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HAEMATOLOGICAL EFFECTS OF HOMOLOGOUS SERIES OF DICHLOROETHYLARYLAMINES (AROMATIC NITROGEN MUSTARDS)

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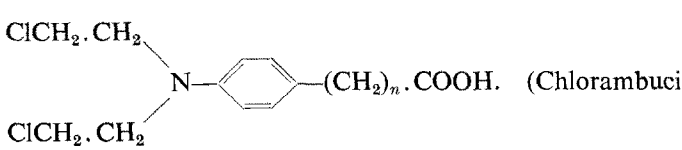
Abstract—The effects of three homologous series of dichloroethylarylamines (aromatic nitrogen mustards): (1) a series of phenylalkanoic acid derivatives, (2) phenoxyalkanoic acid derivatives, and (3) phenyl-α-aminoalkanoic acid derivatives, on the numbers of circulating blood cells and of platelets in the blood of rats have been investigated. Maximum activity in depressing the number of circulating lymphocytes and neutrophils is shown in series (1) by the phenylacetic acid derivative, in (2) by the phenoxypropionic acid derivative, and in (3) by the phenylalanine derivative.

The relation between the haematological activity and chemical reactivity of the compounds is discussed.

IN DESCRIBING the preparation of aryl-2-halogenoalkylamines ("aromatic nitrogen mustards") Everett *et al.*[1] pointed out that most substances which contain a di-2-chloroethylamine group possessing a certain level of chemical reactivity are cytotoxic to proliferating tissues. Their use as chemotherapeutic agents in neoplastic diseases is limited, however, by the lack in many of them of sufficiently greater specific action on malignant tissue than on some normal proliferating tissues, particularly those of the haemopoietic system. Indeed their therapeutic application has so far shown most promise in the treatment of diseases of this system, particularly the leukaemias.

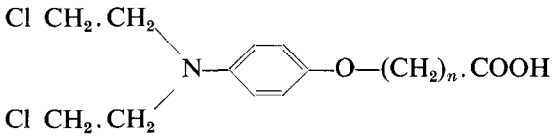
A large number of "aromatic nitrogen mustards" have been prepared with the object of obtaining more selective action on neoplastic tissue[1–4] and it was often found advantageous to introduce potential water solubility into the molecule by means of the carboxylic acid group (usually solubilized as the Na salt). Chlorambucil (I: $n = 3$) which is widely used therapeutically, particularly in the treatment of chronic lymphatic leukaemia, is a compound of this type. Another compound which is in use therapeutically is the derivative of the naturally occurring amino acid phenylalanine, Merphalan (Sarcolysin)[3, 5] III, $n = 1$. The haematological effects in the normal rat of homologous series of compounds related to these drugs, and also of a series of phenoxyalkanoic acids have now been investigated. These series may be depicted as:

(I) Phenylalkanoic acid series[1]

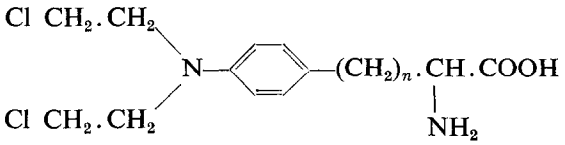


(Chlorambucil: $n = 3$).

(II) Phenoxyalkanoic acid series[2]



(III) Phenyl-α-aminoalkanoic acid series[2]



(Merphalan (Sarcolysin): $n = 1$)
(Aminochlorambucil $n = 2$)

In previous work[6] on the effects of a series of compounds related to Myleran which is used in treatment of chronic myeloid leukaemia, it was pointed out that in spite of the difference in pattern of blood counts in the rat compared with the human pattern the effect of the drug on the normal rat can give a reasonable indication of its behaviour in the clinical treatment of leukaemia. The pattern of blood response of the normal rat to a single dose of a chemical has been found of considerable help as a guide in the selection of substances for clinical trial against leukaemia, lymphomas and other neoplastic diseases.[7]

EXPERIMENTAL

Rats of a Wistar albino colony were used. They were kept in separate cages and fed a constant "rat cake" diet during the period of the experiment. Compounds were administered by intraperitoneal injection either dissolved in, or in the form of a fine suspension in, arachis oil.

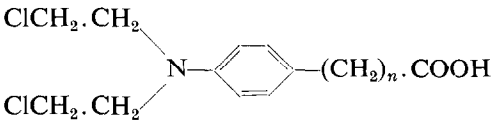
Blood counts

For blood counts, blood was taken from a tail vein direct into the pipette in which it was diluted in the usual manner.

Four rats were usually used for each compound tested and their individual response curves plotted as numbers of blood cells against time. A mean curve giving the percentage of normal count (before injection) against time was then compiled from these individual results.

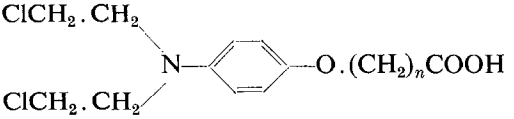
Toxicity

Tests to obtain the approximate toxic dose of the compounds were carried out by intraperitoneal injection in female rats. Groups of three rats were usually used for each dose level tested. The animals were fed a constant "rat cake" diet and weighed daily. The approximate toxicity figures given (Tables 1, 2 and 3) are based on the smallest dose which would kill the three rats tested

TABLE 1. EFFECT OF SERIES (I) 

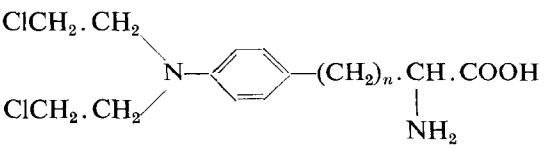
ON THE CIRCULATING LEUCOCYTES IN THE RAT

n	Code no.	Dose (*d*) (mg/kg)	% Fall from normal (*F*) Lymphocytes (*L*)	Neutrophils (*N*)	*L*/*d*	*N*/*d*	Approx. toxic dose (mg/kg)
0	CB 1078	150	33	15	0·2	0·1	>150
1	CB 1331	8 10	90 98	75 85	11·3 9·8 mean 10·5	9·4 8·5 mean 9·0	15
2	CB 1332	16	62	48	3·8	3·0	>20
3	CB 1348	12·5	85	75	6·8	6·0	18
4	CB 1356	20	50	40	2·5	2·0	50

TABLE 2. EFFECT OF SERIES (II) 

ON THE CIRCULATING LEUCOCYTES IN THE RAT

n	Code no.	Dose (*d*) (mg/kg)	% Fall from normal (*F*) Lymphocytes (*L*)	Neutrophils (*N*)	*L*/*d*	*N*/*d*	Approx. toxic dose (mg/kg)
1	CB 1360	10	50	40	5·0	4·0	30
2	CB 1364	5	70	65	14·0	13·0	10
3	CB 1365	6	60	54	10·0	9·0	14
4	CB 1378	10	80	70	8·0	7·0	16

TABLE 3. EFFECT OF SERIES (III) 

ON THE CIRCULATING LEUCOCYTES IN THE RAT

n	Code no.	Dose (*d*) (mg/kg)	% Fall from normal (*F*) Lymphocytes (*L*)	Neutrophils (*N*)	*L*/*d*	*N*/*d*	Approx. toxic dose (mg/kg)
0	CB 1447	20	40	20	2	1	>100
1	CB 3007	10	87	90	8·7	9·0	12
2	CB 1385	12·5	95	55	7·7	4·0	25
3	CB 1494	10	80	45	8·0	4·5	12

RESULTS

Fig. 1 shows the blood response pattern to one member of each of the three series of compounds. In each representative the carboxylic acid group is separated from the benzene ring by the same number of atoms.

The general pattern is the same for all three compounds. For the first few days after treatment there is a fall in all blood components followed by a rapid recovery,

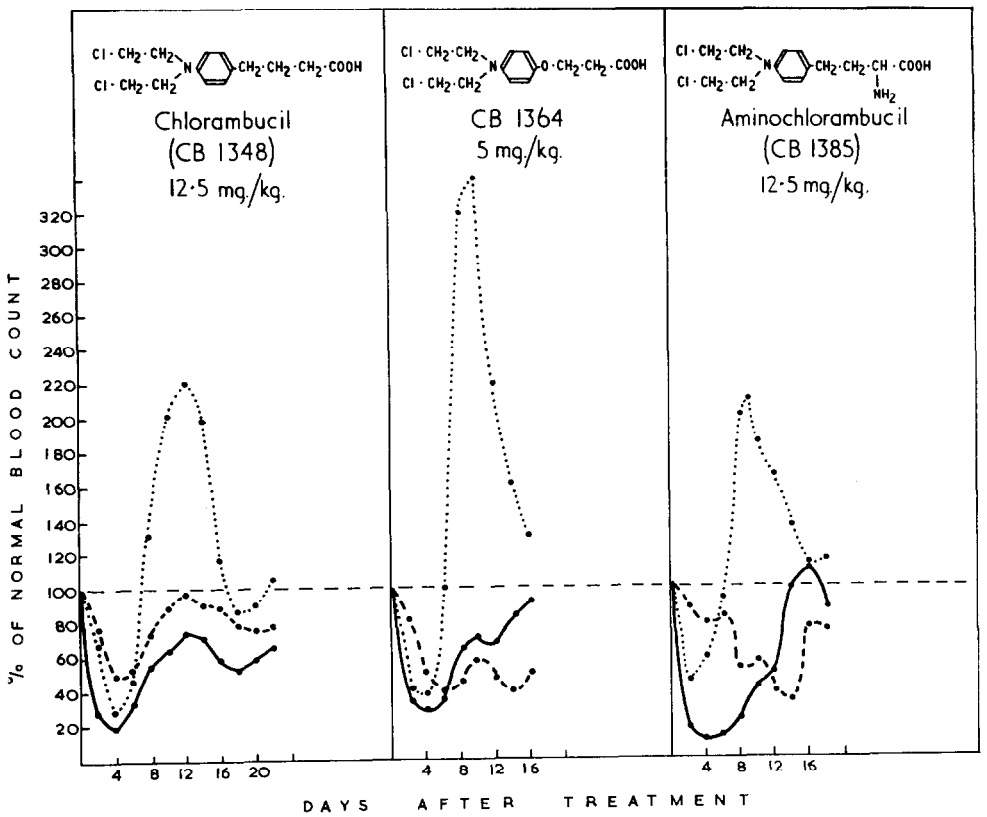


FIG. 1. Blood response patterns of the normal rat to Chlorambucil, 4-N : N-di-(2-chloroethyl) aminophenoxypropionic acid (CB 1364) and Aminochlorambucil. ——— lymphocytes; · · · · neutrophils; — — — platelets.

which in the case of the neutrophils leads to a very large increase in the numbers circulating in the blood, so that there is very marked neutrophilia at about 8–12 days after treatment. The excessive number of circulating neutrophils then falls sharply, and normal values for most blood components are restored by about 20 days after administration of the single dose of the drug.

The relative effects on the two types of leucocytes, viz. lymphocytes and neutrophils, is very similar for Chlorambucil and CB 1364 although with the latter compound only about half the dose is required to produce the same depression. Aminochlorambucil, however, shows a relatively much greater depressive action on lymphocytes than on neutrophils. This more marked action on lymphocytes is also shown by the next higher member of the series, CB 1494 (see Table 3).

Based on these blood response patterns, in an attempt to compare directly the "absolute" activity of the various compounds on the circulating blood cells, an index has been devised consisting of the quotient of the maximum percentage fall from normal values and the dose in mg/kg body weight used to produce this fall. The percentage fall is denoted in the case of the lymphocytes by L, and for the neutrophils by N, the dose being designated d. The index thus becomes L/d, N/d or P/d if platelets are being considered. Although rather crude the index does allow some measure of direct comparison of the absolute haematological activity of different types of compounds. It does not take into account the duration of action in response to a single dose. For instance a compound may produce the maximum fall in lymphocytes in 2, 3, 4 or more days after administration, and this difference is not taken into account in the L/d figure. For true assessment of activity the blood response patterns as shown in Fig. 1 are of course indispensable. Again, the index must of course always be considered together with the toxicity of the compound if it is to be of any use in assessing the therapeutic usefulness of a potential cancer chemotherapeutic agent.

A similar index (F/d) applied only to the neutrophils was used in comparing the haematological activities of the homologous series of compounds related to Busulphan (Myleran).[6] Using the modified nomenclature now described Myleran has an index $L/d = 2{\cdot}3$; $N/d = 8{\cdot}8$. When this is compared with that of Chlorambucil ($L/d = 6{\cdot}8$; $N/d = 6{\cdot}0$) it is seen that the selectively greater effect of Myleran on the myeloid rather than on the lymphoid system is well indicated by the much higher value for the N/d index compared to the L/d value. (This can also be expressed by the low L/N value of 0·26.)

The effects of the three series of compounds (I), (II) and (III) on the circulating leucocytes of the rat are summarised in Tables 1, 2 and 3.

In series (I) (Table 1) maximum activity against both types of leucocytes is shown by the phenylacetic acid derivative (CB 1331: $n = 1$). When $n = 2$ the activity is diminished but returns in Chlorambucil ($n = 3$). The last member of the series ($n = 4$) shows very much reduced activity.

In series (II) the highest activity is shown by the second member (CB 1364: $n = 2$) and then again activity falls rapidly as the value of n increases.

All members of both series show a slightly greater depressive action on lymphocytes than on neutrophils (L/N about 1·2).

In the amino acid series (III) greatest activity against both types of leucocytes is shown by the phenylalanine derivative, Merphalan ($n = 1$). In the higher members of this series, however, activity against lymphocytes does not fall off as in series (I) and (II), although activity against neutrophils does. Thus aminochlorambucil ($n = 2$) is about twice as active in depressing the number of circulating lymphocytes as in depressing the neutrophils ($L/N = 1{\cdot}9$).

The activity against leucocytes of the three series of compounds in relation to their chemical reactivity is illustrated in Fig. 2. The method of assessing chemical reactivity is described in an Appendix by W. C. J. Ross. The very low activities shown by the first members of series (I) and (III) can probably be related to their low chemical reactivity[8] since high biological activity is usually not obtained in compounds which show a percentage hydrolysis of less than 20.[9]

The activity of the most active member of the series (I) and (II) also probably bears some relation to the chemical reactivity. The compound (II-3) is more active than (I-1)

corresponding with the hydrolysis of 70 per cent against 40 per cent. In the higher members of the series, however, although these all have about the same chemical reactivity, their biological activity appears also to depend on the number of carbon atoms separating the —COOH group from the aromatic nitrogen mustard grouping, activity falling off as this distance is increased.

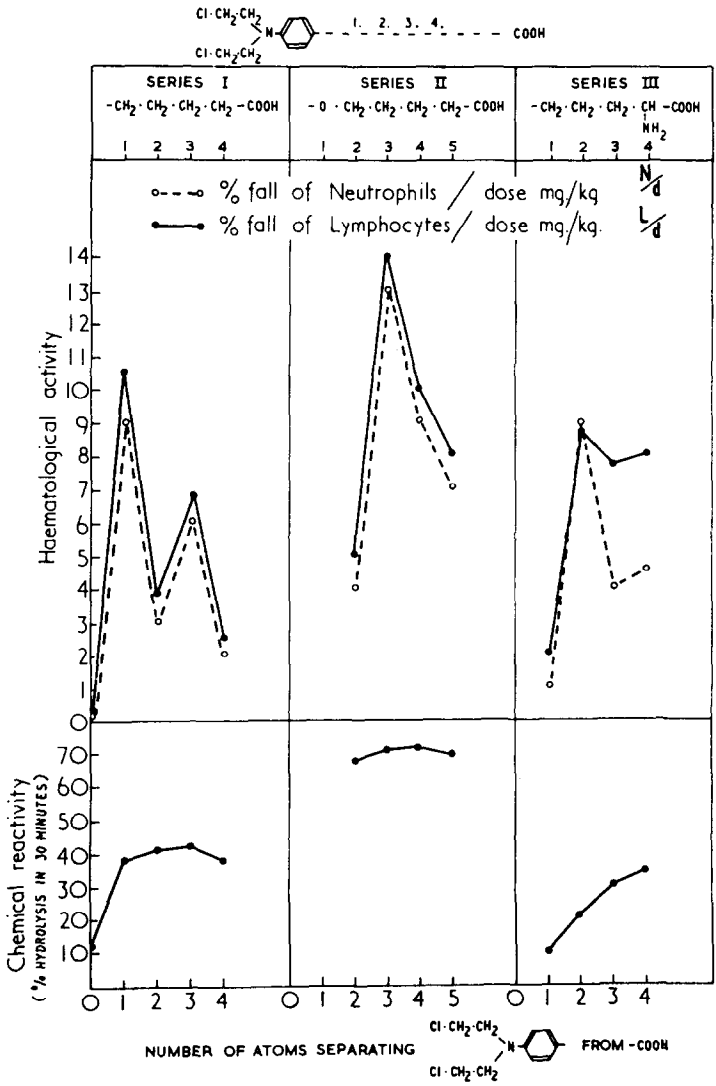
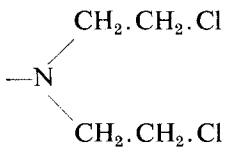


FIG. 2. Haematological activity and chemical reactivity in homologous series of dichloroethylarylamines: (I) phenylalkanoic acid series; (II) phenoxyalkanoic acid series; (III) phenyl-α-aminoalkanoic acid series.

In series (III) although the activity against neutrophils shows a similar falling off in ascending the series, that against lymphocytes remains high. Possibly in this case continuing increase in chemical reactivity compensates to some extent for the fall expected with increasing chain length.

DISCUSSION

In each of the three series of compounds tested the haematological activity of the individual members may be considered in terms of two factors: (a) chemical reactivity of the dichloroethyl amino group—



and (b) the distance of this group from the terminal carboxylic acid (—COOH) group of the compound. It would appear that compounds must have a necessary minimum of chemical reactivity in order to show appreciable biological activity, but once this critical level is surpassed the haematological activity is dependent more on the nature of the molecule as a whole particularly the length of the alkanoic acid side chain.

The toxicity and tumour-inhibitory activity against the Walker rat carcinoma follow much the same pattern as the activity on the blood. In series (I) Chlorambucil was the member originally selected for clinical trials since it appeared to show a rather better inhibitory activity against the Walker carcinoma than the rather more haematologically active CB 1331[1] (Table 1). The most active compound of the phenoxyalkanoic acid series (II) is CB 1364 ($n = 2$). This compound also has a high activity against the transplanted Walker rat carcinoma but is also one of the most toxic.

In series (III) the carbon atom to which the —COOH and —NH_2 groups are attached is asymetrically substituted. The compounds described here are all the racemic (DL) forms. The most active compound of this series Merphalan (Sarcolysin) has been resolved into its optical isomers and the L-form, Melphalan, which is thus a derivative of the naturally-occurring L-phenylalanine, is almost twice as active as Merphalan in depressing the circulating leucocytes. The biological action of derivatives of this "L-phenylalanine mustard" Melphalan are described by Elson *et al.*[10]

The most interesting feature of series (III) is the maintenance of high activity against circulating lymphocytes in the higher members of the series. The activity against neutrophils does show a considerable fall in the last two members of the series and thus a compound like aminochlorambucil ($n = 2$) shows a relatively specific action in reducing the numbers of circulating lymphocytes proportionately much more than the number of neutrophils. Elson[11] and Gerhartz[12] have shown that a dose of 12·5 mg/kg aminochlorambucil which results in a greater depression of circulating lymphocytes than the same dose of Chlorambucil causes less damage to the bone marrow. With Chlorambucil there is an 80 per cent depression of granulopoiesis at about 4 days after administration of the drug whereas a much milder depression (40 per cent) occurs with Aminochlorambucil.

In considering the mechanism of action of Chlorambucil and Busulphan, Elson *et al.*[13] considered that whilst Busulphan may exert its biological action mainly by decreasing mitotic frequency, Chlorambucil damages cells about to divide and cells in mitosis. It also damages adult lymphocytes. It would appear probable, therefore, that the greater lymphocyte depressing action of aminochlorambucil may be mainly due to its greater destructive action on the circulating lymphocytes.

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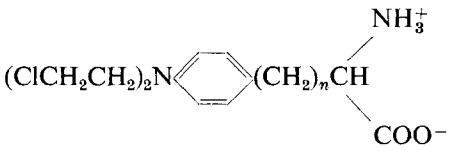
APPENDIX

*Chemical Reactivity in Relation to Structure of Acid Derivatives of Dichloroethylarylamines**

The relative chemical reactivity of the aromatic nitrogen mustards discussed in this paper may be assessed by determining the rates of hydrolysis under standard conditions. In the examination of a large group of compounds of widely differing solubility it has proved convenient to measure the amount of reaction which occurs when a solution of the compound in 50% aqueous acetone is heated under reflux (66 °C) for $\frac{1}{2}$ hr.[14] The figures obtained do not represent true rate values since no allowance has been made for the pH change during the reaction or for the effect of increasing chloride ion concentration in retarding the rate as time proceeds, though the correction for these factors will be quite small under the conditions chosen. Dr. Davis is at present measuring the rates of reaction of a number of derivatives in purely aqueous solution at constant pH and at high dilution using automatic titration equipment. These new values, which will be reported later, will more closely indicate the rate at which the agents react under physiological conditions. Nevertheless the values obtained using aqueous acetone are a measure of the relative reactivity.

The rates of hydrolysis of the free acidic derivatives and of their sodium salts have been reported.[1, 2] For the present purpose the rates for the sodium salts are the more relevant, for the phenylalkanoic and phenoxyalkanoic acids will be present in the anionic form at physiological pH. In the case of the amino acid derivatives the rate for the free acid will be relevant in the present discussion since under the conditions of hydrolysis and under physiological conditions the compounds will be present as the zwitterion form:



As previously indicated[1] the rate of reaction of a mustard may be followed by measuring the liberation of hydrogen ion or of chloride ion, but in the case of the sodium salts of acidic derivatives the two values are not identical because of a self esterification reaction which will probably not be significant when the agent reacts in a mixed biological system. In a table of reactivities published earlier[9, 15] figures were given for the chloride ion production from phenylalkanoic acid salts

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and for the hydrogen ion production from phenoxyalkanoic acid salts. Whilst these values were adequate for comparing reactivities within a series, it is necessary to consider the chloride ion production alone if compounds in different series are to be compared. Table 4 gives the relevant values for the salts of acids now under consideration.

TABLE 4

Compounds	% liberation of chloride ion in ½ hr, in 50% aqueous acetone at 66 °C from the sodium salt
$(ClCH_2CH_2)_2N$–C$_6$H$_4$–$(CH_2)_nCOOH$ (I)	
$n = 0$	15
1	39
2	41
3	42
4	39
$(ClCH_2CH_2)_2N$–C$_6$H$_4$–$O(CH_2)_nCOOH$ (II)	
$n = 1$	68
2	70
3	71
4	70

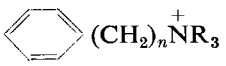
In the phenylalkanoic acid series the proximity of the carboxylate ion to the benzene ring in the *p*-aminobenzoic acid derivative (I, $n = 0$) has the expected effect of slightly lowering the chemical reactivity as compared with the unsubstituted aniline derivative (hydrolysis under standard conditions, 20 per cent[8]). When this ionized group is separated from the ring by a methylene chain all subsequent compounds have reactivity comparable to that of the *p*-toluidine "mustard" (hydrolysis, 38 per cent).[8] The carboxylate group in the phenoxyalkanoic acid series is sufficiently far removed to have no appreciable effect on reactivity and all compounds have comparable reactivity of the same order as that of the *p*-anisidine "mustard" (hydrolysis, 58 per cent[8]). The greater reactivity of acids (II) as compared with acids (I) is a consequence of the greater electron releasing capacity of an oxygen atom as compared with a methylene group.

Table 5 shows the rates of hydrolysis of the amino acid mustards.

TABLE 5

Compound	% liberation of chloride ion in ½ hr in 50% aqueous acetone at 66°C from the free acid
$(ClCH_2CH_2)_2N$–C$_6$H$_4$–$(CH_2)_nCH(NH_2)COOH$	
$n = 0$	10
1	22
2	30
3	34

The predominating influence of the positive charge on the nitrogen atom in the zwitterionic form of the amino acid derivative has the effect of reducing chemical reactivity and this effect falls off more slowly with distance than does the effect of a carboxylate ion. A similar gradual falling off of the effect of a charged nitrogen atom has been observed[16] in the direction of nitration of a series of aromatic amines of structure:



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