Biochemical Pharmacology, 1961, Vol. 8, pp. 343–357. Pergamon Press Ltd., Printed in Great Britain.

SOME INTERACTIONS OF NITROGEN MUSTARDS WITH CONSTITUENTS OF HUMAN BLOOD SERUM

J. H. LINFORD

Department of Biochemistry, University of Manitoba

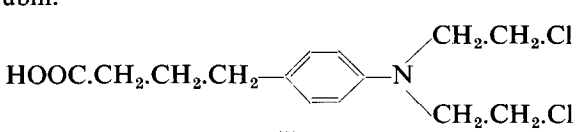
(*Received* 8 *February* 1961; *accepted* 1 *July* 1961)

Abstract—Three compounds of the nitrogen mustard class: *p*-N : N-di (B-chloroethyl) aminophenylbutyric acid, (I), 4-N : N-di (B-chloroethyl) amino-2-methyl-2′-carboxyl azobenzene, (II), and N : N-di (B-chloroethyl)-N′ : O-propylenephosphoric acid ester diamide, (III), have been studied. The rates of their hydrolyses in aqueous solutions and the rates of chemical condensation with proteins of human blood serum *in vitro* have been measured. These reactions proceed more rapidly with compound (I) than with (II) and (III). The compounds (I) and (II) are firmly adsorbed onto protein. They do not react with the bilirubin or lipochrome constituents of serum. The rate of hydrolysis of Chlorambucil is decreased in the presence of serum.

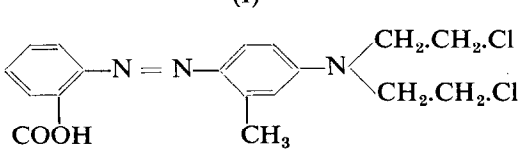
INTRODUCTION

THE chemical properties of the nitrogen mustards have been studied by a number of authors. Ross[1] compared the rates of hydrolysis of a number of nitrogen mustards in acetone–water solvent; the rate of hydrolysis increased in parallel with the ability of these compounds to cause regression of the Walker rat tumour. Stacey *et al.*[2] and Alexander and Cousens[3] reacted bovine serum albumin (BSA) in 0·5 M sodium bicarbonate solution with concentrations of alkylating agents in excess of the number of reactive carboxyl groups present in the protein. A 3% solution of crystallized BSA was made 0·12 M with respect to the bifunctional alkylating agents that were used. The rates of condensation were not determined. About 50 per cent of the available carboxyl groups were esterified by the nitrogen mustards. These authors demonstrated that all the different types of alkylating agents tested esterify carboxyl groups, only the epoxides react extensively with amino groups.

The present paper reports on investigations of some reactions of the following three drugs, (I), (II) and (III) of the nitrogen mustard type with dilute alkalis, serum proteins and bilirubin.

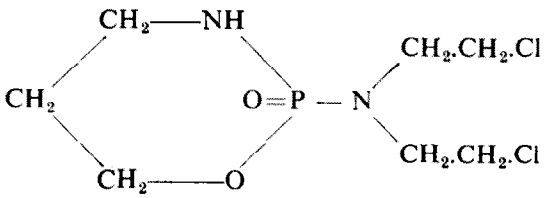


(I)



(II)

A

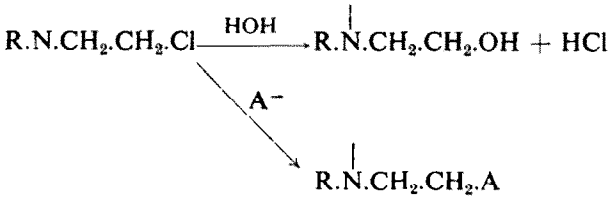


(III)

The molecular weights are as follows: acid form of (I), 304; sodium salt of (II), 402; (III), 261.

These compounds are in clinical use under the names of: (I) CB 1348 and Chlorambucil; (II) CB 1414; (III) B 518 and Endoxan. Compounds (II) and (III) differ from (I) in that they are considered to require modification of the molecule by body processes before the terminal chlorine atoms become reactive.[4–7]

The carboxylic compounds CB 1348 (I) and CB 1414 (II) are moderately soluble in water in the form of the sodium salt of the acid. B 518 is readily soluble (III). These compounds react with anions in solution to form esters; when the anion is an hydroxyl ion the alcohol derivative of the mustard is formed. The former process is usually referred to as alkylation of the acceptor molecule, the latter process as hydrolysis of the alkylating agent. The reactions may be represented by the general equation:



(IV)

The phenomena that were investigated included the absorption spectra in suitable solvents and the degree to which hydrolysis occurred under various conditions. These properties were useful for detection and for measurement of amount of the compounds. Studies of hydrolysis rates were made as quantitative knowledge might be of value in interpreting clinical results. It was also necessary to know how to prepare and to preserve solutions of the drugs in the active and hydrolysed forms.

The extent to which the alkylating agents reacted with the constituents of blood serum, for example by esterification of the carboxyl groups of protein, was of interest because this may be a source of loss of the active drug administered to patients. Furthermore, quantitative studies of these reactions would provide information on the activities of these drugs with respect to compounds of biological importance. Studies on the degree of adsorption of this type of drug to the colloidal protein phase of serum were undertaken because such an occurrence might limit the distribution of the drug.

REAGENTS AND APPARATUS

Absorption spectra were measured with a Hilger Uvispek photoelectric spectrophotometer. Titration procedures and pH measurements were made with a Beckmann H2 glass electrode pH meter. Ultrafiltration was carried out under nitrogen pressures of 40 lb/in^2 through "Visking" cellulose acetate membrane.

Volumetric solutions of 0·025 N sodium hydroxide and 0·050 N hydrochloric acid were prepared. For this purpose British Drug Houses Volumetric Solution 0·1 N hydrochloric acid was used as the primary standard. Concentrations of 0·5 M and less of sodium bicarbonate were prepared. Absolute ethyl alcohol was used as a protein precipitant. Solutions of 0·3 N barium hydroxide and 5 per cent zinc sulphate heptahydrate were prepared for use as a neutral protein precipitant.[8] These solutions were standardized relative to one another with the pH-meter by titrating the barium hydroxide solution into the zinc sulfate solution. One solution was adjusted by dilution, if necessary, so that equal volumes of each yielded a mixture having a pH value between 7 and 8. It was important to titrate the barium hydroxide into the zinc sulfate, as the former when present in excess appeared to be adsorbed onto the barium sulfate–zinc hydroxide precipitates. Bilirubin was obtained from Homburg of Frankfurt. Compound (I) was obtained from the Chester Beatty Institute, London, and from Burroughs Wellcome & Company; Compound (II) was obtained from the Chester Beatty Institute; Compound (III) was obtained from Asta-Werke A.G. The human blood serum was obtained by pooling the excess from samples taken for routine laboratory examinations. It was available from 2 to 3 hr after withdrawal from the patients.

EXPERIMENTAL TECHNIQUES

Detection and measurement of alkylating agents

In aqueous solution as the sodium salt, compounds (I) and (II) possess characteristic absorption spectra which served for their identification and measurement. Compound (I) has a maximum at 258 mμ of optical density 1·55 for a concentration of 30 μg per ml. Compound (II) has a maximum at 410 mμ of optical density 1·75 for a concentration of 30 μg per ml. These values are not affected by changes in the pH of the solvent from 7 to 11·5, nor are they affected by changes in sodium bicarbonate content from 0·025 M to 0·5 M.

In alcohol solution the optical density of (I) is increased to 1·85 but the curve shape is changed very little. The absorption maximum of (II) is shifted to 395 mμ with optical density 2·00. These values refer to concentrations of 30 mμ per ml.

The optical densities, to the measurable limit, were proportional to the concentrations of (I) and (II). Measurements of solutions that had been filtered or extracted from blood serum were compared to blank procedures of the same type carried out in the absence of the drug. Control solutions of the drug in aqueous solvent were processed in the same way. The absorption difference between a drug solution and its blank was compared with the controls by the method of Allan.[9]

Titration methods were also applicable because compounds (I), (II) and (III) are capable of undergoing hydrolysis in alkaline solution; equation (IV) refers. A sample of drug was allowed to hydrolyse completely in the presence of 10 ml of 0·025 N sodium hydroxide. The difference between the amount of standard (0·050 N hydrochloric acid required to titrate the alkali alone and that required to titrate

the alkali plus the added sample was equivalent to the amount of acid liberated. The conditions that brought about complete hydrolysis of each compound are discussed below.

Rates of hydrolysis in aqueous solutions

The rates of hydrolysis were ascertained by measurements of the acid released, equation (IV). Solutions of 75 mg (I), or of 100 mg (II), or of 65 mg (III) in 50 ml of alkaline solvent were prepared and placed in a constant temperature bath. Immediately, and at measured intervals of time, 10 ml volumes were withdrawn, pipetted onto crushed ice in a beaker and titrated at once with 0·050 N hydrochloric acid. The pure solvent was also titrated. The decrease with time in the amount of standard acid required to titrate each 10 ml sample to pH 6·5 ,was equivalent to the amount of acid liberated in the solution in that time. The solvents are listed in Table 1. In some instances the drug was dissolved in alkali and the solution was quickly adjusted to a lower pH for the rate experiment.

The above solutions contained $0{\cdot}50 \times 10^{-4}$ mole of drug in 10 ml. The acid released during hydrolysis did not alter appreciably the pH of 0·025 N sodium hydroxide or bicarbonate in the course of the experiments. Mixtures of sodium hydroxide and hydrochloric acid that were used to obtain pH values of 7·5 and 5 were appreciably reduced in pH by the amount of acid released from the drug. The drugs were allowed to react with these solvents in separate 10 ml volumes, the pH of which was adjusted to the starting value whenever it dropped 0·5 units.

Rates of alkylation in blood serum

The amount of compound (I), (II) or (III) which had condensed with serum protein, at a given time, was determined by alcoholic precipitation of the protein. Of the initial amount of drug, the fraction that was chemically combined was precipitated with the protein but the free drug remained in the supernatant alcohol. The amount in alcohol solution was determined by one of two methods.

For spectroscopic measurement of rates of alkylation, a 10 mg quantity of (1) or (II) was dissolved in 0·5 ml of 0·5 M sodium bicarbonate at 37 °C and diluted at once to 10 ml with serum at 37 °C. Immediately and at measured intervals of time 0·5 ml of the serum solution was diluted to 5·0 ml with ethanol. The mixture was stirred and centrifuged, then 1·0 ml of the supernatant was again diluted to 5·0 ml, or as necessary, with ethanol. The concentration of drug in the final solution was determined spectroscopically.

To study the effect of increased bicarbonate concentration, the drug was dissolved directly by shaking in 10 ml of serum made 0·5 M with respect to sodium bicarbonate. Measurements were made also on solutions of 150 mg of drug in 10 ml of serum, by an additional ten-fold dilution of the supernatant with ethanol.

Experiments were also carried out with the hydrolysed form of (I). Eight milligrams were dissolved in 2·0 ml of 0·5 M sodium bicarbonate and allowed to stand at 37 °C for 2 hr, by which time the chlorine atoms were replaced by hydroxyl groups. Eight millilitres of serum were then added. Over a period of 24 hr at 37 °C, samples were tested by alcohol extraction for content of free drug as described above.

In all methods, standard solutions were prepared with water in place of serum and controls were prepared with serum but no alkylating agent.

The second method of determining the quantity of drug in the alcohol supernatant, based on titration measurements, was developed for two reasons. It was used to study reactions of compound (III) which in solution was transparent to ultraviolet and visible light. Again, whereas light absorption measurements, when applicable, yielded values of both hydrolyzed and unreacted active alkylating agent that remained in solution, titration measurements would determine only the amount of unreacted active alkylating agent. A comparison of results obtained by light absorption methods and by titration methods would indicate the rate of hydrolysis of the drug in the presence of serum protein and the buffering salts.

To measure with the titration method the rate of alkylation of serum protein by (III), 65 mg were dissolved in 1·0 ml water and made up to 10 ml with serum maintained at 37 °C; the serum was $2{\cdot}5 \times 10^{-2}$ M with respect to the drug. Immediately and at measured intervals of time 2·0 ml of the serum was pipetted into 8·0 ml of ethanol. The mixture was stirred for 2 min and centrifuged. The precipitate was washed with 2·0 ml ethanol. The combined supernatants were poured into a conical flask and 10 ml of 0·025 N sodium hydroxide was added. The mixture was heated in a bath of boiling water for 60 min. A distillation head and condenser were fitted to collect the alcohol. Titration of the solution in the flask was then carried out with standard (0·052 N) hydrochloric acid. Control titrations were made on serum samples in the absence of the drug. The difference between the amount of acid required to titrate the zero-time sample and that required to titrate a control of serum only, was equal to 100 per cent of the drug in solution in the serum. Using the quantities and concentrations of the experiments this was 2·0 ml of 0·052 N acid. As alkylation proceeded the difference decreased if less drug was present in free solution in the active form.

The rate of alkylation of serum protein by compound (I) was measured by the titration method and by absorption spectroscopy. Compound (I) (215 mg) was dissolved in 3·0 ml of 0·5 M sodium bicarbonate and made up to 25 ml with serum at 40 °C. The serum was, therefore, $2{\cdot}85 \times 10^{-2}$ M with respect to (I) and 0·085 M with respect to sodium bicarbonate, assumng an initial bicarbonate content of 0·025 M. Two-milliliter samples were withdrawn and extracted for titration measurements as described for compound (III) except that hydrolysis in the alcohol–sodium hydroxide solution was carried out at 65 °C for 2 hr. In the presence of bicarbonate, the point of neutralization of the sodium hydroxide was taken as pH 8·5. For each 2·0 ml sample of serum withdrawn for titration, an additional 0·4 ml was also withdrawn and pipetted into 3·6 ml of ethanol. This was again diluted fifty-fold and the amount of (I) in solution was measured spectroscopically. Control titration and absorption measurements were carried out on a 25 ml volume of the serum maintained at 40 °C with no drug.

The drug was also measured spectroscopically in the chemically bound form. The protein precipitate from each 0·4 ml sample of serum was dissolved in 2·5 ml of 0·025 N sodium hydroxide and further diluted twenty-five-fold with water. The minimal quantity that could be detected was limited by the absorption of the protein. The maximum optical density due to protein that could be permitted was approximately 1·2, which is the value obtained when protein from 0·4 ml serum was dissolved in 15 ml of alkaline solvent. To detect (I) it must be present in concentrations of 5–10 μg per ml; thus about 100 μg must be combined in 0·4 ml of serum.

Physical binding of CB 1348 *(I) and CB* 1414 *(II) to protein*

To investigate the occurrence of physical binding when compounds (I), (II) or (III) were in solution in serum, a small fraction of the solvent phase was separated from the colloidal protein phase by ultrafiltration through "Visking" cellulose acetate membrane. The amount of drug present in the filtrates from (I) and (II) was determined spectroscopically; that from (III) was measured by titration.

A series of concentrations of each drug was prepared in 10 ml volumes of serum. Two millilitres of filtrate were collected in a graduated tube in 6 hr. Experiments with (II) were carried out at 40 °C and at 5 °C, with (I) at 5 °C. No alkylation occurred during this time. The initial concentrations of drugs (I) and (II) in serum were checked by an alcohol extraction as described above. A second 0·5 ml of the serum solution was left in proximity to the filter during the experiment and the free drug concentration was checked again at the end of the filtration period.

In the case of compound (I), experiments were also made with the hydroxyl form of the drug. Five milligrams were dissolved in 2·0 ml of solvent which was 0·025 N sodium hydroxide and 0·13 M sodium chloride. 1·0 ml of the solution was immediately diluted to 10 ml with serum at 5 °C, the remaining 1·0 ml was kept at 37 °C for 2 hr to hydrolyze, then made up to 10 ml with serum. The initial concentrations were checked by alcohol extraction and both solutions were ultrafiltered.

To measure the degree of physical binding of (III) to serum protein, three solutions were prepared of 40 mg, 20 mg, and no drug in 2·0 ml of water, which were made up to 12·0 ml with serum. From each solution 2·0 ml was removed for assay, the remaining 10 ml were subjected to ultrafiltration to obtain 2·0 ml of filtrate from each sample. The six 2·0 ml samples were mixed with 8·0 ml of ethanol and assayed for drug content by hydrolysis and titration as described above.

The degree of binding to protein as determined by the ultrafiltration method was the average result of 6 hr of contact. To determine whether rapid binding did occur, the protein in the serum was precipitated chemically immediately after the addition of drugs (I) or (II). The Somogyi method was used to keep the pH above 7·5. Volumes of serum–drug solution of 2·5 ml were stirred into 5·0 ml of 0·3 N barium hydroxide solution. Five millitres of an equivalent concentration of zinc sulphate were added and the mixture was stirred for 2 min, while 2·5 ml of serum and 2·5 ml of an aqueous standard solution of drug were treated in the same manner. A second 2·5 ml of the standard was diluted with 10 ml of water. After centrifuging, the concentrations of drug in the supernatant liquids were determined spectroscopically.

Interaction of CB 1348 *(I) with bilirubin and lipochrome constituents of serum*

The distribution of the bilirubin and lipochrome constituents, which are responsible for the yellow color of serum, was of interest in connection with the methods of precipitating protein from serum. When a fresh solution of (I) in serum was treated with the Somogyi reagent the drug was removed and so were the yellow constituents, along with the protein. When a similar fresh solution was heated with alcohol to remove protein, the drug remained in the supernatant as did also the yellow constituents. Experiments were carried out to determine whether a direct reaction was taking place between (I) and the lipochrome materials.

For the first experiment, 10 ml of serum was pipetted into 40 ml of ethanol, the mixture was stirred and centrifuged, the supernatant was decanted into a flask and

distilled under reduced pressure until about 6 ml of liquid remained. This was made up to 9 ml with water. To this yellow solution was added 1·0 ml of a solution which contained 1·5 mg (I) in 0·5 M sodium bicarbonate; 2·5 ml of this mixture was treated with 5·0 ml of barium hydroxide and 5·0 ml of zinc sulphate solution as for the removal of protein. The inorganic precipitant removed the bilirubin and lipochrome fractions completely, leaving a clear, colorless supernatant. In the same way, 2·5 ml of a standard aqueous solution of (I), which contained 30 μg per ml, was treated. The concentrations of drug in the supernatant liquids were measured spectroscopically.

For the second experiment a solution of 80 mg per cent bilirubin in 1·85 per cent sodium carbonate was prepared, which also contained 1·5 mg (I) in 10 ml. This solution and an aqueous standard of (I) were cleared by the Somogyi reagent as above and the colorless supernatants were examined spectroscopically.

RESULTS AND DISCUSSIONS

Rates of hydrolysis in aqueous solutions

Solution in alkali of the acid form of (I) resulted in the immediate release of 1 mole of hydrogen ion per mole of drug as the sodium salt was formed. The slower hydrolytic process was made evident by production of two additional moles of hydrogen ion. The titration measurements which indicated these processes in 0·025 N sodium hydroxide at 37 °C are shown in Fig. 1. During each titration the sample became cloudy

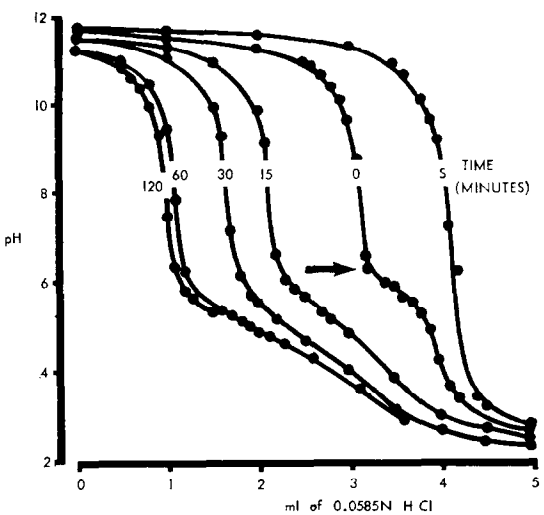


FIG. 1. Measurement of hydrolysis at pH 11·5 of CB 1348 in 0·025 N sodium hydroxide by titration of 10 ml volumes of $0{\cdot}50 \times 10^{-2}$ M solution with standard acid. (S) is solvent only. Numbers on curves are time in minutes at 37 °C.

at pH 6·2 because of formation of the non-ionized acid (I). This occurrence was shown also by the break in the titration curve at pH 6·2 as indicated by the arrow, Fig. 1. Similar experiments with compounds (I), (II) and (III) in a number of solvents yielded reactions which were all first order. Compound (II) began to precipitate from

solution as the acid form at pH 7·5. The degree of dependence of the rate constant on the temperature and the ion species present is shown in Table 1.

The rate constants of CB 1414 (II) and B 518 (III) at 37 °C are one-fiftieth or less that of the CB 1348 (I) reaction. The temperature coefficient of reaction of (1) is large; no appreciable hydrolysis occurred when stored at 5 °C for 24 hr. From the rate constants at 25 °C and 37 °C the activation energy of the hydrolytic reaction of (I) was calculated to be 23 kcal/mole.

The presence of 0·13 M sodium chloride did not alter significantly the hydrolytic reaction rate of (I). This indicates the reaction to be irreversible and it must be accompanied by a large decrease in standard free energy.

TABLE 1. EFFECT OF TEMPERATURE, pH AND COMPOSITION OF SOLVENT ON THE RATE OF HYDROLYSIS OF CERTAIN NITROGEN MUSTARDS

Drug	Temperature (°C)	pH	Solvent	Rate constant k
CB 1348	37	5	0·025 N NaOH 0·050 N HCl	0
CB 1348	37	7·5	0·025 N NaOH 0·050 N HCl	0·008
CB 1348	37	11·5	0·025 N NaOH	0·03
CB 1348	37	11·5	0·025 N NaOH 0·13 M NaCl	0·027
CB 1348	25	11·5	0·025 N NaOH	0·006
CB 1348	37	8·5	0·025 M $NaHCO_3$	0·04*
CB 1414	37	11·5	0·025 N NaOH	0·0005
B 518	37	11·5	0·025 N NaOH	0·0004

* Alkylation of bicarbonate anion to form an insoluble condensation product also occured.

At 37 °C, hydrolysis of (I) in 0·025 N sodium hydroxide, and in 0·025 M sodium bicarbonate, was complete in 120 min. In 0·025 N sodium hydroxide the hydrolysis of (1) at 60 °C was complete in 30 min; that of (III) at 100 °C was complete in 60 min. Rate constants were not measured at these latter two temperatures but this information was used in the hydrolytic and titration methods of assay.

Rates of alkylation in blood serum

The rate of condensation of (I) with serum at 37 °C is shown in Fig. 2(a). These data represent the differences in the amounts recovered in the alcohol soluble form, as measured spectroscopically. The zero time recoveries corresponded closely to 100 per cent concentration; in comparison with a standard aqueous solution they showed a small increase which corresponded to a 5 per cent decrease in solvent volume caused by precipitation of the protein. The reaction rate was not affected by the change from 0·025 M to 0·5 M concentration of sodium bicarbonate in the serum.

The logarithm of the concentration of unreacted compound (I) is plotted against

time in Fig. 2(b). The graph is a curve which may consist of two straight lines. Assuming the first linear portion to represent the reaction of a small amount of drug with excess of protein, the rate constant k is 0·0010 at 37 °C. At 25 °C the rate constant dropped to 0·0003. At 5 °C no detectable degree of alkylation occurred in 24 hr. The reaction has a marked temperature coefficient, the activation energy is 25 kcal/mole.

Of the compound (I) that was hydrolyzed prior to mixing with serum, 96 per cent was recovered after 17 hr at 37 °C. This confirmed that the decrease in the amount of (I) extractable by alcohol was due to an alkylation reaction.

Solutions in serum of CB 1414 (II) of concentration $4{\cdot}0 \times 10^{-3}$ M were subjected to alcohol extraction as for (I). By spectroscopic measurements, no appreciable change in the concentration occurred over a period of 24 hr at 37 °C. No alkylation of serum protein had occurred.

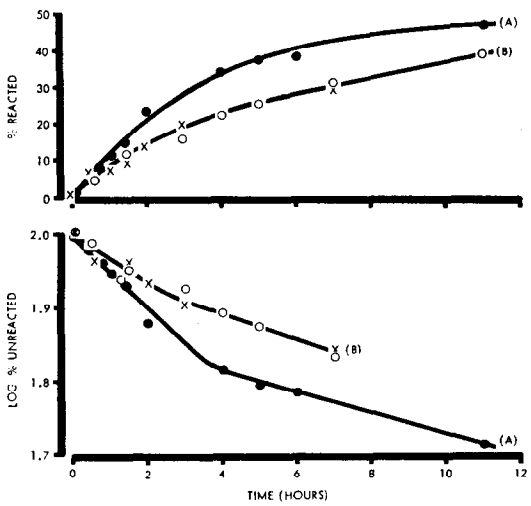


FIG. 2(a). Graphs showing the rate of the alkylation reaction of CB 1348 with serum protein, at 37 °C
FIG. 2(b). Data of Fig. 2(a) plotted as logarithm of percentage of unreacted CB 1348 in relation to time in hours. Curves A, concentration $5{\cdot}0 \times 10^{-2}$ M; curves B, concentration $0{\cdot}26 \times 10^{-2}$ M CB 1348 in serum. ○-○-○ and -●-●-● serum 0·5 M with respect to added bicarbonate. ×-×-× serum 0·025 M with respect to added bicarbonate.

Solutions in serum of B 518 (III) at concentrations of $2{\cdot}5 \times 10^{-2}$ M were subjected to alcohol extraction, By the titration method, recoveries over a period of 24 hr at 37 °C were 98–102 per cent. No alkylation of serum protein had occurred and no hydrolysis of the drug had taken place.

The rate of alkylation of serum protein by compound (I), the only one of the three drugs to react at a measurable speed, was determined by both titration and absorption measurements on the alcohol extracts. The difference between the two rate curves as plotted in Fig. 3 is the rate of hydrolysis of (I) ($2{\cdot}85 \times 10^{-2}$ M) at 40 °C in blood serum made 0·085 M with respect to sodium bicarbonate. In the presence of the serum 10 per cent of the drug was hydrolysed in 6 hr; in aqueous 0·025 M bicarbonate hydrolysis was complete in 2 hr. We may assume either that the thermodynamic activity

of the bicarbonate was reduced, or that the drug was protected, by the presence of serum protein.

The rate of alkylation by (I) as shown in Fig. 3, curve A was checked by re-dissolving the protein residues, followed by spectroscopic measurement of the amount of drug chemically combined with the protein. The results corresponded exactly with curve A on a relative scale. On an absolute scale, the amount condensed with the protein residue at 22.5 hr corresponded to 51 per cent of the added drug; curve A records 65 per cent of the added drug in the combined form at this time. The absorption coefficient of the drug in the chemically combined form may be less than the value in the free form; an optical density of 1·03 for a drug concentration of 30 μg per ml. in the combined state, would yield a rate curve identical with curve A.

No alkylation reaction with whole red cells occurred at 37 °C when (I) was incubated for 20 hr in a suspension of red cells in 1 % sodium chloride of volume equal

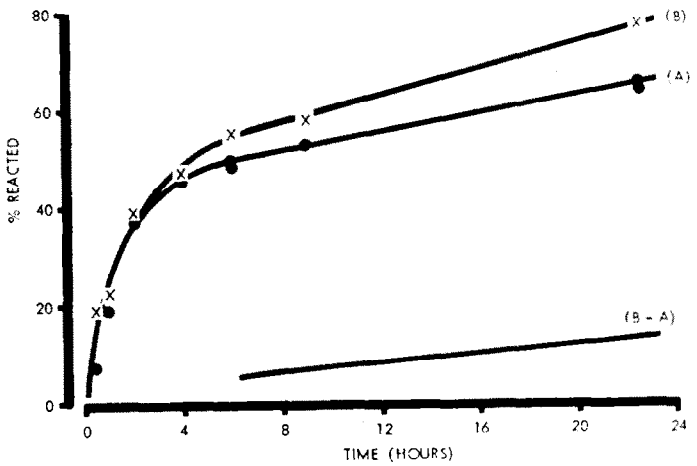


FIG. 3. Comparison of measurements of rate of reaction of CB 1348 2·85 × 10^{-2} M in blood serum (0·085 M sodium bicarbonate) at 40°C. Curve A, rate of alkylation reaction by absorption method; curve B, rate of alkylation reaction plus hydrolysis reaction by titration method. The numerical difference between curves B and A is plotted as measured from the graphs.

to the serum removed. At the end of the period 100 per cent of the drug present was recovered by alcohol extraction.

Interaction of (I) with bilirubin lipochromes

Solutions of (I) in aqueous bicarbonate, in the presence of a lipochrome extract of serum and in the presence of bilirubin, were treated with the Somogyi reagent. The solutions were rendered colorless by the reagent but the absorption of the supernatant liquid did not differ significantly from aqueous standards used as controls. No interaction of (I) with the bilirubin, or the lipochrome fractions of serum, could be detected.

Physical binding of CB 1348 (I), *CB* 1414 (II) *and B* 518 (III) *to protein*

The results of the ultrafiltration of solutions of compound (II) in blood serum are

shown in Table 2 and Fig. 4. Table 2 refers to measurements made over a wider range of concentrations, Fig. 4 refers to a second series of experiments over a range of lower cencentrations. No alkylation reaction occurred under the experimental conditions.

The values in Table 2 show that 95–99 per cent of the drug was not in free solution. As the total amount present could be extracted by alcohol it may be assumed that the drug was adsorbed to the protein. The degree of adsorption was the same at 5 °C and 40 °C. The relative amount of drug in the bound form decreased rapidly at drug concentrations greater than 15 mM.

TABLE 2. DISTRIBUTION OF SODIUM SALT OF CB 1414 (II) IN BLOOD SERUM BETWEEN PROTEIN-BOUND AND FILTRABLE STATES

40 °C				5 °C			
Bound (m-moles/l.)	(%)	Filtrable (m-moles/l.)	(%)	Bound (m-moles/l.)	(%)	Filtrable (m-moles/l.)	(%)
1·02	99·3	0·007	0·7				
2·38	99·4	0·014	0·6				
3·46	99·2	0·029	0·8	3·24	99·3	0·022	0·7
3·67	98·9	0·040	1·1	5·88	99·5	0·031	0·5
4·70	99·2	0·039	0·8	5·97	98·8	0·069	1·2
5·28	98·7	0·066	1·3	11·7	98·2	0·210	1·8
9·75	98·6	0·135	1·4	13·6	96·1	0·528	3·9
12·8	97·0	0·380	0·3	13·9	96·5	0·488	3·5

The results of the second series of experiments are plotted in Fig. 4(a), curve A, the shape of which corresponds to an adsorption isotherm. This is borne out by the plot in Fig. 4(b), curve A, which shows an approximately linear relationship of the logarithms of the concentrations in the solid and solution phases. At concentrations above 4 mM the relative amount bound to protein appeared to increase. This may indicate the occurrence of denaturation of the protein.

The results of ultra-filtration of solutions of compound (I) in blood serum are shown in Table 3. The experiments were carried out at 5 °C at which temperature no alkylation reaction occurred.

The measurements demonstrated that CB 1348 (I) was adsorbed to a high degree in the presence of protein but the amount bound decreased more rapidly with increase of drug concentration than was the case with compound (II). This suggested that the benzene ring was responsible for the adsorption. Also, replacement of the active chlorine atom of (I) by hydroxyl greatly reduced the degree of physical binding.

The ultrafiltration of solutions of B 518 (III) of molarities $1{\cdot}4\times10^{-2}$ M and $0{\cdot}7\times10^{-2}$ M in blood serum yielded filtrates which did not differ appreciably in concentration from the initial serum solutions. No measurable adsorption had occurred, but a reduction of concentration of less than $1{\cdot}0\times10^{-3}$ M would not be detected.

Chemical precipitation of the serum proteins by the Somogyi reagent in the presence of (I) and (II), removed the drugs to a great extent. At concentration $0{\cdot}5\times10^{-2}$ M in serum, compound (I) was removed completely since the resultant spectrum was

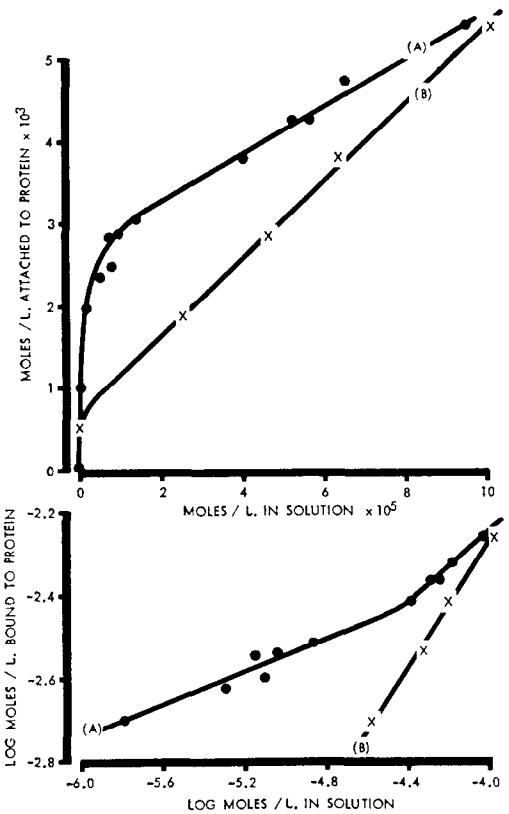


FIG. 4(a). Adsorption isotherms of CB 1414 at room temperature relating molar concentration in free solution to the number of moles bound in one litre of human blood serum.
FIG. 4(b). Date of Fig. 4(a) plotted as logarithm of quantity of CB 1414 bound to protein in relation to logarithm of concentration in free solution. Curves A, equilibrium concentration of free CB 1414 determined by ultrafiltration. Curves B, concentrations of free CB 1414 determined by chemical precipitation of protein.

TABLE 3. DISTRIBUTION OF SODIUM SALT OF CB 1348 (I) IN BLOOD SERUM AT 5 °C BETWEEN PROTEIN-BOUND AND FILTRABLE STATES

Bound (m-moles/l.)	(%)	Filtrable (m-moles/l.)	(%)
0·822	100	0·0	0·0
2·21	97·1	0·066	2·9
3·45	94·9	0·184	5·1
3·88	92·3	0·324	7·7
4·37	92·8	0·338	7·2
6·38	86·8	0·972	13·2
6·90	80·9	1·63	19·1
*0·32	37	0·55	63

* Chlorine atoms in (I) replaced by hydroxyl groups.

indistinguishable from that of serum treated in the absence of the drug (Fig. 5). Only a small change in light absorption occurred when the aqueous control solution was subjected to the Somogyi reagent.

In the control solutions that contained drug but no serum, the salt precipitate decreased the volume of liquid from the 12·5 ml total of drug solution plus reagents to 10·5 ml. When solutions of (I) of concentration greater than 40 μg per ml were treated with the Somogyi reagent a perceptible quantity of drug adhered to the barium sulfate–zinc hydroxide precipitate. Of a total of 375 μg of (I) present in 2·5 ml of solution, 11 per cent was extracted by alcohol from the salt precipitate; on the basis that 89 per cent of the drug remained in the supernatant liquid, the volume of solvent calculated from optical density measurements of (I) agreed with the measured volume of 10·5 ml.

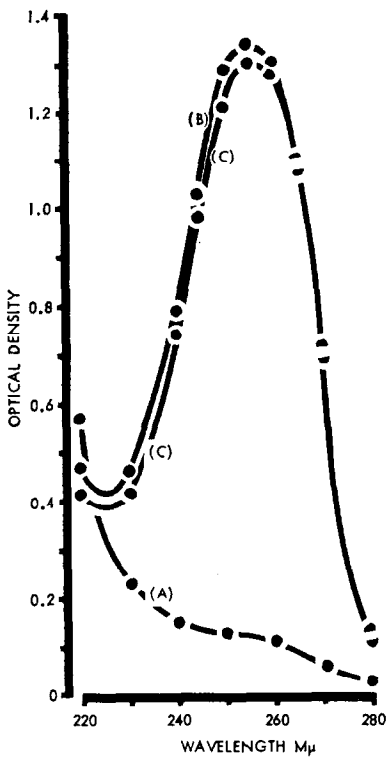


FIG. 5. Graphs showing the adsorption to the protein components in human blood serum of CB 1348 in 0·50 $\times$ 10^{-2} M solution. Curve A, absorption spectrum of supernatant after precipitation of serum proteins by Somogyi reagents. Curve B, absorption spectrum of supernatant after treatment of aqueous control solution by Somogyi reagents. Curve C, aqueous control solution untreated.

When serum solutions were treated with the Somogyi reagent, the serum–salt precipitate reduced the liquid volume from 12·5 ml to 7·5 ml. The concentration changes were corrected for this decrease of solvent volume.

The protein–salt precipitates were extracted with alcohol. The precipitate from a freshly prepared drug solution yielded 100 per cent of the drug content of the serum.

The precipitate from a solution of (I) in serum which had been incubated at 37 °C for 6 hr yielded 50 per cent of the drug present.

The distribution of (II) between the solvent phase and the colloidal protein phase of serum, as determined by chemical precipitation of the protein, is shown by curves B of Figs. 4(a) and 4(b). Compared to the values obtained by ultrafiltration, a greater proportion was in the solvent phase for the same initial concentration of (II) in the serum. This probably was caused by the dilution of the serum which occurred when the Somogyi reagents were added. In comparison with the values obtained by ultrafiltration, the rate of increase of adsorbed drug with increase of drug concentration was greater. This indicates greater adsorption of (II) to denatured (precipitated) protein than to protein in the native state. The change in the slope of curve A, Fig. 4(b), at concentrations greater than 4×10^{-3} M is in the direction of the slope of curve B. This suggests that high concentrations of (II) act as a protein denaturant.

No adsorption of (I) to red cells occurred. A freshly prepared solution of (I) in whole blood was centrifuged to separate the plasma and red cell components. All the drug was in the serum phase.

CONCLUSIONS

Certain effects of the structures of alkylating compounds, on properties which may be involved in the clinical applicability of these compounds, have been demonstrated. The three compounds of the β-chloroethylamine type that were studied have analogous clinical application but differ among themselves in their reactivities *in vitro* in some respects. Compounds (II) and (III) differ from (I) in that they undergo hydrolysis in aqueous alkaline solution at a much slower rate. In parallel with this difference in reactivity, compound (I) undergoes a condensation reaction with serum protein whereas compounds (II) and (III) do not react appreciably. The same activation energy of 24 kcal applies to both the hydrolytic and alkylation reactions of (I). These reactions appear to be accompanied by a large decrease in standard free energy, because neither the rate nor the extent of the hydrolytic reaction are affected by the presence of an excess of chloride ion, and the alkylation of blood serum by 0·0026 M (I) proceeds in the presence of the chloride content of serum, which is approximately 0·1 M.

As clinical effectiveness must depend upon the occurrence of the alkylation reaction, compounds (II) and (III) may be modified within the body or may react more rapidly with groups other than the carboxyl of protein. In this connection, the hydrolytic and serum protein condensation reactions of compound (I) are probably sources of waste of the administered drug. The half-life of the condensation reaction is about 6 hr; the rate of hydrolysis in the presence of serum protein is relatively unimportant. The condensation reaction has a high temperature coefficient which could conceivably favor specific tissues. *In vitro* no alkylating reaction occurred between compound (I) and the bilirubin or lipochrome components of blood serum, nor with red cells in suspension in saline.

The proteins of blood serum adsorb compounds (I) and (II) rapidly, and at concentrations comparable to those prevailing *in vivo* adsorption is nearly complete. No adsorption of (III) could be detected. The binding forces appear to be van der Walls type since the benzene rings of the drugs are involved and the bonds are broken

by solvents such as ethanol. This physical binding must limit the extent of diffusion of (I) and (II) but at certain locations they must be released to penetrate cell membranes. No adsorption of (I) or (II) to whole red cells could be detected.

The methods which were developed have enabled measurements to be made of the rate and extent of reaction of these alkylating agents with serum protein in colloidal suspension. Applied to other tissues and particles, similar methods may be of use in determining whether such drugs exhibit selectivity of action. The methods are not sufficiently sensitive to investigate reactions of the drugs in concentrations approaching those that may be present during clinical use of the drug (less than 0·03 mg per ml of serum). The titration method of detecting changes in drug concentration required a minimum amount of from 2 to 3 mg (Fig. 1). This amount could be reduced to one-fifth by use of a more sensitive pH-meter fitted with an external short-range scale. Assay of the free drug (I) in serum by alcohol extraction was not applicable to concentrations of less than 0·3 mg per ml of serum; assay of the protein-condensed form in alkaline solution was limited to the same degree. These limitations due to extraneous absorption might be overcome by the use of a double-beam spectrophotometer which would be able to compare the drug solution with a reference solution of low transmission.

Experiments are being made at present to overcome the limitations so that the alkylation reaction of small amounts of (I) with blood serum can be followed to completion and the rate of disappearance of administered drug from the blood may be studied.

Acknowledgement—This work was supported by a grant from the National Cancer Institute of Canada.

REFERENCES

1. W. C. J. Ross, *Ann. N. Y. Acad. Sci.* **68,** 669 (1958).
2. K. A. Stacey, M. Cobb, Sheila F. Cousens and P. Alexander, *Ann. N. Y. Acad. Sci.* **68,** 682 (1958).
3. P. Alexander and Sheila F. Cousens, *Biochem. Pharmacol.* **1,** 25 (1958).
4. W. C. J. Ross and C. P. Warwick, *Nature, Lond.* **176,** 298 (1955).
5. W. C. J. Ross and G. P. Warwick, *J. Chem. Soc.* 1364, 1719. 1724 (1956).
6. N. Brock and H. Wilmanns, *Ger. Med. Monthly* **111,** 189 (1958).
7. H. Arnold, F. Bourseaux and N. Brock, *Nature, Lond.* **181,** 931 (1958).
8. M. Somogyi, *J. Biol. Chem.* **160,** 69 (1945).
9. W. M. Allan, *J. Clin. Endocrin.* **10,** 71 (1950).