Biochemical Pharmacology, 1963, Vol. 12, pp. 317–324. Pergamon Press Ltd., Printed in Great Britain.

THE INFLUENCE OF pH ON THE REACTIVITY OF CHLORAMBUCIL

J. H. LINFORD

Department of Biochemistry, University of Manitoba, Winnipeg, Canada

(*Received* 8 *November* 1962; *accepted* 4 *December* 1962)

Abstract—The reactivity of chlorambucil in aqueous solution, as measured by the rate of the alkylation reaction with haemoglobin, decreases with decrease of pH below 7. Both the solubility of chlorambucil and the degree of dissociation of the carboxyl group in chlorambucil also decrease in solutions of acid pH. A study of the related changes in these three factors indicates that activation of the molecule to the carbonium ion form is brought about by an approach of the carboxyl group to the chloro group. On this basis an explanation is offered of the effect of length of alkanoic acid chain and its point of attachment to the benzene ring on the reactivity of compounds in this series. These considerations may also explain the greater reactivity of the bifunctional mustards.

CHLORAMBUCIL has been useful in the treatment of malignant lymphomas[1] and is in current clinical use. It is usually administered by mouth and must pass through the stomach before exerting the observed biological effects. To understand how the active form persists in the gastric juice, a study of its chemical reactivity at acid pH was undertaken.

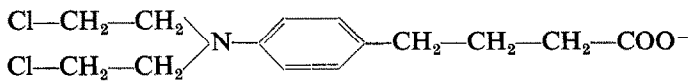
The form of chlorambucil that occurs in aqueous solution is dependent upon the hydrogen ion concentration of the medium. At alkaline pH values the compound dissolves readily and ionic species (I) is present. When the pH is reduced to less than 7 the non-ionic species (II) begins to form as indicated by titration curves and by an increase in the concentration in the organic phase when the compound is partitioned between benzene and water.[2, 3] The non-ionized form is of limited solubility in water. In the region of pH 2 the chlorambucil again dissolves readily in aqueous solvents as the cationic form (III); this point is also indicated by changes in the solubility in organic solvents and by changes in the absorption spectrum.[3] Chlorambucil hydrolyses in aqueous solution of pH 7 or greater to yield the hydroxyl derivative (IV).[2–4]

Chlorambucil reacts with haemoglobin in solution,[5] presumably by alkylation of the ionized carboxyl groups. This explanation is likely since Stacey *et al.* and Alexander *et al.* have shown that this agent reacts readily with the ionized carboxyl groups of bovine serum albumin and does not react so extensively with amino and imidazole groups.[6–8] These workers used concentrations of the mustard 50 times greater than was used in the work to be described.

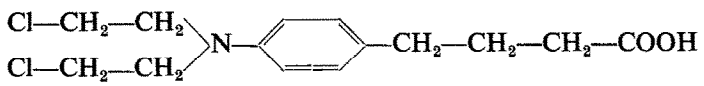
The half-reaction time either for the hydrolysis reaction or for the alkylation of haemoglobin is 0·4 hr and is independent of the concentration of protein.[5] It may therefore be assumed that the latter alkylation reaction is of the S_N1 type and the rate is controlled only by the relatively slow intermediate activation step, in which (V) is

formed. This alkylation reaction was therefore studied to determine the effect of pH on the rate of formation of the activated form (V).

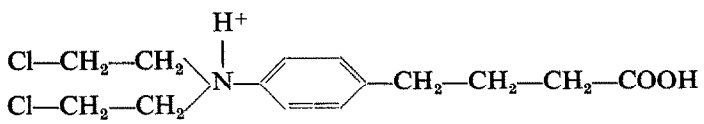
Haemoglobin, of molecular weight 64,450, contains $8{\cdot}38 \times 10^{-7}$ equivalent of acid carboxyl groups per mg, associated with residues of aspartic and glutamic acids.[9] Chlorambucil, of molecular weight 304, contains two reactive chloro groups per



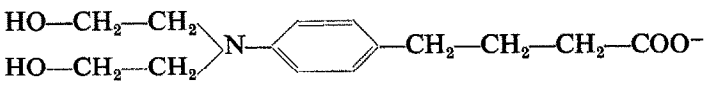
(I)



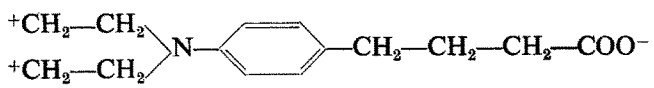
(II)



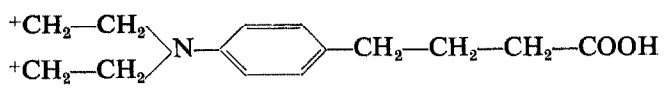
(III)



(IV)



(V)



(VI)

molecule, or $65{\cdot}8 \times 10^{-7}$ equivalents per mg. With respect to the carboxyl groups, 1·0 mg of chlorambucil is therefore equivalent to 7·8 mg of haemoglobin. Haemoglobin also contains 38 histidine, 44 lysine, and 12 arginine residues per molecule, or 5·9, 6·8, and $1{\cdot}8 \times 10^{-7}$ equivalents per mg, respectively.

METHODS

Preparation of reactants. Haemoglobin solutions were prepared from human adult red cells which had been washed with 0·9% saline. To determine the haemoglobin content of the preparation, 0·5 ml was lysed with 4 volumes of water, 0·020 ml of this solution was converted to cyanmethaemoglobin, and the absorbance at 540 mμ was measured. To prepare the chlorambucil solutions, 18 mg was dissolved in 3·0 ml of

0·5 M sodium bicarbonate. Of this solution 0·5 ml was diluted 400-fold with ethanol to check the initial concentration by measurement of the absorbance.[2, 3] To prepare the reaction mixture, 1,200 mg of haemoglobin (approximately 4 ml of red cells) was diluted with 6 ml of water. To this was added 2·0 ml of the chlorambucil solution and 2·0 ml of 1 M potassium dihydrogen phosphate. The pH was adjusted to the required value with 1 N potassium hydroxide, and the volume was made up to 20·0 ml with water. The final solution therefore contained 0·6 mg chlorambucil/ml and 60 mg haemoglobin/ml. All solutions were prepared just prior to use.

Measurement of rate of alkylation reaction. The mixture was shaken at 37°, and the rate of alkylation reaction was measured by assay of the concentration of unreacted chlorambucil at intervals of time. For each assay two 1·0-ml volumes of the reaction mixture were removed. The protein was precipitated from each sample in 10 ml of ethanol and the unreacted chlorambucil in the alcohol, after evaporation of this solvent, was dispersed in 5·0 ml of an aqueous solution of pH 3·6. One aqueous solution was extracted three times with 5·0 ml of benzene, the other with the same volume of ethyl acetate, The organic solvents were evaporated and the residues dissolved in ethanol for absorbance measurements. The difference in the amounts extracted by the two organic solvents was a measure of the degree of hydrolysis of chlorambucil that had taken place during the course of the alkylation reaction.[3, 5]

Measurement of extent of dissociation of the carboxyl groups of chlorambucil. The pH at which the nondissociated state (II) began to form from an alkaline solution of (I) was determined by electrometric titration and by absorbance measurements. For the titration experiment 39 mg of chlorambucil was dissolved in 25 ml of 0·025 N sodium hydroxide. One 10-ml volume was titrated immediately with 0·055 N hydrochloric acid, a second volume was titrated after shaking for 2 hr at 37° to form species (IV), The solvent also was titrated. For the absorbance measurements, 7 to 10 mg of chlorambucil was dissolved in 10 ml of 5 N hydrochloric acid; 1·0-ml samples were adjusted to pH values 2·0 to 9·0 with a solution of 3 M potassium monohydrogen phosphate in 2·5 N potassium hydroxide and were then made up to 50 ml with water. Both the absorbance and the pH of each final solution were measured. The decrease in absorbance was caused by the precipitation from solution of the acid form (II); the absorption measurements were therefore corrected for scattered light by the method of Allan.[10]

Measurement of amount of chlorambucil in cation form (III). The pH at which the cationic state (III) began to form in solution was determined by the absorbance method described above except that the solutions were made up to 20 ml with water, and an equal volume of alcohol was added. The pH was adjusted again if necessary, and the final volume was made up to 50 ml with vol./vol. alcohol–water. To obtain solutions at low pH, water or 5 N hydrochloric acid was used in place of the alkaline phosphate solution. In the presence of the alcohol no precipitation of undissolved or protonated chlorambucil occurred. Both the absorbance and pH of the final solutions were measured.

RESULTS

Influence of pH upon the rate of alkylation of haemoglobin

All the alkylation reactions between haemoglobin and chlorambucil were first order and, with the relative concentration of haemoglobin to chlorambucil that was used, the degree of hydrolysis was negligible at the termination of the reaction. The rate constants were determined from the linear plots of the logarithms of the concentration of unreacted chlorambucil against time and were converted into half-reaction times in hours. A plot of the time course of the reaction at pH 8·5 is shown in Fig. 1. Replacement of the phosphate buffer at pH 8·5 with bicarbonate solution produced no change in the rate constant. In the pH range 7·0 to 9·5 the half-reaction time changed only from 0·39 to 0·36 hr, but at 6·8 a marked dependence of reaction rate upon pH became apparent, as shown in Fig. 2. At pH 4·5 the half-reaction time was 2·4 hr.

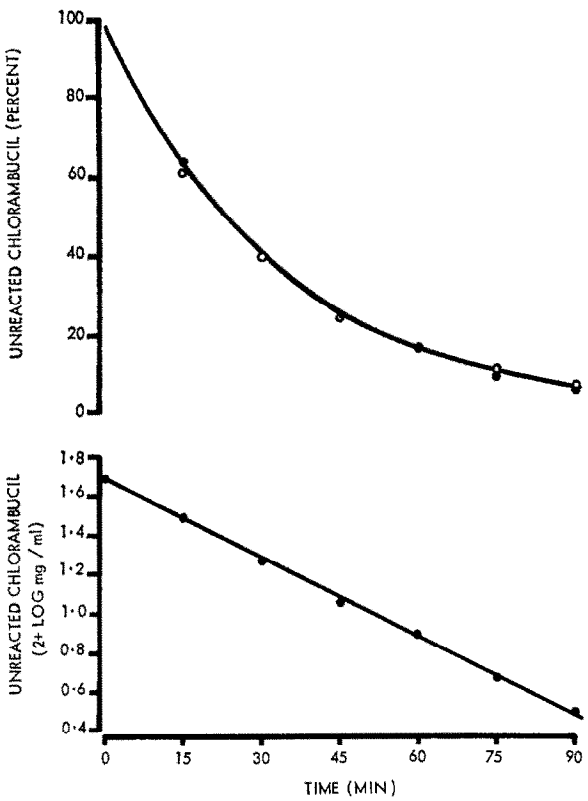


FIG. 1. Recovery of chlorambucil at intervals of time from a reaction mixture containing haemoglobin (60 mg/ml) and chlorambucil (0·6 mg/ml) at pH 8·5 at 37°; ○, benzene extraction; ●, ethyl acetate extraction.

Formation of undissociated chlorambucil (II) and cationic form (III)

The results of the titration of an alkaline solution of chlorambucil, with acid, are shown in Fig. 3. The break at pH 6·8 in the titration curve C is associated with the commencement of protonation of the carboxyl group of the chlorambucil, which

began to precipitate from solution at this pH in the form (II). By inspection, the acid pK_a of the compound is in the region of 5·8. The hydroxyl derivative possessed solubility properties similar to the chloro form as shown by curve H. The displacement of curves C and H from curve S, which was the titration of the solvent only, is a measure of the acid released during the process of solution and hydrolysis.[2]

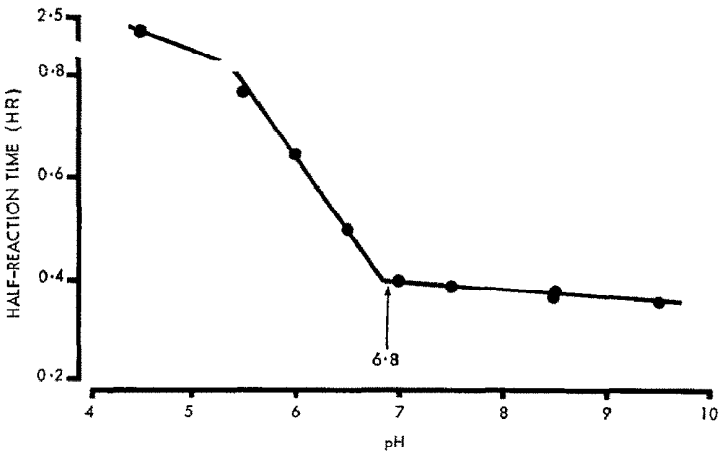


FIG. 2. Change in half-reaction time with change of pH. Conditions as for FIG. 1.

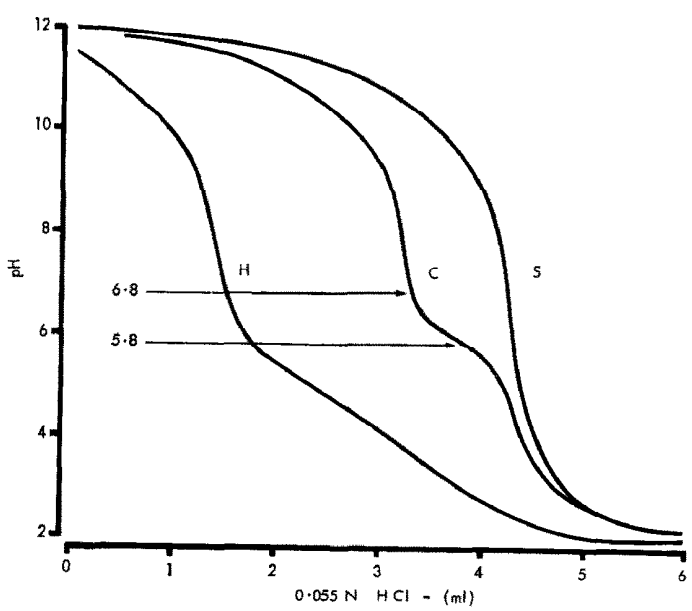


FIG. 3. Titration of 10-ml volumes of: S, 0·024 N sodium hydroxide; C, 0·024 N sodium hydroxide containing 15·6 mg of chlorambucil; H, 0·024 N sodium hydroxide containing 15·6 mg of hydrolsyed chlorambucil.

The influence of pH upon the absorbance in aqueous solution and in aqueous alcohol solution is shown in Fig. 4. In aqueous solution the decrease in absorbance, caused by precipitation of the undissociated form (II), is evident at pH 6·8. This agrees with the result of the titration experiment. In aqueous alcohol solution a marked decrease in absorbance is apparent at pH 2·5, which is attributable to the formation of the cationic state (III). From curve B. Fig. 4, the acid pK of the tertiary N atom is in the region of 1·3.

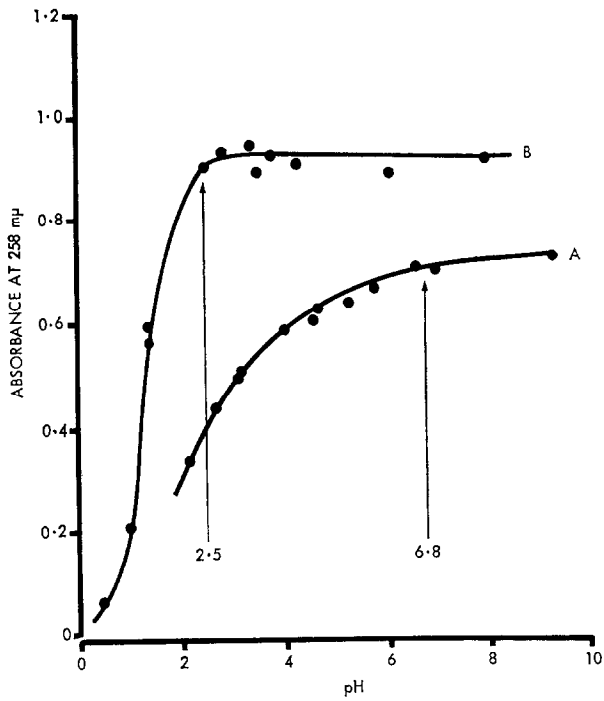


FIG. 4. Effect of change of pH on the absorbance at 258 mμ of a solution of chlorambucil (14 μg/ml): **A**, in aqueous phosphate buffer; **B**, in phosphate buffer mixed with an equal volume of alcohol.

DISCUSSION AND CONCLUSIONS

The decrease in the rate of alkylation of protein, with decrease of pH as shown in Fig. 2, may be compared to the decrease in the degree of ionization of the carboxyl group of the chlorambucil as calculated from the pK value of 5·8. The rate of alkylation of protein dropped by 50% over the pH range 6·8 to 5·4, whereas the proportion of chlorambucil in the ionized carboxyl state dropped by 70%. At pH 4·5 the rate of alkylation was 17% of the rate at pH 6·8, but only 5% of the chlorambucil was in the anionic form. However, the release of the chloro atom may take place more readily at lower pH. It is therefore not possible to distinguish whether the decrease in reactivity of the chlorambucil at lower pH is a result of the complete inactivity of form (II) or of a slower rate of activation of (II) to form (VI).

The dependence of the rate of formation of (V) upon the degree of ionization of the carboxyl group implies that an inductive effect exists between the ionized carboxyl

group and the chloro groups. This effect is more likely to occur by direct approach of these groups to one another, as shown in Fig. 5, than by transmittance through the carbon chains of the molecule. In the aminophenoxy-alkanoic acid series, of which chlorambucil is a member, the activity is at a maximum for a single carboxyl group when it is in the *ortho* position to the mustard chains and again when the *para* position

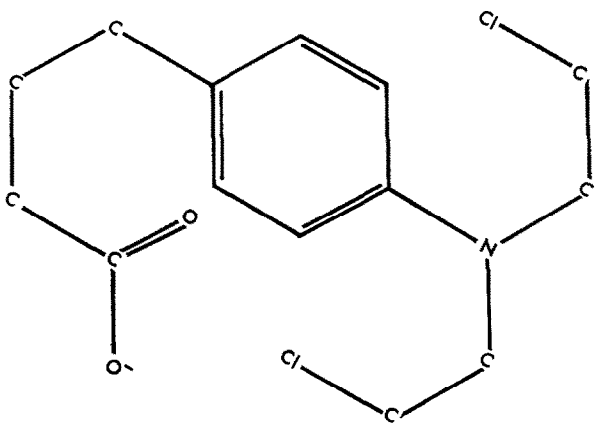
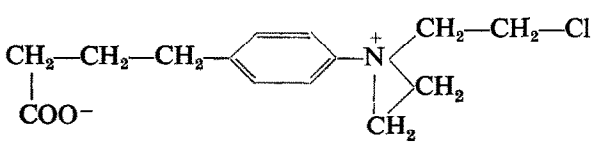


FIG. 5. Spatial configuration of the chlorambucil molecule in which carbonium ion formation may occur.

is occupied by a 3 or 4 atom alkyl carboxyl chain.[4] These circumstances favor the close proximity of the chloro and carboxyl groups. Furthermore, repulsive forces between the two similar chloroalkyl chains attached to the N atom would assist the molecule to spend more time in the configuration of Fig. 5. In this connection the reactivities of the bifunctional mustards are known to be greater than those of the monofunctional derivatives.[4] The chloroethyl groups would be activated one at a time and forms of type (VII) could come into existence.



(VII)

The relationship of the solubility of chlorambucil, initially present at concentration of 20 μg/ml, to the degree of dissociation of the carboxyl group can be deduced from Fig. 4. Over the pH range from 6·8 to 5·4 the amount of chlorambucil in solution decreased to 90%. At pH 4·5 about 80% of the chlorambucil remained in solution. It appears possible that in the presence of the protein the chlorambucil remained molecularly dispersed over the pH range that was used. In the reaction mixture with protein the chlorambucil was initially present at a concentration of 600 μg/ml and fell during the reaction to 30 μg/ml. At acid pH a relatively large amount of chlorambucil should therefore be thrown out of solution in comparison with the weaker solutions of Fig. 4. However, the reactions were first order at all pH values tested. If a

saturated solution of chlorambucil had been formed one would expect a constant reaction rate.

Changes in the degree of ionization of the protein as a result of the change in pH would not be expected to affect the mechanism of activation of the chlorambucil; in any case the reacting centers of the protein were present in 12-fold excess with respect to their carboxyl groups alone. In the pH range above 5·5, over 85% of the protein carboxyl groups were ionized, and at pH 4·5 at least 40% were ionized. No nucleophilic groups from the lysine or arginine residues were present at pH below 8·5, but the 38 histidine residues, of pK 5·6 to 7·0, would have been reactive down to pH 4·5.

The formation of the cationic state (III) in dilute alcohol solution at pH 2·5 ,is in close agreement with the solubility effects in aqueous solutions. Therefore the decrease in the rate of alkylation of protein by chlorambucil at pH values higher than 2·5 cannot be attributed to protonation of the tertiary N atom of the chlorambucil.

The results indicate that the active form of chlorambucil may be retained in the body for several hours in media of pH 5·5 to 4·5, and that it will remain molecularly dispersed in the presence of protein.

Acknowledgements—Professor Paul Hagen read the manuscript and made valuable suggestions with regard to interpretation of the results of this study. Technical assistance was given by Mrs. Marian Boroditsky. The work was supported by a grant from the National Cancer Institute of Canada.

REFERENCES

1. Symposium on Comparative Clinical and Biological Effects of Alkylating Agents, *Ann. N.Y. Acad. Sci.* **68**, 657–1266 (1958).
2. J. H. LINFORD, *Biochem. Pharmacol.* **8**, 343 (1961).
3. J. H. LINFORD, *Biochem. Pharmacol.* **11**, 693 (1962).
4. W. C. J. ROSS, *Biological Alkylating Agents*. Butterworths, London (1962).
5. J. H. LINFORD, *Canad. J. Biochem.* **41**, (4), (1963).
6. K. A. STACEY, M. COBB, S. F. COUSENS and P. ALEXANDER, *Ann. N.Y. Acad. Sci.* **68**, 682 (1958)
7. P. ALEXANDER and S. F. COUSENS, *Biochem. Pharmacol.* **1**, 25 (1958).
8. P. ALEXANDER, *Ciba Foundation Symposium on Drug Resistance in Micro-organisms*. Churchill, London (1957).
9. G. BRAUNITZER, R. GEHRING-MÜLLER, N. HILSCHMANN, K. HILSE, G. HOBOM, V. RUDLOFF and B. WITMANN-LEIBOLD, *Hoppe-Seylers Z. physiol. Chem.* **325**, 283 (1961).
10. W. M. ALLAN, *J. clin. Endocr.* **10**, 71 (1950).