DEPENDENCE OF *in vitro* ENZYMIC HYDROLYSIS OF ALKYL(2-BENZOYLOXYETHYL)DIMETHYLAMMONIUM BROMIDES ON THE ALKYL LENGTH

Eva OLASZOVA[a1], Ingrid PAULIKOVA[a2], Otto HELIA[a3], Emil SVAJDLENKA[b1], Ferdinand DEVINSKY[b2] and Ivan LACKO[b3]

[a] *Department of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Comenius University, 832 32 Bratislava, Slovak Republic; e-mail:* [1] *olaszova@kbm.fpharm.uniba.sk,* [2] *paulikova@kbm.fpharm.uniba.sk,* [3] *helia@kbm.fpharm.uniba.sk*

[b] *Department of Chemical Theory of Drugs, Faculty of Pharmacy, Comenius University, 832 32 Bratislava, Slovak Republic; e-mail:* [1] *svajdlenka.kchtl@fpharm.uniba.sk,* [2] *devinsky@fpharm.uniba.sk,* [3] *lacko.kchtl@fpharm.uniba.sk*

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Microsomal esterases were used in the study of the *in vitro* enzymic hydrolysis of the ester bond in alkyl(2-benzoyloxyethyl)dimethylammonium bromides. These compounds are potential "soft" disinfectants, easily biodegradable to nontoxic biologically inactive hydrolytic products, namely substituted choline and benzoic acid. Formation of the latter product was used to monitor the kinetics of the reaction. It has been found that the rate of enzymic hydrolysis is substantially influenced by different length of the alkyl chain on the ammonium nitrogen. At the same time, interspecies (rat–mouse) and interorgan (liver–kidney) variability has been observed.

Key words: Quaternary ammonium salts; Soft drugs; Enzymic hydrolysis; Microsomal esterases.

Due to their high environmental adaptability, microorganisms are able to acquire resistance against antimicrobial agents. Therefore, research efforts are constantly devoted to a search for effective disinfectants with suppressed side effects.

At present, efforts concentrate on synthesis of the so-called "soft" drugs. Their molecules contain groupings similar to biomolecules and acceptable to biological systems (*e.g.*, ester bonds). After their therapeutic effect, the "soft" drugs are quickly decomposed (by hydrolysis, in most cases) to metabolites with low biological activity and toxicity. Typically, the deactivation of "soft" compounds is easily predicted and controlled.

The quaternary ammonium salts (QAS), synthesized by Csiba *et al.*[1] also belong to the soft drug class. These compounds, derived from 2-(dimethylamino)ethyl benzoate acid, are isosteric and/or isoelectronic with the common "hard" surface-active quaternary ammonium disinfectants. They are characterized by the general formula C_6H_5–$COO(CH_2)_2$–$N^+(CH_3)_2R$ Br^- (R = straight-chain alkyl with n = 1,… carbon atoms). In

further text, we will designate these molecules with symbol BCH*n* (the parent structure of the homologous series is benzoylcholine).

The antimicrobial effect of QAS is based on their physicochemical properties. Their ammonium functional groups bind to the negatively charged phosphate groups of phospholipids, whereas their nonpolar parts penetrate into the hydrophobic membrane core. This results in a loss of membrane semipermeability, leakages of intracellular components, inhibition of metabolic reactions as glucolysis or oxidative phosphorylation, changes in protein structure and final cell death. Thus, QAS could be characterized as membrane-active compounds[2], and an optimum balance of their hydrophobic and hydrophilic characteristics is substantial for the desired effect[3].

The antimicrobial activity of the newly synthesized QAS was investigated in a previous paper[1]. The results show a high activity against the microorganisms tested (*S. aureus, E. coli, C. albicans*). An activity increase with the alkyl length was found. A good activity was in this case associated with alkyl chains ranging from decyl to octadecyl. The most sensitive organism was *S. aureus* and the most resistant one *E. coli*. For *E. coli*, the antimicrobial activity was nonlinearly dependent on the alkyl length, whereas for the Gram-positive *S. aureus* and *C. albicans*, the authors[1] observed in general a high activity (with the exception of hexyl and octyl derivatives), not much influenced by the alkyl length. In all the cases studied, the most effective were the dodecyl and tetradecyl derivatives (n = 12, 14).

For a complete characterization of new compounds, knowledge of their behaviour in living systems and of their interaction with enzyme systems is necessary. The structure of model substrates can be influenced by biotransformation at several sites. The most sensitive site is probably the ester bond.

Helia and coworkers[4] studied the hydrolytic activity of microsomal esterases isolated from different organs of two animal species using (2-benzoyloxyethyl)trimethylammonium bromide as substrate.

In this work, we have examined the reaction rate of *in vitro* enzymic hydrolysis in dependence on the alkyl length in alkyl(2-benzoyloxyethyl)dimethyl bromides by microsomal fractions of murine and rat livers and kidneys.

EXPERIMENTAL

Model Substrates

The alkylammonium salts have been synthesized in the laboratory of F. Devínsky at the Faculty of Pharmacy, Comenius University, Bratislava. All substrates were white crystalline substances, chromatographically pure, well soluble in polar and insoluble in nonpolar solvents. They have been stored in a vacuum desiccator above P_4O_{10}. The substrate solutions were freshly prepared by dissolution in distilled water at laboratory temperature. For BCH14, BCH16 and BCH18 heating to 40–60 °C and intense shaking was necessary. Basic characteristics of the substrates used in this study are summarized in Table I.

Reagents

4-Iodobenzoic acid was a product of BDH, England, methanol of HPLC grade was purchased from Sigma Chemie, Germany. All the other reagents were obtained from Lachema, Brno, Czech Republic and were of reagent grade purity (with the exception of benzoic acid, which had the sL 3 purity).

Preparation of Microsomal Fractions

The liver and kidney tissue was obtained from male Wistar rats (weighing 150–180 g) or male Balb mice (approximately 20 g). Microsomal fractions were isolated by differential centrifugation of a 20% homogenate of the particular organ tissue in 0.25 mol l^{-1} saccharose according to Remmer and coworkers[5]. The protein concentration was determined by the method of Lowry *et al.*[6].

Conditions of Incubation

The incubation was done under aerobic conditions in a Dubnoff incubator at 37 °C with intense mixing and aeration. First, the reaction mixture containing 0.1 mol l^{-1} potassium phosphate (pH 7.4) buffer, 1 mmol l^{-1} substrate and 40 μmol of Mg^{2+} ions was preincubated for 5 min. Then, the reaction was started by addition of the microsomal fraction in an amount corresponding to 15 or 5 mg of protein for rats or mice, respectively. Total volume of the incubation mixture was 3.5 ml. After the specified incubation period, 1 ml of incubation mixture was removed and the reaction in this sample stopped by cooling to 0 °C and by denaturation using 100 μl of 1 mol l^{-1} HCl.

Isolation and Determination of the Hydrolytic Product (Benzoic Acid)

To the sample, the internal standard of 4-iodobenzoic acid in reagent grade methanol was added in an amount corresponding to the amount of liberated benzoic acid. The acidity of the resulting reaction mixture was adjusted to pH 2.3–3.0 with 1 mol l^{-1} HCl and benzoic acid was extracted three

TABLE I
Characteristics of $C_6H_5COO(CH_2)_2N^+(CH_3)_2(CH_2)_{n-1}CH_3Br^-$

Code	*n*	M.w.	c_c [a], mol^{-1}
BCH1	1	288.18	–
BCH2	2	302.21	–
BCH3	3	316.23	–
BCH4	4	330.26	–
BCH6	6	358.34	$1.51 . 10^{-1}$
BCH8	8	286.39	$4.82 . 10^{-2}$
BCH10	10	414.44	$1.22 . 10^{-2}$
BCH12	12	442.49	$2.31 . 10^{-3}$
BCH14	14	470.55	$7.93 . 10^{-4}$
BCH16	16	498.60	$8.96 . 10^{-5}$
BCH18	18	526.66	$1.50 . 10^{-4}$

[a] c_c Critical micellar concentration.

times with 5 ml of chloroform. The collected extracts were evaporated to dryness on a rotatory evaporator, dissolved in methanol (HPLC grade) and analyzed with HPLC as described in an earlier paper[4].

Enzyme Kinetics

Enzyme activity of microsomal esterase was expressed as the quantity of liberated benzoic acid in nmol min^{-1} mg^{-1} of protein. For evaluation, we used calibration curves correlating the benzoic acid concentration with the peak areas for benzoic and 4-iodobenzoic acids. The calibration curves were analyzed with linear regression, giving the linear correlation coefficient $r = 0.997$.

RESULTS AND DISCUSSION

We have studied the *in vitro* enzymic hydrolysis of the ester bond in model QAS substrates in both liver and kidney microsomes of rats and mice. The rate of hydrolysis of homologues with alkyls of different lengths was also compared for different species and organs. As shown in Fig. 1, in all cases, there are obtained nonlinear relations between the reaction rate and alkyl length, showing maxima for different substrates, most often for BCH2 and BCH8.

The rates of enzymic hydrolysis in rat liver (Fig. 1a) vary for different substrates between 0.24 and 3.42 nmol min^{-1} mg^{-1}. The curve has alternating maxima and minima, with maxima found for BCH2 > BCH4 > BCH8 and minima for BCH3 and BCH6. After the last maximum for BCH8, the rate of enzymic hydrolysis decreases in the order BCH10 > BCH12 > BCH14, and becomes roughly stabilized of the minimum value for BCH16 and BCH18.

In rat kidneys, the rates of enzymic hydrolysis range from 0.07 to 0.52 nmol min^{-1} mg^{-1} with maxima for substrates BCH2 > BCH8 > BCH4. From BCH10 to BCH16, the rates gradually decrease, with an interesting increase for BCH18 (Fig. 1b).

The enzymic hydrolysis rates in murine liver have similar values around 70 nmol min^{-1} mg^{-1} for BCH1–BCH10, with low maxima for BCH8 > BCH2 (see Fig. 1c). Starting from BCH10, the rates drop rapidly to the value of 7.09 nmol min^{-1} mg^{-1} for the BCH18 substrate.

In murine kidney, the observed relationhip is bell-shaped (Fig. 1d) with maximum value found for BCH10 and with an increase in hydrolysis rate for BCH18. The reaction rates vary between 0.35 and 3.03 nmol min^{-1} mg^{-1}.

A comparison of curves obtained for rat and murine liver and for rat kidney shows several common features. In all three cases, there are more or less pronounced maxima for substrates BCH2, BCH4 and BCH8. However, the relative intensity of these three maxima is different. Substrates BCH1, BCH3 and BCH6 lie at the minima. For both rat liver and kidney, the reaction rates in the maxima decrease in the mentioned order, whereas for murine liver they increase. After reaching a maximum for BCH8, the rate of hydrolysis gradually decreases in the order BCH10, BCH12, BCH14, BCH16 and BCH18, with an exception of the increase for BCH18 in rat kidney. In the case of

murine kidney, the curve has a shape differing from all the three above mentioned. The only resemblance is the increase in hydrolysis rate for BCH18. This can be used as a basis for evaluation of interpecies variability in kidney.

The obtained results were subjected to a statistical analysis using the Student *t*-test. In microsomal fractions of rat liver and kidney and of murine liver, the overall shape of the curves obtained (Fig. 1a–1c) roughly corresponds to a hyperbolic decrease with the alkyl length. The reaction rate decreases/increases between neighboring homologues are not statistically significant on the $P = 0.05$ level, but the differences were confirmed experimentally. In murine kidney, the maximum for BCH10 is statistically significant when compared with substrates BCH1 and BCH16–BCH18.

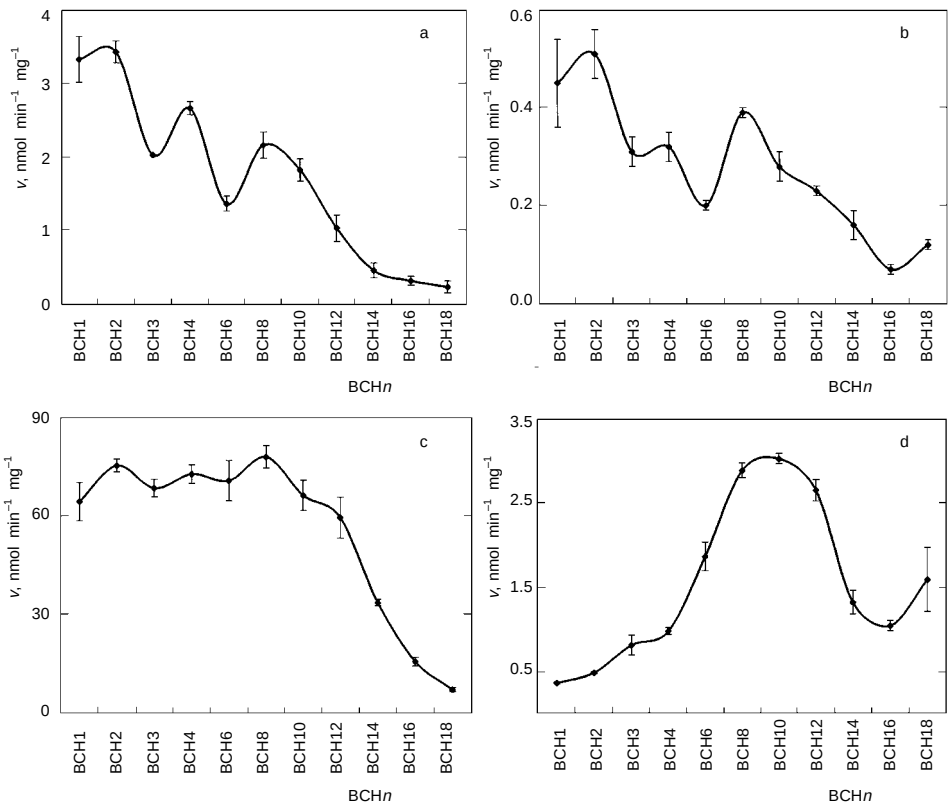

FIG. 1

Influence of the alkyl length in the substrate on the rate of enzymic hydrolysis in: a rat liver, b rat kidney, c murine liver, d murine kidney. The values are averages from 3–5 experiments. In each experiment, microsomes isolated from at least five animals were used. The standard deviation is indicated by the bar

A comparison for all studied organs shows that the overall rate of enzymic hydrolysis is highest in murine liver. In rat liver and murine kidney, the reaction rates are about 20–30 times slower compared to murine liver. Ester bonds of model substrates were cleaved with lowest velocity in murine kidney preparations.

Comparing different organs, the enzymic hydrolysis has been found faster in liver. On an interspecies level, it has higher reaction rates for mice.

These conclusions correlate with kinetic studies of Helia and coworkers[4], who have determined K_M and V_{max} for enzymic hydrolysis of BCH1 by microsomal fractions from different rat and murine organs. On the basis of these K_M values, one can conclude that the affinity to BCH1 follows the order: rat kidney (K_M = 0.7 mM) = (murine kidney?) > rat liver (K_M = 1.09 mM) > murine liver (K_M = 1.58 mM). Interestingly, the affinity to BCH1 substrate decreases with increasing rate of hydrolysis of this substrate.

We expect that in rat liver and kidney and in murine liver we deal with microsomal esterases differing in affinity to particular alkylammonium salts, but with comparable properties. Similar conclusions are found in literature[4]. The enzymic hydrolysis of model substrates in murine kidney indicates the presence of an esterase with different properties. A similar anomalous behaviour was noted previously also for K_M of BCH1 hydrolysis[4].

The rate of enzymic splitting of the ester bond is influenced also by the general amphiphilicity of the molecule, not solely by the presence of the lipophilic alkyl chain. We can expect, on the basis of structural resemblance of higher homologues to the phosphatidylcholine membrane components, that the lipophilic part of the molecule penetrates into the microsomal membrane. Also, the antimicrobial effect of this group of QAS has a similar basis. Such penetration, as well as possible formation of micelles, can influence the enzymic hydrolysis considerably. (Similar phenomena were quoted by Arndt and Krisch[7], who found a decrease in the carboxylesterase-catalyzed hydrolysis of aliphatic esters with increasing alkyl length.)

Micelle formation strongly inhibits enzymic hydrolysis (in contrast to chemical hydrolysis). Abrupt changes in the observed curves suggest micelle formation as one of the causes of the rapid decrease of hydrolysis, catalyzed by microsomal cholinesterase (CHE), E.C.3.1.1.7./8. (ref.[8]). Taking into account the known structure of the active site of CHE and chemical mechanisms of ester hydrolysis[9], we can conclude that the micelle dimensions are too large to allow the micelle to react in a complementary way with the enzyme active site. This makes formation of a stabilized transition state impossible. The catalytic triad of the active site cannot function as proton pendulum and, consequently, the ester bond degradation in these substrates slows down dramatically.

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REFERENCES

1. Csiba I., Devinsky F., Lacko I., Mlynarcik D.: *Cesk. Farm.* **1986**, *35*, 61.
2. Herold F. M. in: *Advances in Microbial Physiology* (A. H. Rose and J. E. Wilkinson, Eds), p. 455. Academic Press, London 1970.
3. Devinsky F., Lacko I., Mlynarcik D., Racansky V., Krasnec E.: *Tenside Detergents* **1985**, *22*, 10.
4. Helia O., Paulikova I., Svajdlenka E., Devinsky F., Lacko I., Olaszova E.: *Pharmazie* **1995**, *50*, 705.
5. Remmer M., Greim H., Schenkamn J. B., Estabrook V.: *Methods Enzymol*. **1955**, *10*, 706.
6. Lowry O. H., Rosenbrough N. J., Randall R. J., Farr A. L.: *J. Biol. Chem*. **1951**, *193*, 265.
7. Arndt R., Krisch K.: *Eur. J. Biochem*. **1973**, *36*, 129.
8. Jbilo O., Lhermite V., Talesa V., Toutant J. P., Chatonnet A.: *Eur. J. Biochem*. **1994**, *225*, 115.
9. Voet D., Voet G. J.: *Biochemistry*, p. 373. Wilye, New York 1990.