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THE RECOVERY OF FREE CHLORAMBUCIL FROM SOLUTION IN BLOOD SERUM

J. H. LINFORD

Department of Biochemistry, University of Manitoba, Winnipeg, Canada

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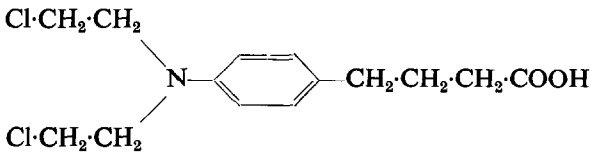
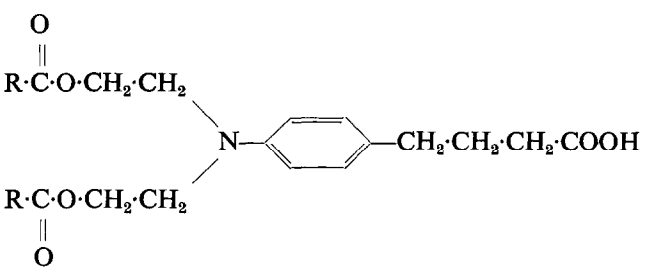
Abstract—A method has been developed for the isolation and measurement of chlorambucil present in serum or plasma in the chemically uncombined form at concentrations of 2 μg/ml or greater. Applied to the condensation reaction of chlorambucil with human serum, and dog serum and plasma, *in vitro*, the method has shown the reaction to be first order throughout its course, when the initial concentration of drug is less than 0·6 mg/ml. The value of the rate constant of the condensation reaction in dog serum is three times that of the reaction in human serum; this may be attributed to different sites of adsorption of the chlorambucil. The applicability of the method has been indicated by assay of active and hydrolyzed chlorambucil in the circulatory plasma of dogs.

CHLORAMBUCIL (I) is an alkylating agent of the nitrogen mustard type, which reacts with the protein of human blood serum to form a condensation product. The reaction approximates to first-order kinetics and has a large temperature coefficient. In 24 hr at 37 °C, 65 per cent of chlorambucil condenses with protein; at 40 °C close to 80 per cent reacts.[1] Stacey *et al.*[2] as well as Alexander and Cousens,[3] showed that the product of alkylation of protein is a diester of two carboxyl groups, which may be attached to the same polypeptide chain or may cross-link two different chains (II).



(I)



(II)

Hydrolysis of the salt of chlorambucil occurs in alkaline solution, with replacement of the chlorine atoms by hydroxyl groups. In aqueous sodium hydroxide or bicarbonate

at 37 °C the hydrolytic reaction is complete in 2 hr; slow alkylation of bicarbonate ion also occurs to form an insoluble condensation product. In the presence of blood serum at pH 8·5 or less, the hydrolysing reaction is very slow. Experiments using ultrafiltration have shown that chlorambucil at low concentration is adsorbed to a high degree to the protein fraction of serum; the hydroxyl derivative is less firmly adsorbed.[1, 4] These physically bound forms are readily extracted with alcohol, and references in this paper to "free" amounts of the drug include the physically bound form in contradistinction to the chemically bound or condensed form.

In order to measure the concentration of circulating chlorambucil in patients undergoing treatment, or in animals under test, some experiments have been undertaken to extract the free chlorambucil from blood serum. The methods and the results are described in the present paper. Because measurements of light absorption were used for detection and assay of the drug in solution, studies were made of the effect of pH and of solvent upon the absorption spectrum. In order to devise some method to remove free chlorambucil from aqueous solution, a knowledge of its solubility properties was required. A method was then devised to recover free drug from human serum *in vitro* and from the serum or plasma of dogs when the drug was present at concentrations of 2μg/ml or greater. With this method a preliminary survey has been made of the rate of disappearance of injected chlorambucil from the circulation of dogs.

MATERIALS AND METHODS

To neutralize strong acid with a minimal increase of volume of solution, an alkaline solution was prepared 3 M with respect to dipotassium hydrogen phosphate and 2·5 N with respect to potassium hydroxide. Of the phosphate, 131 g was dissolved in 100 ml of water; 42 g of the hydroxide (85 per cent) was dissolved in 30 ml of water. The solutions were cooled in ice, mixed slowly, and made up to 250 ml with water.

Absorption spectra

Absorption measurements were made with a Hilger Uvispek spectrophotometer with quartz cells having 1·00-cm length of light path. The absorbance ($\log_{10} I_0/I$) for a 1·00-cm light path is referred to as optical density throughout this paper. The amount of chlorambucil present in aqueous or alcoholic solutions was deduced by direct subtraction of the measured absorptions of the control-serum extract from those of the drug-serum extract. The amount of chlorambucil present was also deduced by applying the method of Allan[5] to the experimental absorption spectrum to correct for extraneous linear absorption or scattering.

Solubility properties

Alkaline aqueous solutions of the reactive (chloro) form of chlorambucil were prepared as the sodium salt, either directly in 0·025 N sodium hydroxide, or first in 0·5 M sodium bicarbonate after which the solution was diluted with water until it was 0·05 M with respect to bicarbonate. When the drug had dissolved at room temperature the solution was immediately chilled in ice-water and kept at 5°. To obtain the hydroxyl form of chlorambucil as the sodium salt, a solution in sodium hydroxide was maintained at 37 °C for 120 min or at 60 °C for 30 min; or a solution in 0·5 M bicarbonate was heated at 100 °C for 30 min before the dilution with water.

To prepare a solution of the active (chloro) form in acid, a weighed amount of chlorambucil was dissolved in 1·0 ml of 2·5 N hydrochloric acid, and this was diluted to 10 ml with distilled water. To prepare a solution of the hydroxyl form in acid a weighed amount in 1·0 ml of 0·5 M sodium bicarbonate was first hydrolysed; this was diluted to 5 ml with water and then to 10 ml with 5 N hydrochloric acid.

Extraction with ethyl alcohol

Alcohol solutions were used as controls for extraction experiments with water-immiscible solvents and as a check on weighing and dilution procedures. For these purposes 0·5 ml of aqueous solution was diluted to a known volume with a minimum of 10 vols of alcohol. If protein was present the supernatant solution was recovered by centrifugation. The concentration was then determined by spectrophotometric absorption measurements.

Extraction with benzene and ethyl acetate

To study the partition of chlorambucil between water and solvents immiscible with water, series of aqueous solutions of both the chloro and hydroxyl forms were prepared within pH ranges 1·5 to 8·5. These solutions, of concentration 1·2 and 0·075 mg/ml, were made first in acid, because no hydrolytic action occurred at low pH values. The pH was then adjusted to the required higher value by titration of 1·0 ml of solution with the alkaline phosphate-potassium hydroxide solution. The alkaline solution was diluted before use if convenient, but conditions were so chosen that the volume of solution at the desired pH could be brought up to 5·0 ml with water.

Two series of each of the chlorine and hydroxyl forms of chlorambucil were prepared and one 5·0-ml sample of solution of each form was extracted with benzene and one with ethyl acetate, by shaking in a stoppered test tube with 5·0-ml, 3·0-ml, and 2·0-ml vols in succession. In later experiments three successive 5·0-ml vols of organic liquid were used. The organic solvent was removed by pipet and was evaporated under reduced pressure on a hot-water bath. The residue was maintained for a further 15 min in a vacuum desiccator under suction from a water pump, and was then dissolved in 5·0 ml of ethanol. Spectrophotometric absorption measurements were made on the alcohol solutions, diluted as necessary.

Extraction of chlorambucil from blood serum

From the results of the solubility experiments and the fact that benzene and ethyl acetate are able to remove physically bound chlorambucil from protein, a technique was developed for isolating and measuring chlorambucil present in blood serum at concentrations of 2 μg/ml or greater.

To 1·0 ml of serum, 0·1 g of sodium chloride and 1·0 ml of 5 N hydrochloric acid were added. The mixture was stirred, then heated in a bath of boiling water for 5 min. The tube was cooled, and the pH of the contents was adjusted to a value of 3·6 with the alkaline phosphate-potassium hydroxide solution. The volume was made up to 5·0 ml with distilled water. This aqueous solution was extracted three times with 5·0-ml vols of benzene which were pooled, and evaporated under reduced pressure. The residue was kept 15 min in a vacuum desiccator to remove traces of benzene vapor. The residue was dissolved in 3·0 ml of absolute ethanol, or more if required, and the chlorambucil content was measured spectroscopically.

The added sodium chloride resulted in lower background of the optical density of the control solution, probably by reducing the amount of water and water-soluble material in the benzene. The acid served the purpose of stopping the chlorambucil-protein condensation reaction, which does not occur at low pH values, and the short period of heating the protein solution in the presence of acid prevented the formation of thick gels during the benzene extraction. This partial hydrolysis did not liberate chemically combined chlorambucil in a form that is soluble in benzene or ethyl acetate. The aqueous phase contained highly absorbing material, which was readily soluble in alcohol, so care was taken that no trace of the water layer was removed with the benzene.

To extract the hydroxyl form of chlorambucil the solvent ethyl acetate was substituted for benzene.

Later, an alternative method of extracting the serum was developed that eliminated the use of the 5 N hydrochloric acid but required two steps involving vacuum distillation. To 1·0 ml of serum 20 mg of sodium bicarbonate and 10 ml of ethanol were added. The mixture was stirred, centrifuged, and the alcohol decanted from the precipitate. The latter was washed with a further 2 ml of ethanol. The combined alcohol solutions were evaporated to dryness under reduced pressure. To the residue was added 5·0 ml of 0·1 M phosphate buffer, pH 3·6, which was extracted with benzene or with ethyl acetate as described.

A comparison of the rates of condensation of chlorambucil with the protein of dog serum and dog plasma and with the protein of human serum was made *in vitro* under conditions as similar as possible. The 10-ml sample of serum or plasma was obtained 15 min after withdrawal of blood from the subject, and to this was added 2·0 ml of 0·5 M sodium bicarbonate in which 6 mg of chlorambucil had been dissolved within the previous 2 min. The solutions of drug were then incubated at 40 °C, and samples were withdrawn at intervals for assay of free chlorambucil by the benzene extraction method.

Assay of free, active chlorambucil in the circulation of dogs

The drug was prepared for injection into a 10-kg dog by dissolving 15 mg of chlorambucil in 1·5 ml of 0·5 M sodium bicarbonate and warming at 37 °C for 3 min. This solution was then immediately mixed with 5 ml of heparinized plasma, obtained from blood freshly drawn from a leg vein. This drug preparation was reinjected into the leg vein as soon as mixing was complete. The quantity of drug, 1·5 mg/kg dog weight, was calculated to produce a concentration in the plasma of about 40 μg/ml.

The concentration of drug in plasma was determined from 3-ml samples of blood, which were withdrawn from the other leg. One control sample was taken prior to the injection; the injection lasted 20 sec. Between the injection and withdrawal of the first blood sample, 3 min was allowed to elapse in order to permit complete mixing. Other blood samples were withdrawn at 3-min intervals for 18 min, then at 30 and 60 min after the injection. By centrifuging and pipetting, 1·0 ml of heparinized plasma was obtained from each sample and was assayed as described for blood serum.

A comparison was made of the rates at which the active chloro form and the inactive hydroxyl derivative of chlorambucil were removed from the circulating system of the same dog. For these experiments the acid form of the active drug, 1·5 mg/kg of weight of the dog, was dissolved in 1·0 ml of 1 N potassium hydroxide. To retain the active

form this was chilled immediately, adjusted to pH 9 with ice-cold 1 N hydrochloric acid, and mixed with 5 ml of whole blood freshly drawn from the dog. To obtain the hydroxyl form, the solution in potassium hydroxide was shaken at 60 °C for 30 min. The whole blood, which contained the drug in solution, was reinjected into the leg vein of the dog, and samples were withdrawn for assay as described. Ethyl acetate extraction was used for both forms of the drug.

RESULTS AND DISCUSSION

Absorption spectra

The absorption spectra of chlorambucil in ethanol and in 0·1 M sodium bicarbonate are shown in Fig. 1. No appreciable change in the absorption of aqueous solutions

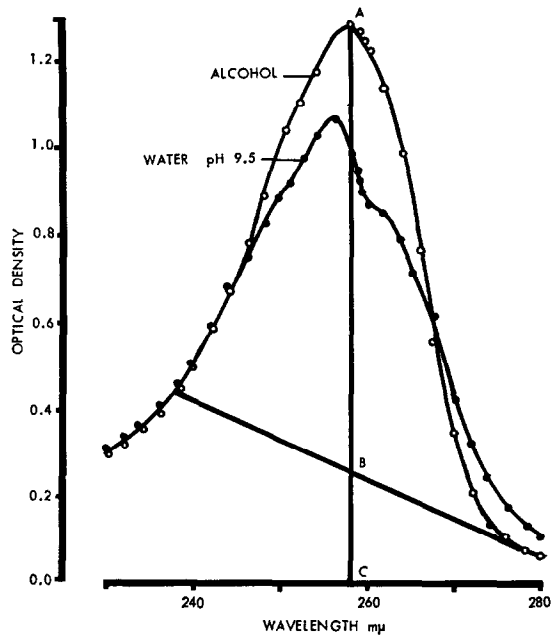


FIG. 1. Absorption spectra of chlorambucil in alcohol and in water solvents. Concentrations 20·8 μg/ml.

occurred between pH 7 and 11·5. In alcohol solution at concentration of 30 μg/ml the maximal optical density at 258 mμ is 1·85. In water solution in the form of the sodium salt, at pH 7 or greater, the same concentration has a maximal optical density of 1·55 at 256 mμ. The difference in absorption intensity may arise because chlorambucil is not ionized in alcohol but is in the anionic state in the aqueous solution. No difference in the shape of the absorption curve in alcohol was discernible when the crystalline form (I) was dissolved directly in alcohol and when an alkaline solution in water was diluted with 9 vols of alcohol.

The application of the Allan method[5] to the analysis of absorption spectra to determine the amount of chlorambucil present is illustrated in Fig. 1. With reference to the values in alcohol solution, the point B is one-half the sum of the optical densities

at 238 and 278 mμ. With pure chlorambucil the distance AC is 1·25 times the distance AB. To analyse impure extracts, the optical density value AB is obtained from the experimental absorption curve and is multiplied by the factor 1·25 to give the optical density at 258 mμ attributable to chlorambucil only, although extraneous absorption that is a linear function of wavelength may also be present. For analyses of absorption curves in water solvent, values at 234, 256, and 278 were used; the ratio AC to AB is 1·30 at pH 9·5.

Chlorambucil dissolved readily in aqueous solutions at pH 2·0, but the optical density was reduced to one-third that of the same concentration of the anionic form and decreased further as the pH value was adjusted down to 0·5. The shape of the curve changed only in the region of the inflexion at 261 mμ (see Fig. 1). On the other hand dilution of a concentrated aqueous solution of chlorambucil at pH 2 with 9 vols of alcohol yielded an absorption intensity 20 per cent greater than that of a corresponding concentration of drug that had been dissolved directly in alcohol. In alcohol containing 20 per cent or more of water this absorption intensity was greatly reduced.

The results indicated that, in aqueous solution at pH values less than 2·0, the cation of chlorambucil is formed by attachment of a proton to the nitrogen atom. In solution in alcohol the hydrogen chloride form of chlorambucil has an absorption intensity appreciably greater than that shown by chlorambucil. In alcohol containing 20 per cent or more of water, ionization to the cationic state occurs, and the absorption intensity is decreased.

That the absorption band of chlorambucil is associated with the benzene ring is indicated by comparison with other compounds that contain this ring.[6–8] The unshared electron pair on the nitrogen atom of chlorambucil does not appear to influence the absorption; however a positive charge on the nitrogen atom does reduce the capability of the ring to absorb light in aqueous solution.

Solubility properties

As the sodium salt, chlorambucil was soluble in aqueous solvents; titration of the chloro and hydroxyl forms in alkaline solution with dilute hydrochloric acid showed that the solubilities of these two forms differed very little. The chloro form precipitated perceptibly at pH 6·2, the hydroxyl form at pH 5·8, as the un-ionized carboxylic acids. At pH 2·0 the drug was redissolved as the hydrogen chloride salt in the cationic state. Chlorambucil in both the chloro and hydroxyl forms was soluble in alcohol; aqueous solutions or suspensions at any pH value will remain in solution or be dissolved upon the addition of alcohol.

The results of the partition experiments are shown in Fig. 2. The hydroxyl form was insoluble in benzene and was soluble in ethyl acetate only in the un-ionized state. The chloro form was soluble in benzene in the un-ionized carboxyl form and in the cationic state and was soluble in ethyl acetate in the ionized and un-ionized states. The pH range at which the ionized carboxyl states of the chloro and hydroxyl forms existed in aqueous solutions, as deduced from these experiments, agreed with that deduced by the titration experiments.

At the optimum pH of 3·6, about 80 per cent of the drug was extracted from the 5 ml of aqueous phase by successive 5·0- 3·0- and 2·0-ml vol of the organic solvent. A second series of extractions removed 80 per cent of the remaining drug from the aqueous phase. In later experiments using three successive 5·0-ml vols of organic

solvent, 88 per cent of the chlorambucil present in 5·0 ml of aqueous solution at pH 3·6 was extracted.

In addition to the major changes in solubility noted above, reference to Fig. 2 shows minor changes in the partition between aqueous and organic phase, with change of pH of the former. These variations are not of practical importance in the extraction process, but they have persisted throughout a number of trials and appear to indicate subtle changes with pH in the chlorambucil-solvent relationship. It is of

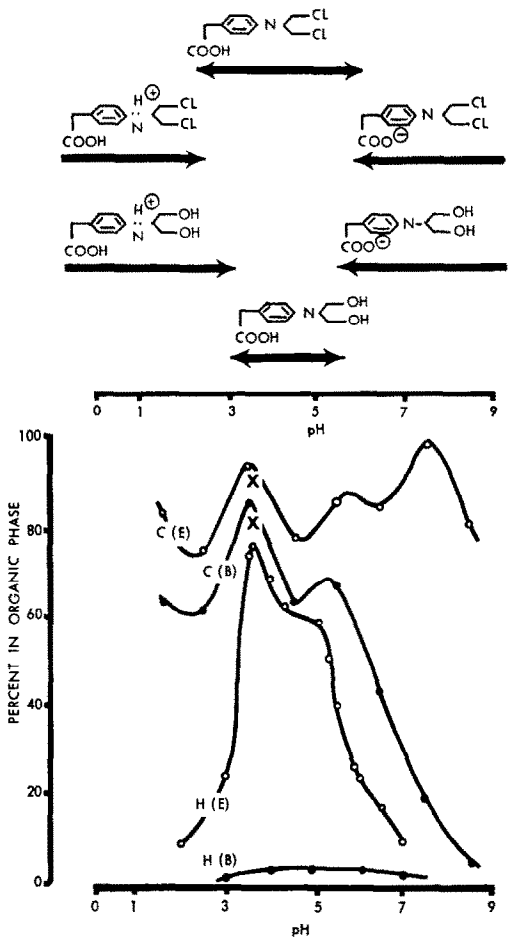


FIG. 2. Partition of chlorambucil, C, and the hydroxy derivative, H, between aqueous solvents at various pH values and the organic solvents ethyl acetate (E) and benzene (B). Formulae indicate the ionic state of chlorambucil in the aqueous solvent. Points X refer to use of blood serum in place of water.

interest to note that the nonsolubility of the hydroxyl form in benzene, in contrast to the solubility of the chloro form, is paralleled by a low degree of adsorption of the hydroxyl form to protein, in contrast to the high degree of adsorption of the chloro form.[1] Both processes involve Van der Waal's forces, which, in the case of the protein binding, have been associated with the benzene ring.

The extraction of free chlorambucil from human blood serum

The benzene and ethyl acetate extractions of chlorambucil from serum by the first technique described in the section on methods gave recoveries comparable with those from aqueous solutions. In Fig. 2 the recoveries from serum are plotted as X.

The benzene extract contained little besides the free chlorambucil. The absorption spectra of extracts from serum containing chlorambucil and of extracts of the serum only are shown in Fig. 3 (top). The difference between the two spectra closely resembles the absorption curve of the pure drug. Curve A represents a concentration in alcohol of 1·3 μg/ml. Curve B represents a concentration of 2·4 μg/ml. The total volume of alcohol in which the residue from the benzene extraction was dissolved was 4·0 ml; a minimum of 2·5 ml was required to fill the cells. The serum thus contained extractable amounts of 5·2 and 9·6 μg per ml for A and B, respectively. The use of microabsorption cells would increase the sensitivity of the measurements, as would the conversion of the chlorambucil to the protonated nitrogen form by the addition of 5 N hydrochloric acid to the residue prior to the addition of the alcohol. The absorption of the control serum and, consequently, the background absorption of the serum-drug solution increases with time of incubation.

With the benzene extraction method, the condensation reaction of chlorambucil in human serum was followed over a long period of time. The results are shown in Table 1.

TABLE 1. MEASUREMENT OF RATE OF CONDENSATION OF CHLORAMBUCIL WITH HUMAN BLOOD SERUM AT 39 °C BY ASSAY OF FREE CHLORAMBUCIL WITH BENZENE EXTRACTION METHOD

Initial concentration of chlorambucil: 0·620 mg/ml.

Time (hr)	Free amount (mg/ml)	Percentage of free chlorambucil (time zero = 100)	Condensed amount (mg/ml)	Percentage of condensed chlorambucil (time zero = 0)
0	0·530	100	0	0
2	0·457	86·2	0·073	13·8
4	0·399	75·4	0·131	24·6
6·5	0·309	58·4	0·221	41·6
11·5	0·224	42·3	0·306	57·7
28	0·080	15·0	0·450	85·0
31	0·068	12·8	0·462	87·2
71	0·027	5·1	0·503	94·9
95	0·016	3·0	0·514	97·0
143	0·009	1·7	0·521	98·3

The recovery was 86 per cent at zero time and probably 65–70 per cent toward the end of the period of incubation, when only small amounts of free chlorambucil were present. The condensation reaction was first order with a rate constant $k = 0{\cdot}0014$ at 39 °C, with time measured in minutes. At these concentrations a linear relationship of logarithm of concentration to time was found throughout the reaction. This was not the case with the earlier results[1] obtained with ten times this drug concentration.

The fact that the chloro form of chlorambucil was soluble in both benzene and ethyl acetate, whereas the hydroxyl form was soluble in ethyl acetate only, enabled the rate of hydrolysis of chlorambucil to the hydroxyl form in serum to be checked. A solution

containing 0·58 mg of chlorambucil/ml of serum was prepared and incubated at 39 °C. At various intervals 1·0-ml samples were withdrawn for assay by extraction with benzene or with ethyl acetate.

The amount extracted by benzene decreased with time to a value of 8·2 per cent in 48 hr; the amount extracted by ethyl acetate approached 16 per cent in the same time. The results are plotted in the lower half of Fig. 3 as the percentage condensed with protein *versus* time. The upper curve B is the sum of the reactions due to both condensation and hydrolysis; the lower curve E represents the course of the reaction due to condensation only. The rate constant of the reaction at 39 °C was $k = 0{\cdot}0013$, with time measured in minutes.

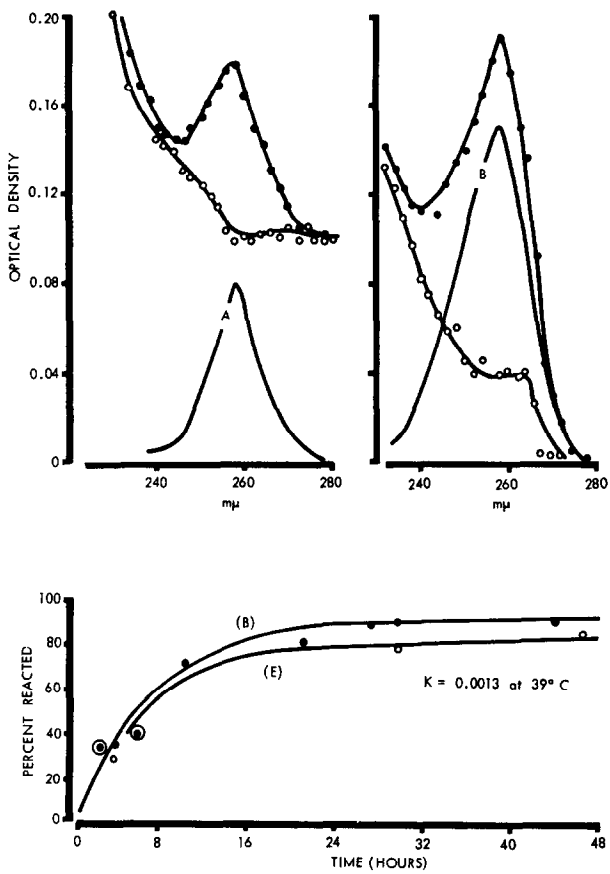


FIG. 3 TOP. Solid dot: absorption spectra in alcohol of chlorambucil obtained by benzene extraction of blood serum at pH 3·6. Circle dot: spectra of extracts of serum only are plotted as control. Curve A, concentration in alcohol 1·3 μg/ml from a serum concentration of 5·2 μg/ml. Curve B concentration in alcohol 2·4 μg/ml from a serum concentration of 9·6 μg/ml. Serum concentrations not corrected for (88%) extract efficiency.

BOTTOM. Rate of condensation of chlorambucil with protein in human blood serum as at 39 °C, determined by extraction of free drug with benzene (B) and with ethyl acetate (E).

These results agree reasonably well with previously determined values of the rate constant[1] and confirm that the degree of hydrolysis of chlorambucil is small in the presence of blood serum.

The extraction of free chlorambucil from dog plasma

The rate of combination of chlorambucil with dog plasma *in vitro* was measured. The results are listed in Table 2.

The reaction is first order, $k = 0{\cdot}0036$, when time is measured in minutes. Since this rate constant is much greater than that found for the condensation reaction of

TABLE 2. RATE OF CONDENSATION OF CHLORAMBUCIL WITH DOG PLASMA AT 39 °C BY THE BENZENE EXTRACTION METHOD

Time (hr)	Extracted amount (mg/ml)	Extracted (%)	Condensed amount (mg/ml)	Condensed (%)
0	0·630	100	0	0
0·5	0·524	83·1	0·106	16·9
1·5	0·470	74·6	0·160	25·4
2	0·433	68·5	0·197	31·5
3	0·331	52·4	0·299	47·6
4	0·287	45·5	0·343	54·5
5	0·283	45·0	0·347	55·0
21·5	0·110	17·4	0·520	82·6

chlorambucil with human serum, a comparison was made of the rates of condensation with the protein of dog serum, of dog plasma, and of human serum. The results are listed in Table 3, which confirms that a marked difference in dog and human serum protein exists with respect to the rate of condensation of chlorambucil *in vitro*.

TABLE 3. COMPARISON OF THE RATES OF CONDENSATION OF CHLORAMBUCIL AT 40 °C WITH DOG PLASMA AND DOG SERUM AND WITH HUMAN SERUM BY THE BENZENE EXTRACTION METHOD

Time (hr)	Percentage of chlorambucil in condensed form		
	Dog plasma	Dog serum	Human serum
0	0	0	0
0·5	42·5	49·4	
1·5	56·5		8·0
2·0	64·3	66·4	6·0
3·0	72·0	70·0	11·8
5·0	81·7		19·6
7·0	87·9	85·1	33·1
23·0	94·2	93·9	77·5
	$k = 0{\cdot}0038$	$k = 0{\cdot}0032$	$k = 0{\cdot}0011$

Assay of free chlorambucil in dog plasma in vivo

Three dogs, designated A, B, and C, were injected with chlorambucil, and samples of plasma were obtained for assay by benzene extraction. Dog C was treated twice, with an interval of 3 days between the two experiments.

The results are plotted in Figs. 4 and 5. The chlorambucil disappeared rapidly from the circulation, as only 20 to 25 per cent of the administered concentration was present in the first sample taken 3 min after the injection. The linear relationships that existed between the logarithms of the concentration of drug and the logarithms

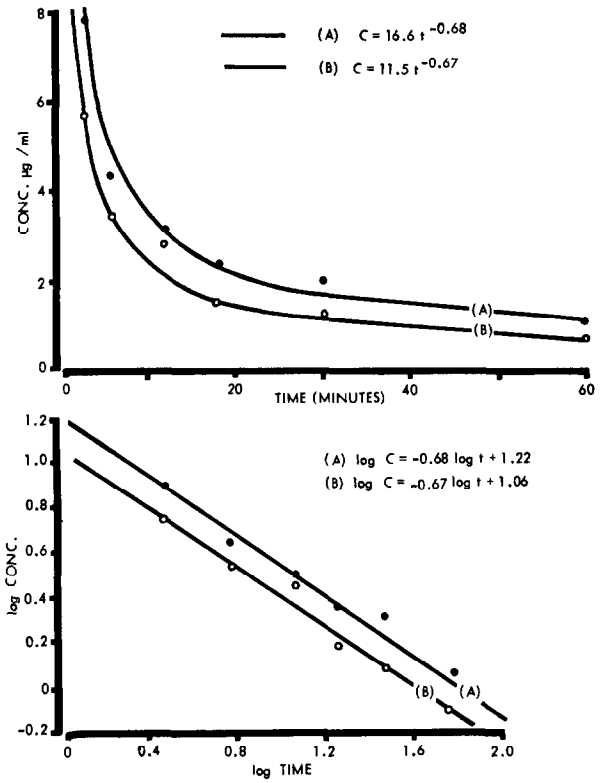


FIG. 4. Rate of disappearance of chlorambucil from the circulating plasma of dogs A and B. The quantity injected produced a level of approximately 40 µg/ml of dog plasma.

of the time after injection enabled the equations to be deduced. The form of the disappearance curve was the same in all cases and is described by the general expression relating concentration C to time t:

$$C = kt^n$$

The value of k varied little, the exponents n were similar for dogs A and B, but differed in the case of dog C; repeated experiments on dog C yielded similar values of n.

These *in vivo*-experiments show a more rapid disappearance of the drug from the circulation and a different relationship between concentration and time, compared with the condensation reaction with protein *in vitro*. A more rapid condensation reaction could have occurred with other substances, but the form of the equation suggested a loss by other than chemical effects. A comparison was made of the rates

at which chlorambucil and its inactive hydroxyl derivative were removed from the circulation of the same dog (D) under similar conditions, as described in the methods section. Identical amounts of the chloro and hydroxyl forms were recovered at 4 min after the injection. The disappearance of the chloro form followed the time pattern of the other dogs, $n = -0{\cdot}75$. The hydroxyl form disappeared more rapidly; thus only a trace could be detected at 12 min, and after that no measurable quantity was recovered.

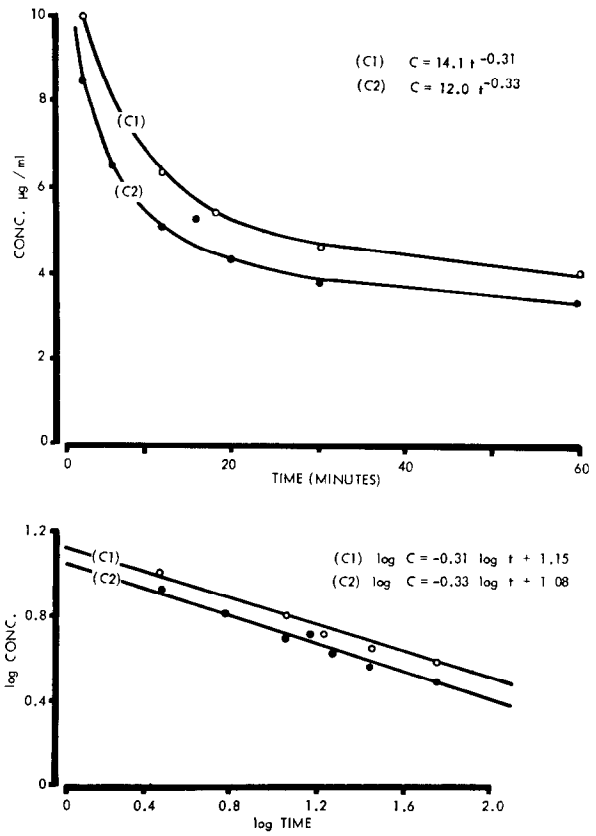


FIG. 5. Rate of disappearance of chlorambucil from the circulating plasma of dog C (two experiments). The quantity injected produced a level of approximately 40 μg/ml of dog plasma

The more rapid disappearance of the inert hydroxyl form is consistent with the smaller extent of physical binding of this derivative to the plasma proteins. This is further indication that the removal of the drug from the circulation is an immediate physical effect and is not attributable to a rapid chemical condensation.

With reference to the studies of dog plasma *in vivo*, the control extract that was obtained from the blood sample removed prior to injection was found in some cases to absorb at the extreme ultraviolet end of the spectrum to a greater degree than did the extract containing chlorambucil. It appeared that the background, which was caused by scattering particles and absorbing material, was not the same in the presence and absence of the drug. It had been noted earlier[1] that chlorambucil altered the surface

properties of precipitated serum protein. During the process of washing the protein that had been precipitated by alcohol, in a second volume of alcohol, it was noted that protein heated with drug was more granular and difficult to pack into the centrifuge. The presence of the drug may alter the size of the particles in the present case under the conditions of the extraction; therefore, the concentration of chlorambucil in the benzene extracts was estimated from the measured absorption spectra by Allan's method,[5] with no direct use of the control extract.

CONCLUSIONS

Procedures have been devised for the assay of small amounts of chlorambucil down to 2 μg/ml and have been applied to the condensation reactions in human serum and in dog serum and dog plasma *in vitro*. These experiments have confirmed earlier conclusions[1] that the rate of hydrolysis of chlorambucil in the presence of serum protein is slow. At concentrations of chlorambucil in serum of 0·6 mg/ml and lower, the condensation reaction is first order throughout its course for both dog and human serum; this finding indicates that the reactive protein groups are present in excess in both cases. Since the rate of hydrolysis is low under these conditions, the chlorambucil spends the greater part of its time in the adsorbed state. Yet the rate constant for the chemical reaction with the protein is approximately three times larger for dog serum than for human serum. As this constant represents the reaction rate at unit drug concentration, the time required for the presumably adsorbed mustard molecule to react with the reactive protein center must be shorter in the case of dog serum. With reference to Fig. 6, reaction (i) is fast, and reaction (ii) could be

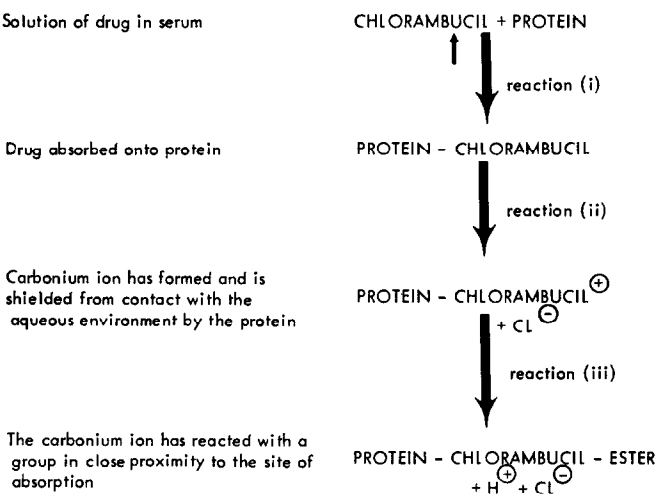


FIG. 6. Hypothetical stages in the interaction of protein with chlorambucil in solution in blood serum.

relatively fast; it is not likely that reaction (ii) would be greatly affected by the nature of the physically bound protein. Reaction (iii), which entails the collision of the carbonium ion with a reactive group in the protein, would be governed by the site of adsorption with respect to the availability of the carboxyl groups. It is possible that large differences in reaction rate could occur at this stage, which would be the slowest

rate-governing stage. This type of experiment may be of value in detecting differences in protein structure.

A study has been made of the concentrations of free chlorambucil in the plasma of dogs *in vivo*, immediately after injection of the drug. There appeared to be no correlation between the rate of disappearance of chlorambucil *in vivo* and the condensation reaction with blood plasma *in vitro*. The former experiments have shown that a consistent pattern of concentration change exists, but the rapid rate of disappearance and the form of the relationship of concentration to time indicated that dispersion or diffusion, rather than a chemical reaction with blood protein, is involved. The more rapid disappearance of the inactive hydroxyl derivative, which is also less firmly adsorbed to protein, as compared with the active drug, supports this conclusion.

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