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DRUG RESISTANCE IN TRYPANOSOMES; SELECTIVE INTERFERENCE WITH TRYPANOCIDAL ACTION

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Selective reversal of the trypanocidal action of carboxylated arsenicals by *p*-aminobenzoic acid and of melaminyl arsenicals and diamidines by melamine has been demonstrated *in vivo* and *in vitro*. The structural specificity of these reversal phenomena is high, and suggests preferential adsorption of the antagonist during a reversible primary drug fixation stage. Thiols antagonize neutral, carboxylated and melaminyl aromatic arsenicals equally, but not diamidines; *p*-aminobenzoic acid antagonism is specific for carboxylated arsenicals, and melamine antagonizes only the melaminyl arsenicals and the diamidines. These reversals reflect the pattern of cross-resistance behaviour and suggest that cellular structures associated with a reversible stereospecific drug adsorption phase are modified during the development of resistance.

Antagonism of the chemotherapeutic activity of one substance by another was originally termed "therapeutic interference" by Browning and Gulbransen (1922), who made experiments on the reversal of the trypanocidal action of acriflavine by parafuchsin. This and later examples of the effect have been reviewed in detail by Schnitzer and Grunberg (1957) and by Albert (1949, 1951).

The present work is based on the observation in trypanosome infections of antagonism of butarsen by *p*-aminobenzoic acid (Williamson and Lourie, 1946), antagonism of melarsen oxide by Surfen C (Williamson and Lourie, 1948), and antagonism of diamidines and melaminyl arsenicals by melamine (Williamson, 1954). The more extended experiments now reported indicate a high degree of structural specificity in these reversal phenomena, many of which are reproducible on isolated parasites *in vitro*.

METHODS

Trypanosome Strain.—The strain of *Trypanosoma rhodesiense* used throughout these experiments was isolated from man in 1923 (Yorke, Adams, and Murgatroyd, 1929), and subsequently maintained by blood passage in mice.

Estimation of Interference.—Mice weighing 18 to 25 g. were injected intraperitoneally with a suspension of 20,000 trypanosomes, and about 43 hr. later microscopical examination of fresh tail blood showed an average count of 1 to 10 parasites/field (1/6 in. objective; ×10 ocular).

Four or five mice were used for each test group, and each mouse was injected intraperitoneally with a neutral solution of the antagonist (0.5 ml./20 g. body weight), followed 2 to 3 min. later by an intraperitoneal ED90 dose (0.5 ml./20 g.) of the trypanocide. In each experiment, four or five infected mice were treated with the trypanocide only as controls. LD10 doses of test antagonists were also determined where necessary. ED90 and LD10 are defined by Williamson and Rollo (1959).

After treatment, blood examinations were made at intervals of 30 min. or 1 hr., and, if necessary, at intervals of 24, 48, and 72 hr. The criterion of negativity throughout was the absence of trypanosomes in thirty microscope fields selected at random.

For analysis of the extent of antagonism of a fixed dose of butarsen by varying doses of *p*-aminobenzoic acid, mice were infected as above, and, 48 hr. later, blood films were made and stained. Immediately afterwards, batches of five mice were given intraperitoneal doses (0.5 ml./20 g.) of sodium *p*-aminobenzoate followed 2 to 3 min. later by the intraperitoneal injection (0.5 ml./20 g.) of a solution of the sodium salt of butarsen. A batch of five mice treated with the drug alone served as controls. Times of treatment of each batch were noted, and, exactly 1 hr. later, stained blood films were made from the tail blood of the mice in each batch. Differential parasite counts on the stained films gave infection concentrations in terms of parasites/4,000 erythrocytes. Mean parasite concentrations for each batch of five mice were calculated and the standard error of these mean values estimated. The proportion (x) of trypanosomes killed at each treatment level was obtained from the ratio $x=(A-B)/A$, where $A=$

mean initial count and B=mean count after treatment. The amount of inhibition (I) of the trypanocidal action of the drug was estimated from the ratio: $I = [x_D - x_{CT}]/x_D$, where x_D is the ratio x for the arsenical alone, and x_{CT} the ratio for the combined treatment by arsenical and antagonist. In batches of mice where the concentration of antagonist was sufficient to produce permanent inhibition, this ratio (x) was found to be greater than 1 by a factor representing multiplication of the trypanosome population during the hour elapsing between treatment and examination. This factor was found to have a mean value of 1.25 for the highest concentration of *p*-aminobenzoate at which antagonistic activity was maximal. The proportionate inhibitions at lower concentrations were divided by 1.25 to give the corresponding values representing a stationary population. The percentage inhibition of the arsenical was assumed to be a measure of the amount of *p*-aminobenzoic acid adsorbed on the site of action of the drug.

The "interference activity" of compounds tested against butarsen is expressed in terms of activity/mole, taking the activity of *p*-aminobenzoic acid as 100, and was calculated in the following way. The average time of persistence (in hr.) of parasites in the peripheral blood of mice subjected to combined treatment, in excess of the persistence time of parasites in mice treated with butarsen alone, was divided by the molar concentration of the interfering compound, and this quotient was expressed as a percentage of the maximum quotient, calculated similarly, for *p*-aminobenzoic acid. The time of persistence with each compound was measured in two ways: (A) for persistence of parasites in undiminished numbers, and (B) for persistence of parasites in diminished numbers. Activities/mole were calculated using (A) and (B), and, taking the corresponding values for *p*-aminobenzoic acid (1,536 and 3,072 respectively) as 100, the relative activity/mole of each compound tested was found approximately to correspond for both calculation procedures.

In batches of four or five mice, the persistence times were generally found to be uniform to within 1 hr., particularly in the case of (A), and the control mice used in each experiment and treated with the ED90 of butarsen showed a uniform disappearance of parasites within 0.5 to 1.0 hr. after treatment.

The "effective interfering dose" (the minimum dose necessary to produce maximum interference) was determined for each of the compounds related to *p*-aminobenzoic acid.

Throughout, the molar concentrations referred to are those of the test solutions administered intraperitoneally in doses of 0.5 ml./20 g.

Trypanocidal Action in Vitro.—This was measured by the technique of Yorke and Murgatroyd (1930) in the manner described by Williamson and Rollo (1959).

Organic Preparations.—Sodium pyruvate: prepared by the method of Robertson (1942). p-*Aminocinnamic acid*: from ethyl *p*-nitrocinnamate, hydrolysed to *p*-nitrocinnamic acid, thence reduced by the method of Schofield and Simpson (1945), to give yellow-brown crystals of *p*-aminocinnamic acid. M.P. (dec.)=176°, M.P. (lit.)=175–6°. Found: C=66.53%; H=5.81%; N=10.3%. Calc. for $C_9H_9O_2N$: C=66.24%; H=5.56%; N=8.59%. p-*Aminomethylbenzoic acid*: by the method of Blicke and Lilienfeld (1943). The acid, M.P.=230°, was insoluble in ether, chloroform, acetone, and benzene (Günther, 1890), and was used without further purification. p-*Aminobenzamide*: *p*-nitrobenzamide was prepared from *p*-nitrobenzoyl chloride by the method of Reichenbach and Beilstein (1864), and was then reduced by either (i) catalytic hydrogenation or (ii) stannous chloride. Method (i) gave, on recrystallization from water, yellow-brown flakes of *p*-aminobenzamide. M.P.=180° (M.P. (lit.)=180°, 182.9°). Found: C=61.51%; H=5.75%; N=21.0%. Calc. for $C_7H_8ON_2$: C=61.75%; H=5.92%; N=20.58%. Method (ii) gave a similar product, M.P.=182.5°.

Determination of p*K Values.*—p*K* values of the acidic groups (and in some cases of the basic groups also) of *p*-substituted aromatic acids and phenylarsonates were determined at 19° to 20° by potentiometric titration with the glass electrode of the sodium salts, either in water or in 50% (v/v) aqueous methanol. The method has been adopted for relatively insoluble members of compound types of therapeutic interest such as the sulphonamides (Bell and Roblin, 1942) and the aminoacridines (Albert and Goldacre, 1943).

All pK_a values were corrected for variation in ionic strength, salt hydrolysis, Na^+ error, and the effect of alcohol.

Surface Tension Determination.—Correlation of antibacterial action and surface activity is largely dependent on measurements of surface tension made over a range of concentrations in the appropriate culture medium (Stacey and Webb, 1947). The measurements recorded here were made in solutions of rabbit serum and physiological saline, using the dynamic maximum bubble-pressure method of Sugden (1922, 1924). This technique overcomes a number of objections inherent in static methods (Neurath and Bull, 1938; Gaddum, 1931), and its application to measurements in serum is discussed in detail elsewhere (Williamson, 1959). The apparatus was calibrated and checked against distilled water, absolute ethanol (redistilled), acetone (A.R.), and benzene (A.R.). The temperature throughout was controlled at 20°.

In all experiments the given value for the surface tension is the mean of at least three determinations, each determination involving four scale readings. The value of H for the saline and serum/saline solvents was about 12.0 cm., corresponding to about 72 dynes/cm., and the means are accurate to ±0.3 dyne/cm.

Cell-free rabbit serum was obtained by centrifuging blood obtained by cardiac puncture, which had been

left for an hour at 37° to clot. A well-defined minimum in the surface tension of solutions of rabbit serum in 0.85% aqueous NaCl occurred at approximately 15% (v/v) serum; above 40% (v/v) serum the surface tension became constant and equal to that of undiluted serum. A concentration of 1% (v/v) serum gave a surface tension close to that of undiluted serum; the measurements recorded here were therefore made in a solution of this strength, in which protein molecular association, considered to be responsible for the observed surface tension minimum, would be unlikely to operate.

As far as possible all test solutions were adjusted to *p*H 7.0; the *p*H of solutions in serum-saline mixtures was determined with the glass electrode. A surface tension/*p*H curve was constructed for a series of 1% serum solutions in buffered saline (Williamson, 1959), in order to correct the surface activity in unbuffered test solutions, in the following way. The surface tension of a sample of 1% serum/saline dilution used for the preparation of a batch of test solutions was determined and compared with the value at the corresponding *p*H on the surface tension/*p*H curve, and a correction factor (σ curve value/σ observed value) obtained. The surface tension of the test solution was multiplied by this factor, and the corrected value subtracted from the value of the serum/saline solvent at the corresponding *p*H on the surface tension/*p*H curve. The standard value of 71.9 dynes/cm. for a 1% solution of rabbit serum in 0.85% aqueous NaCl at *p*H 7.0 was taken as a comparative basis for the results.

The above correction procedure is only valid in the *p*H range 6.0 to 10.0, in which surface tension measurements are reproducible, and is based on the assumption that within this range the surface tension/*p*H curves for the solution and the solvent are parallel.

RESULTS

p-*Aminobenzoic Acid/Butarsen Interference*

p-Aminobenzoic acid/butarsen interference could be demonstrated *in vitro* only under limited conditions (Williamson and Lourie, 1946), and therefore analysis of the quantitative relations involved had to be based on mouse experiments which could be less rigidly controlled. Although *p*-aminobenzoic acid produced permanent inhibition of an ED90 dose of butarsen (0.75 mg./kg. body weight), an increase of 0.25 mg./kg. of the arsenical was sufficient to reduce the period of inhibition to only 1 to 2 hr. Analysis of the effect of varying doses of the arsenical combined with a constant dose of *p*-aminobenzoic acid was thus impossible. The antagonistic effects of varying doses of *p*-aminobenzoic acid on the ED90 of butarsen are shown in Table I.

A curve was drawn putting percentage inhibition of the trypanocidal action of 0.015 mg./20 g. mouse body weight of the arsenical solution as the ordinate, and *p*-aminobenzoic acid concentration as the abscissa. This curve seemed to conform to the typical rectangular hyperbola associated with an adsorption process. By putting Gaddum's equation (Gaddum, 1943) into the form of the Langmuir adsorption isotherm: $C_A/b = k + C_A$ (where b represents % inhibition of the arsenical and C_A is the concentration of *p*-aminobenzoic acid, the value of the intercept constant was obtained, and a theoretical curve plotted for comparison with the experimental curve (Fig. 1).

Although the experimental method does not allow rigid control, and the results cannot be considered conclusive, they suggest the possibility that a preferential adsorption of *p*-aminobenzoic acid may be involved in the interference process.

TABLE I

EFFECT OF *p*-AMINOBENZOIC ACID CONCENTRATION ON THE INHIBITION OF THE TRYPANOCIDAL ACTION OF BUTARSEN ON STANDARDIZED *T. RHODESIENSE* INFECTIONS OF MICE

Butarsen dose, 0·5 ml./20 g. of $1{\cdot}18 \times 10^{-4}$ M solution. *p*-Aminobenzoic acid was injected intraperitoneally in a volume of 0·5 ml. The standard error of the mean is given in brackets.

p-Aminobenzoic Acid (M)	Trypanosomes/4,000 R.B.C.		% Inhibition for Stationary Population
	Before Treatment (A)	1 Hr. after Treatment (B)	
1·00	70 (±32)	81 (±42)	96
0·50	182 (±47)	223 (±29)	101
0·125	186 (±17)	246 (±32)	111
0·063	182 (±11)	219 (±47)	100
0·031	164 (±22)	198 (±28)	100
—	255 (±66)	43 (±12)	0
0·016	145 (±16)	113 (±16)	62
0·008	137 (±39)	82 (±35)	46
—	62 (±15)	3 (±2)	0
0·021	335 (±60)	338 (±70)	77
0·004	477 (±88)	346 (±73)	−5
—	502 (±51)	371 (±68)	0

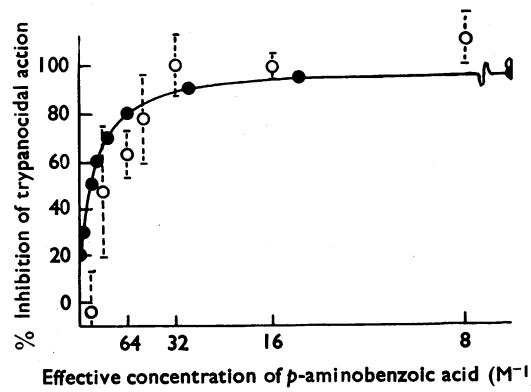

FIG. 1.—Inhibition of trypanocidal action of butarsen by *p*-aminobenzoic acid in mice infected with *T. rhodesiense*, 1 hr. after combined treatment. ●, theoretical value on basis of adsorption isotherm; O, experimental value, with 2× standard error represented by vertical broken line.

As indicated below, this result may be an alternative expression of a normal probability distribution of a drug-susceptibility factor among the large heterogeneous population of parasites in the blood, rather than of receptor sites on a homogeneous parasite surface. The minimum concentration of *p*-aminobenzoic acid required for complete inhibition is 3×10^{-2} M, a molar inhibition ratio of the order of 300:1.

Experimental confirmation that interference takes place in the immediate neighbourhood of the trypanosome was obtained by demonstration of *p*-aminobenzoic acid/butarsen interference when the substances were administered by separate routes, the former subcutaneously and the latter intraperitoneally. This experiment excludes the possibility of extracellular chemical interaction between the drug and its antagonist. Complete and permanent inhibition of the trypanocidal action of butarsen by the prior injection of *p*-aminobenzoate was observed at spacing-intervals ranging from about 30 sec. to 1 hr.

Taking a level of significance for activity/mole as 0.4 (the value for glutathione) for parasite persistence times (A) or (B), no significant interference with the action of butarsen was shown by injection of the following: normal and hypertonic saline, isotonic phosphate buffers (*p*H 4, 5, 6, 7, 8, 9), phosphate-citrate buffers (*p*H 4, 5), borate-succinate buffer (*p*H 5), phthalate buffer (*p*H 5), HCl-saline (*p*H 5), glucose (25% in water), or by LD10 injections of urea, casein hydrolysate, peptone, uracil, xanthine, lactate, pyruvate, succinate, malonate, toluidine blue, ascorbic acid, glutamic acid, aspartic acid, tyrosine, histidine, arginine, glycine, β-aminobutyric acid, methanol, ethanol, mercuric chloride, formaldehyde, phenylguanidine, naphthalene - 2,6 - diamidine, para rosaniline, pentamidine, phenidium (phenanthridinium 897), dimidium, melamine, *o*-nitrobenzoic acid, folic acid, nicotinic acid, and phenylacetic acid.

Table II shows the interference activity against butarsen of compounds related to *p*-aminobenzoic acid and of similar molecular size.

p-Aminobenzoic acid was the only active antagonist which produced complete and permanent inhibition at low concentrations. The structural specificity of the interference effect was high, because isomers of *p*-aminobenzoic acid were inactive, and homologues showed greatly reduced activity. Removal or replacement of either the amino or carboxyl groups in the *p*-aminobenzoic acid molecule also abolished or reduced activity.

TABLE II

INTERFERENCE ACTIVITY AGAINST BUTARSEN OF COMPOUNDS RELATED TO *p*-AMINOBENZOIC ACID

Where the effective interfering dose differs from the LD10, the latter is shown in brackets.

Test Substance	Class	Activity/Mole (*p*-Aminobenzoic Acid = 100) (A)	Activity/Mole (*p*-Aminobenzoic Acid = 100) (B)	Effective Interfering Dose (M^{-1})
p-Aminobenzoic acid	Homologues of *p*-aminobenzoic acid	100	100	32 (0·69)
p-Aminophenylacetic acid		25·0–40·0	25·0	16 (1)
β-(*p*-Aminophenyl)-propionic acid		0·74	1·4	2
γ-(*p*-Aminophenyl)-butyric acid		25–63	100	16 (2)
p-Aminobenzoylglycine		25–50	100	8 (4)
p-Aminocinnamic acid		0·04	0·08	1·41
o-Aminobenzoic acid	Isomers of *p*-aminobenzoic acid	0·00	0·01	1
m-Aminobenzoic acid		0·07	0·06	1
Aniline	Carboxyl group replaced	7·0–10·0	4·1–5·0	4
p-Aminobenzamide		0·33–1·73	0·95–1·65	2
Ethyl *p*-aminobenzoate		4·50	3·65	32
p-Aminobenzenesulphonic acid		0·00	0·00	0·5
Sulphanilamide		6·05–8·0	7·70	8
Sulphadiazine		0·00	0·00	10
p-Aminophenol		0·00	0·00	8
p-Aminophenylarsonate		3·52	2·34	36
p-Toluidine		0·52	0·78	16
p-Phenylene diamine		0·00	0·00	40
Benzidine		0·18	0·52	16
Benzoic acid	Amino group replaced	0·11	0·10	2
p-Acetamidobenzoic acid		0·02	0·02	1
p-Hydroxybenzoic acid		0·20	0·13	2
p-Toluic acid		0·17	0·24	2
p-Nitrobenzoic acid		0·00	0·00	2
p-Hydroxyaminobenzoic acid		0·26	0·25	4
p-Aminomethylbenzoic acid		1·64	1·59	2
β-Indolylalanine	Miscellaneous	>0·23	0·55	10 (20)
Phenylacetic acid		0·13	0·12	2
β-Phenylpropionic acid		2·76	1·55	4
Benzoylglycine		25	20·4	8
N-Phenylglycine		1·25	3·45–5·7	8
Surfen		20–49	5·2–12·7	55

The pK_a values of ionizing groups of active interfering agents and of a number of related trypanocides are given in Tables III, IV and V.

The remaining group of substances which showed significant interference activity against butarsen was a series of surface-active compounds of varying type (Table VI). In some instances, the interference activity on a molar basis was greater than that of *p*-aminobenzoate, but complete and permanent inhibition was never observed.

The molar interference activity (persistence method A) was determined when butarsen was

TABLE III

pK_a VALUES IN WATER

The asterisk refers to results interfered with by precipitation of free acid; †, estimated by extrapolation; ‡, as anhydrous disodium salt.

Compound	Temp. (°)	pK_a (Carboxyl or Arsonic Acid)		Temp. (°)	pK_a (Basic Group)	
		Uncorrected	Corrected		Uncorrected	Corrected
p-Aminobenzoic acid	20	4·85	4·81	20	2·80	2·51
p-Aminophenylacetic acid	19	5·30	5·26	19	3·68	3·54
β-(*p*-Aminophenyl)propionic acid	19	5·48	5·44	19	*ca.* 4·1†	
γ-(*p*-Aminophenyl)butyric acid	20	5·52	5·48	20	*ca.* 4·3†	
p-Aminocinnamic acid	19	4·98	4·94	19	*ca.* 3·7†	
p-Hydroxyaminobenzoic acid	19	*ca.* 5·8*				
Melarsen‡	20	5·20	5·16	19	7·70	7·55
Butarsen	20	4·70	4·66			

TABLE IV

pK_a VALUES IN 50% (V/V) AQUEOUS METHANOL

Values marked with an asterisk are uncorrected; †, indicates sodium *p*-acetamidophenylarsonate; ‡, sodium 4-acetamido-3-hydroxyphenylarsonate.

Compound	Temp. (°)	pK_a (Arsonic or Carboxylic Acid Group)		Estimated pK_a (H_2O)
		Uncorrected	Corrected	
Atoxyl	19	4·85	4·77	3·97
Arsacetin†	19	4·30	4·22	3·42
Orsanine‡	19	4·55	4·47	3·67
Tryparsamide	19	4·90	4·82	4·02
Melarsen	19	5·85	5·77	4·97
Butarsen	19	5·55	5·47	4·67
p-Aminobenzoic acid	19	pK_a (NH_3^+) = 2·72*		
		pK_a (carboxyl) = 5·80*		
p-Aminobenzamide	19	pK_a (NH_3^+) = 2·90*		

TABLE V

pK_a VALUES OF COMPOUNDS RELATED TO *p*-AMINOBENZOIC ACID

References for pK values quoted in this Table and in the text are taken from: Kuhn and Wassermann (1928), Dippy (1939), Landolt-Börnstein (1931, 1936), International Critical Tables (1929), Bell and Roblin (1942), Cohn and Edsall (1943), Pressman and Brown (1943), Albert and Goldacre (1942), Goldacre (1944). Values marked with an asterisk are those determined experimentally (see Tables III and IV).

Acid	pK_a (Carboxyl) of Acid with *p*-Substituent		
	None	–As=O	$-NH_2$
Benzoic	4·20	4·0	4·92, 4·81*
Phenylacetic	4·30	4·35	5·20, 5·26*
β-Phenylpropionic	4·66	4·7	5·42, 5·44*
γ-Phenylbutyric	—	4·9, 4·66*	5·48

given subcutaneously and the surface-active agent intraperitoneally. Three representative compounds (i) cetyltrimethylammonium bromide, (ii) octadecylamine, and (iii) sodium tauroglycocholate gave respectively the values (*p*-aminobenzoate, method A = 100) (i) 2.6, (ii) 1.3, and (iii) 3.9. The values obtained when the compounds and the arsenical were administered by the same route were (i) 68.5, (ii) 144, and (iii) 65.1. Effects other than surface activity alone were thus operative. Table VI also shows the trypanocidal titre *in vitro*, denaturant effect on protein, and approximate critical micelle concentration (estimated from surface tension/concentration curves in 1% serum-saline).

Only feeble surface activity was shown by the related compounds ethyl *p*-aminobenzoate, sulphanilic acid, *p*-aminophenol, benzoic, *p*-hydroxybenzoic, *p*-toluic, *p*-nitrobenzoic, phenylacetic and β-phenylpropionic acids, and by benzoylglycine and phenylglycine.

TABLE VI

PROPERTIES OF SURFACE-ACTIVE COMPOUNDS ANTAGONISTIC TO BUTARSEN

Where the effective interfering dose (EID) differs from the LD10, the latter is shown in brackets; critical micelle concentrations are] estimated from surface tension/concentration curves in 1% serum/saline; N.D. = not determined.

Compound	Interference Activity/Mole (*p*-Aminobenzoic Acid = 100)	Effective Interfering Dose (M^{-1})	Depression at EID (Dynes/cm.)		Trypanocidal Titre ($\times 10^{-3}$)	Lowest Conc. for Protein Interaction (M^{-1})	Approximate Critical Micelle Concentration (M^{-1})
			1% Serum/Saline	0·85% Saline			
Palmitamidine	258	320	5·9	0	4,000	5,120	5,120
Octadecylamine	144	40	—	>5	2,000	640	640
Cetyltrimethylammonium bromide	68·5	100 (200)	17	18·4	64	5,120	5,120
Tauroglycocholic acid	65·1	80 (40)	22·9	25·2	1	>10,240	160
Trimethyloctadecylammonium chloride	48·3	160	10·3	3·4	256	10,240	640
Octadecamethylene diamine	34·8	320	8·1	—	16,000–64,000	640	—
Ricinoleic acid	*ca.* 20	20	>32·3	>35	4	>20,480	5,120
Lauric ,,	*ca.* 20	20	12	—	4	N.D.	—
Palmitic ,,	18·2	20	—	—	4	,,	—
Octadecylguanidine	2·6	46	>5·5	2·8	500	20,480	—
Trimethylnonylammonium iodide	0	160	>3·7	—	64	N.D.	—

The Effect of Aminobenzoate on the Action of Trypanocides of Varying Type

These results are shown in Table VII. The "inhibition time" in Tables VII and VIII is the difference between the average time of persistence of trypanosomes in undiminished or increasing numbers in the peripheral blood of a batch of four or five mice after intraperitoneal doses (0.5 ml./20 g.) of (a) the trypanocide (ED90), and (b) the interfering agent followed by the trypanocide. "Complete" inhibition means an infection course indistinguishable from that in untreated mice.

The results were much more variable with the metal-free and pentavalent arsenical trypanocides than with the trivalent arsenical drugs. The two first groups exert their action much more slowly (5 to 24 hr.) than the trivalent arsenicals in which visible trypanocidal action develops within 1 hr. of treatment.

p-Aminobenzoate interferes unequivocally only with atoxyl and two carboxyl-substituted arsenicals, butarsen and 3-acetamido-4-carboxyphenylarsenoxide. Significant antagonism by *p*-aminobenzoate is observed also with acriflavine, arsacetin (sodium *p*-acetamidophenylarsonate) and tryparsamide.

A wider range of examples of selective interference *in vivo* is shown in Table VIII, which

TABLE VII

EFFECT OF *p*-AMINOBENZOIC ACID ON THE ACTION OF TRYPANOCIDES OF VARYING TYPE

Drugs marked with an asterisk were as used by Yorke and Murgatroyd (1930); †, from Dr. F. Hawking; ‡ Indicates sodium 4-acetamido-3-hydroxyphenylarsonate.

Type	Compound	Dose (mg./kg.)	Inhibition Time (hr.)
Metal-free	Para rosaniline	16	0–19
	Acriflavine	10	19–37
	Suramin	2·7	0–20
	Pentamidine	0·65	0–20
As^{3+}	4-Carboxyphenylarsenoxide	15	13–18
	3-Acetamido-4-carboxyphenylarsenoxide	8·3	Complete
	Butarsen	0·75	,,
	Arsenophenylglycine†	32·5	4–16
	Oxophenarsine	0·75	2
	Reduced tryparsamide*	4·0	2–14
	Dichlorophenarsine (halarsol)*	1·25	1
	Melarsen oxide	0·15	0·5
	Neoarsphenamine	6·25	1
As^{5+}	Atoxyl	150	45
	Arsacetin*	200	20–24
	Tryparsamide	750	19–22
	Orsanine‡	81·5	1–16
	Melarsen	30	3–11

TABLE VIII

SELECTIVE INTERFERENCE WITH TRYPANOCIDAL ACTION *IN VIVO*

Results marked with an asterisk refer to glutathione; † to cysteine.

Interfering Agent	Conc. (M^{-1})	Inhibition Time (hr.)			
		Maphar-side	Butarsen	Melarsen Oxide	Pent-amidine
Thiol	5	1*	1*	1·5*	0†
p-Aminoben-zoic acid	1	2	Complete	1	0–24
Surfen C	55	0	0	5–24	—
	110	—	—	5–24	—
	220	—	—	2–5·5	—
Melamine	4–12	0	0	Complete	24–48
Ammeline	12–24	0	0	1	0
Cyanuric acid	12–24	0	0	0	0
Tretamine	640	—	—	0	—
Surfen	47	1–6	1–5	4	—
	94	—	—	4	—
	188	—	—	2·5	—

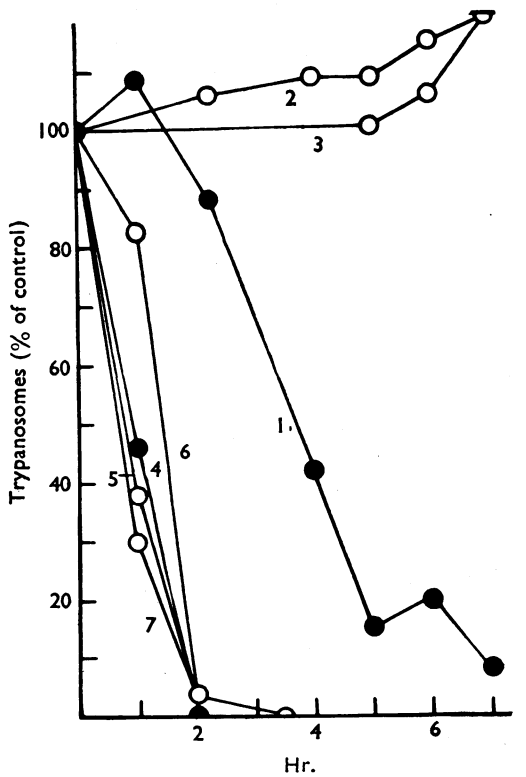

FIG. 2.—Selective interference with the trypanocidal action of melarsen oxide *in vitro*. Medium: 50/50 rabbit serum/0·85% NaCl-0·2% glucose. Temperature: 37°. Trypanosomes: about 1×10^6/ml. The ordinate represents the number of motile surviving parasites as a percentage of the control (medium only). Curves 1–3 from experiment 1. Curves 4–6 from experiment 2. Melarsen oxide 1: 102×10^6 present in all instances. **1** and **4**, melarsen oxide alone; **2**, also melamine 1: 6,080; **3**, also cysteine 0.01 M; **5**, also ammeline 1: 6,040; **6**, also cyanuric acid 1: 5,930; **7**, also *p*-aminobenzoic acid 1: 290.

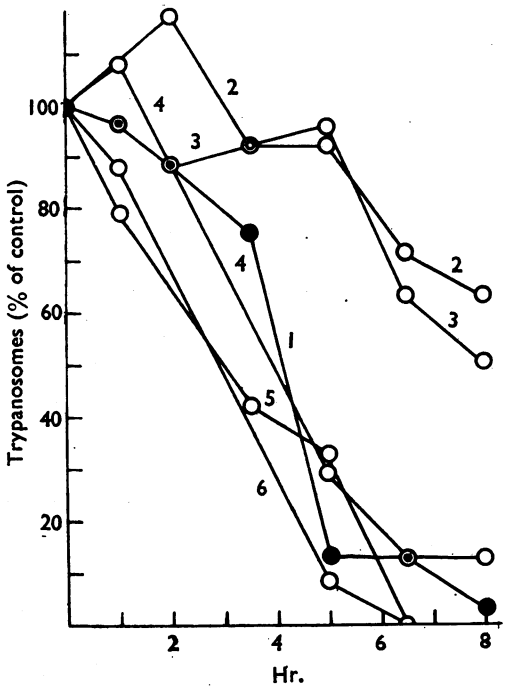

FIG. 3.—Selective interference with the trypanocidal action of pentamidine *in vitro*. Medium : 50/50 rabbit serum/0.85% NaCl-0.2% glucose. Temperature: 37°. Trypanosomes: approx. 1×10^6/ml. The ordinate represents the number of motile surviving parasites as a percentage of the control (medium only). Pentamidine 1: 1.6×10^6 was present in all instances. **1**, pentamidine alone; **2**, also melamine 1: 6,080; **3**, also cysteine 0.01 M; **4**, also ammeline 1: 6,040; **5**, also cyanuric acid 1: 5,930; **6**, also *p*-aminobenzoic acid 1: 290. The interference in **3** is probably due to salt formation.

shows that (a) thiol antagonism is common to all three types of aromatic arsenical but does not occur with pentamidine, (b) *p*-aminobenzoate antagonism is specific for the carboxylated arsenical butarsen, and (c) melamine antagonism is common only to the melaminyl arsenical, melarsen oxide, and to pentamidine.

Melamine antagonism shows structural specificity, because three closely-related substances, ammeline (cyanuric acid diamide), cyanuric acid, and tretamine, are inactive ; three compounds with amidine or guanidine groups (*p*-chlorophenylguanidine, proguanil and palmitamidine) are inactive against pentamidine.

Melamine (M/12) shows the same marked inhibition time (24 to 48 hr.) with decane diamidine, Synthalin B and stilbamidine, as with pentamidine. Melamine antagonism of melarsen oxide and pentamidine is also demonstrable *in vitro* (Figs. 2 and 3). The unexpected antagonism of pentamidine by cysteine *in vitro* is probably caused by salt formation, which could also explain the partial antagonism by *p*-aminobenzoate *in vivo*. The formation of crystalline salts with carboxylic acids is characteristic of the diamidine series (Walker, 1949), and a precipitate forms immediately on mixing dilute neutral solutions of pentamidine and sodium *p*-aminobenzoate.

DISCUSSION

Four likely mechanisms for chemotherapeutic interference may be distinguished: (a) chemical reaction between the drug and its antagonist with formation of an inactive product, (b) oxidation (or reduction) by the antagonist of a substance or redox system which is reduced (or oxidized) by the drug, (c) competition by the substrate for an enzyme inhibited by the drug, which is usually structurally related to the substrate concerned, and (d) with colloidal materials, formation of a biologically inert mixed micelle of drug and antagonist.

A well-known example of mechanism (a) is the formation of inactive thioarsenites from the reaction of excess thiols with trypanocidal arsenoxides (Voegtlin, Dyer and Leonard, 1923). Von Jancsó and von Jancsó (1936) invoked mechanism (b) in their experiments on the reversal of the trypanocidal activity of phenylarsenoxides and phenylstibinoxides by a large number of varied compounds sharing the property of exhibiting a redox potential more positive than −0.06 V (*p*H 7.0). Mechanism (c) is closely related to the competitive inhibitions observed with isolated enzyme systems, where there is a direct relation between the concentrations of inhibitor and substrate, which are often closely related in structure. Sulphanilamide/*p* - aminobenzoate antagonism in certain bacteria (Woods, 1940) is a classical example. The fourth type of mechanism, (d), has been proposed by Albert (1949) for drugs or antagonists with colloidal properties.

Mechanisms (b) and (c) most probably occur at the parasite cell/medium interface, where the primary interaction between drug and cell takes place. The primary fixation of a number of organic arsenicals is known to be reversible (Hawking, 1938), and Yorke and Murgatroyd (1930) showed earlier that " arsenic-resistance " in trypanosomes is not towards arsenic itself but to the substituted phenyl radical to which the arsenic is attached. The constitution of the phenyl radical is responsible for the primary fixation of the drug ; resistance does not develop to inorganic arsenic compounds such as sodium arsenite. This primary drug fixation concept has been elaborated

by Clark (1933), King and Strangeways (1942), and by Mudd (1945). Mudd (1945) stressed the common *p*-aminophenyl structural basis of a number of biologically active compounds such as sulphonamides, antipyretics, local anaesthetic esters of *p*-aminobenzoic acid and organometallic antiprotozoal agents; to these may be added the antitubercular drug *p*-aminosalicylic acid (Youmans, Raleigh, and Youmans, 1947) which is also trypanocidal (Pick, 1950), and *p*-aminobenzenephosphonic acid which is antibacterial (Bauer, 1941; Kanitkar and Bhide, 1947; Thayer, Magnuson and Gravatt, 1953). The diversity of active substances with the above general configuration implies the existence of a common and widely-distributed cellular reaction site of complementary structure.

Postulation of a primary reversible combination of a drug with a structurally specific site on or in the trypanosome, followed by a separate lethal stage, is the basis of the argument to be developed here for a common mechanism for selective interference phenomena and resistance behaviour in at least four main types of trypanocide.

p-*Aminobenzoic Acid/Butarsen Interference*

Neither an extracellular chemical reaction between the two substances, nor a possible functioning of *p*-aminobenzoic acid as an accessory respiration catalyst, is probable. Veldstra and Havinga (1947) have in fact shown polarographically that no oxidation or reduction of *p*-aminobenzoic acid occurs at voltages up to 0.6 V or 2.8 V respectively, and they consider it unlikely that *p*-aminobenzoic acid can function in a redox system. As *T. rhodesiense* has not yet been cultured satisfactorily on synthetic media, the unequivocal demonstration of *p*-aminobenzoic acid as an essential metabolite for the parasite is not possible; it had no effect in synthetic maintenance media developed by Williamson and Rollo (1952). Surface activity determinations indicate that the interference effect is unlikely to result from mixed micelle formation.

Hawking (1938) demonstrated a phase of primary adsorption conforming to the Langmuir adsorption isotherm in the trypanocidal action *in vitro* of acriflavine. This interpretation, as Ipsen (1941) emphasizes in connexion with haemolytic activity, may be due rather to an alternative expression of a normal probability distribution with respect to heterogeneous factors, such as resistance, among the parasites. A similar demonstration in terms of the drug competition theory of Gaddum (1943) has been attempted here for the antagonism of butarsen by *p*-aminobenzoic acid. The results (Table I, Fig. 1), though necessarily imprecise, suggest that a competitive adsorption of *p*-aminobenzoic acid may occur; the reversibility, on dilution with serum, of the immobilizing action of high concentrations of *p*-aminobenzoic acid on trypanosomes *in vitro* (Williamson and Lourie, 1946), also implies an initial loose adsorption phase.

Table II shows that, of the seven compounds which produce marked inhibition of the trypanocidal action of butarsen, *p*-aminobenzoic acid is the only one which causes complete and permanent inhibition at low concentrations. Moreover, the interference activity of *p*-aminobenzoic acid is completely abolished by alteration of the relative positions of the substituent amino and carboxyl groups from the *para* to the *ortho* or *meta* configuration. There are no gross differences between the three isomers in respect of physical properties, and chemically they share the amphoteric properties of the amino acids, except that in water a larger proportion of the *meta* acid exists as dipolar ions.

The three homologues show no regular increase or loss of interference activity with increasing length of the aliphatic side-chain (Table II), and none is as active as *p*-aminobenzoic acid. There is no correlation with pK_a (carboxyl); any shift in pK_a value (carboxyl or amino group) is progressive as the series is ascended, and all four compounds are 99% ionized as anions at blood *p*H. Schueler (1947) has indicated that, by virtue of the *n*-butyric acid side-chain which could assume an incomplete hexagonal structure on the basis of the normal carbon bond angle, butarsen may exist in forms where there is a much reduced distance between the arsenoso and carboxyl groups. This distance is likely to be of the order of separation of the amino and carboxyl groups in *p*-aminobenzoic acid (6.7 Å: Roblin and Bell, 1943), and this factor, combined with the similarity in acid strength of the two compounds, may be responsible for the specificity of the interference. Ionization of the amino group is negligible at blood *p*H in this series, and replacement of this group largely abolishes interference activity; it is therefore probable that hydrogen bonding, rather than ionic interaction, occurs at this locus. The importance of the spatial factor is emphasized by the lack of activity of *p*-aminocinnamic acid and the relatively high activity of both *p*-aminobenzoylglycine and benzoylglycine.

Complete removal of the carboxyl group, as in aniline, reduces activity by about 90%. The

activity of aniline is nevertheless marked; interference activity is completely abolished only by (i) replacement of the carboxyl by the sulphonic acid group, as in sulphanilic acid which has a much higher acid strength, (ii) replacement by a second ring structure as in sulphadiazine, (iii) substitution by a weakly acid group as in *p*-aminophenol (pK_a (OH)=10.30), or (iv) substitution by another amino group as in *p*-phenylene diamine, which destroys the heteropolar nature of the molecule and increases the basic strength (pK_a (amino), 6.08).

The activity of atoxyl (*p*-aminophenylarsonate) is of especial interest, since it indicates that this substance may be adsorbed to some extent on the trypanosome surface in the pentavalent form. The use of higher concentrations than those indicated is, of course, limited by the trypanocidal action of the compound, but the molecular configuration bears a close resemblance to *p*-aminobenzoic acid and adsorption at the same site is probable. In addition to the close structural resemblance the ionization at blood *p*H of the polar groups is similar; pK_a (amino, atoxyl)= 3.97, pK_a (carboxyl *p*-aminobenzoic acid)=4.81, pK_a (amino, atoxyl)=2.05, pK_a (amino, *p*-aminobenzoic acid)=2.51.

The bisquinolyl compound Surfen interferes actively not only with butarsen but also with oxophenarsine, and is almost as active as Surfen C in antagonizing melarsen oxide. Aqueous solutions of Surfen are markedly colloidal, and the antagonism probably results from mixed micelle formation.

The results above clearly indicate that the inhibitory action of *p*-aminobenzoic acid on the trypanocidal action of butarsen is highly specific and demands, as far as the experimental procedures described here can ascertain, the optimal assembly of the following factors: (i) a benzene nucleus, (ii) a negatively charged substituent, the pK_a of which is approximately 5.0, (iii) a weakly positive substituent, preferably an amino group, in the *para* position to (ii), the pK_a of which is equal to or less than 2.5, and (iv) the existence, actual or potential, of an inter-charge separation distance of approximately 7 Å.

The inter-charge separation distance, it may be noted, corresponds to twice the separation distance between two alternate side-chains in the polypeptide chain of a protein in the β-keratin configuration (Springall, 1954).

The trypanocidal action of butarsen is exerted very rapidly and depends on the achievement of a peak blood concentration almost immediately after intraperitoneal injection, which is followed within a few minutes by a precipitous fall (Murgatroyd, Russell, and Yorke, 1934). Therefore, with therapeutic interference where trypanocidal activity has been delayed for hours, this activity cannot be due to a trypanocidal concentration of the drug in the surrounding blood; it suggests that the drug is adsorbed at a point other than the vulnerable metabolic site where it exerts its ultimate lethal action, and to which it migrates only when the loosely-held antagonist is eluted as a result of the decreasing external bulk concentration. As Hurst (1943) points out, the lack of trypanocidal activity in phenyl-*pp'*-diarsenoxide suggests that the site of ultimate lethal action is not directly accessible to the external drug phase, since the availability of the arsenoso group —As:O is increased in this compound. In the case of *p*-aminobenzoic acid, where complete and permanent abolition of trypanocidal activity is effected, the antagonist-receptor affinity is high and at least equal to that of butarsen. On the basis of the results obtained here, it is not possible to establish directly whether the antagonism takes place at the trypanosome surface or intracellularly, though the results with compounds showing incomplete interference suggest the latter site.

Interpretation of *in vivo* experiments on interference must take into account the operation of "detoxicant" mechanisms. Since *p*-acetamidobenzoic acid is inactive, the acetylation process cannot affect the interference mechanism in the case of *p*-aminobenzoic acid, though it may well accelerate the loss of activity in *p*-amino-substituted compounds showing incomplete interference. In the case of *p*-aminomethylbenzoic acid, by analogy with maphenide, the basic group is probably rapidly oxidized *in vivo*, thus contributing to the low activity displayed.

Operation of the β-oxidation mechanism is also unlikely in the case of *p*-aminobenzoic acid and its homologues, since β-(*p*-aminophenyl)propionic acid, which should oxidize to the active *p*-aminobenzoic acid, is found in fact to have less than 2% of the activity of *p*-aminobenzoic acid. Reduction mechanisms do not seem to be important *in vivo*, since *p*-nitrobenzoic acid, which is reduced to *p*-aminobenzoic acid, has no interference activity. Hydrolysis of aromatic acid amides occurs *in vivo* and might be expected to confer higher activity on *p*-aminobenzamide, but the activity of this compound is much less than that of *p*-aminobenzoic acid. Apart from affecting rates of excretion, it would seem unlikely that *in vivo* detoxicant processes influence trypanocidal interference.

Surface Active Antagonists of Butarsen

As this interference is demonstrable only when the drug and antagonist are given by the same route, it seemed unlikely that facilitated preferential adsorption on the trypanosome of these compounds occurred by virtue of their surface-active polar-non-polar configuration. As Table VI shows, there is no correlation between interference activity and surface tension depression in saline or serum-saline solutions, trypanocidal titre, and the ability to flocculate serum protein. For the more soluble compounds, minima in the surface tension/concentration curves in serum-saline solutions were used as approximate estimates of critical micelle concentration. At the concentration used in the interference experiments, all the compounds formed visibly colloidal aqueous solutions. Micellar aggregation has been reported for compounds such as bile salts (Rains and Crawford, 1953), and the importance of critical micelle concentration in the detergent and biological action of compounds like cetyltrimethylammonium bromide and related substances is discussed in detail by Glassman (1948), Colichmann (1950), and Pankhurst (1953). Although there is no clear correlation between interference activity and critical micelle concentration (Table VI), it seems highly probable that the effective concentration of the injected butarsen is lowered by non-specific physical admixture with micelles of the antagonist.

Effect of p-*Aminobenzoic Acid on Trypanocides other than Butarsen*

Table VII shows that *p*-aminobenzoic acid interferes most effectively with carboxylated phenylarsenoxides and with atoxyl, as might be expected from the considerations above. Antagonism by *p*-aminobenzoic acid of the trypanocidal action of atoxyl has also been noted by Moshkovskiy and Stoyanova (1947). Atoxyl has been shown above to interfere with butarsen, and *p*-aminobenzoic acid is known to interfere with the antibacterial action of atoxyl (Hirsch, 1942). The latter result was confirmed by Peters (1943), who emphasized the importance of the structural factor in this effect; other pentavalent arsenical drugs such as acetarsol (3-acetamido-4-hydroxyphenylarsonic acid) and tryparsamide had no antibacterial activity and the bacteriostatic action of atoxyl, like that of sulphanilamide, was reversed by procaine (2-diethylaminoethyl *p*-aminobenzoate) and by methionine, but not by *o*- or *m*-aminobenzoic acid or by methyl 3-amino-4-hydroxybenzoate.

The pentavalent aromatic arsenicals circulate in the blood for 5 to 24 hr. before the reduced forms accumulate at trypanocidal levels. During this period, the arsonic acid group in all five drugs tested will be ionized like the carboxyl group of *p*-aminobenzoic acid (Table IV), but only atoxyl possesses the necessary intact primary *p*-amino group. An analogous competitive inhibition has been described by Seaman (1952) for the aliphatic pentavalent arsenical, arsonoacetate, on the succinic dehydrogenase of *Tetrahymena geleii*, S, where combination with "carboxyl affinity points" occurs without affecting thiol groups.

The selective interference experiments described here and summarized in Table VIII, together with the cross-resistance analyses reported earlier (Williamson and Rollo, 1959) and the data integrated by Schnitzer and Grunberg (1957), enable further inferences on trypanocidal drug resistance mechanisms to be made.

Selective permeability has been invoked frequently in the past to explain acquirement of resistance in trypanosomes, but there is no convincing evidence for this, and it is difficult to find a basis in terms of physical diffusion to the complex and highly selective patterns of resistance of which the trypanosome is capable. The cell phenomenon of pinocytosis (Fell, 1958), which seems to operate for the absorption of fat by *T. lewisi* and *T. equiperdum* (Wotton and Halsey, 1957), is an additional impediment to acceptance of the permeability thesis.

The trypanocidal drugs considered here are all aromatic compounds carrying substituents which are either ionized at blood *p*H or are capable of hydrogen bonding. There are relatively few kinds of chemical grouping in the cell with which trypanocides are likely to form a durable attachment, but these groups are capable of sufficient diversity of arrangement to account for the degree of structural specificity which is observed in drug action, resistance and interference behaviour. Assumption of a structurally specific and reversible primary fixation stage and a separate subsequent lethal stage accords with the facts so far assembled. The nature of the cellular "receptor" groups will be considered in a later report, but it is reasonable to assume that the trypanocides which are ionized at blood *p*H will combine with oppositely charged ionized groups in trypanosome cell material. This is the basis of the "isoelectric point shift" resistance theory of Schueler (1947), who postulated that such a shift "would be indicative of changes in the relative number of

positive or negative charges (for example, free amino or carboxyl groups) of the constituent proteins of the trypanosomes, a change which might very greatly affect the fixation of ionizable drug molecules." Such an alteration in receptor distribution could result from changes in the degree of folding of protein polypeptide chains or of nucleic acids (Goldacre and Lorch, 1950; Alexander, 1952; Kopac, 1947).

Aromatic arsenical drugs are generally conceded to be lethal by virtue of covalent binding of trivalent arsenic to essential cellular thiol groups. Little is known of the nature of any similar specific lethal mechanism for metal-free trypanocides, except that thiol combination is unlikely to be involved; there is, for example, no thiol antagonism of acriflavine (Strangeways, 1937; Schleyer and Schnitzer, 1948; Ercoli, Gosford, Carminati, Kley and Schwartz, 1951) or of stilbamidine or suramin (Ercoli *et al.*, 1951; Sen, Dutta and Ray, 1955). Thus reversal of trypanocidal action by thiols can be used to differentiate two stages in the action of three chemically distinct groups of aromatic arsenical. Table VIII shows that thiols antagonize the neutral oxophenarsine, the anionic butarsen, and the cationic melarsen oxide equally, but do not interfere with pentamidine. Oxophenarsine is known to be antagonized selectively by methyl *p*-hydroxybenzoate (Schleyer and Schnitzer, 1948), but not by *p*-aminobenzoic acid or melamine. Butarsen is antagonized selectively by *p*-aminobenzoic acid but not by methyl *p*-hydroxybenzoate (Schnitzer and Grunberg, 1957) nor by melamine. Melarsen oxide is antagonized by melamine but not *p*-aminobenzoic acid. These antagonisms resemble the malonate reversal of thiol poisons on succinic dehydrogenase (Potter and DuBois, 1943), and the pyruvate protection of carboxylase inactivation by organic arsenicals (Stoppani and Actis, 1952).

Thus each type of arsenical, neutral, anionic and cationic, appears to have a different structurally specific initial attachment site on the trypanosome cell. This corresponds to the observed resistance behaviour where an oxophenarsine-resistant strain is sensitive to butarsen and to melarsen oxide, a butarsen-resistant strain is resistant also to oxophenarsine but not to melarsen oxide, and a melarsen oxide-resistant strain is resistant to oxophenarsine but not to butarsen.

However, there are several anomalies not easily explicable on the assumption of either modified, and therefore resistant, primary receptors or of a changed relative proportion of anionic and cationic receptors. The cross-resistance between the cationic acriflavine and the neutral drugs such as oxophenarsine and tryparsamide is an obvious example. Despite the possibility of common factors in other contexts such as antimitotic activity, involving combination with nuclear material where reaction with thiols may be important (Bell, 1958), the trypanocidal action of acriflavine, unlike the arsenicals, is not reversed by thiols. A change in the relative proportion of anionic and cationic receptors in a strain made resistant to an ionic trypanocide would be expected to produce an increased sensitivity to ionized drugs with a charge of the opposite sign. In fact, a strain fast to the cationic stilbamidine is fast also to the anionic dye pontamine sky blue 5BX; Schumacher and Schnitzer (1956) have reported a butarsen-fast strain of *T. equiperdum* which, unlike the butarsen-fast *T. rhodesiense* used here, became cross-resistant to the cationic drugs Mel B and stilbamidine.

The suggestive but obscure interrelationships of para rosaniline, butarsen, *p*-aminobenzoic acid and the acriflavine-atoxyl group, which Schnitzer and Grunberg (1957) have noted, cannot be illuminated further by the present results; in addition, these results do not demonstrate cross-resistance of butarsen to para rosaniline or antagonism of para rosaniline by *p*-aminobenzoic acid in such a clear-cut fashion as the experiments of Schumacher and Schnitzer (1956) and Schnitzer and Schumacher (1956).

On the information presented here, the "two-stage" theory of trypanocidal action accords reasonably well with the observed facts. The specific melamine antagonism of the melaminyl arsenicals and antimonials and the diamidines reflects the cross-resistance between the groups and suggests that a common receptor group is modified during the development of resistance. It may be noted that melamine, like the diamidines (Walker, 1949), is capable of forming crystalline salts with carboxylic acids (Steele, Glover and Hodgson, 1952), and that the melamine interference is unlikely to be due to a redox effect because polarographic measurements show that pentamidine does not have a redox potential between +0.2 and −1.9 V against a saturated calomel electrode (H. Campbell, personal communication). At blood *p*H, both melamine (pK_a= 9.0, Dixon, Woodberry and Costa, 1947) and pentamidine (pK_a=11.40) are fully ionized, and the melaminyl substituent of melarsen (Table III) is about 70% ionized as the cation.

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