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ENZYME ACTIVATED ANTI-TUMOUR AGENTS—III. HYDROLYSIS OF CONJUGATES OF *p*-HYDROXYANILINE MUSTARD IN AQUEOUS SOLUTION

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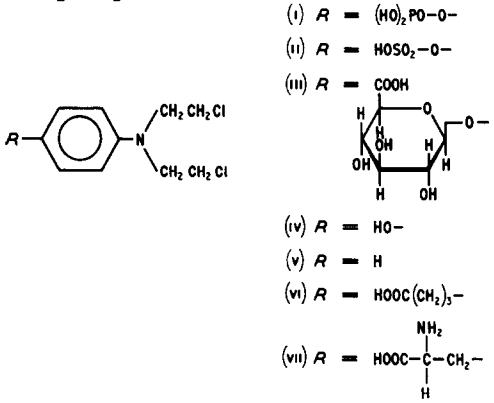
Abstract—The rates of hydrolysis of a series of aromatic nitrogen mustards, in aqueous solution at physiological temperature and pH, have been determined. Identical rates were observed when the hydrolysis reaction was followed either by titration of acid released or by estimation of residual alkylating activity using the Epstein reagent.

In this laboratory a study has been made [1–4] of the *O*-phosphate (AMPh, I), *O*-sulphate (AMSu, II) and *O*-glucuronide (AMGl, III) of *p*-hydroxyaniline mustard (AMOH, IV) [5] which were synthesised by Bukhari *et al.* [6], as potential anti-tumour agents for neoplasms containing high levels of phosphatase, sulphatase and glucuronidase, respectively. It was proposed that selectivity could be achieved by the release of the rapidly hydrolysing and potently cytotoxic AMOH specifically in the tumour by the action of the appropriate deconjugating enzymes. The activity of aniline mustard (AM, V) [7] against certain mouse plasma cell tumours is attributed to metabolism to AMGl in the liver followed by deconjugation to AMOH in the high glucuronidase environment of the tumour [8–11].



In the course of this work it was necessary to assess the chemical reactivity of this group of alkylating agents since this property is likely to strongly influence their pharmacological properties and biological activity. Although the conjugates are ionised at pH 7.4, and therefore would be expected to be less toxic than AMOH due to their rapid excretion, it is possible that the chemical reactivity of these agents could influence their selectivity. For example, a positive correlation between chemical reactivity and antitumour activity has been demonstrated for some members of the aromatic mustard series [12–14].

The hydrolysis of alkylating agents in aqueous solution may be followed by estimation of released hydrogen [7, 12–16], or chloride ion [17–19] or by estimation of residual alkylating activity. The most common reagent for the estimation of alkylating agents is 4-(*p*-nitrobenzyl)pyridine (Epstein reagent, NBP) [20]. The reaction involves the alkylation of NBP to form a pyridinium salt which has an intense purple colour at alkaline pH. This method has been widely used for the estimation of alkylating agents in biological materials [18, 21–28] and, to a lesser extent, for the determination of their rates of hydrolysis [14, 17, 29, 30]. However, the validity of this method for the quantitative estimation of alkylating agents in both biological materials [31] and aqueous solution [13, 32] has been questioned. The major criticism of the use of the Epstein reagent in the context of this work is that it determines the total amount of alkylating molecules present and fails to discriminate between the bifunctional mustard, which has anti-neoplastic activity, and the monofunctional 2-chloroethyl-2-hydroxyethylamine, formed by the primary hydrolysis reaction, which has not [13, 33, 34].

In the present paper we have compared the Epstein method with titration of released acid in determining the rates of hydrolysis of the AM derivatives which are under investigation in this laboratory.

MATERIALS AND METHODS

Alkylating agents. AM (*p*-di-2-chloroethylaniline), AMOH (*p*-di-2-chloroethylaminophenol, hydrochloride), AMPh (*p*-di-2-chloroethylaminophenyl phosphate, dicyclohexylamine salt and disodium salt), AMSu (*p*-di-2-chloroethylaminophenyl sulphate, monohydrate, sodium salt), AMGl (*p*-di-2-chlorothylaminophenyl-D-glucopyranosiduronic acid, tetra-*n*-butyl ammonium salt), and chlorambucil (*p*-di-2-chloroethylaminophenyl butyric acid, sodium salt) were a gift from the Chester Beatty Research Institute to whom we wish to express our thanks. Chlorambucil (free acid, VI) [19] and melphalan (L-*p*-di-2-chloro-

ethylaminophenylalanine, VIII) [35] were a gift from Burroughs Wellcome & Co., to whom we are also grateful.

Mustard hydrolysis. The term *hydrolysis*, used here, applies to the hydrolysis of the chloroethyl moieties of the mustards. The removal of the ester moiety is referred to as *deconjugation* and was shown to be enzyme-dependent.

The rates of hydrolysis of AM, AMOH, AMPh, AMGl, AMSu, chlorambucil and melphalan (the latter two agents, the hydrolysis rates of which have been determined elsewhere [13, 30], were included as standards), were determined by automatic titration of released acid and by estimation of residual alkylating ability. The alkylating agents were dissolved in a small volume of suitable solvent before addition to the aqueous solution. AMPh (dicyclohexylamine salt and disodium salt), AMSu, AMGl, chlorambucil (sodium salt) were dissolved in water, AM in acetone, AMOH in dimethylsulphoxide, chlorambucil (free acid) in ethyl alcohol, and melphalan in NaOH (0.1 M). Solvent added did not exceed 10% v/v.

1. *Automatic titration*

Alkylating agents—AM, AMOH, AMPh (dicyclohexylamine salt), AMGl, AMSu, chlorambucil (sodium salt), and melphalan, dissolved in the appropriate solvent, were added to sodium perchlorate solution (0.1 M, 10 ml) at 37° and pH 7.4 to give a final known concentration of approximately 2×10^{-3} M with the exception of AM which, due to its limited solubility in aqueous solution, was 2×10^{-4} M. AMOH solution was neutralised by the addition of an equivalent amount of NaOH (0.1 M). The reaction was followed to effective completion (99.9 per cent) by titration of the released acid with NaOH (0.01 M for AM, 0.1 M for the other agents) using an Automatic Titrator (Radiometer, Copenhagen) at a constant pH of 7.4 at 37°. Sodium perchlorate, a non-nucleophilic salt, was included to increase the conductivity of the aqueous solution.

2. *Determination of residual alkylating activity*

Epstein reaction. Alkylating agent in aqueous solution (0.5 ml) was incubated with Epstein reagent (BDH, 2% w/v in 90% ethylene glycol/10% acetate buffer, 0.5 M, pH 4.6, 1 ml) in stoppered tubes at 80°. Duplicate tubes were removed at 5-min intervals up to 30 min and cooled in ice. Triethylamine/acetone (50% v/v, 3 ml) was added and the extinction at 570 nm determined immediately against a reagent blank using an SP 1800 (Unicam) or CE 272 (Cecil Instruments) spectrophotometer. Maximum extinction for each of the mustards was observed following an incubation period of 20 min which was used for the rest of this work. Calibration curves for each of the alkylating agents obeyed Beer's Law over the range $5 \times 10^{-6} - 2 \times 10^{-4}$ M and molar extinction coefficients were very similar (ϵ = approximately 3×10^4).

Hydrolysis. Alkylating agents AM, AMPh (dicyclohexylamine salt and disodium salt), AMGl, AMSu, chlorambucil (free acid) dissolved in the appropriate solvent, were added to phosphate buffer (0.01 M, pH 7.4) at 37° to give a final concentration of approximately 2×10^{-4} M with the exception of AM which, due to its very low solubility in this system, was 4.7×10^{-5} M. The solutions were incubated in a shaking water-bath at 37°. Duplicate samples, removed at time zero and subsequently at suitable time intervals over at least one half-life, were assayed for alkylating agent concentration. The pH of the incubation solution was constant over the period of the hydrolysis reactions studied.

RESULTS

The order of each mustard hydrolysis reaction was determined graphically. According to kinetic theory [36] the integrated rate equation for the first-order reaction A → products can be written $\log (a/a - x) = kt/2.303$, where a is the initial concentration of A at time zero, x is the decrease in A at time t, and k is the first-order rate constant for the reaction. Thus plotting $\log (a/a - x)$ against t yields a straight line whose slope is equal to $k/2.303$. The half-life, $t_{1/2}$, of A (time required for the concentration of A to halve) is given by the equation $t_{1/2} = 0.693/k$. Plots of $(a/a - x)$ against t were linear over the initial 50 per cent of the hydrolysis reaction indicating a unimolecular mechanism typical of the aromatic nitrogen mustards [7, 12, 13, 14, 30]. Identical plots were obtained when the reaction was followed by Epstein and titration methods. The reaction rate coefficients and half-lives given in Table 1 were derived from lines of best fit, obtained by least squares regression analysis, for the initial 50 per cent of the reaction. Values for chlorambucil and melphalan (included as known standards) are in good agreement with published work [13, 30]. A progressive increase in reaction rate was observed when the reaction was followed over longer periods due to the increasing con-

Table 1. First-order rate coefficients and half-lives for the mustard hydrolysis reactions

	Rate coefficient (10^5 sec^{-1})		Half-life (min)	
	Titration	Epstein	Titration	Epstein
AM	19.78	18.68	58	63
AMOH	90.89	101.70	13	11
AMPh	91.35	93.27	13	12
AMGl	54.40	58.37	21	20
AMSu	16.97	16.94	70	69
Chlorambucil	41.97	45.93	28	25
Melphalan	19.19	20.22	60	58

Table 2. Alkylating agent LD_{50} values in mice

	LD_{50} (μmoles/kg)
AM	518
AMOH*	52
AMPh†	221
AMGl‡	658
AMSu§	650

* Hydrochloride.
† Dicyclohexylamine salt.
‡ Free acid.
§ Sodium salt.

tribution of the more reactive 2-chloroethyl-2-hydroxyethylamine, produced by the primary hydrolysis reaction. The parameters given in Table 1 do not, therefore, accurately describe the kinetics of the entire hydrolysis reaction but are extremely useful, in biological terms, as an estimate of the maximum rate of hydrolysis of the bi-functional anti-neoplastic species.

DISCUSSION

The results described in this paper have established two main points.

(1) Identical rate coefficients are obtained when the hydrolysis of the aromatic nitrogen mustards in aqueous solution are determined by automatic titration of released acid and estimation of residual alkylating activity using the Epstein reagent.

(2) The half-lives of the AM derivatives, in aqueous solution at physiological temperature and pH, range from 12 to 70 min. AMOH, AMPh and AMGl hydrolyse considerably faster than AM and AMSu and the reaction rate correlates quite well with the Hammet constant [37] for the *para*-substituent (where available) as was observed for other members of this series [19].

Despite the similar short half-lives of AMPh, AMGl and AMOH, the two conjugates are much less toxic than AMOH in mice. The LD_{50} values for these compounds in Balb/c$^-$ [6] and Balb/c* female mice are shown in Table 2. The biological effects of these agents are less likely to correlate with their chemical reactivity than is found with other members of this series [12–14] due to the influence of complicating factors. The conjugates will be largely ionised at physiological pH and their failure to permeate cell membranes will result in their rapid excretion and low toxicity. AMGl, for example, has very little cytotoxicity against bacterial cells in the absence of deconjugating enzymes [38] despite its chemical reactivity, presumably due to its failure to enter the cells. Conversely the action of deconjugating enzymes on AMPh and AMGl results in liberation of the lipid-soluble and highly reactive AMOH which is potently cytotoxic. Serum phosphatases have been shown to deconjugate AMPh and Estracyt [39]. The fact that AMGl is less toxic than AMPh may be due to the fact that serum glucuronidase activity is much lower than that of the phosphatases.* The pharmacokinetics of these agents are at present under study.

The toxicity of AMSu appears to be rather high in the light of its low chemical reactivity and its resistance to deconjugation by aryl sulphatases [1, 2]. It remains a possibility that AMSu is a substrate for an unspecified sulphatase.

* Unpublished results.

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