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ADSORPTION TO HUMAN RED BLOOD CELLS OF CHLORAMBUCIL AND OTHER BIOLOGICAL ALKYLATING AGENTS

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Abstract—Utilizing the red blood cell as a model system, a study was made of the uptake of several alkylating agents and of certain biologically inert derivatives of these agents. The results suggest that the chloroethyl group binds to the surface of the cell presumably by virtue of its fat solubility. Adsorption or uptake by the cell takes place independently of alkylation, which may follow at a slower rate. The binding associated with the presence of the chloroethyl group is of a specific type which involves a surface solubility action. This has a potentially disruptive effect on the cell surface which is not associated with the binding of the substance by the Van der Waals type of adsorptive forces. When the molecule that is bound to the cell surface by the action of the chloroethyl group also carries a negative charge, the cell membrane may undergo complete disruption. From the pharmacological point of view, the binding of drugs to the cell may be of importance because the process serves to remove the drug temporarily from the action of nucleophilic substances in the body fluids. Of the compounds tested, only those with a net negative charge were able to bring about hemolysis after adsorption to the red cell.

CHLORAMBUCIL is one of a class of compounds that can alkylate nucleophilic centres in aqueous solution under physiological conditions of temperature and pH. The members of the class differ widely among themselves in chemical constitution, but they react with cellular systems *in vivo* to produce similar characteristic cytostatic, mutagenic and carcinogenic effects which suggest that genetic centres within the chromosomes are attacked by the alkylating agents. However, the sequence of events which leads to these effects is not fully understood.[1] The nature of the interaction of these biologically active compounds with the cell surface is therefore of interest.

Previous work has shown that the compound, chlorambucil or *p*-*N*,*N*-di-(β-chloroethyl) aminophenylbutyric acid (Fig. 1, compound I), will initiate lysis of red blood cells in suspension in isotonic buffer, provided that the ratio of chlorambucil to red cell concentration is above a minimum value.[2] The present paper shows that certain alkylating agents adsorb to the red cell surface, that the adsorption is a consequence of the presence of the chloroethyl group in the drug molecule, and that the adsorbed compound may bring about the disruption of the cell membrane. The red blood cell was used in this work because the degree of cell lysis is readily measurable.

MATERIALS AND METHODS

Red cells were obtained from heparinized blood (0·1 mg/ml) freshly drawn from

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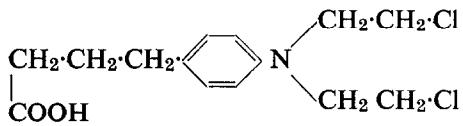

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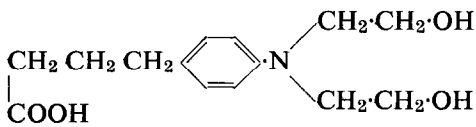

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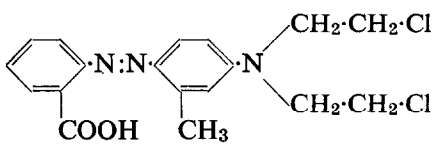

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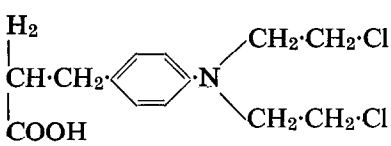

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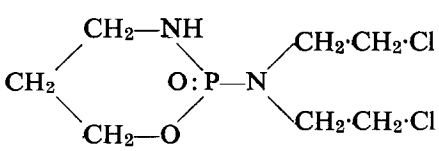

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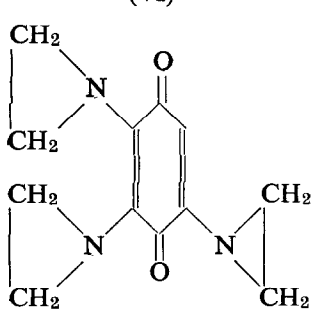

FIG. 1—*Continued on page 2725*

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(VII)

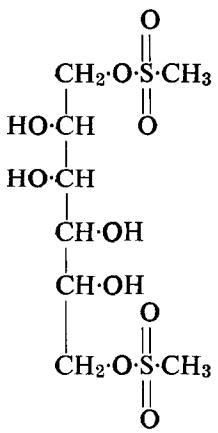

FIG. 1. Structural formulae of the biological alkylating agents tested for hemolytic activity. I. Chlorambucil; III, azo-mustard; IV, phenylalanine mustard; V, cyclophosphamide; VI, Trenimon; VII, mannitol myleran. II, An inactive hydroxyl derivative of I, is also representative of the hydrolysis products of III, IV and V.

healthy young adults. The blood was centrifuged for 5 min in a clinical centrifuge, the plasma and buffy coat were removed, and the cells were washed twice in 2 vol. of an isotonic buffer solution; this contained 0·11 M sodium chloride, 0·02 M glucose, and 0·02 M phosphate, pH 7·5. The second wash was carried out at 4° at 10,000 rpm.

The red cells were suspended in a buffered isotonic solution which was also of known molarity with respect to the drug or derivative under test. The concentration of the drug, about 0·005 M, made a negligible contribution to the osmolarity of the medium. The drug uptake was measured by centrifuging an aliquot of the suspension to remove the cells, and then measuring the concentration of the agent remaining in the supernatant solution by methods described in each section below.

Chlorambucil. Chlorambucil was supplied by Burroughs Wellcome & Company, Ltd.

Three isotonic buffer systems were prepared for test. One was 0·02 M with respect to phosphate, pH 7·5, 0·02 M with respect to glucose, and 0·11 M with respect to sodium chloride. A second isotonic buffer consisted of Tris (hydroxymethyl) aminomethane, 0·02 M, and sodium chloride, 0·14 M. An alkaline aqueous solvent was prepared of 0·5 M sodium bicarbonate to which was added a small volume of 0·5 M sodium carbonate to bring the pH to 9·0. A second aqueous solvent was prepared of 0·11 M sodium chloride and 0·02 M glucose which, when mixed with the alkaline bicarbonate solution, 9 vol. to 1 vol., yielded a third buffered medium isotonic to red blood cells.

To prepare a solution of chlorambucil (I) as the sodium salt, a weighed quantity was dissolved in a measured volume of the alkaline bicarbonate solvent by shaking for 2 min at 37°. This was then diluted 10-fold with the saline–glucose mixture; this stock solution was isotonic and in the presence of red cells had a pH of 8·5. A series of chlorambucil concentrations was then prepared from this stock solution using saline–glucose–bicarbonate buffer as the diluent. An aliquot of each member of the series

was diluted with alcohol and the optical density was measured at 258 mμ to determine the chlorambucil concentration.[3] Chlorambucil solutions were used immediately after preparation.

To prepare the hydrolysis product of chlorambucil (the dihydroxyethyl derivative, Fig. 1, compound II, a solution of I in 1·0 ml of 0·15 N sodium hydroxide was kept at 37° for 2 hr. The pH was adjusted to 7 with 0·15 N hydrochloric acid and diluted 10-fold with the alkaline isotonic saline–glucose–bicarbonate medium.

To measure the extent of lysis of red blood cells in a given medium, a series was prepared of ten 5·0-ml volumes of the isotonic saline–glucose–bicarbonate solution, which contained concentrations of the alkylating compound under test varying from zero to 1·0 mg/ml. To each volume, 0·5 ml of a packed suspension of washed red cells was added and the resulting suspension was kept at 37° in a shaker bath. Identical tubes were kept at 4°, and these tubes were inverted at intervals. The cell concentration was 8·5 to 9 per cent. After a measured time interval, an aliquot was centrifuged and the optical density of the supernatant layer was read at 540 mμ; this value was compared with that of a hemolysate produced by adding 0·5 ml cells to 5·0 ml water. No corrections were made for the change in solvent volume brought about by cell lysis.

Chlorambucil is recovered from an aqueous solution of pH 3·5 by extraction with benzene or with ethyl acetate. The dihydroxyethyl derivative (II) is not soluble in benzene, but is soluble in ethyl acetate.[3] A comparison of the recoveries in these two solvents enabled a measurement to be made of the extent of hydrolysis of the chlorambucil in the course of the experiment. These tests were made either on an aliquot of the supernatant of the cell suspension or on an aqueous solution of the residue remaining after a solution in aqueous alcohol was evaporated to near dryness at 37° under reduced pressure.

The combined amount of chlorambucil (I) and its dihydroxyethyl derivative (II) was measured by diluting an aliquot of the supernatant with 9 vol. of ethanol and measuring the absorption spectrum of the solution at 258 mμ. To measure the adsorbed drug as well as that in free solution, an aliquot of the cell suspension was added to 9 vol. of ethanol, centrifuged to remove the protein precipitate, and the optical density was measured against that of a control suspension treated in the same way.[2]

Azo-mustard. The compound, 4-di-2″-chloroethylamino 2-methylazo-benzene-2-carboxylic acid (azo-mustard or CB1414; Fig. 1, compound III), was generously supplied by Professor W. C. J. Ross of the Chester Beatty Research Institute. The acid form (III) was converted to the sodium salt by the following procedure*: 3 g CB1414 was dissolved in 100 ml methanol and to this was added 7·9 ml of 1 N sodium hydroxide. The liquid was removed on a flash evaporator at room temperature and 50 ml benzene was added to the sticky residue. In the presence of a trace of methanol, the residue went into solution in the benzene. The benzene was then removed with the flash evaporator and the residue was dissolved in 50 ml chloroform with the addition of a trace of methanol. This solution was filtered and the chloroform was allowed to evaporate in a large desiccator connected to a water pump. The residue was ground with sodium-dried ether, the solid was filtered off and stored in a vacuum desiccator over calcium chloride.

*W. C. J. Ross, personal communication.

The hydroxyethyl derivative of CB1414 was prepared by dissolving 80 mg of the sodium salt of (III) in 10 ml of 0·15 N sodium hydroxide and refluxing the solution at 100° for 0·5 hr. Drops of strong hydrochloric acid were added to bring the pH to 9·4. The volume was brought up to 40 ml with saline–glucose–phosphate buffer of pH 9·2.

The concentration of azo-mustard in a given reaction mixture was measured spectroscopically in acid–alcohol solution.[4]

L-*Phenylalanine mustard.* L-Phenylalanine mustard or melphalan or *p*-di-(β-chloroethyl) aminophenylalanine (Fig. 1, compound IV) was obtained from Burroughs Wellcome & Company as Alkeran. The compound is soluble in aqueous solvents to a concentration of 1 mg/ml and has a characteristic absorption spectrum between 220 and 300 mμ, with a single sharp maximum at 258 mμ.

The drug concentration in a given reaction mixture was measured spectrophotometrically in aqueous buffer.

Cyclophosphamide. The cyclophosphamide, or *N,N*,-di-(β-chloroethyl)-N^1,*O*-propylene phosphoric acid ester diamide (Fig. 1, compound V), was obtained from Asta-Werke A.G. Brackwede, Germany. The compound is readily soluble in aqueous media. It does not absorb light in the visible or ultraviolet range; therefore, assay of an aliquot of a given reaction mixture was carried out by the colorimetric reaction of Tan and Cole.[5]

Trenimon. The compound Trenimon, or 2,3,5-trisethylenimino-1,4-benzoquinone (Fig. 1, compound VI), was supplied by Farhenfabriken Bayer, A.G., Leuverkusen, Germany, through the courtesy of F.B.A. Pharmaceuticals Ltd., Montreal. Trenimon is readily soluble in aqueous solvents, in alcohol and in benzene, and possesses a characteristic absorption spectrum having a single maximum at 335–345 mμ, depending upon the solvent which was used to assay the compound.

Mannitol myleran. The compound mannitol myleran, or 1,6-(dimethanesulphonyl)-1,6-dideoxymannitol (Fig. 1, compound VII) was the only biological alkylating agent of the sulphonic acid ester series tested. Mannitol myleran is readily soluble in water. The drug was assayed by measurement of the acid released during the hydrolytic reaction of mannitol myleran in 0·025 N sodium hydroxide at 37° for 90 min.

RESULTS

Chlorambucil. The relationship between the concentration of chlorambucil and the extent of hemolysis of a cell suspension of 8–9 per cent in isotonic buffer is shown in Fig. 2. No lysis was observed in those cells exposed to the dihydroxyethyl derivative of chlorambucil, thus the chloroethyl groups appear to be necessary for the lytic action of the parent compound. Osmotic fragility tests[6] were carried out on the cells that survived the treatment with chlorambucil. If the cells had undergone appreciable lysis, the survivors showed an increase in osmotic fragility. If the chlorambucil concentration was below that required to cause lysis, the fragility of the cells was normal.

Temperature influenced both the rate and extent of lysis, but only to a limited extent. As shown in Fig. 3, 95 per cent hemolysis was produced in 2 hr at 37°, while 79 per cent hemolysis was produced in 2 hr at 4° and it took slightly longer to produce lysis at the lower temperature. In these experiments, an 8 per cent cell suspension was used with a drug concentration of 1·0 mg/ml.

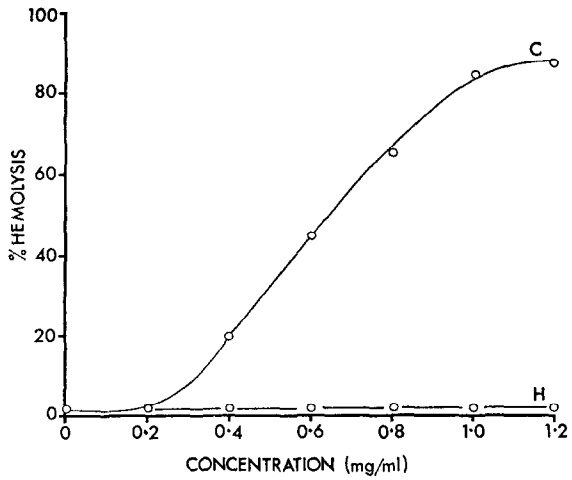

FIG. 2. Hemolysis of an 8% red cell suspension in isotonic buffer after 30 min at 37° with fixed concentrations of C, chlorambucil (I) and of H, hydroxy derivative (II).

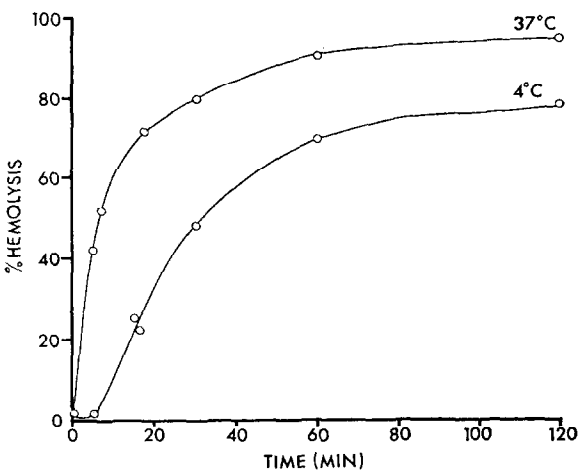

FIG. 3. Effect of temperature on the rate of hemolysis of an 8% red cell suspension in isotonic buffer produced by chlorambucil (I) at a concentration of 1·0 mg/ml.

At 4°, the alkylating action of chlorambucil is very slow, there being no detectable amount of hydrolysis or of ester formation with simple anions in 10 hr.[7] The total amount of chlorambucil recoverable from the 8 per cent red cell suspension was 100 per cent over the drug range from 200 to 1000 μg/ml. More than 96 per cent of this was benzene soluble, indicating that the active drug was completely recoverable within experimental error. The experiments at 4°, therefore, indicate that an alkylation reaction was not involved in the lytic process.

To compare the physical adsorption of chlorambucil (I) and of its dihydroxyethyl derivative (II) under conditions in which no lysis occurs, an initial concentration of 500 μg/ml of either (I) or (II) and a 30 per cent suspension of red cells was used. The results are shown in Fig. 4. Under these conditions, the amount of chlorambucil (I)

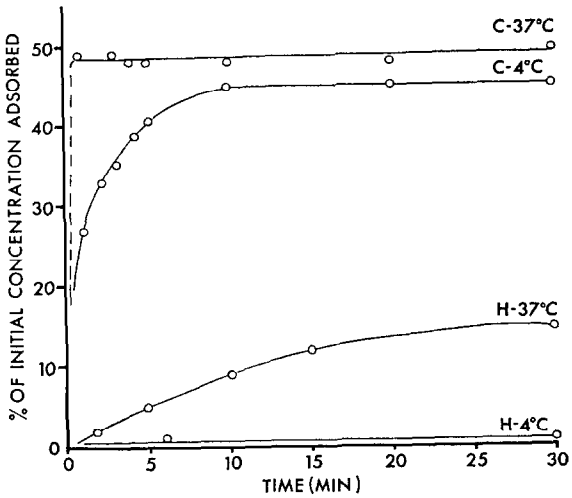

FIG. 4. Effect of temperature on the adsorption of C, chlorambucil (I), and of H, the hydroxy derivative (II), to a 30% red cell suspension. Initial concentration of both forms of chlorambucil in the suspension medium is 0·5 mg/ml.

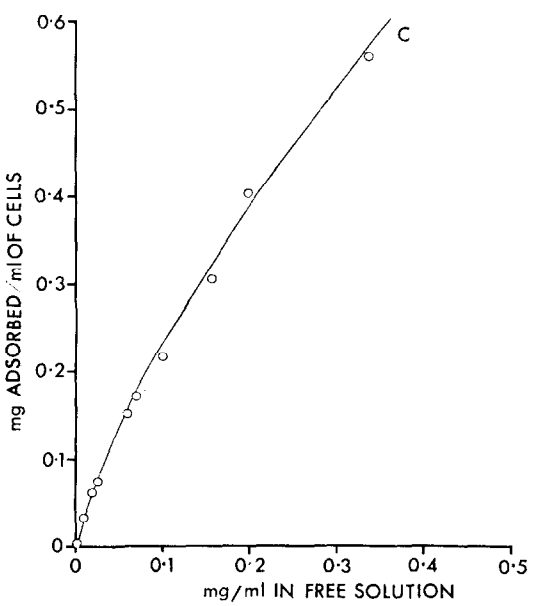

FIG. 5. Distribution of chlorambucil (I) between free solution and the red cell after 30 min of contact of drug with a 30% red cell suspension at 4°.

which is rapidly taken up by the red cells approaches 50 per cent of the initial drug concentration. There is little difference in the uptake at 4° compared to that at 37°; therefore. alkylation plays little or no part in this process. On the other hand, the uptake of the dihydroxyethyl derivative of chlorambucil (II) is much less than that of chlorambucil and occurs very slowly. This strongly indicates that the chloroethyl group is directly involved in the interaction of the drug (I) with the cell.

The effect of the initial concentration of chlorambucil (I) on the uptake at 4° by red cells is shown in Fig. 5. Assuming that 1·0 ml of packed red cells contains 10^{10} cells, 3×10^7 molecules of adsorbed chlorambucil per cell appear to be in equilibrium with $1{\cdot}2 \times 10^{17}$ molecules per ml of free solution (0·2 mM concentration). This equilibrium is not affected by change of pH between 7·0 and 8·5, nor by the nature of the suspending buffer medium. No changes in the adsorptive or lytic phenomena were

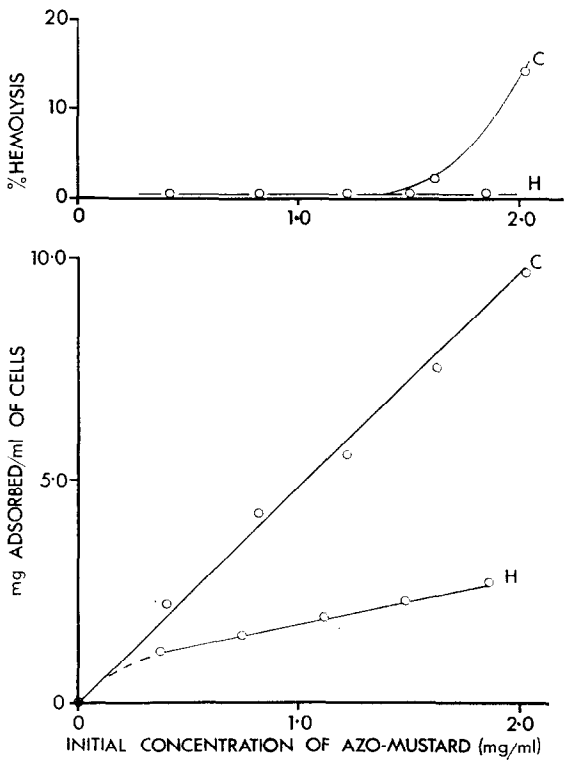

FIG. 6. Upper graph, hemolytic action of azo-mustard after 0·5 hr at 37°; lower graph, adsorption of azo-mustard at different initial concentrations of the drug. C, biologically active compound; H, inactive hydroxy derivative; 9% red cell suspensions were used for both experiments.

observed when chlorambucil solution was prepared by initial solution of the active drug in 0·025 N sodium hydroxide at 4° followed rapidly by adjustment of pH with an isotonic buffer. The buffer solutions used could be 0·02 M phosphate or Tris buffer made up to isotonic concentration with sodium chloride, or could be a saline–glucose–phosphate isotonic medium.

The chlorambucil which is bound to the cell surface is protected from hydrolytic alkylating reactions with the suspending medium. In nonprotein media, chlorambucil undergoes an alkylation reaction by an SNI reaction with a half-reaction time of 0·5 hr; in 20 hr no trace of the active drug is evident. Chlorambucil binds firmly to bovine serum albumin (BSA); 50 per cent of the drug is recoverable at the end of 4 hr and 20 per cent is recoverable at the end of 24 hr, when the suspending medium contains 100 mg of BSA Fraction V per ml. A similar quantity of hemoglobin does not retard the rate of loss of chlorambucil and the drug does not bind to the hemoglobin.[8] To complement these results, the rate of loss of chlorambucil from whole plasma and from a 15 per cent suspension of red blood cells in saline was determined by measuring the amount recoverable by benzene extraction. In whole plasma, 86 per cent of active drug is present at the end of 20 hr; from the red cell suspension, 80 per cent is recoverable after 0·5 hr and 19 per cent is recoverable at the end of 20 hr. When the red blood cells were themselves suspended in plasma, 78 per cent of chlorambucil was recovered at the end of 20 hr. The initial chlorambucil concentration was 0·5 mg per ml.

Alcohol removes chlorambucil which is adsorbed to the red cell[2]. A suspension of 9 per cent hematrocrit was prepared in an isotonic medium also containing 100 μg chlorambucil per ml. By absorption measurement of an aliquot of the supernatant aqueous medium after dilution with 9 vol. of ethanol, 11 per cent of the drug was adsorbed to the cells within 2 hr at 37°. On the other hand, when an aliquot of the suspension itself was added to 9 vol. of ethanol, the supernatant liquid contained 10 μg of chlorambucil per ml.

Azo-mustard (*CB*1414). Azo-mustard is strongly adsorbed to protein[9] and preliminary experiments revealed a high degree of adsorption to red blood cells. Furthermore, the sodium salt of the active chloroethyl form is converted to the less soluble acid form at a pH below 9. The measurements of the extent of binding and of the lytic activity of the compound were therefore carried out at pH 9·2 on a 9 per cent cell suspension. The degree of lysis was determined by measurement of released hemoglobin at 580 mμ because in this region the optical absorption of the drug is negligible.

The results, which are shown in Fig. 6, reveal that the adsorption to red cells is markedly reduced when the chloroethyl group is replaced by the hydroxyl group. At a concentration greater than 1·5 mg/ml, the chloroethyl derivative lyses red cells but the hydroxyethyl derivative does not.

L-*Phenylalanine mustard.* When phenylalanine mustard was initially dissolved in 0·5 M bicarbonate, the solution possessed no alkylating action. Solutions were therefore prepared in the isotonic saline–glucose–phosphate medium. This agent was found to be lytic and to be adsorbed to red blood cells in suspension at 37° and pH 7·5, as shown in Fig. 7, but to a lesser extent than either chlorambucil or azo-mustard.

Cyclophosphamide. Solutions were prepared in isotonic saline–glucose–phosphate. Lysis of a 9 per cent red blood cell suspension was negligible at drug concentrations up to 2 mg per ml. Using 30 per cent red cell suspensions and cyclophosphamide concentrations of 0·5 or of 1·0 mg/ml, 56 per cent of the drug was immediately removed from the supernatant medium. At 4°, 53 per cent was immediately removed. No further changes in the concentrations of the drug in the supernatant medium occurred over a further time interval of 90 min.

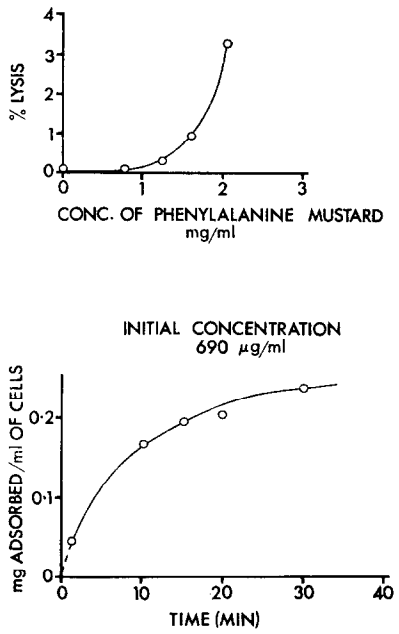

FIG. 7. Upper graph, hemolytic action of phenylalanine mustard after 0·5 hr at 37°; 9% red cell suspensions were used. Lower graph, rate of adsorption of phenylalanine mustard from a solution of initial concentration of 0·7 mg/ml to a 30% red cell suspension.

Trenimon. Trenimon, up to concentrations of 2 mg per ml, showed no lytic activity, but the red cells in suspension rapidly became dark. In 0·5 hr at 37°, 69 per cent of the Trenimon present at a concentration of 90 μg per ml was taken up by a 9 per cent red blood cell suspension.[10] Further experiments were carried out with a 4 per cent cell suspension. In this case, the initial Trenimon concentration of 100 μg per ml showed a loss of 37 per cent in 0·5 hr and of 46 per cent in 24 hr. Samples of the suspension were lysed for spectrophotometric studies of the hemoglobin content. With time, the intensities of the absorption maxima of the hemoglobin at 545 and 580 mμ were diminished and a new absorption maximum appeared at 630 mμ. These changes indicate that the Trenimon, which had passed readily into the red cell, had brought about a degree of formation of methemoglobin by the direct or indirect oxidizing action of the quinone groups of the Trenimon.

An aliquot of the supernatant of the 4 per cent red cell suspension was diluted with 9 vol. of ethanol. As noted above, the Trenimon concentration was found to be 3·7 μg/ml. When 1 vol. of the cell suspension (allowing for the volume occupied by the cells) was added directly to 9 vol. of ethanol, the concentration of Trenimon in solution was 3·6 μg/ml.

Mannitol myleran. After 0·5 hr at 37° no mannitol myleran had been removed from solution by red cells (18 per cent suspension) and no measurable degree of lysis occurred over a period of 16 hr at 37°.

DISCUSSION

In the experiments described, the drug or derivative in the aqueous suspension medium was diminished in amount by the presence of red blood cells. The term

"adsorption" has been used to refer to this uptake of the compounds by the cells, but the processes may include not only surface attachment but partial or complete penetration of the cell membrane.

Of the series of drugs and their derivatives which were tested, adsorption to the red cell surface occurred in all instances in which the chloroethyl group was present. On general chemical evidence we may assume this group to be fat-soluble; as a specific example, the compound chlorambucil (I) which contains two chloroethyl groups is itself entirely fat-soluble in the non-ionized state. We may, therefore, assume that the chloroethyl groups are involved in the process of adsorption of the agent to the cell by a surface-solubility effect; a hydrophobic type of bond is formed. A compound which is in itself fat-soluble, under the conditions of contact with the red cell, will pass readily through the cell membrane as shown by the behavior of the drug Trenimon.

When the chloroethyl group was absent or was replaced by the hydroxyethyl derivitive, the amount of adsorbed agent decreased markedly. However, a degree of adsorption did occur and this is most marked in the case of the hydroxyethyl derivative of the azo-mustard, which contains two (planar) rings in the molecule. Surface Van der Waals forces are probably acting in these circumstances.

Lysis of the cell was brought about by high concentrations of phenylalanine mustard, chlorambucil and azo-mustard, but not by their hydroxyethyl derivatives. Lysis was not brought about by cyclophosphamide. Thus, an intact chloroethyl group was required but, in addition, it was necessary that the adsorbed molecule carry a charge. Referring to Fig. 1, compounds I, III and IV are negatively charged in the aqueous medium. Phenylalanine mustard (IV) carries both positive and negative charges; in this case, the lytic action is weak and the extent of adsorption is also relatively low, which may indicate that the positive charge is incompatible with a penetration of the cell surface by the chloroethyl group. However, steric effects may be important. Alkylation was not involved in the lytic phenomenon because lysis occurs at a temperature of 4°, under which condition alkylation does not occur. The lysis of the cell membrane may require a high degree of binding of the agent, and this may be brought about by the combined action of hydrophobic and coulombic forces of attraction.

From one compound to another there is no correspondence between the onset of lysis and the amount of drug adsorbed to a given volume of cells, which may be evidence that only the drug adsorbed by the surface-solubility effect can condition the cell for lysis. Two mg of the hydroxyethyl derivative of azo-mustard is adsorbed to 1 ml of cells with no evidence of lysis (Fig. 6); on the other hand, lysis occurs when 0·3 mg chlorambucil is adsorbed to 1 ml of cells (compare Fig. 2 and Fig. 5).

The need for a penetration of the cell surface in the manner which we have designated as "surface-solubility" to bring about a disruption of the cell membrane, is given further support by the effect of secondary conditions noted in the experiments with chlorambucil. At 4°, the lytic activity is decreased relative to that at 37°, and a decrease of approximately the same magnitude occurs in the degree of adsorption. It was also found that prior storage of red cells at 4° increased the lytic action of chlorambucil at 37° relative to cells that were stored at 37°. The same treatment produced a corresponding increase in the extent of adsorption of chlorambucil to the precooled cells.

From the experiments of Pethica and Schulman,[11] lysis appears to depend upon a lowering of the interfacial tension of the red cell, which can be brought about by both non-ionic and ionic detergents. The ionic detergents appear to act by penetration of a cholesterol residue within a cholesterol–phospholipid–lipoprotein complex. The necessary association of lysis with the presence of an ionic charge, in the limited series of compounds used in the present study, suggests that the surface activity of these substances in the uncharged state is small. However, the lytic process does serve to indicate that the fat-soluble chloroethyl group is able to penetrate a component of the cell surface.

The compounds which possess in the same molecule both the hydrophobic chloroethyl group and the hydrophilic charged grouping would be expected to concentrate at the boundary between lipid and aqueous phases.

In two instances the amount of drug which was recovered from a sample of the supernatant aqueous medium, taken from a red cell suspension, was compared with that recovered by adding a corresponding volume of the suspension itself directly to alcohol. In the case of the drug Trenimon, the amount recovered (which was 37 per cent of that initially present in the suspension) was not altered by the change of method, possibly because the action of the alcohol in coagulating the cell material trapped the drug within the cell. On the other hand, in the case of chlorambucil, where the drug appears to be taken up at the membrane, all of the drug initially present in the suspension was recovered by the alcohol treatment.

The lysis of red cells occurred at drug concentrations much greater than would be obtained from doses in clinical use. The lytic phenomenon may therefore be of little direct clinical significance. However, the use of the red blood cell as a model system is convenient because the lytic phenomenon can provide insight into the consequences of the adsorptive phenomenon which very likely has clinical importance; a sublytic damage to the cell membrane may occur in the presence of very small amounts of drug.

A second consequence of the adsorption of the alkylating agents to the cell membrane, which may be of pharmacological interest, is the evidence that the drugs so adsorbed are protected to a degree from the attack of nucleophilic substances in the aqueous medium surrounding the cell. Many of these agents disappear very rapidly from the plasma after intravenous injection; the drugs may owe their biological activity to the fact that they are initially dispersed while being maintained in a chemically reactive state, rather than being dissipated by random reactions with irrelevant biological material.

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