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ARYL-2-HALOGENOALKYLARYLAMINES—XXI.*

THE DESIGN OF AGENTS TO EXPLOIT DIFFERENCES IN CELLULAR OXIDATION-REDUCTION POTENTIALS

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Abstract—It should be possible to obtain compounds of greater carcinostatic specificity by exploiting the lower oxidative capacity of some cancer cells as compared with normal cells. The preparation of various 2-halogenoethylamino-derivatives that are more reactive in their reduced form is described in the present paper. Oxidation–reduction potentials and rates of hydrolysis under standard conditions have been determined and a preliminary screening of the new compounds against the transplanted Walker rat carcinoma has been undertaken.

A LARGE number of so-called 'biological alkylating agents' that are effective carcinostatic agents when assayed against the especially sensitive transplanted Walker rat carcinoma are now known.[1–5] If such agents are to be useful against the more resistant tumours it would seem necessary to exploit some characteristic feature of the cancer cell in order to localize the cytotoxic effect. One property of neoplastic cells which has already received some consideration is the lower pH relative to most normal cells.[6, 7]

It has been established that tumours tend to be deficient in cofactors concerned with electron transport (compare Weinhouse[8]) and this can lead to a lower activity of various oxidative enzyme systems—indeed, such a deficiency in lactic acid dehydrogenase activity may well be responsible for the lower pH already referred to. Morton[9] remarked on the increase in nicotinamide mononucleotide adenylyl transferase activity as cells differentiate from embryonic to adult types, and has indicated that the increase is paralleled by changes in nicotinamide nucleotide co-enzyme concentrations. Co-enzymes of this type are implicated in the majority of enzymically catalyzed oxidations within cells, and the known low level of these cofactors in de-differentiated neoplastic tissues is probably responsible for their lowered oxidative capacity. Adamson and Fouts[10] demonstrated that certain kinds of neoplastic liver tissue lack the ability to metabolize a variety of drugs. Although they tend to associate the loss of enzyme activity with a deficiency of enzyme protein in the cancerous liver their conclusions about the therapeutic implications of lowered oxidative capacity are of importance in the design of more specific carcinostatic agents. They suggest the examination of compounds that can be detoxified by oxidation in normal cells but which would be cytotoxic towards the cancer cells that cannot effect this detoxication. It is of interest in this connection that Cater[11] drew attention to the lower oxidation-reduction potential of tumour tissue relative to most normal tissue and Cater and

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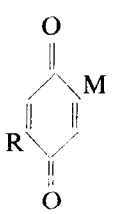
Phillips[12] have established significant differences in potential between Walker tumour tissue and normal tissue in the same animal.

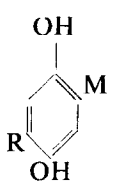
In view of these established cellular differences it was felt that if one could design an alkylating agent that was chemically more reactive in its reduced form, greater carcinostatic specificity would be achieved. A wide range of possible structures would have to be explored since one must expect that the metabolism of some structures will be more complex than a simple reversible oxidation–reduction process. There is, also, no clear indication of the oxidation–reduction potential difference within cells. Cater and Phillips[12] measurements indicating values of 0–150 mV for Walker tumour tissue and 200–300 mV for muscle tissue in the same rat probably refer to the extracellular situation, but they do give some guide as to the properties desirable in agents designed to exploit oxidative differences. It will clearly not be advantageous to employ agents which, by virtue of their oxidation–reduction potential, will be in the same form in both types of tissue. This fact may account for the relatively poor chemotherapeutic index of some di-2-chloroethylaminoazobenzene derivatives which were examined some years ago[13] as agents that would be activated by *in vivo* reduction—one of the most effective compounds, CB 1414, is included in Table 3.

Only if equilibrium conditions are rapidly achieved within cells will values of oxidation–reduction potentials be strictly relevant and since rates of transformation may also be of importance it was deemed advisable to test the oxidized and the reduced form of any given structure. Some preliminary attempts to obtain agents which are chemically more reactive, and by implication biologically more active, in the reduced form are described in the present paper.

Considerations of the mechanism of alkylation indicated that the reduced forms of various di-2-chloroethylarylamino-compounds would be the more reactive. This is because the activation of the halogen atom depends on electron release from the nitrogen atom and a reduced form of carrying structure necessarily leads to the greater basicity of this centre. A representative system in which this situation arises is the benzoquinone–hydroquinone pair (I, R=M*) and (II, R=M) of which the latter would be expected to be the more reactive (compare Ross[1]). These compounds and some related derivatives have now been examined and the expected reactivity differences confirmed.



(I)



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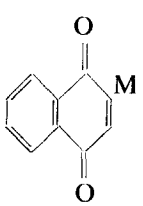


(III)

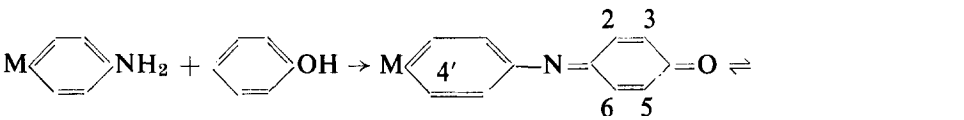
MATERIALS

Benzoquinone readily condenses with di-2-chloroethylamine and under oxidizing conditions gives the disubstituted product (I, R=M) and this on catalytic reduction affords the hydroquinone (II, R=M). When monosubstituted benzoquinones are

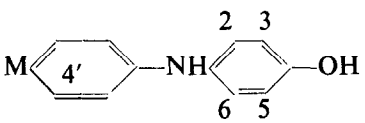
* Throughout this paper M refers to the di-2-chloroethylamino group: $-N(CH_2CH_2Cl)_2$

employed only one di-2-chloroethylamino group is introduced, and in this way (I, R = Me, MeO, and Cl) have been prepared. 1,4-Naphthoquinone similarly reacts to give (III).

Another type of carrying structure which should give compounds with oxidation–reduction potentials in the range indicated by Cater and Phillips[12] is the indoaniline system (V). Considerable difficulty attended the synthesis and characterization of this type of compound since reaction products were highly coloured and the required derivative generally had no definite melting point. Nevertheless a general method was evolved for the oxidative coupling of 4-amino-NN-di-2′-chloroethylaniline (IV) with phenols in acid solution. A mixture of intensely coloured products was obtained and this could be resolved chromatographically.



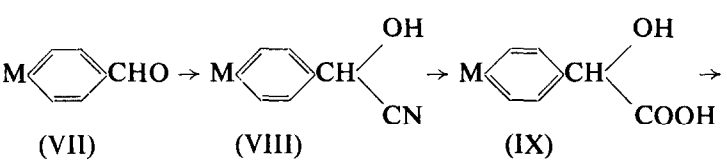
(IV) (V)



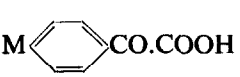
(VI)

Yields were variable, generally about 20% of theory, but up to 50% could be obtained when the phenolic component was 2,6-xylenol giving (V, 3,5-Me_2). It also proved possible to obtain (V) by condensation of nitrosophenol with NN-di-2-chloroethylaniline in strong sulphuric acid solution but this method did not have general applicability. Catalytic reduction of the indoanilines (V) gave the readily oxidized non-crystalline leuco-bases (VI) which formed solid hydrochlorides, the composition of which tended to be variable due to ready loss of hydrogen chloride.

In view of the preferential formation of lactic acid in cancer cells it was considered of interest to prepare the lactic acid–pyruvic acid analogues (IX) and (X). Using deliberately mild conditions 4-di-2′-chloroethylaminobenzaldehyde (VII) was converted successively into the cyanhydrin (VIII) and the mandelic acid derivative (IX). This acid could only be isolated as an unstable benzene complex but it gave good crystalline piperidine and dicyclohexylamine salts. Mild oxidation of the sodium salt of (IX) in aqueous acetone with potassium permanganate gave the benzoylformic acid (X) characterized as its dicyclohexylamine salt and its 2,4-dinitrophenylhydrazone.

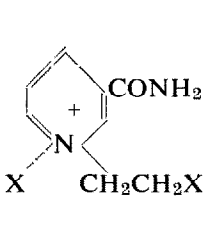


(VII) (VIII) (IX)

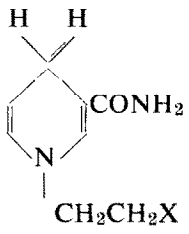


(X)

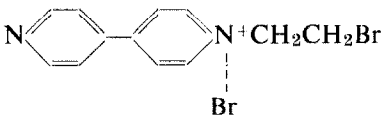
The preparation and properties of (XI and XII, X = Cl) have recently been described by Friedman *et al.*[14] A more convenient preparation of (XI, X = Br) by the direct reaction of ethylene dibromide with nicotinamide and its reduction to give (XII, X = Br) using sodium borohydride in saturated aqueous sodium bromide is described below. Under similar conditions 4,4'-dipyridyl condensed with ethylene dibromide to give the monosubstituted derivative (XIII).



(XI)



(XII)



(XIII)

All melting points are corrected.

2,5-*Bis*(*di*-2'-*chloroethylamino*)1,4-*benzoquinone* (I, R = M)

A mixture of di-2-chloroethylamine hydrochloride (36 g) and aqueous sodium hydroxide (100 ml, 2 N) was extracted with ether (2 × 200 ml) and the extract was dried over anhydrous magnesium sulphate. After adding dioxan (100 ml) the ether was removed by distillation and the solution was added to benzoquinone (10·8 g) in dioxan (100 ml). The mixture was stirred at room temperature overnight in a stream of oxygen. Methanol (150 ml) was then added and the solid collected by filtration.

2,5-*Bis*(*di*-2'-*chloroethylamino*)-1,4-*benzoquinone* formed red flattened needles, m.p. 132–133°, from chloroform–methanol (1 : 2) (Gauss and Petersen[15] give m.p. 132–133°).

2,5-*Bis*(*di*-2'-*chloroethylamino*)-1,4-*dihydroxybenzene* (II, R = M)

The above quinone (1 g) in ethyl acetate (100 ml) was hydrogenated over a Raney nickel catalyst. Evaporation of the filtered solution gave a quantitative yield of the *hydroquinone* derivative which formed flattened needles, m.p. 128–130°, from ethyl acetate–light petroleum (b.p. 60–80°) (Found: C, 43·0; H, 5·2; Cl 36·3; N, 7·2. Calc. for $C_{14}H_{20}Cl_4N_2O_2$ C, 43·1; H, 5·2; Cl, 36·4; N, 7·2%). The hydroquinone formed a *diacetate*, m.p. 155–160°, rhombs from ethyl acetate–light petroleum (1 : 3) (Found: C, 45·6; H, 5·2; Cl, 29·4; N, 6·0. Calc. for $C_{18}H_{24}Cl_4N_2O_2$: C, 45·6; H, 5·1; Cl, 29·9; N, 5·9%).

The following were similarly prepared from the appropriate quinone.

2-*Methyl*-5-(*di*-2'-*chloroethylamino*)-1,4-*benzoquinone* (I, R = Me), scarlet red flattened needles, m.p. 128–130°, from ethyl acetate–light petroleum (b.p. 40–60°) (Gauss and Petersen give m.p. 133–135°) (Found: C, 50·9; H, 5·1; Cl, 27·2; N, 5·5. Calc. for $C_{11}H_{13}Cl_2NO_2$: C, 50·4; H, 5·0; Cl, 27·1; N, 5·4%). The *diacetate* of the corresponding *hydroquinone* formed rhombic prisms m.p. 83–85°, from chloroform–light petroleum ether (b.p. 60–80°) (Found: C, 51·7; H, 5·7. Calc. for $C_{15}H_{19}Cl_2NO_4$: C, 51·7; H, 5·5%).

2-*Methoxy*-5-(*di*-2′-*chloroethylamino*)-1,4-*benzoquinone* (I, R = MeO), brick red plates, m.p. 184–185° (decomp.) from ethyl acetate (Found: C, 47·6; H, 4·8; Cl, 25·4; N, 5·2. Calc. for $C_{11}H_{13}Cl_2NO_3$: C, 47·5; H, 4·7; Cl, 25·5; N, 5·0%). The corresponding *hydroquinone* formed small prisms, m.p. 86–88°, from ethyl acetate–pentane (Found: C, 47·3; H, 5·6; Cl, 25·5; N, 5·1. Calc. for $C_{11}H_{15}Cl_2NO_3$: C, 47·1; H, 5·4; Cl, 25·3; N, 5·0%).

2-*Chloro*-5-(*di*-2′-*chloroethylamino*)-1,4-*benzoquinone* (I, R = Cl), deep red purple plates, m.p. 165–167°, from ethyl acetate (Found: C, 43·0; H, 3·7; Cl, 37·4; N, 5·0. Calc. for $C_{10}H_{10}Cl_3NO_2$: C, 42·5; H, 3·5; Cl, 37·7; N, 5·0%). The corresponding *hydroquinone* formed rhombs, m.p. 70–72°, from ethyl acetate–pentane (Found: C, 42·2; H, 4·3; N, 5·2. Calc. for $C_{10}H_{12}Cl_3NO_2$: C, 42·2; H, 4·2; N, 4·9%).

2-(*Di*-2′-*chloroethylamino*)-1,4-*naphthaquinone* (III), small deep orange plates, m.p. 165–167° from ethyl acetate (Found: C, 55·9; H, 4·3; Cl, 24·3; N, 4·8. Calc. for $C_{14}H_{13}Cl_2NO_2$: C, 56·4; H, 4·4; Cl, 23·8; N, 4·7%).

N-(4′-*Di*-2″-*chloroethylaminophenyl*)*quinoneimine* (V)

Method 1. Zinc dust was added to a solution of 4-nitroso-NN-di-2′-chloroethylaniline (5 g) in aqueous hydrochloric acid (40 ml, 2 N) until the colour was practically discharged. The mixture was filtered and the residue washed with hydrochloric acid (10 ml, 2 N). After adding phenol (1·88 g) to the filtrate a solution of chromic acid (2·66 g) in water (5 ml) was added with stirring and cooling so that the temperature never rose above 20°. A benzene–ethyl acetate (1 : 1) extract of the reaction mixture was dried over anhydrous potassium carbonate and passed through a column of activated alumina. The first deeply coloured eluates contained a purple solid which formed flattened needles having a greenish glint from chloroform–pentane. Yield 1·4 g. The compound has no definite m.p. but shrinks and decomposes about 90–93°. It forms deep purple solutions in benzene, ethyl acetate and chloroform and red solutions in pentane and ether (Found: C, 59·8; H, 5·0; Cl, 22·0; N, 8·6. Calc. for $C_{16}H_{16}Cl_2N_2O$: C, 59·4; H, 5·0; Cl, 21·9; N, 8·7%).

Method 2. A finely ground mixture of NN-di-2-chloroethylaniline (4·36 g) and 4-nitrosophenol (2·46g) was added slowly to a vigorously stirred mixture of concentrated sulphuric acid (19·5 ml) and water (5·5 ml) that was kept at 20°. When addition was complete the mixture was stirred for a further 15 min, water (11 ml) was then added with cooling and the mixture was stirred for ¾ hr at 30–35°. The solution was then poured into a mixture of ice (500 g) and ethyl acetate (500 ml). The ethyl acetate layer contained the *indoaniline* derivative which was purified by chromatography as above.

4′-(*Di*-2″-*chloroethylamino*)-4-*hydroxydiphenylamine* (VI)

The above indoaniline (1 g) in ethanol (30 ml) was hydrogenated over a palladium–charcoal (5% Pd) catalyst. The solution was rapidly filtered with exclusion of air and evaporated under reduced pressure. After dissolving the residue in dry ether, hydrogen chloride was passed in and the almost colourless and slightly deliquescent solid was collected. Analysis of the product, which melted indefinitely at 60–70°, proved that it was the *sesqui-hydrochloride* of (VI)) (Found: C, 50·3; H, 5·4; Cl, 30·8; N, 7·5; equiv. by potentiometric titration, 251. Calc. for $C_{16}H_{18}Cl_2N_2O$. 1½ HCl: C, 50·6; H, 5·2; Cl, 32·6; N, 7·4%; equiv. 253.

The following were similarly prepared from the appropriate phenols:

N-(4′-*Di*-2″-*chloroethylaminophenyl*)-3-*methylquinoneimine* (V, 3-Me) which formed small deep blue plates, decomp. c. 80–90°, from ether–pentane (Found: C, 60·5; H, 5·5; Cl, 21·7; N, 8·3. Calc. for $C_{17}H_{18}Cl_2N_2O$: C, 60·5; H, 5·4; Cl, 21·0; N, 8·3%).

4′-(*Di*-2″-*chloroethylamino*)-4-*hydroxy*-3-*methyldiphenylamine* (VI, 3-Me), obtained as a *dihydrochloride* which formed small plates, m.p. 167–175, from ethanol–ether (Found: C, 50·1; H, 5·5; Cl, 36·0; N, 6·4; equiv. 212. Calc. for $C_{17}H_{20}Cl_2N_2O{\cdot}2HCl$: C, 49·6; H, 5·4; Cl, 34·4; N, 6·8; equiv. 206).

N-(4′-Di-2″-*chloroethylaminophenyl*)-3,5-*dimethylquinoneimine* (V, 3,5-Me_2). This formed small deep purple plates, m.p. indef., from ether–pentane (Found: C, 61·9; H, 5·8; Cl, 20·5; N, 7·7. Calc. for $C_{18}H_{20}Cl_2N_2O$: C, 61·6; H, 5·7; Cl, 20·2; N, 8·0%).

4′-(*Di*-2″-*chloroethylamino*)-4-*hydroxy*-3,5-*dimethyldiphenylamine* (VI, 3,5-Me_2)

An air dried specimen of the compound prepared as described above proved to be a *sesquihydrochloride*; it formed small plates, m.p. 165–168°, from ethanol–ether (Found: equiv. 264. Base. $1\frac{1}{2}$ HCl requires equiv. 272). On drying this material at 55°/0·1 mm for several hours, hydrogen chloride was lost and a *monohydrochloride* was formed (Found: C, 56·1; H, 5·9; Cl, 27·3; N, 7·1. Calc. for $C_{18}H_{22}Cl_2N_2O{\cdot}HCl$: C, 55·5; H, 6·0; Cl, 27·3; N, 7·2%). If a solution of the reduction product of the quinoneimine was saturated with dry hydrogen chloride and the product collected and washed with saturated ethereal hydrogen chloride a *dihydrochloride* was formed (Found: C, 51·0; H, 6·1; Cl, 34·5; N, 6·9. Calc. for $C_{18}H_{22}Cl_2N_2O{\cdot}2HCl$: C, 50·8; H, 5·7; Cl, 33·3; N, 6·6%). The m.p. of these various hydrochlorides is not definite since hydrogen chloride is readily lost on heating.

N-(4′-*Di*-2″-*chloroethylaminophenyl*)-1,4-*naphthaquinoneimine* (VI, 2,3-benz)

This compound, obtained by using α-naphthol as coupling component, formed reddish purple prismatic needles having a greenish glint from chloroform–pentane; the m.p. was indefinite (Found: C, 64·4; H, 4·9; Cl, 19·8; N, 7·9. Calc. for $C_{20}H_{18}Cl_2N_2O$: C, 64·4; H, 4·9; Cl, 19·0; N, 7·5%). The leuco-base formed an amorphous *monohydrochloride* of indefinite m.p. (Found: equiv. 414. Calc. for $C_{20}H_{20}Cl_2N_2O{\cdot}HCl$: 411·5).

4-(*Di*-2′-*chloroethylamino*)*mandelic acid* (IX)

4-(Di-2′-chloroethylamino)benzaldehyde (4·8 g), potassium cyanide (4·0 g), and glacial acetic acid (2 ml) in ether (100 ml) were stirred for 2 days at 25°. Sufficient water was then added to dissolve the inorganic salts and then an excess of anhydrous sodium carbonate was added to dry the ethereal layer. Fuming hydrochloric acid (30 ml) was added to the ether extract and the ether was removed by drawing a stream of air through at room temperature. Next day the acid solution was concentrated on a flash evaporator at 60°. After dilution with water the solution was extracted with ether and the acid fraction was obtained by washing with sodium carbonate solution. This acid fraction solidified in contact with benzene. 4-(*Di*-2′-*chloroethylamino*)-*mandelic acid* formed plates, m.p. 79°, from benzene. On washing the crystalline product with light petroleum or on exposure to air benzene of crystallization was lost giving a resinous product. A sample of the crystals had a titration equivalent of 386

($C_{12}H_{15}Cl_2NO_3$ requires equiv. 292 and 1 : 1 benzene complex requires equiv. 370); the pK_a of the acid in 50% aqueous acetone was 4·85. The acid was characterized by preparing the *piperidine salt*, prisms, m.p. 126–128°, from ether–methanol (Found: C, 54·1; H, 6·9; N, 7·3. Calc. for $C_{17}H_{26}Cl_2N_2O_3$: C, 54·1; H, 7·0; N, 7·4%) and the *dicyclohexylamine salt*, small prisms, m.p. 171°, from acetone (Found: C, 60·8; H, 8·6; N, 6·1. Calc. for $C_{24}H_{38}Cl_2N_2O_3$: C, 60·9; H, 8·1; N, 5·9%).

4-(*Di*-2′-*chloroethylamino*)*benzoylformic acid* (X)

The mandelic acid derivative (1·48 g) and aqueous sodium hydroxide (4 ml, N) in acetone (40 ml) was cooled below 10° and finely powdered potassium permanganate (0·42 g) was added during ½ hr to the rapidly stirred solution. Stirring was continued until no permanganate colour was detected on spotting the solution on to filter paper. Water and ether were added to the reaction mixture; the ether layer contained a little aldehyde. The filtered aqueous layer was acidified with hydrochloric acid (5 ml, N) and extracted with ether. On evaporating the dried extract the acid was obtained as a yellow gum. It formed a *2,4-dinitrophenylhydrazone*, deep red flattened needles, m.p. 206–207°, from ethanol (Found: C, 45·9; H, 3·7; N, 14·4. Calc. for $C_{18}H_{17}Cl_2N_5O_6$: C, 46·0; H, 3·7; N, 14·9%) and a *dicyclohexylamine salt*, prisms, m.p. 150–152°, from ether–methanol (Found: C, 60·9; H, 7·4; N, 6·4; Calc. for $C_{24}H_{36}Cl_2N_2O_3$: C, 61·1; H, 7·7; N, 6·0%).

N[1]-2′-*Bromoethyl-3-carboxyamidopyridinium bromide* (XI)

Nicotinamide (15 g), ethylene dibromide (25 ml), and acetone (180 ml) were heated under reflux for 3 days. The solid which separated was collected at intervals. The combined precipitate was extracted with hot acetone and then recrystallized from methanol, the *pyridinium bromide* formed flattened needles, m.p. 192–194° (Found: C, 31·5; H, 3·2; ionic Br, 25·8; N, 8·6. Calc. for $C_8H_{10}Br_2N_2O$: C, 31·0; H, 3·3; ionic Br, 25·75; N, 9·0%).

N[1]-2′-*Bromoethyldihydronicotinamide* (XII)

The above pyridinium bromide (7 g) was dissolved in water (15 ml) and this solution was saturated with sodium bromide. A slight excess of sodium borohydride was added to the rapidly stirred solution keeping the temperature below 10°. The precipitated *dihydro-compound* was collected by filtration, washed with iced water, and then with dry ether and transferred to a desiccator. It reoxidized readily on exposure to air especially when heated and the m.p. was indefinite at about 90–100°. (Found: C, 41·4; H, 5·1; Br, 34·7; N, 12·0. Calc. for $C_8H_{11}BrN_2O$: C, 41·5; H, 4·8; Br, 34·6; N, 12·1%). An aqueous alcoholic solution of the dihydro-compound rapidly reduced aqueous silver nitrate to metallic silver.

N-2-Bromoethyldipyridinium bromide (XIII)

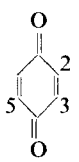
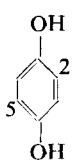
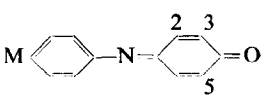
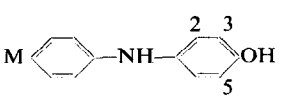
When dipyridyl was heated with an excess of ethylene dibromide in acetone solution a solid slowly separated during several days. It formed prisms, decomp. above 250°, from acetone–methanol. Analysis showed that it was mainly the mono-substituted derivative (Found: C, 39·0; H, 3·5; Br, 47·0, N, 8·1; ionic Br, 23·6. Calc. for $C_{12}H_{12}Br_2N_2$: C, 41·9; H, 3·5; Br, 46·5; N, 8·2; ionic Br, 23·25%).

METHODS

Determination of chemical reactivity (Rates of hydrolysis)

The rates of hydrolysis of the new 2-chloroethylarylamines were determined essentially by the method already described by Ross.[16] Sufficient of the compound to develop acidity equivalent to 20 ml N/10 alkali on complete hydrolysis was dissolved in aqueous acetone (1 : 1) and after adjusting the pH to 7 by potentiometric titration with 0·1 N aq. NaOH plus an equivalent volume of acetone the solution was heated under reflux for ½ hr. Re-titration of the cooled solution to pH 7 then indicated the amount of hydrogen ions liberated. Only in the case of the two acidic derivatives (IX) and (X) would there be expected to be any difference between the rate of liberation of hydrogen and chloride ions and in these cases the amount of chloride ion released was also determined by titration with 0·1 N silver nitrate using a chromate indicator.

TABLE 1. EXTENT OF HYDROLYSIS IN ACETONE–WATER (1 : 1) AT 66° DURING ½ hr

Structure	Substituent(s)	H^+ liberated* (%)	Cl^- liberated* (%)
	2,5-M_2	25·5	
	2-Me, 5-M	5·4	
	2-MeO, 5-M	1·5	
	2,3-benz, 5-M	1·0	
	2,5-M_2	52·5	
	2,5-M_2; diacetate	20·8	
	2-MeO, 5-M	80	
	none	7	
	3-Me	10	
	3,5-Me_2	10·5	
	2,3-benz	7·5	
	none. 1½ HCl	43†	
	3-Me. 2 HCl	56·6†	
	3,5-Me_2. 1½ HCl	51·4†	
	2,3-benz. HCl	57†	
$MC_6H_4CHOH.COOH$	benzene complex	22	34
$MC_6H_4CO.COOH$	dicyclohexylamine salt	1	1

* Based on complete hydrolysis of the 2-chloroethyl groups.

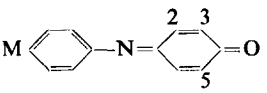
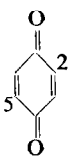
† These values are relatively low because of the appreciable concentration of chloride ions at the start of the reaction.

The values obtained are given in Table 1. The relative alkylating ability of the two nicotinamide derivatives (XI, X = Cl) and (XII, X = Cl) has already been studied by Friedman *et al.*[14] and it was not deemed necessary to repeat the measurements with the new bromo-derivatives.

Determination of oxidation–reduction potentials

These potentials were determined essentially by the method of Fieser and Thompson[17] which involved titrating the leuco-bases with standard potassium molybdicyanide in an aqueous ethanolic phosphate buffer. The results were checked by reducing the indoaniline derivative *in situ* using a 5% Pd-C catalyst, sweeping out the excess of hydrogen with purified nitrogen, and titrating as before. The results are given in Table 2. Difficulty was experienced with the quinone-hydroquinone pairs due to precipitation of the oxidized form even at high dilution.

TABLE 2. OXIDATION–REDUCTION POTENTIALS MEASURED AT 25° IN 37% AQUEOUS ETHANOL, 0·047 M KH_2PO_4, 0·047 M Na_2HPO_4 (pH = 7·6)

Structure	Substituent(s)	$E_m^{7\cdot6}$ (mV)
	none	200*
	3-Me	160*
	3,5-Me_2	110*
	2,3-benz	40*
	none	275†
	2-MeO, 5-M	24

* These values were independently confirmed by Dr. G. D. F. Jackson.

† Hewitt[18] gives $E_m^7 = 290$ mV.

Cytostatic assays

The LD_{50} and MED (dose to produce 90% inhibition of the growth of the transplanted Walker rat carcinoma) were determined by the combined toxicity-therapeutic activity comparative assay method described by Connors *et al.*[7] The results expressed in μmoles/kg are given in Table 3 together with data for some of the more efficient 'aromatic nitrogen mustards' and triethylenemelamine.

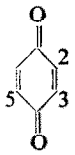
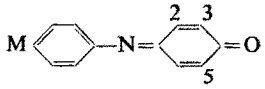
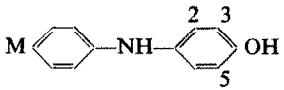
DISCUSSION

Chemical Reactivity

In order to be able to exploit any difference in oxidizing capacity between normal and cancer cells it is necessary to have agents that are more effective in a lower state of oxidation. There is a correlation between the chemical reactivity of 2-chloroethylarylamines and their cytostatic potentiality (Ross[1]). Table 1 shows that the reduced forms of three types of structure have higher rates of hydrolysis and should be more effective tumour growth inhibitors. Thus the first requirement for selective activity can be achieved with this group of compounds.

The increase in rate of hydrolysis on passing from the quinone (I, R = MeO) to (II, R = MeO) is 5·4 to 80% but it is considerably less on passing from (I, R = M) to (II, R = M). If one compares the reactivity of the other quinones it will be seen that the reactivity of (I, R = M) is higher than would have been anticipated. There is

TABLE 3. CYTOSTATIC ASSAYS

Structure	Substituent(s)	Vehicle for i.p. administration	LD_{50} (μmole/kg)	MED (μmole/kg)	Index LD_{50}/MED
	2,5-M_2	10% acetone–arachis oil	100	17	5·9
	2-Me, 5-M	arachis oil	16·8	ca. 8	ca. 2
	2-MeO, 5-M	arachis oil	27	14	1·9
	2-Cl, 5-M	arachis oil	46	inactive	
	2,3-benz, 5-M	arachis oil	1900	ca. 1000	1·9
	2,5-M; diacetate	arachis oil	37	ca. 10	ca. 3·7
	2-MeO, 5-M	arachis oil	32	18	1·8
	none	10% acetone–arachis oil	331	93	3·6
	3-Me	10% acetone–arachis oil	208	ca. 148	1·4
	3,5-Me_2	10% acetone–arachis oil	256	162	1·6
	2,3-benz	10% acetone–arachis oil	188	inactive	
	none	arachis oil	197	44	4·5
	3-Me	arachis oil	240	197	1·4
	3,5-Me_2	arachis oil	361	266	1·4
	2,3-benz	arachis oil	216	inactive	
$MC_6H_4CHOHCOOH$	piperidine salt	water	472	101	4·7
$MC_6H_4CHOHCOOH$	dicyclohexylamine salt	arachis oil	376	254	1·5
$MC_6H_4CO.COOH$	dicyclohexylamine salt	10% aq-ethanol	378	170	2·2
(XI)		water	1450	inactive	
(XII)		oil	980	inactive	
(XIII)		water	320	inactive	
PhM		arachis oil	645	60	10·8
$C_{10}H_7M(\beta)$		arachis oil	4050	363	11
MC_6H_4(2-Me)N:NC_6-H_4(2′-COOH)(CB 1414)		arachis oil	53	24	2·2
$MC_6H_4(CH_2)_3$-COOH (chlorambucil)		arachis oil	58·2	8·6	7
MC_6H_4-p-$CH_2CH(NH_2)COOH$ (melphalan)		arachis oil	14·7	1·64	9
MC_6H_4-o-$CH_2CH(NH_2)COOH$ (merophan)		water	11·5	0·79	14
Triethylenemelamine (TEM)		water	5·5	0·58	9·5
$NH(CH_2CH_2Cl)_2$.HCl (nor-HN2)		water	760	200	3·8

evidence that in the hydrolysis of this compound, which can be regarded as a vinylogue of an amide, di-2-chloroethylamine is released and this would, of course, increase the apparent hydrolysis rate. After hydrolysis under standard conditions, potentiometric titration indicates the liberation of 0·25 equivalents of a base of $pK_a = 9·2$ which is similar to that of di-2-hydroxyethylamine (9·3).

The indoaniline derivatives have relatively low rates of hydrolysis but the leuco bases obtained on reduction are considerably more reactive. The increase is actually greater than that indicated in Table 1 for it was not convenient to use the unstable resinous free bases. The initial presence of chloride ion has the effect of suppressing the ionisation of the di-2-chloroethylamine and lowers the observed rate.

When the acidic derivatives (IX) and (X) are hydrolysed in aqueous solutions containing no added nucleophile the carboxyl group competes with water for reaction with the carbonium ion thus reducing the amount of hydrogen ion liberated. The alkylating ability towards complex biological systems is more correctly indicated by the rate of liberation of chloride ions.

Oxidation–reduction potentials

The results in Table 1 show that the first requirement, that is, the design of agents that are more effective alkylating agents in the reduced form is possible. It is now necessary to consider to what extent the various agents will be in the more reactive form in cancer cells as compared with normal cells. As already stated there is no clear indication of oxidation–reduction potential differences between the two types of cell but as a starting point one can use the figures cited by Cater and Phillips.[12] For the purposes of calculation one can assign a potential of 250 mV to the normal cell and 150 mV to the cancer cell. In Table 4 we have calculated—assuming equilibrium con-

TABLE 4. RELATIVE ALKYLATING ABILITY OF DRUGS IN NORMAL AND CANCER CELLS

E_m^7 of drug (mV)	Reduced form in normal cell (%)	Reduced form in cancer cell (%)	Alkylating ability in cancer cell/normal cell if reactivity of reduced form/oxidized form =			
			2	5	10	∞
300	98	99·999	1	1·02	1·02	1·02
250	50	99·95	1·33	1·67	1·82	2
200	2	98	1·94	4·55	8·3	49
150	0·05	50	1·5	3·0	5·5	1000
100	0·001	2	1·02	1·08	1·18	2000

ditions—the percentage of drugs of a given E_m^7 value that is in the more reactive form in each type of cell. The end columns show the relative alkylating ability of the drug when one assigns different ratios for the reactivity of the reduced form as compared with the oxidized form.

If the oxidized form is completely unreactive then drugs with low potentials would appear to be the most useful but it must be remembered that the absolute amount of the drug in the reactive form falls sharply as the E_m^7 value decreases: only 2% of the drug in the cancer cells is reactive if the E_m^7 is 100 mV. For the more usual situation where the ratio of reaction rates is finite, one finds a peak of relative reactivity at about 200 mV. If the reduced form is ten times as reactive as the oxidized form then

the drug is 8·3 times as effective in alkylating the cancer cell. For this reason we have aimed at E_m^7 (values) in the region of 200 mV in this preliminary study.

The unsubstituted indoaniline derivative (V) has $E_m^{7\cdot6} = 200$ mV and this is reduced by substitution in a manner similar to that observed for indophenols[19] (Table 2). Preliminary data on the quinone derivatives also indicates that the oxidation–reduction potential of benzoquinone is reduced by the type of substitution made in the present study. No values are yet available for the mandelic–benzoylformic acid system but the related lactic–pyruvic acid system has $E_m^7 =$ about -180 mV.[19]

Carcinostatic assays

In studies of this kind it is necessary to be able to compare the carcinostatic specificity of drugs. This can be done in a tentative way by deriving a chemotherapeutic index. In the present experiments rats bearing transplanted Walker tumours receive graduated doses of agent and the LD_{50} and the dose to produce 90% inhibition of tumour growth (minimum effective dose MED) are determined in the same group of animals. The chemotherapeutic index is defined as LD_{50}/MED; the values of the two parameters are expressed as μmoles/kg in Table 3 since this facilitates comparisons of effectiveness of different structures. The higher the value of the index, which relates overall host toxicity to tumour growth inhibitory activity, the greater is the carcinostatic specificity of the agent. Table 3 includes the value of this index for some clinically useful drugs, e.g. chlorambucil has index = 7, melphalan = 9, and TEM = 9·5 in this system.

Quinones

Of the five quinones examined three have an index of about 2 and one is inactive. The most effective compound (I, R = M) could conceivably owe its superior index (5·9) to *in-vivo* release of nor-HN2 which has a comparable index (see p. 979). The greater potency of the quinone may be due to the more effective transport of alkylating potential to a target site. The similarity of the toxicity and index for (I, R = MeO) and (II, R = MeO) suggests that equilibrium between the two forms is rapidly reached *in vivo*.

Indoanilines

The values of the index are low for compounds with $E_m^{7\cdot6} = 40$–150 but considerably higher for the parent compound of $E_m^{7\cdot6} = 200$ mV. This could mean that the calculations in Table 4 are relevant and the examination of more compounds with oxidation–reduction potential in this region is clearly indicated. The lower toxicities of the indoanilines as compared with the quinones—except for the sparingly soluble naphthaquinone derivative—is noteworthy and is probably due to the relatively higher toxicity of the quinone carrying structure.

Mandelic–benzoylformic acid derivatives

The importance of the mode of administration is shown in the case of the mandelic acid derivative which exhibits a superior index when given as the water-soluble piperidine salt. The similarity of the toxicity and index of the two dicyclohexylamine salts suggest ready interconvertibility *in vivo*.

Nicotinamide derivatives

The inactivity of the agents (XI, XII, and XIII) is not altogether surprising since few monofunctional alkylating agents have activity against the Walker tumour. The lack of activity even at near toxic dose levels shows that the effect on the tumour is quite distinct from any generalized host toxicity.

CONCLUSIONS

It must be admitted that in this preliminary study no improvement in the index over the values for the most effective di-2-chloroethylarylamines has been achieved. The lack of success is not necessarily due to shortcomings in the method of approach for the following factors have to be considered.

1. The biochemical differences which we are seeking to exploit may not be fully developed in the Walker tumour when we treat it, that is, on the day following implantation. Cater and Phillips' measurements were, of necessity, carried out on well-developed tumours.
2. The oxidation–reduction potentials that we have taken as the basis for this initial work may not be the relevant ones. Table 4 shows that the properties of a drug would be critically dependent on the values of these potentials.
3. The drugs which are administered may be metabolized so that the circulating agent has a less favourable oxidation–reduction potential. For example, results were obtained in the case of certain azo-benzene derivatives[13] which were consistent with the ready oxidation of methyl groups attached to aromatic rings giving carboxylic acids. If such a change occurred with the compounds now under examination there would be a profound effect on the potential.
4. In some instances the carrying structures have considerable inherent toxicity towards the host and this would mask any differential effects on the alkylating moiety of the molecule. This consideration probably applies particularly to the more toxic quinone derivatives.

A feature which emerges from an examination of Table 3 is the relatively small change in chemotherapeutic index despite a wide variation in potency. NN-Di-2-chloroethyl-β-naphthylamine has LD_{50} = 4,050 μM/kg, hydrolysis rate = 15%, and index = 11 whilst *p*-di-2-chloroethylamino-L-phenylalanine has LD_{50} = 14·7 μM/kg, hydrolysis rate = 20%, and index = 9. The difference in potency cannot be due to the small difference in chemical reactivity but probably derives from a facile transport of the amino acid derivative through cellular membranes. There is no indication in these figures that the attachment of an amino acid side chain to a di-2-chloroethylarylamine specifically favours transport into cancer cells. Only by exploiting some established biochemical difference between the two types of cell is one likely to enhance carcinostatic specificity. The only significant increase in chemotherapeutic index recently observed in our test system has resulted from the use of TEM-glucose combinations[7] (index = 28) which were intended to exploit the relatively lower pH of neoplastic tissue. Attempts to take advantage of oxidation-reduction potential differences are continuing with the synthesis of new compounds that should satisfy the requirements laid down in this paper. In particular, it would be advantageous to obtain structures in which there was a greater reactivity difference between the oxidized and the reduced forms.

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