan blue (a) was apparent only on very light infections and at very much higher doses than recorded elsewhere (Morgenroth and Freund, 1924; Ormerod, 1952; Schumacher and Schnitzer, 1956).

Trypanocide	ED90	CD90	LD10
Tryparsamide	750	1,000	2,250
Oxophenarsine	0·75	4·0	10
Acriflavine	5·0	—	20
Proflavine	25	—	25
Melarsen	30	70	340
Melarsen oxide	0·125	1·25	5·0
Sodium *p*-melaminyl-phenylstibonate polymer (MSb)	20	30	1,150
MSb 3	0·25	1·25	25
Synthalin B	7·5	—	7·5
Decane diamidine	12·5	—	12·5
Pentamidine	0·65	12·5	50
Stilbamidine	0·65	2·5	50
Berenil	—	5	100
Surfen	50	200	200
Surfen C	50	200	200
Quinapyramine	1·6	—	15
Cinnoline 528	0·75	—	25
Dimidium	5·0	—	50
Homidium	1·25	2·5	12·5
Para rosaniline	12·5	—	12·5
Butarsen	0·75	5·0	12·5
3-Acetamido-4-carboxy-phenylarsenoxide	17·5	—	17·5
Suramin	1·35	5·0	250
Trypan blue (a)	2,000	—	2,000
(b)	62·5	—	250
Pontamine sky blue 5BX	125	—	125

TABLE II

R_F VALUES OF ACID DYES

(1) Benzyl alcohol/ethanol/10N H_2SO_4 (45/25/15, v/v) on Whatman No. 50 (20°). (2) Butanol/acetic acid/H_2O (40/10/50, v/v) on Whatman No. 1 (28°).

Acid Dye	R_F Value (1)	R_F Value (2)	Trypanocidal Action
Xylene cyanol FF	Approx. 1		Nil
Isamine blue	0·66		,,
Azovan ,,	0·24, 0·63	0·16	,,
Trypan ,,	0·01	0·08	Active
Pontamine sky blue 5BX	0·02	0·04	,,
,, ,, ,, 6BX	0·09	0·12	Nil

Table II shows the R_F values of six sulphonated dyes, of which the last four are closely related in structure. The two trypanocidally active dyes are seen to be the most strongly adsorbed, that is they give the lowest R_F values.

Approximate times taken to produce maximal drug resistance by the "relapse" method (Schnitzer and Grunberg, 1957) were respectively: *atoxyl-resistant*, 5 weeks (Yorke, Murgatroyd, and Hawking, 1932); *butarsen-resistant*, 16 weeks (30 treatments); *melarsen-resistant*, 18 weeks (30 treatments); *acriflavine-resistant*, 56 weeks (76 treatments); *stilbamidine-resistant*, 59 weeks (Fulton and Grant, 1955); *Surfen C-resistant*, 16 weeks (21 treatments); *suramin-resistant*, 36 weeks (originally developed by Hawking (1939) and treated later to maximal resistance levels by Fulton and Yorke (1941)); *pontamine sky blue 5BX-resistant*, 13 weeks (subsequently treated for a further 38 weeks; 80 treatments in all).

No loss of the parabasal body was apparent in any of the fully-developed resistant strains except in the Surfen C-fast strain, where the proportion of trypanosomes with a parabasal body decreased *pari passu* with acquisition of resistance until the loss was complete when the strain became fully resistant.

Table III shows the cross-resistance pattern obtained by treatment of the various strains with the series of drugs and doses listed in Table I.

DISCUSSION

The cross-resistance pattern revealed in Table III conforms in the main to the grouping of trypanocidal drugs according to resistance behaviour which has been established by the work of Ehrlich, Franke, and Roehl (Ehrlich, 1907), Browning (1908), Yorke and Murgatroyd (1930), Yorke, Murgatroyd, and Hawking (1931, 1932), and King and Strangeways (1942). We would emphasize that the results in Table III have been obtained using offshoots of one parent strain only, the Liverpool *T. rhodesiense* strain, and our results are comparable not only among themselves but with those of other workers who have used the same strain, for example, Yorke and Murgatroyd (1930), Yorke *et al.* (1931, 1932), Lourie and Yorke (1938), Hawking (1938), Fulton and Yorke (1941), and Fulton and Grant (1955).

Ehrlich and his collaborators distinguished four main classes of trypanocide: (i) parafuchsin (para rosaniline) and related green, blue, violet and red triphenylmethane dyes (Table III. triphenylmethane dyes group), (ii) benzidine azo dyes (trypan red, trypan blue, and trypan violet

TABLE III

ACTIVITY OF TEST DRUGS AGAINST RESISTANT STRAINS OF *T. RHODESIENSE*

Degrees of resistance: −, normally sensitive; +, fast to ED90 only; ++, fast to CD90 only; +++, fast to LD10. N.D. indicates not done; (a), trypan blue sample (a). The asterisks refer to responses also found *in vitro*.

Drug Group	Drug	Ionization at Blood *p*H	Response of Strain Resistant to							
			Atoxyl	Acriflavine	Melarsen	Stilbamidine	Surfen C	Butarsen	Suramin	Pontamine Sky Blue 5BX
Neutral aromatic arsenicals	Tryparsamide	Feeble	+++	++	+++	+++	−	+++	−	+++
	Oxophenarsine		+++*	+++	+++	+++	−	+++	−	+++
Melaminyl arsenicals and antimonials	Melarsen	Cationic	−*	−	+++	+++	+	−	−	−
	Melarsen oxide		−*	N.D.	+++*	++*	+	−	−	N.D.
	MSb		−*	,,	+++	+++	++	−	−	,,
	MSb3		−*	,,	+++	+++	++	−	−	,,
Acridine derivatives	Acriflavine		+++	+++	+++	+++	+	+++	−	−
	Proflavine		N.D.	+++	+++	+++	N.D.	N.D.	N.D.	−
Diguanidines and diamidines	Synthalin B		−	−	+++	+++	−	−	−	−
	Decane diamidine		−	−	+++	+++	−	−	−	−
	Pentamidine		−	−	+++*	+++*	+++	−	−	−
	Stilbamidine		−	N.D.	+++	+++	+++	−	−	N.D.
	Berenil		N.D.	−	N.D.	+++	N.D.	N.D.	N.D.	−
6-Aminoquinoline and 6-aminocinnoline derivatives	Surfen		−	−	−	+++	+++	−	−	−
	Surfen C		−	−	−	+++	+++	−	−	−
	Quinapyramine		−	+++	−	−	+++	−	−	−
	Cinnoline 528		−	−	++	+++	N.D.	−	−	−
Phenanthridinium derivatives	Dimidium		−	−	++	+++	+++	−	−	−
	Homidium		N.D.	−	−	+++	N.D.	−	N.D.	−
Triphenylmethane dyes	Para rosaniline		−	−	+++	+++	+++	±	−	−
Carboxylated aromatic arsenicals	Butarsen	Anionic	−	−	−	−	−	+++*	−	−
	3-Acetamido-4-carboxyphenylarsenoxide		−*	N.D.	N.D.	−	N.D.	+++	−*	N.D.
Sulphonated naphthylamine derivatives	Suramin		−	−	−	−	−	−	+++	−
	Trypan blue		− (a)	−	N.D.	+	− (a)	− (a)	± (a)	+
	Pontamine sky blue 5BX		−	−	−	++	N.D.	−	−	+++

(Table III, sulphonated naphthylamine derivatives group), (iii) heterocyclic orthoquinonoid dyes of tricyclic anthracene structure containing one or two heteroatoms in the *meso* position (for example, derivatives of pyronine, acridine, selenopyronine, phenoxazine, phenothiazine, and phenoselenazine) (Table III, acridine derivatives group), and (iv) aromatic arsenicals of the atoxyl type (Table III, neutral aromatic arsenicals group). They showed that strains resistant to (i) or (ii) were sensitive to members of all the other groups. Mutual cross-resistance occurred between groups (iii) and (iv), but strains resistant to either group were still sensitive to groups (i) and (ii).

Yorke and his colleagues showed that resistance to aromatic arsenicals was not towards arsenic itself but to the non-arsenical part of the molecule, and King and Strangeways (1942) were able to subdivide aromatic arsenicals into three groups: (*a*) having hydrophilic substituents feebly ionized at blood *p*H (Table III, neutral aromatic arsenicals group), (*b*) lacking hydrophilic or having lipophilic substituents, and (*c*) having substituents which ionize as anions at blood *p*H (Table III, carboxylated aromatic arsenicals group). Trypanosomes resistant to (*a*) (atoxyl-type) were sensitive to (*b*) and (*c*), but strains resistant to (*c*) (butarsen-type), although sensitive to (*b*), were resistant also to (*a*).

To these three sub-classes, of which (*b*) and (*c*) represent Ehrlich's "avid" arsenicals (those active against strains resistant to the atoxyl-type), we can add a fourth (*d*) comprising aromatic arsenicals with a melaminyl substituent ionizing as the cation at blood *p*H. Strains resistant to (*a*) and (*c*) are sensitive to (*d*) (Williamson and Lourie, 1948); this is also an "avid" type of arsenical. Strains resistant to (*d*) are resistant to (*a*) but not to (*c*) (Table III). Schnitzer and Grunberg (1957) have indicated that strains resistant to these "avid" arsenicals seem invariably to show cross-resistance to acriflavine-type and to para rosaniline-type dyes, although atoxyl-resistant strains which are resistant also to acriflavine-type dyes are sensitive to para rosaniline. The connexion between cross-resistance behaviour and

selective interference with trypanocidal action is discussed by Williamson (1959). Schnitzer and Grunberg (1957) have compiled evidence which seems to "indicate that *p*-rosaniline occupies a key position in the pattern of cross-resistance of the arsenicals."

A number of other groups of metal-free trypanocides can also be distinguished on the basis of cross-resistance behaviour: (i) a group of symmetrical sulphonated naphthylamine derivatives which includes suramin and trypanocidal benzidine azo dyes, (ii) the diamidines, and (iii) a group of 6-aminoquinoline derivatives and related compounds. All three groups are active against trypanosomes resistant to aromatic arsenicals of the atoxyl type.

In group (i), suramin-resistance (Morgenroth and Freund, 1924) is difficult to induce and is highly specific; its only known cross-resistance is to the benzidine azo dyes trypan red and trypan blue; strains resistant to trypan blue are resistant to suramin (Leupold, 1924).

In group (ii), diamidine resistance was reported by Lourie and Yorke (1938), who showed that strains resistant to Synthalin (an aliphatic diguanidine) and to undecane diamidine showed cross-resistance to other aliphatic diguanidines and to aliphatic and aromatic diamidines, but were sensitive to suramin and to aromatic arsenicals. A strain made resistant to the aromatic diamidine stilbamidine (Fulton and Grant, 1955) is, however, cross-resistant to a much wider variety of trypanocides (notably to the melaminyl group), and is sensitive only to suramin, quinapyramine, and carboxylated aromatic arsenicals (Williamson and Rollo, 1952) (Table III). The new diamidine, berenil, like the other members of the group, is active against the acriflavine- and pontamine sky blue 5BX-fast strain, and, as Hawking (1958) has also shown, is inactive against a stilbamidine-fast strain.

The group of trypanocidal derivatives of 6-aminoquinoline and 6-aminocinnoline (group iii) is not strictly homogeneous chemically, but there are several aspects of resistance behaviour in this group which are of interest, despite the incomplete nature of the evidence. Surfen and Surfen C are symmetrical bisquinoline compounds, with a 6,6′ linkage through urea in the former and through melamine in the latter; cinnoline 528 has a similar symmetrical biscinnolinium structure linked in the 6,6′ positions by a guanidine group. These three drugs are uniformly active against trypanosomes resistant to neutral and anionic arsenicals and to acriflavine, and also to suramin and acid dyes, but are inactive against strains resistant to stilbamidine. Cinnoline 528, but not Surfen or Surfen C, is inactive against a melarsen-resistant strain. A Surfen C-fast strain showed no cross-resistance to neutral and anionic arsenicals, to aliphatic amidines and guanidines, or to suramin and acid dyes; it is, however, cross-resistant to melaminyl arsenicals and antimonials, aromatic diamidines, quinapyramine, dimidium, and para rosaniline.

Although we can discern a pattern in the resistance behaviour of the melaminyl arsenicals, the diamidines, the Surfens and cinnoline 528 in the common presence of amidine type $=N-\overset{|}{C}=N-$ linkages, capable of tautomerism and thus peculiarly prone to hydrogen-bond formation, it is difficult to construct an appropriate receptor structure, modification or loss of which would account for all the observed facts. On this basis why should the Surfen C-fast strain be able to differentiate between aliphatic and aromatic amidines while the melarsen-fast strain does not? Is this another aspect of the sensitivity to neutral aromatic arsenicals which the Surfen C-fast and aliphatic amidine-fast strains display, unlike strains fast to other basic metal-free trypanocides such as acriflavine, stilbamidine, and styryl-6-aminoquinolines (Browning, Cohen, Ellingworth, and Gulbransen, 1929)?

Quinapyramine is an asymmetrical 6-aminoquinoline derivative, and the cross-resistance shown to it by the Surfen C-fast strain can be referred to the 4,6-diaminoquinaldine moiety which is common to both compounds. Quinapyramine is active against all the other resistant strains in Table III except the acriflavine-fast strain. It is also inactive against a strain of *T. congolense* resistant to cinnoline 528, but an analogous quinapyramine-fast *T. congolense* is sensitive to cinnoline 528 (Williamson and Lourie, unpublished observations). A quinapyramine-fast strain of *T. equiperdum* (Ormerod, 1952) was resistant to the phenanthridinium drug dimidium, to acriflavine and to stilbamidine, but was sensitive to suramin, trypan blue, and *p*-hydroxyphenylarsenoxide.

Cross-resistance between quinapyramine and phenanthridinium compounds is of considerable importance in animal trypanosomiasis in the field, but available evidence from this source is often contradictory and difficult to establish. This situation, coupled with the difficulty of producing experimental phenanthridinium resistance in laboratory trypanosome strains, does not facilitate the formulation of rules for the resistance behaviour

of these two important drug groups. Such indications as there are point to a non-reciprocal cross-resistance between quinapyramine and dimidium; strains resistant to quinapyramine are cross-resistant to dimidium but not conversely (Wilson, 1949; Macaulay and Shaw, 1950; Robson and Wilde, 1954; Evans, 1956). Dimidium and homidium, which differs from dimidium only in having an ethyl- instead of a methyl-substituted quaternary nitrogen atom, have similar patterns of activity against all the resistant strains tested except the melarsen-fast strain.

We have attempted to extend the evidence for an ionic basis of the trypanocidal action and resistance behaviour of acidic and basic drugs (King and Strangeways, 1942; Schueler, 1947) by using as wide a variety of anionic trypanocides as possible. Unfortunately, of the series of acid dyes tested, several of which had been reported as trypanocidal, only two, trypan blue and pontamine sky blue 5BX, were active against our parent *T. rhodesiense* strain. Because of the relative inactivity of a sample of trypan blue used in the early part of this work, evidence of cross-resistance to trypan blue in some instances (Table III) is not so clear-cut as could be desired. However, within this group of metal-free anionic trypanocides, we have found several features of novel interest. Of the six sulphonated dyes listed in Table II, four are closely related chemically, trypan blue, azovan blue, pontamine sky blue 5BX, and pontamine sky blue 6BX [C.I. No. 518 (1st Edn.); 24410 (2nd Edn.)]. Trypan blue and azovan blue, having a central dimethylbenzidine moiety, differ only in the distribution of sulphonic acid substituents; the former is the bis-3,6-disulphonate, and the latter the bis-2,4-disulphonate. Pontamine sky blue 5BX and pontamine sky blue 6BX form a similar pair, having a central dimethoxybenzidine moiety, and again differ only in that the former is the bis-3,6-disulphonate and the latter the bis-2,4-disulphonate. In both these pairs, only the bis-3,6-disulphonate-substituted dye is trypanocidal; it is also the member which is most strongly adsorbed on cellulose (Table II), and in this respect recalls the relationship between trypanocidal action and cotton substantivity noted by Quastel (1931) in series of aminobenzoyl-substituted derivatives of naphthylamine disulphonates related to suramin. Sulphonated dyes generally are taken up readily by wool, which is a protein with basic groups, but the uptake of these dyes by cellulose is structurally much more limited and specific. The size, shape, and composition of the non-sulphonated part of the dye play a large part in the degree of its ultimate adsorption. In the case of the 2,4- and 3,6-disulphonated dyes considered here, there is the possibility of a solubility difference between the two groups which would be likely to affect their adsorbability on both protein and cellulose structures (W. Bradley, personal communication). The enhancement of cotton substantivity in azo dyes by the *s*-triazine nucleus (Bradley, 1958) which occurs in the melamine trypanocides suggests a further link between trypanocidal action and affinity for cellulose.

The cross-resistance pattern (Table III) of the trypanocidal sulphonated naphthylamine derivatives shows that although cross-resistance exists between suramin and trypan blue, and to a small extent between trypan blue and pontamine sky blue 5BX, there is no cross-resistance between suramin and pontamine sky blue 5BX, although the latter differs from trypan blue only in having dimethoxy instead of dimethyl substituents on the central benzidine moiety. A further unexpected feature in this group is that the stilbamidine-resistant strain, although normally sensitive to suramin, is cross-resistant to some degree to both the benzidine azo dyes, trypan blue and pontamine sky blue 5BX. Later (Williamson, 1959b) it will be shown that the stilbamidine-resistant strain is resistant *in vitro* also to another acid dye, indigo carmine, and that the degree of resistance, as with a number of other ionic trypanocides, is a function of the *p*H of the culture medium. Uptake in these instances is determined by the degree of ionization of a cellular receptor.

One of the more plausible current theories of the resistance mechanism in trypanosomes (Schueler, 1947) implies that the ionic properties of a drug molecule control to a large extent the subsequent cross-resistance behaviour of a strain made resistant to it. For example, the strain made resistant to the atoxyl-tryparsamide-oxophenarsine-type of arsenical, in which the substituents are hydrophilic but ionize only feebly or not at all, is normally sensitive to trypanocides with ionizable substituents (anionic or cationic). This scheme is, however, an oversimplification, as it fails to explain (Table III) why (i) the cationic arsenic-free Surfen C gives rise to a resistant strain which is sensitive to arsenicals of the atoxyl type and to aliphatic diamidine-type drugs, but resistant to quinapyramine, while the similarly cationic arsenic-free stilbamidine produces a strain resistant to the two former groups but sensitive to quinapyramine; (ii) the cationic arsenic-free acriflavine produces resistance to atoxyl-type arsenicals and

quinapyramine only; (iii) the three strains made resistant to the anionic drugs butarsen, suramin, and pontamine sky blue 5BX are not cross-resistant to each other; and (iv) of four strains made resistant with cationic drugs, one, the stilbamidine-fast, is cross-resistant to anionic benzidine azo trypanocides. Other inconsistencies in the theory have been noted by Wagner, Pedal, and Schöneberger (1954).

The resistance produced by stilbamidine apparently involves a more fundamental change in the drug "receptors" than is implied by the suggestion of Schueler (1947) of a shift in the isoelectric point of protein in resistant strains. If this mechanism operated in the stilbamidine-fast strain, the decrease in net negative charge of the receptor structure, as manifested by resistance to cationic drugs, should be accompanied by an increased net positive charge tending to produce an increased sensitivity to anionic trypanocides, instead of the resistance which is actually found. In this respect the strain differs from the quinapyramine-resistant strain of *T. equiperdum* described by Ormerod (1952), which, though resistant also to stilbamidine, was sensitive, or possibly hypersensitive, to the anionic trypan blue and suramin.

From Table III it seems that a number of drug adsorption sites are likely to be common or adjacent to each other. The largest "receptor area" would appear to be anionic in nature, and may well consist of free phosphoric acid groups of nucleic acids, because cationic trypanocides such as aromatic diamidines, acriflavine, triphenylmethane dyes, and phenanthridinium drugs do in fact combine with nucleic acid *in vitro*.

The difference in action of aliphatic and aromatic diamidines on the metabolism of *T. evansi* (Marshall, 1948) may be reflected in the marked difference in resistance behaviour between the stilbamidine-fast and the Synthalin- and undecane diamidine-fast strains described by Lourie and Yorke (1938). Unlike the first of these strains, the two last were both normally sensitive to an atoxyl-type arsenical, dichlorophenarsine (halarsol). Further, the Surfen C-fast strain, which is fully resistant to pentamidine and stilbamidine, is sensitive to the aliphatic compounds Synthalin and decane diamidine, and also to aromatic arsenicals of the atoxyl type.

Changes in cell metabolism whereby drug-sensitive enzyme reactions are modified, by-passed or eliminated may well be linked to changes in structures associated with drug uptake. Some changes in the metabolism of *T. rhodesiense* induced by resistance to melarsen have been demonstrated (Williamson, 1953), but nothing in this work or in the work of others (Fulton and Christophers, 1938; Harvey, 1948, 1949; von Brand, Tobie, Mehlman, and Weinbach, 1953; Tobie and von Brand, 1954) has yet revealed any far-reaching differences in intermediary metabolism to correspond to the relatively enormous losses in drug sensitivity found in resistant trypanosomes.

One of the oldest and still one of the most refractory problems in trypanocidal drug resistance is the nature of the cross-resistance between acriflavine and atoxyl-type aromatic arsenicals. Thiol compounds can reverse the trypanocidal action of the arsenicals but not of acriflavine, and further evidence of a separation in the function of a thiol-type arsenical "receptor" is provided by the Surfen C-fast strain which is resistant to acriflavine but not to atoxyl-type arsenicals. Possibly the action of acriflavine involves oxidation-reduction changes in the thiol receptor to which the arsenic is ultimately linked by covalent bonds. Albert (1951) has indicated that antibacterial acridines can be divided into two classes, (i) mainly aminacrine and its derivatives, which resist oxidation by light and air and iron-catalysed hydrogen peroxide, and (ii) mainly 2-aminoacridine derivatives, which are susceptible to these oxidizing agents. Resistance in bacteria develops only with this latter class. It may be relevant that the active antibacterial substance aminacrine, unlike proflavine and acriflavine which belong to class (ii), has no trypanocidal action.

There is no detectable difference in the thiol content of normal and arsenical-resistant trypanosomes (Harvey, 1948), but Hawking (1938) has shown that resistant trypanosomes can be killed by a much smaller amount of intracellular arsenic or acriflavine than normal trypanosomes. This suggests that in the resistant parasite a proportion of metabolically active thiol groups has been masked by some form of structural rearrangement; a masking effect of this sort would not normally be detectable by ordinary chemical methods of thiol analysis.

Results of selective interference experiments (Williamson, 1959a) confirm the observations presented here, which suggest that stereospecific structural changes associated with initial drug uptake occur in resistant trypanosomes. The complexity of these changes appears to be of similar degree, if not of similar nature, to that of antigen-antibody interaction.

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