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Degradation of chlorambucil in aqueous solution—influence of human albumin binding

H. EHRSSON†, U. LÖNROTH, I. WALLIN, M. EHRNEBO*, S. O. NILSSON, *Karolinska apoteket, Box 60024, S-104 01 Stockholm, Sweden*

Chlorambucil, an alkylating agent of the nitrogen mustard type, is rapidly decomposed in pure aqueous solutions (Owen & Stewart 1979; Ehrsson et al 1980b) while an increased stability has been observed in the presence of albumin (Linford 1963; Hopwood & Stock 1971/72; Ehrsson et al 1978). Since the binding of alkylating agents to plasma albumin might significantly affect the stability of the compounds in vivo a more detailed study of this phenomenon is warranted. In the present study the rate constants for chlorambucil and its significant metabolite, phenylacetic mustard (Newell et al 1979), when bound to albumin have been evaluated. The degradation rate has been studied using high performance liquid chromatography (Ehrsson et al 1980b) or gas chromatography with selected ion monitoring (Ehrsson et al 1980a). The degree of protein binding has been established using a modified ultrafiltration technique.

Materials and methods

Human albumin, essentially fatty acid free (Sigma, St Louis, U.S.A.) was dissolved in isotonic phosphate buffer pH 7·35 (NaCl concn 0·095 M). Blood was obtained from healthy drug-free volunteers and collected in tubes containing heparin. The plasma was separated by centrifugation and stored at −20 °C until assayed. Cellophane dialysis tubing (10 mm folded width) was obtained from Union Carbide Corp., Chicago, U.S.A. Chlorambucil was kindly supplied by Burroughs Wellcome, London, U.K. Phenylacetic mustard was synthesized according to Everett et al (1953), recrystallized twice from toluene-n-hexane (m.p. 104 °C, reported 105 °C). The purity was checked by t.l.c. on silica gel $60F_{254}$ with methylene chloride–ethyl acetate (1 : 1) as eluting solvent.

Degradation studies. A solution of chlorambucil or phenylacetic mustard in ethanol was evaporated to dryness and the residue was thoroughly mixed with plasma or albumin solution giving a final alkylating agent concentration of 25 $\mu g\ ml^{-1}$. The mixture was incubated at 25·0 ° ± 0·1 °C or 37·0 ° ± 0·1 °C. At appropriate times aliquots were separated and extracted as described (Ehrsson et al 1980a). The albumin solutions were analysed by h.p.l.c. (Ehrsson et al 1980b) and the plasma by g.c. with selected ion monitoring (Ehrsson et al 1980a).

Ultrafiltration. The ultrification studies were performed at 25 ° ± 2 °C. The dialysis tubing, previously soaked in distilled water for 15 min and excess water removed with cotton-wool, was shaped into a U-bend and placed with the open ends over the lip of a tube. Ultrafiltration was carried out using 1 ml of plasma or albumin solution containing 25 $\mu g\ ml^{-1}$ of the alkylating agent (prepared as described above). After an initial centrifugation (800 g, 10 min) the ultrafiltrate was discarded, the tubing removed, wiped free of moisture on the external surface, placed in a centrifuge tube containing 1 ml of an albumin solution (40 $mg\ ml^{-1}$) and centrifuged again (800 *g*, 20 min). The distance between the lower part of the dialysis tubing and the albumin solution was about 2 cm. The amount of ultrafiltrate (about 50 mg) was determined by weighing. The concentration of the alkylating agent on both sides of the dialysis membrane was assayed. No binding of

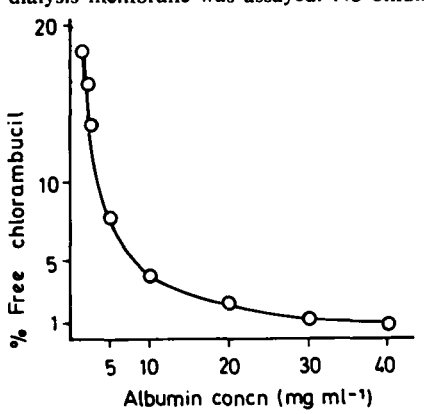

FIG. 1. Influence of albumin concentration on per cent free chlorambucil. Chlorambucil concentration: $8{\cdot}2.10^{-5}$ M. Temperature: 25 °C. Each value is a mean of four determinations.

* Present address: Galenus AB, Box 9045, S-580 09 Linköping, Sweden.
† Correspondence.

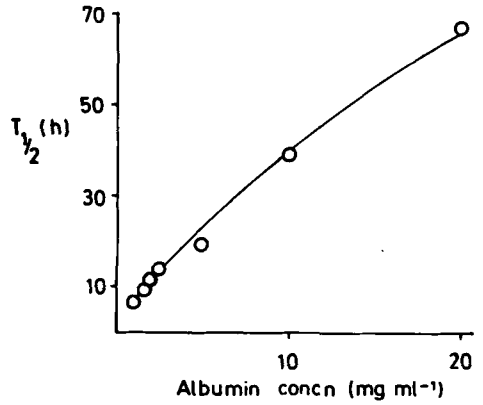

FIG. 2. Degradation rate of chlorambucil—influence of albumin concentration. Chlorambucil concentration: $8{\cdot}2.10^{-5}$ M. Temperature: 25 °C.

the alkylating agents to the dialysis membrane could be observed as established by ultrafiltration of the compounds dissolved in isotonic phosphate buffer pH 7·35. The ultrafiltration procedure resulted in a change of the plasma pH from 7·9 to 8·0.

Results and discussion

The degradation of nitrogen mustards in aqueous solutions is generally supposed to proceed via the formation of a cyclic aziridinium ion which is the rate determining step in the decomposition of the aromatic mustards (Owen & Stewart 1979; Ehrsson et al 1980b). The rate constant for the degradation of chlorambucil in albumin solution can be expressed by the equation:

$$k_{obs} = k_1 f_{bound} + k_2 f_{free} \quad .. \quad .. \quad (1)$$

which can be rearranged to:

$$k_{obs} = k_1 + f_{free}(k_2 - k_1) \quad .. \quad .. \quad (2)$$

where k_1 and k_2 are the pseudo first-order rate constants for the degradation of protein-bound and free chlorambucil, respectively, f_{bound} is the fraction of chlorambucil which is bound to albumin, and f_{free} is the fraction of free chlorambucil in solution. Thus k_1 and k_2 can be evaluated by determining k_{obs} for different fractions of free chlorambucil.

The fraction of free chlorambucil was varied by the use of different concentrations of albumin at a constant ligand concentration.

The protein binding studies were performed by a modified ultralfitration technique. Since chlorambucil is rapidly decomposed when present in a pure aqueous solution ($t_{\frac{1}{2}}$ = 0·45 h in phosphate buffer pH 7·35; and 15·8 h in albumin 45 mg ml^{-1}, at 37 °C and chlorambucil concn $8{\cdot}2 \times 10^{-5}$ M), the ultrafiltrate was collected in an albumin solution and determined by weight difference. The analysis of chlorambucil must be carried out using a selective analytical technique since the degradation product 4-{*p*-[di(2-hydroxyethyl) amino]phenyl} butyric acid has a much lower binding to albumin than the parent compound (Linford 1961). The influence of albumin concentration on the per cent free chlorambucil is shown in Fig. 1. The percentage free chlorambucil was 0·94 ± 0·15% (n = 5) at an albumin concentration of 40 mg ml^{-1} ($6{\cdot}15 \times 10^{-4}$ M), the total concentration of chlorambucil being $8{\cdot}2.10^{-5}$ M. The influence of albumin concentration on the degradation rate is shown in Fig. 2. Albumin concentrations <20 mg ml^{-1} were used because higher concentrations gave inconveniently long degradation rates (a change of albumin concentration from 1·6 to 20 mg ml^{-1} resulted in an increase of the half life from 9·9 h to 67 h). The degradation curves according to pseudo first-order kinetics were linear in all cases.

Table 1. Degradation and protein binding of chlorambucil and phenylacetic mustard.

Compound	Phosphate buffer isotonic pH 7·35 $t_{\frac{1}{2}}$ (h)	Albumin 20 mg ml^{-1} in phosphate buffer pH 7·35 isotonic $t_{\frac{1}{2}}$ (h)	Albumin 20 mg ml^{-1} in phosphate buffer pH 7·35 isotonic % free nitrogen mustard
Chlorambucil	2·0	65	2·3
Phenylacetic mustard	2·6	44	3·4

Nitrogen mustard concentration: $8{\cdot}2.10^{-5}$ M.
Temperature: 25 °C.

The data plotted according to equation 2 gave a straight line. Evaluation of the rate constant k_1 by linear regression analysis gave $k_1 = 5{\cdot}66 \times 10^{-3} \pm 1{\cdot}21.10^{-3}$ h^{-1} (r = 0·9906, n = 24). Evaluation of k_2 from the plot gave a value of $3{\cdot}34 \times 10^{-1} \pm 0{\cdot}10 \times 10^{-1}$ h^{-1} which is in good agreement with the value obtained separately in isotonic phosphate buffer pH 7·35 ($k = 3{\cdot}56 \times 10^{-1} \pm 0{\cdot}07 \times 10^{-1}$ h^{-1}, r = 0·9976, n = 13). Hence chlorambucil is about 100 times more stable when bound to albumin than when unbound in solution. Since the degradation rate of chlorambucil is increased markedly with increasing dielectric constant of the solvent (Owen & Stewart 1979), it can be concluded that the nitrogen mustard part of chlorambucil when bound to albumin is in a chemical environment with solvating properties quite different from those in pure aqueous solution. The protein binding and the degradation rate for phenylacetic mustard, a significant metabolite of chlorambucil in man (Newell et al 1979) is given in Table 1. Phenylacetic mustard is protein bound to a less degree than chlorambucil. Evaluation of the rate constant for the protein bound form of phenylacetic mustard gave $k_1 = 7{\cdot}30 \times 10^{-3} \pm 1{\cdot}75 \times 10^{-3}$ h^{-1} (r = 0·9706, n = 16) while the rate constant in isotonic

phosphate buffer pH 7·35 is $2·74 \times 10^{-1} \pm 0·04 \times 10^{-1}$ h^{-1} ($r = 0·9911$, $n = 11$). Thus, the lower stability of phenylacetic mustard in albumin solution compared with chlorambucil is most likely due to the fact that phenylacetic mustard is bound to a less extent to albumin.

The binding of chlorambucil ($8·2 \times 10^{-5}$ M) in plasma from five healthy drug-free volunteers was 98·98 ± 0·05%, the mean albumin concentration being 46 mg ml^{-1}. Albumin seems to play a major role for the binding of chlorambucil in plasma since this is in good agreement with the values obtained in buffer solutions containing albumin (99·06 ± 0·15; albumin concentration 40 mg ml^{-1}, pH 7·35). The degradation half life was 17·78 ± 1·58 h at 37 °C which is in agreement with the value obtained in an albumin solution (15·8 ± 0·2 h, 45 mg ml^{-1} of albumin, pH 7·35, 37 °C).

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Nicotinic release of noradrenaline in the presence of tetrodotoxin from sympathetic nerve terminals in rabbit isolated atria

C. SARANTOS-LASKA*, M. W. MC-CULLOCH, M. J. RAND, D. F. STORY, *Department of Pharmacology, University of Melbourne, Victoria, 3052, Australia*

It has been suggested that nicotinic cholinoreceptor agonists release noradrenaline in sympathetically innervated tissues by initiating action potentials in the terminal axons. The main evidence for this is that during exposure of sympathetically-innervated tissues to nicotinic agonists, antidromic impulses have been detected in sympathetic nerve trunks (Ferry 1963; Cabrera et al 1966; Davey et al 1968; Haeusler et al 1968; Krauss et al 1970; Bevan & Haeusler 1975). The role of propagated action potentials in these nicotinic responses has been examined in experiments with tetrodotoxin, which selectively blocks axonal conduction in various nerve-muscle preparations (Gershon 1967); however, these experiments have yielded conflicting results. Thus it has been found by some workers that tetrodotoxin blocks the responses of isolated tissues to nicotinic agonists (Bell 1968; Endoh et al 1970), whereas others did not observe a blocking action (Haeusler et al 1968; Krauss et al 1970; Su & Bevan 1970; Westfall & Brasted 1972; Fozard & Mwaluko 1976); furthermore, Furchgott et al (1975) found that tetrodotoxin blocked the responses to low but not to high concentrations of nicotine in the rabbit ear artery.

In the present experiments, the effects of tetrodotoxin were examined on noradrenaline release from rabbit isolated atria elicited by a range of concentrations of nicotine and by electrical field stimulation of the sympathetic nerve terminals. The efflux of tritium label from the tissue after labelling the noradrenaline stores with (−)-[^{3}H]noradrenaline was taken as the index of noradrenaline release. The atria were exposed to nicotine (10, 50 or 100 μM) for 3 min or field stimulation (1 ms monophasic square waves of supramaximal voltage at 5 Hz for 30 s) in either the absence or presence of tetrodotoxin (0·9 μM). The experimental details are as described by Sarantos-Laska et al (1980). Nicotine- or stimulation-induced efflux of tritium label was calculated by subtracting the efflux of radioactivity determined immediately before stimulation or exposure to nicotine from that determined during these procedures. The nicotine- and stimulation-induced effluxes of radioactivity were expressed as percentages of the tissue content of radioactivity immediately before each period of evoked release.

The results are summarized in Table 1. The amounts of noradrenaline released from the atria by field stimulation and by the low concentration of nicotine (10 μM) were similar, and in both cases the effects were substantially reduced by tetrodotoxin, to 10 and 39%, respectively, of control values. The higher concentrations of nicotine (50 and 100 μM) released much greater amounts of noradrenaline, and in these cases tetrodotoxin failed completely to reduce the effects.

The present findings that tetrodotoxin blocked the noradrenaline-releasing action of a low but not of high concentrations of nicotine are in accord with those of Furchgott et al (1975) who measured contractile responses of the rabbit ear artery, but not in accord with the results of Fozard & Mwaluko (1976) who did

* Correspondence.