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Pathways of decomposition of propylbenzilylcholine mustard in neutral and alkaline solution

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High-voltage electrophoresis has been used to follow the decomposition of propylbenzilylcholine mustard (PrBCM) in aqueous solution. Dilute solutions of PrBCM in 10 mM phosphate buffer, pH 7·5, or Krebs-Henseleit solution allowed to stand for 1 h at room temperature (22–24 °C) contain mainly the aziridinium ion derivative. At pH 7·5 the concentration of this ion declines slowly, giving rise first to the *N*-hydroxyethyl derivative and then ultimately, following hydrolysis of the ester bond, to *NN*-bis(2-hydroxyethyl)propylamine and benzilic acid. In contrast, in 5 mM NaOH the ester bond undergoes rapid hydrolysis, so that the major species present after 15 min at room temperature is the *N*-hydroxyethylaziridinium ion. This ion then undergoes slow reaction with hydroxyl ion to yield the same final decomposition product, *NN*-bis(2-hydroxyethyl)propylamine, as is observed at pH 7·5.

Benzilylcholine mustard (BCM) (Gill & Rang 1966) and its *N*-n-propyl homologue (PrBCM) (Young et al 1972) are potent alkylating antagonists at the muscarinic receptor. The ability to form a covalent bond with the receptor has made the tritium-labelled derivatives (Rang 1967; Burgen et al 1974) of particular value for binding studies using intact tissues (Cuthbert & Young 1973; Burgen et al 1974; Taylor et al 1975; Robinson et al 1975; Ward & Young 1977), in studies on solubilized receptor material (Fewtrell & Rang 1973; Alberts & Bartfai 1976; Birdsall et al 1979) and for autoradiographic localization of muscarinic receptors in the c.n.s. (Rotter et al 1979 a,b,c,d). The pharmacologically active intermediate is the aziridinium ion (Gill & Rang 1966), which forms in aqueous solutions of 2-haloalkylamines at a pH where an appreciable fraction of the amine is in the unprotonated form (Golumbic et al 1946; Hanby et al 1947). Over a period of 1–2 h at pH 7·5 at room temperature the aziridinium ion is the major species present in aqueous solutions of BCM and PrBCM and it has been generally presumed that in the dilute solutions employed the decrease in the concentration of this ion with time is due mainly to reaction with hydroxyl ion to form the *N*-hydroxyethyl derivative. This presumption is based on the known behaviour of model compounds such as *NN*-bis(2-chloroethyl)methylamine (mustine) (Hanby et al 1947). However, this is not the only pathway open for the decomposition of BCM and PrBCM, since these compounds are aminoethylesters, which as a class are known to undergo ready hydrolysis, which may be markedly pH dependent (Chuchani 1968). The products of this scission may retain appreciable biological activity, but not at the same sites as the parent compounds. Acetylcholine mustard, for example, undergoes rapid hydrolysis at alkaline pH (Clement & Colhoun 1975a) and Colhoun and his collaborators have shown that the aziridinium ion analogue of choline so formed has long-lasting effects at cholinergic nerve terminals (Clement & Colhoun 1975a), which may result primarily from irreversible inhibition of choline transport (Clement & Colhoun 1975b; Rylett & Colhoun 1977, 1980). Reversible inhibition of choline acetyltransferase has also been observed (Rylett & Colhoun 1979).

In the course of studies on the mobility of [^{3}H]PrBCM and its aziridinium ion derivative on high voltage electrophoresis, we observed that solutions of [^{3}H]PrBCM at pH 7·5 which had been allowed to stand at room temperature for several days appeared to contain only minor amounts of products with the ester bond intact. In view of the complications which could arise if ester bond scission occurred at an early time, leading to the presence of a second aziridinium ion species in solution, the pathways and relative rates of decomposition of [^{3}H]PrBCM in aqueous solution have been investigated in more detail. To do this advantage has been taken of the fact that the tritium atoms in [^{3}H]PrBCM reside in the *N*-propyl residue (Burgen et al 1974) and that on high-voltage paper electrophoresis the mobility of the amine derivatives

formed after hydrolysis of the ester bond is much greater than that of the parent aminoesters. The results of this study are presented here.

MATERIALS AND METHODS

[^{3}H]Propylbenzilylcholine mustard ([^{3}H]PrBCM)

[^{3}H]PrBCM (1·2 Ci mmol^{-1}) was synthesized as described previously (Burgen et al 1974). The tritium atoms reside in the 2,3-positions in the *N*-n-propyl residue. Purified samples were obtained by thin layer chromatography on silica gel G (Merck, Type 60) using ethylacetate–acetic acid, 99·5:0·5 v/v, as solvent. The plates were prerun in the solvent before application of the sample. The major band (R_F 0·8), which had the same R_F as authentic PrBCM, was eluted with ethanol and stored at −20 °C. As noted by Rotter et al (1979a) samples purified in this way are much less stable chemically than ethanolic solutions of the impure reaction product. To minimize this problem purified samples were used within 2 days of preparation.

[^{3}H]Propylbenzilylcholine was kindly provided by Dr N. J. M. Birdsall and [^{14}C]choline by Dr K. J. Martin.

Reaction conditions and determinations of aziridinium ion

[^{3}H]PrBCM was diluted into the appropriate reaction medium to give a final concentration of approximately 0·1 mM and the solution allowed to stand at room temperature (22–24 °C). Krebs-Henseleit solution was gassed throughout with 95% O_2–5% CO_2. The reaction was terminated by dilution into at least 6 volumes of ice-cold 60 mM citric acid–phosphate buffer, pH 3·0.

Aziridinium ion formation in solutions of PrBCM (final concentration 0·8 mM) was measured by the thiosulphate titration method (Golumbic et al 1946), as described by Young et al (1972).

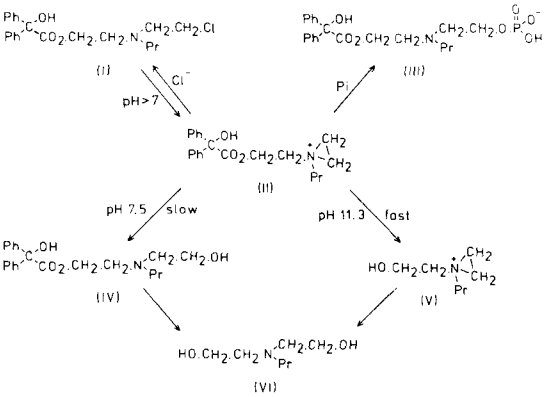
High-voltage electrophoresis

High-voltage electrophoresis (50 V cm^{-1}) was carried out for 1 h on Whatman No. 1 paper in 60 mM citric acid–phosphate buffer, pH 3·0, using a Shandon-Southern L24 apparatus. Samples of [^{3}H]PrBCM or reaction products were always diluted into citric acid–phosphate buffer (cf. above) before the application of aliquots to the paper. After electrophoresis the paper was allowed to air-dry at room temperature before tritium-containing spots were detected using a Packard model 7200 radiochromatogram scanner.

Two different versions of the L24 electrophoresis apparatus were used. With one of these (used for the experiments in Figs 1d and 2b) under the conditions employed there was an appreciable endoosmotic flow of buffer, as demonstrated by rerunning samples of low mobility with the polarity reversed. This had the effect that peaks which had remained near the start with the first apparatus showed some mobility in the other version. However, the pattern of peaks remained the same and all comparisons were made on samples run on parallel tracks, unless specifically noted otherwise.

RESULTS AND DISCUSSION

The probable pathways of [^{3}H]PrBCM decomposition in aqueous solution are set out in the reaction scheme.



In 10 mM phosphate buffer, pH 7·5, the major product present in a solution of 0·1 mM [^{3}H]PrBCM (I) allowed to stand for 1 h at room temperature is the aziridinium ion derivative (II), which on electrophoresis has a greater mobility than the parent mustard (I) (Fig. 1a). On reaction with thiosulphate the aziridinium ion (II) is converted into the adduct (VII) (Golumbic et al 1946), which at pH 3·0 has one positive and one negative charge and consequently remains near the origin on electrophoresis (Fig. 1a, bottom trace).

If solutions of [^{3}H]PrBCM (I) in phosphate buffer are allowed to stand longer than 1 h then further peaks become apparent. The pattern at 6·5 h is shown in Fig. 1b. One component of the leading double peak is almost certainly the aziridinium ion (II), since it disappears after reaction with thiosulphate (Fig. 1b, lower trace). The remaining peak with appreciably mobility is most likely the *N*-hydroxyethyl derivative (IV), resulting from reaction of the aziridinium intermediate (II) with

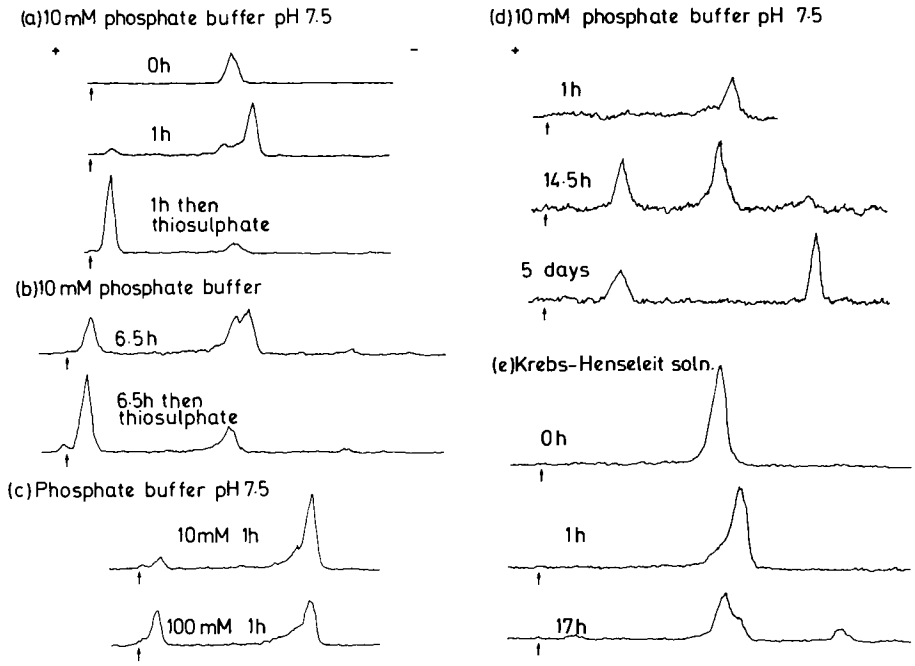
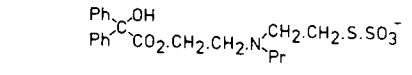
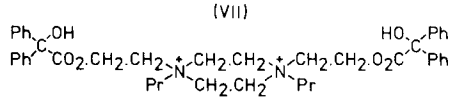


FIG. 1. Decomposition of [^{3}H]PrBCM in phosphate buffer and Krebs-Henseleit solution. The traces are scans for radioactivity after high-voltage electrophoresis. The reaction conditions are given under Methods. Where thiosulphate is indicated on the traces, sodium thiosulphate (final concentration 1·7 mM) was added and the solution allowed to stand for 1 h. The buffer for electrophoresis was 60 mM citric acid-phosphate, pH 3·0. The traces in (d) were obtained on a second version of the electrophoresis apparatus, which produced some apparent changes in mobility (see Methods).

hydroxyl ion. This identification is supported by the self-consistency of the reaction scheme and by earlier studies on nitrogen mustards, such as mustine, made under similar conditions (Hanby et al 1947).



(VII)



(VIII)

The low mobility peak evident after 6·5 h in phosphate buffer (Fig. 1b, upper trace) is most probably the result of reaction of the aziridinium intermediate (II) with phosphate anion (Pi) to yield the adduct (III). This assignment is supported strongly by the observation that the peak becomes more prominent if the concentration of phosphate is increased from 10 to 100 mM (Fig. 1c), when it represents 43% of the total area under the two peaks on the trace as compared with 10% in 10 mM phosphate. Further, the low mobility peak is not evident on electrophoresis of solutions of [^{3}H]PrBCM allowed to stand in Krebs-Henseleit solution, where the phosphate concentration is only 1·2 mM.

If the solution of [^{3}H]PrBCM in 10 mM phosphate buffer is allowed to stand for 14·5 h, then there is little evidence of any aziridinium ion (II) remaining (Fig. 1d) and the major peaks are the phosphate adduct (III) (the reason for the apparent increased mobility is given under METHODS) and the presumed *N*-hydroxy derivative (IV). There is in addition some indication of a small high-mobility peak. After 5 days this peak has increased markedly, while that assigned to the *N*-hydroxyethyl derivative (IV) has disappeared (Fig. 1d). This is consistent with a slow hydrolysis of the ester bond to yield the diethanolamine derivative (VI) and benzilic acid, which carries not tritium atoms and hence is not apparent on scans for radioactivity. Supporting evidence for the identity of the high-mobility peak with compound (VI) from the pathway of [^{3}H]PrBCM decomposition in alkaline solution is presented below.

The high-mobility peak is also evident if [^{3}H]PrBCM in Krebs-Henseleit solution is allowed to stand at room temperature for 17 h (Fig. 1e). the feature of note in these traces, however, is the much slower decay of the aziridium ion (II) concentration

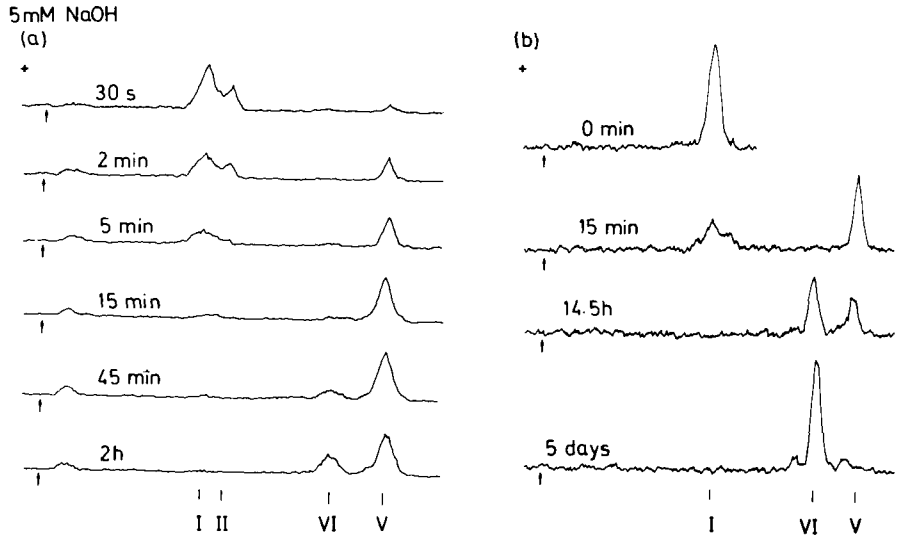


FIG. 2. Decomposition of [^{3}H]PrBCM in 5 mM NaOH. The traces are scans for radioactivity after high-voltage electrophoresis. The reaction conditions are given under Methods. The buffer for electrophoresis was 60 mM citric acid-phosphate, pH 3·0. The traces in both (a) and (b) are from two separate experiments. The alignment was checked with 15 min samples. The roman numerals below the traces identify the peaks with the compounds in the reaction scheme.

compared with 10 mM phosphate buffer at the same pH. This is in accord with previous measurements of the rate of decay of the thiosulphate titre in solutions of PrBCM in the two media (Young et al 1972) and is probably due to the presence of 120 mM chloride ion in the Krebs-Henseleit solution, which can effect a reversal of the cyclization reaction, leading to reformation of the parent chloroethyl compound (I) (Bartlett et al 1947; Hanby et al 1947).

If [^{3}H]PrBCM (I) is diluted into 5 mM NaOH instead of Krebs-Henseleit solution or phosphate buffer at pH 7·5, the time-course and sequence of the reactions changes dramatically (Fig. 2). Within 30 s there is evidence of a high-mobility peak (Fig. 2a), although the major peak is presumably the parent chloroethyl compound (I), with a lesser amount of the aziridinium ion (II). By 5 min the high-mobility peak is the major radioactive species present and at 15 min, in this experiment, it was virtually the only peak observable. The mobility of this peak was comparable with that of [^{14}C]choline and, taken with the well known susceptibility of aminoethyl esters to hydrolysis in alkaline solution (Chuchani 1968), this suggests that it represents, at least in large part, the aziridinium ion analogue of propylcholine (V). The possibility that a second compound with an identical mobility could be present is discussed below.

Strong evidence that the major part, if not all, of the radioactivity in the high-mobility peak after 15 min in 5 mM NaOH can be ascribed to the presence of the *N*-hydroxyethylaziridinium ion (V), is provided by direct measurement of the time course of the formation and decay of aziridinium ion in a solution of PrBCM in 5 mM NaOH (Fig. 3). The maximum yield is obtained by 25 min and the subsequent decline in the concentration is relatively slow. The time course of aziridinium ion formation may be slower in the titration experiment (Fig. 3) than is indicated in the electrophoresis experiment (Fig. 2a), but this can be explained by the necessity of using a higher concentration of PrBCM, 0·8 mM, in the titration experiment to obtain a reasonable accuracy. At this concentration the PrBCM does not dissolve completely, a clear solution only being

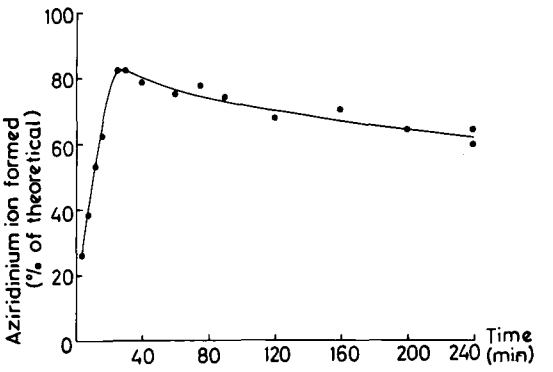


FIG. 3. Time-course of changes in the aziridinium ion concentration in a solution of 0·85 mM PrBCM in 5 mM NaOH at 24 °C.

obtained as formation of aziridinium ion (or ester hydrolysis) proceeds. The same behaviour, with a similar time-course, is observed in 10 mM phosphate buffer, pH 7·5 (Young et al 1972).

The continued presence of aziridinium ion in the solution of PrBCM in 5 mM NaOH at a time when electrophoresis indicates that the initial medium-mobility entities have been converted completely into a single high-mobility peak is strong evidence for the hydroxyethylaziridinium ion (V). In the reaction scheme this is shown as resulting from hydrolysis of the aziridinium ester (II). Certainly this reaction does take place, as is evidenced by the rapid disappearance of the peak ascribed to compound (II) between 30 s and 15 min (Fig. 2a). However, it is possible that hydrolysis of the parent chloroethyl compound (I) could also occur and this would not be apparent if the *N*-2-chloroethyl-*N*-2-hydroxyethylpropylamine formed had a very similar mobility to the hydroxyethylaziridinium ion (V). Further experiments using thiosulphate would be necessary to resolve this question.

Quaternary aminoalkyl esters do not usually undergo hydrolysis as rapidly in basic solution as their tertiary counterparts (Chuchani 1968). This is borne out by the relatively slow hydrolysis of [^{3}H]propylbenzilylcholine in 5 mM NaOH (Fig. 4). With this compound (structure (II) with two–CH_3 groups in place of the -CH_2- residues in the aziridinium ring) it is difficult to see what changes NaOH could bring about under the conditions employed other than scission of the ester bond. Consequently the similarity of the changes in electrophoretic mobility on hydrolysis in 5 mM NaOH with those observed with [^{3}H]PrBCM in the same medium lends

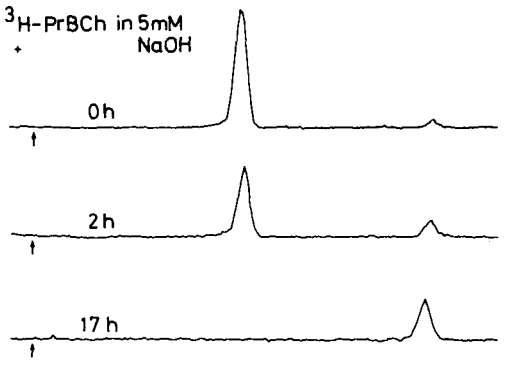


FIG. 4. Decomposition of [^{3}H]propylbenzilylcholine in 5 mM NaOH. The traces are scans for radioactivity after high-voltage electrophoresis. The reaction conditions are given under Methods. The buffer for electrophoresis was 60 mM citric acid-phosphate, pH 3·0.

further support to ester bond hydrolysis as the first step in the decomposition in alkaline solution. The relatively rapid hydrolysis of the quaternary aziridinium ester (II) is explained by the existence of an equilibrium with the open chain carbonium ion tautomer, -N(Pr).CH_2.CH_2^+ (Hanby et al 1947).

Solutions of [^{3}H]PrBCM in 5 mM NaOH allowed to stand for periods longer than 15 min show a further change in the band pattern observed after electrophoresis. By 2 h a peak of slightly lower mobility than the hydroxyethylaziridinium ion (V) is clearly evident (Fig. 2a). At 14·5 h it is the major radioactive entity and by 5 days it is the only peak observed (Fig. 2b). This change is consistent with the reaction of the aziridinium ion (V) with hydroxyl ions to yield the diethanolamine derivative (VI). Electrophoresis in parallel of solutions of [^{3}H]PrBCM allowed to stand for 5 days in 10 mM phosphate buffer, pH 7·5, or 5 mM NaOH shows the high-mobility bands to have the same mobility, consistent with their chemical identity (see reaction scheme).

The pathways of decomposition of [^{3}H]PrBCM proposed above are in good accord with the experimental evidence. It must be emphasized, however, that it is likely that this scheme only holds for dilute solutions of [^{3}H]PrBCM. We have reported previously that in more concentrated solutions of [^{3}H]PrBCM, 2·1 mM, allowed to stand for 1 h in 10 mM phosphate buffer, pH 7·5, another peak appears with slightly lower mobility than the aziridinium ion (II) (Burgen et al 1974). It seems quite likely that this additional peak could represent the piperazinium dimer (VIII) (*cis* and *trans*). The concentration dependence of the bimolecular reaction leading to the formation of the dimer from mustine has been reported by Hanby et al (1947) and in concentrated solutions the dimer can be the major decomposition product (Golumbic et al 1946; Hanby et al 1947). A similar observation has been made with acetylcholine mustard (Hirst & Jackson 1972). In the dilute solutions of [^{3}H]PrBCM, approximately 0·1 mM, used in the present study formation of the dimer (VIII) would be expected to be minimal and consequently at pH 7·5 the *N*-hydroxyethyl derivative (IV) will almost certainly by the initial decomposition product. However, it should be borne in mind that mustard dimers form readily in concentrated solutions in ethanol (Hanby & Rydon 1947) and it is quite possible that small amounts of the dimer could be generated during preparative procedures such as the elution of bands from thin layer chromatograms with ethanol. It is just possible that the small

thiosulphate-unreactive peak in Fig. 1a (bottom trace), which in other experiments has been observed to be relatively resistant to ester bond hydrolysis in neutral solution, could be the dimer. The mobility on electrophoresis would correspond with the secondary peak noted in more concentrated solutions of [^{3}H]PrBCM in phosphate buffer (Burgen et al 1974).

In summary, in dilute solutions of [^{3}H]PrBCM in phosphate buffer, pH 7·5, or Krebs-Henseleit solution the decline of the concentration of the cyclic aziridinium ion (II), once formed, is slow. However, if the pH is allowed to rise then the rate of ester bond hydrolysis increases markedly and the amount of the aziridinium ion (II) present at any time may be small. Instead, the aziridinium ion of a choline mustard analogue (V) may be the major chemically reactive species present in solution.

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