cytotoxicity (26). However, our results do not support this mechanism insofar as they show a lack of correlation between incorporation of FUra into mRNA, which decreased 4-fold in the presence of Urd, and the toxicity of FUra, which remained unchanged. On the other hand, the amount of [^{3}H]FUra incorporated into the rRNA + tRNA fraction appears to have *increased* by about 25 % in the presence of Urd. We believe the most likely cause of this phenomenon is the generation of a small amount of [^{3}H]FUrd from an enzymatically catalysed transribosylation reaction between [^{3}H]FUra and Urd. The resulting [^{3}H]FUrd should then become just as readily incorporated into rRNA, as is [^{3}H]Urd (Fig. 5). Analogous transribosylation reaction between FUra and deoxyribonucleosides have been demonstrated previously (30).

Abbreviations

FUra, 5-fluorouracil; FdUMP, 5-fluoro-2'-deoxyuridylate; FdUrd, 5-fluoro-2'-deoxyuridine; FUrd, 5-fluorouridine; FUMP, fluorouridine-5'-phosphate FUDP, fluorouridine-5'-diphosphate; FUTP, fluorouridine-5'-triphosphate; dThd, thymidine; dTMP, thymidine-5'-phosphate; TTP, thymidine-5'-triphosphate; dUrd, 2'-deoxyuridine; dUMP, 2'-deoxyuridine-5'-phosphate; Urd, uridine; 5,10-CH_2H_4folate, 5,10-methylenetetrahydrofolate; dTMP synthetase, thymidylate synthetase; F-RNA, fluorouracil-containing RNA.

References

(1) Heidelberger, D., Chaudhuri, N. K., Danneberg, P., Moren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Pleven, E., Scheiner, J. (1957) Nature 179, 663–666.
(2) Ansfield, F. J. (1973) Chemotherapy of Malignant Neoplasms, Charles C. Thomas, Springfield, Illinois.
(3) Cullen, S. I. (1979) J. Am. Geriatr. Soc. 27, 529–533.
(4) Heidelberger, C. (1975) in Antineoplastic and Immunosuppressive Agents (Sartorelli, A. C. and John, D. G. Eds.) Vol. 38, Part II. Springer Verlag, New York.
(5) Heidelberger, C., Danenberg, P. V., Moran, R. G. (1983) Adv. Enzymol., 54, 57-119.
(6) Myers, C. E. (1981) Pharmacol. Rev. 33, 1–20
(7) Cohen, S. S., Flaks, J. G., Barnes, H. D., Loeb, M. R., Lichtenstein, J. (1958) Proc. Nat'l. Acad. Sci., USA 44, 1004–1012.
(8) Danenberg, P. V. (1977) Biochim. Biophys. Acta. 473, 73–92.
(9) Santelli, G., Valeriote, F. (1978) J. Nat'l. Cancer Inst. 61, 843–847.
(10) Martin, D. S., Stolfi, R. L., Sawyer, R. C., Nayak, R., Spiegelman, S., Young, C. W., Woodcock, T. (1980) Cancer (Phila.) 45, 1129–1134.
(11) Lockshin, A., Moran R. G., Danenberg, P. V. (1979) Proc. Nat'l. Acad. Sci. USA 76, 750–754.
(12) Mulkins, M. A., Heidelberger, C. (1982) Cancer Res. 42, 956–964.
(13) Grindey, G. B., Nichol, C. A. (1972) Cancer Res. 32, 527–531.
(14) Moran, R. G., Spears, C. P., Heidelberger, C. (1979) Proc. Nat'l. Acad. Sci. USA 76, 1456–1460.
(15) Washtien, W. L., Santi, D. V. (1979) Cancer Res. 39, 3397–3404.
(16) Palmiter, R. D. (1974) Biochemistry 13, 3606–3615.
(17) Lee, S. Y., Mendecki, J., Brawerman, G. (1971) Proc. Nat'l. Acad. Sci. USA, 68, 1331–1335.
(18) Eiden, J. J., Nichols, J. L. (1973) Biochemistry 12, 3951–3956.
(19) Wilkinson, D. S., Crumley, J. (1976) Cancer Res. 36, 4032–4038.
(20) Perry, R. (1963) Exp. Cell Res. 29, 400–405.
(21) Lindberg, U., Persson, T. (1972) Eur. J. Biochem. 31, 246–254.
(22) Houghton, J. A., Houghton, P. J., Wooten, R. S. (1979) Cancer Res. 39, 2406–2413.
(23) Balzarini, J., DeClercq, E., Mertes, M. P., Shugar, D., Torrence, P. F. (1982) Biochem. Pharmacol. 31, 3673–3682.
(24) Au, J. L.-S., Rustum, Y. M., Ledesma, E. J., Mittelman, A., Creaven, P. J. (1982) Cancer Res. 42, 2930–2937.
(25) Maybaum, J., Ullman, B., Mandel, H. G., Day, J. L., Sadee, W. (1980) Cancer Res. 40, 4209–4215.
(26) Carrico, C. K., Glazer, R. I. (1979) Cancer Res. 39, 3694–3701.
(27) Plagemann, P. G. W. (1971) J. Cell Physiol. 77, 241–258.
(28) Baumunk, C. N., Friedman, D. L. (1971) Cancer Res. 31, 1930–1935.
(29) Premveer, Reddy, G. (1982) Biochem. Biophys. Res. Commun. 109, 908–915.
(30) Evans, R. M., Laskin, J. D., Hakala, M. T. (1980) Cancer Res. 40, 4113–4122.

An Efficient In Vitro Assay for Acetylcholinesterase Reactivators Using Immobilized Enzyme

Andy M. Trammel, John E. Simmons, Ronald T. Borchardt[1, 2]

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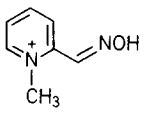
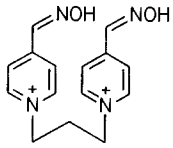
Abstract: A new and efficient *in vitro* assay for evaluating reactivators of organophosphate-inhibited acetylcholinesterase has been developed. Low density polyethylene beads (4 mm) were functionalized to terminal aldehydes and used to immobilize acetylcholinesterase (AChE, *Electrophorus electricus*, E.C. 3.1.1.7) via a stable Schiff base link. AChE activity in columns containing immobilized enzyme could be continuously monitored spectrophotometrically in a closed loop flow system using acetylthiocholine and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Immobilized enzyme exhibited good esterase activity (0.5 units/bead), which could be retained on storage at −16°C for four months. The kinetics for substrate hydrolysis were flow-rate dependent below substrate saturation levels. This system allowed for independent inhibition and reactivation of the enzyme. Immobilized enzyme could be inhibited with diisopropylfluorophosphate (DFP) and 20–90 % of original activity restored with several oximes in less than 20 minutes. The extent of reactivation was dependent on the concentration of the reactivators. This system has advantages over previously reported procedures, because hydrolysis of substrate due to reactivator is minimized and inhibitor-reactivator interactions are eliminated, subsequently minimizing the need to correct experimental results.

[1] The Center for Biomedical Research, The University of Kansas, 2099 Constant Ave., Campus West, Lawrence, KS 66044
[2] To whom correspondence should be addressed.

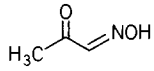
Organophosphate-inhibited acetylcholinesterase (AChE, E.C. 3.1.1.7) can be reactivated by a variety of oximes (e.g., **1–3**). The activities of these reactivators have been evaluated in whole animal studies (1, 2), in assays employing purified AChE (3–8), and by direct reaction with organophosphates (9, 10). Commonly, these reactivators are evaluated *in vitro* using purified AChE that has been inhibited by an organophosphate such as diisopropylfluorophosphate (DFP).



2-Hydroxyimino-methy-1-methyl-pyridinium iodide (2-PAM)
1



1,1'-Trimethylene-bis(4-formyl-pyridinium bromide) dioxime (TMB-4)
2



Monoisonitroso-acetone (MINA)
3

A convenient assay for measuring AChE activity is that described by Ellman et al. (11) which utilizes acetylthiocholine as enzyme substrate and measures spectrophotometrically (412 nm) the formation of thiocholine by its reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The major problem with using the Ellman technique in screening oxime reactivators is the fact that oximes nonenzymatically hydrolyze acetylthiocholine at a rate which is dependent on oxime concentration and oxime structure (8). Evaluating the effectiveness of oxime reactivators is also complicated by the fact that phosphorylated oximes, which result from either the direct reaction of oxime with excess inhibitor of the reactivation step itself, can bind to AChE and serve as potent rephosphorylating agents (12, 13). A third problem in evaluating AChE activity results from the reversible inhibition of the enzyme by the oximes themselves.

In an attempt to overcome these problems we have investigated the use of immobilized AChE to measure the effectiveness of oxime reactivators. The advantage of incorporating immobilized AChE into the assay lies in the fact that reactivation of the inhibited enzyme can be carried out in the absence of substrate (acetylthiocholine), chromogen (DTNB), and excess or residual inhibitor (DFP). Any excess oxime, as well as phosphorylated oxime, can then be washed from the system prior to measuring reactivated enzyme activity. Hence the inhibition, reactivation and measurement of AChE activity can be carried out as separate and discrete steps. It should be noted that AChE found in the central nervous system and the electric eel organ occurs largely in a membrane bound state. Enzyme immobilized on a synthetic matrix may mimic that found in a biological membrane.

Ngo, Laidler, and Yam (14) reported the covalent attachment of AChE to polyethylene tubing. We report here the immobilization of electric eel-AChE on 4 mm polyethylene beads and use of the Ellman assay to measure the effectiveness of oximes **1-3** at regenerating DFP-inhibited, immobilized enzyme.

Materials and Methods

Materials

Purified AChE (E.C. 3.1.1.7, *Electrophorus electricus)* was obtained from Boehringer Mannheim (specific activity 1000 units/mg). Low density polyethylene beads (irregular 4 mm) and DFP were supplied by Aldrich Chemical Company. Acetylthiocholine, DTNB, 3-(N-morpholino)propanesulfonic acid (MOPS) and 1,1'-trimethylene-bis(4-formylpyridinium bromide) dioxime (TMB-4, **2**) were supplied by Sigma Chemical Company and used without further purification. Monoisonitrosoacetone (MINA, **3**) was obtained from Pfaltz & Bauer, Inc., and required purification via column chromatography (Brinkmann Silica gel 60, 70–230 mesh, 100:1 load ratio, 8 % acetone/methylene chloride v/v eluting solvent) to achieve the desired purity. 2-PAM (**1**) was synthesized according to the procedure of Poziomek, Hackley, and Steinberg (15).

Heptanesulfonic acid was obtained from Eastman Kodak Co. A Gilson Minipuls 2 peristaltic pump and Holochrome variable UV-Vis detector were used in the closed loop flow-through system (Fig. 1). HPLC analysis was performed on a Beckman system Model 342 using an Ultrasphere 5 micron ODS RP column (5 mm × 15 cm), a Kratos 769Z variable UV detector (295 nm) and isocratic elution at 1 ml/min with 80 % acetonitrile/0.01 M heptanesulfonic acid adjusted to pH 3.5 with acetic acid.

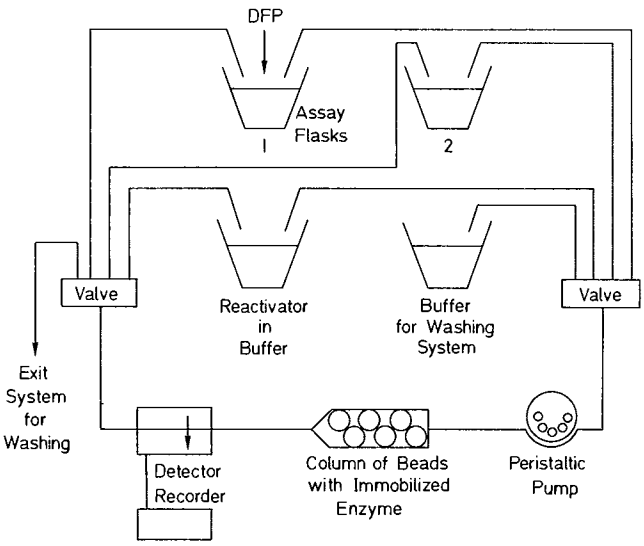


Fig. 1 Schematic of closed loop, flow-through system for immobilized acetylcholinesterase assay.
Flasks 1 and 2 contain substrate (ATC, 10^{-3}M) and chromogen (DTNB, 5×10^{-4} M) in MOPS buffer (0.1 M, pH 7.8) at 37 °C. The peristaltic pump was run at a flow rate of 5.8 ml/minute and the detector was set at 412 nm.

AChE Immobilization

The AChE was immobilized using a modification of the technique reported by Ngo, Laidler, and Yam (14) (Fig. 2). The modifications were as follows: treatment of the beads with thionyl chloride, ethylenediamine and required rinses were carried out under a nitrogen atmosphere; the beads were treated with 10 % glutaraldehyde for three hours with one

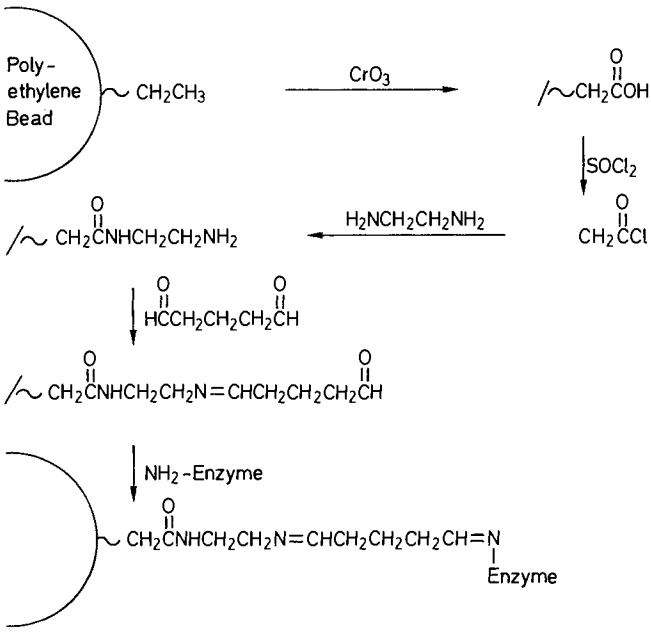


Fig. 2 General scheme for immobilization of AChE on polyethylene beads.

solution change; the attachment of glutaraldehyde to the bead could be monitored for completeness by incubating the beads in the presence of DTNB, since any noncapped amino groups on the bead surface reacted with DTNB resulting in the generation of a chromophore which was monitored at 412 mm; the enzyme was loaded onto the beads in a two hour room temperature incubation; and MOPS buffer (0.1 M, pH 7.8) replaced phosphate buffer throughout.

Beads containing immobilized enzyme were stored at −16 °C in MOPS buffer containing 40 % glycerin. The stability of the enzyme activity was effected by freeze-thaw cycles, therefore the beads were frozen in small batches. The activity was stable for up to four months when stored as described. Enzyme activity per bead averaged 0.5 units/bead; however, significant bead to bead variation was observed. Under normal assay condition (0.1 M MOPS buffer, pH 7.8, 37°C) 1.5–2 %/h loss of enzyme activity was observed.

Measurement of AChE Activity

The enzyme activity assay was based on the Ellman technique (11). Acetylthiocholine (in 90 % ethanol) and DTNB (in 95 % ethanol) were added to 30 ml of MOPS buffer as above to give concentrations of 1×10^{-3} M and 5×10^{-4} M, respectively. Figure 1 is a schematic of the closed loop flow-through system employed in the assay. The peristaltic pump was routinely run at 5.8 ml/min and the column effluent monitored at 412 nm. The column was a polypropylene cylinder which contained immobilized enzyme beads packed with alternating glass beads which optimized surface area and flow characteristics. A solution in the flask containing substrate and chromogen was directed via switching valves through the column. The flask contents were cycled such that a stable baseline rate of enzyme activity was observed on the recorder trace. An excess of DFP (0.572 mM) was added and after all enzyme activity ceased (approximately 5 minutes), the system was flushed with fresh buffer via switching valves.

Reactivators were routinely prepared in 200 ml volumes; however, some of the highest concentrations of 2-PAM and MINA were prepared in 100 ml volumes to conserve material. As mentioned earlier, phosphorylated oximes are potent enzyme inhibitors. To reduce the possibility of the phosphorylated oximes inhibiting the reactivated enzyme, the eluent was passed out of the system for the first five minutes of exposure to the reactivators. A fresh solution of reactivator was then recycled for the remaining exposure time. We observed no difference between the above procedure and pumping reactivator through the column in a single-pass fashion for the entire 45 minute exposure time. In the case of 2-PAM, we also examined the concentrations of reactivator (295 nm), via HPLC, both entering and exiting the column and found no difference.

The detector output was recorded as absorbance units vs time which was converted to rate of substrate hydrolysis using the conversion of Ellman (11):

$$\frac{\Delta \text{ Abs/min}}{1.36 \times 10^4} = \text{moles thiocholine/l} \cdot \text{min}.$$

The substrate concentration routinely used in the assay was at saturation level, and the flow rate was an intermediate value. There was no apparent effect of flow rate on the regeneration of DFP-inhibited immobilized enzyme.

Results

Use of the Ellman technique to screen oxime reactivators is complicated by the fact that oximes nonenzymatically hydrolyze acetylthiocholine at a rate which is dependent on oxime concentration (Fig. 3). To avoid this problem AChE was immobilized on polyethylene beads and incorporated into a closed loop flow-through screening assay for oxime type reactivators of organophosphate-inhibited enzyme. The immobilized enzyme retained activity (0.5 units/bead) for up to four months when stored at −16 °C. There was minimal loss

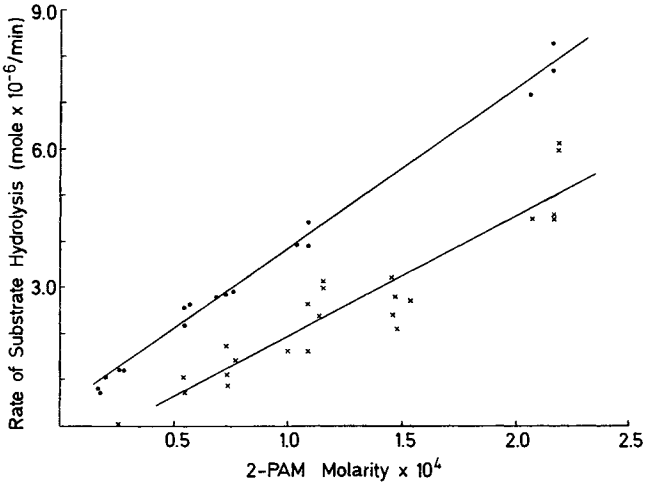


Fig. 3 Enzymatic and nonenzymatic hydrolysis of acetylthiocholine.
x—x, rate of acetylthiocholine (7.5×10^{-4} M) hydrolysis in the presence of varying concentrations of 2-PAM.
●—●, rate of acetylthiocholine (7.5×10^{-4} M) hydrolysis in the presence of varying concentrations of 2-PAM and soluble DFP-inhibited AChE (0.15 units).

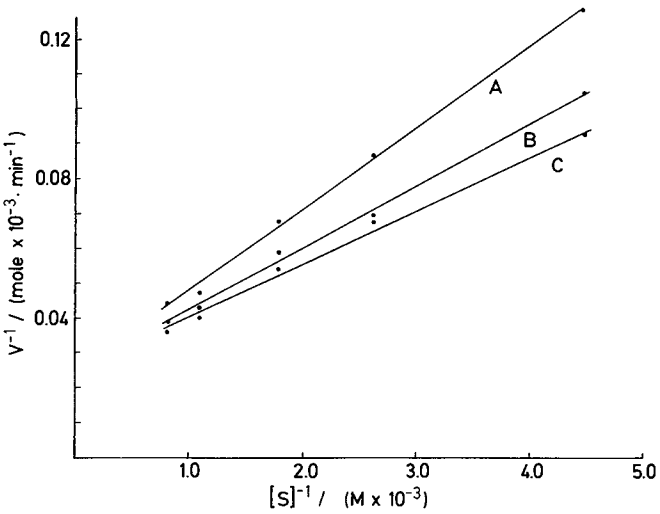


Fig. 4 Lineweaver-Burke plot of AChE activity at various flow rates of substrate and chromogen.
A – 14 ml/min, Vmax – 4.08×10^{-6}, Km – 6.35×10^{-4};
B – 8.7 ml/min, Vmax – 4.07×10^{-6}, Km – 7.21×10^{-4};
C – 4.2 ml/min, Vmax – 4.09×10^{-6}, Km – 9.52×10^{-4}.
Data points represent single determinations. Experimental conditions are identical to those described in Figure 1, except that variable concentrations of acetylthiocholine were employed.

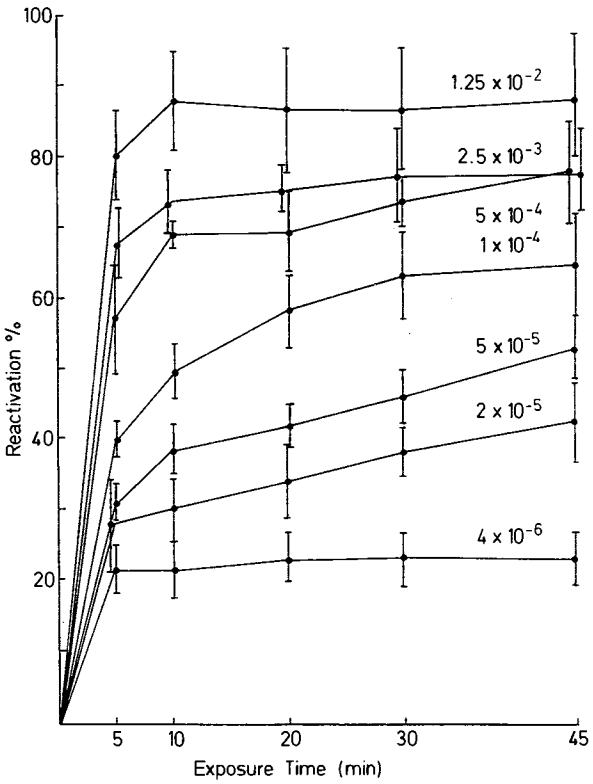


Fig. 5 Effects of varying 2-PAM concentrations on reactivation of acetylcholinesterase.
Concentrations expressed in molarity (M). AChE was inactivated with DFP (6×10^{-4} M). Assay conditions were as outlined in Figure 1. Treatment with varying concentrations of 2-PAM were started at time 0. At 5, 10, 20, 30 and 45 minutes, the immobilized enzyme was washed free of reactivator, then substrated and chromogen (Flask 2, Fig. 1) were cycled through the system to determine the amount of regenerated enzyme activity. Following the measurement of regenerated AChE activity, exposure to regenerator was resumed. Data points represent a minimum of three replications.

of enzyme activity (1–2 %/hr) under normal assay conditions (37 °C). At low substrate concentrations the rate of AChE activity appears to be diffusion controlled (Fig. 4) which was consistent with the results reported by Ngo, Laidler, and Yam (14) using enzyme immobilized on poly-ethylene tubing. We observed 10 % aging (i.e. spontaneous hydrolysis of inhibited enzyme to a nonreactivatible form) of the phosphorylated enzyme after 30 minutes exposure to DFP (data not shown). Following the initial two minute buffer wash, spontaneous reactivation of the inhibited enzyme contributed 10–15 % of original activity and did not increase further (data not shown).

The flow-through system illustrated in Figure 1 permits separate operations to measure AChE activity, to inhibit the enzyme with DFP, to reactivate the enzyme with an oxime, and to measure the reactivated AChE activity. Figure 5 illustrates the reactivation of DFP-inhibited immobilized AChE by various concentrations of 2-PAM. Reactivation to 90 % of initial activity could be achieved routinely. In Figures 6 and 7 the effectiveness of TMB-4 and MINA as reactivators are compared to 2-PAM. TMB-4 was found to be more active and MINA less active than 2-PAM.

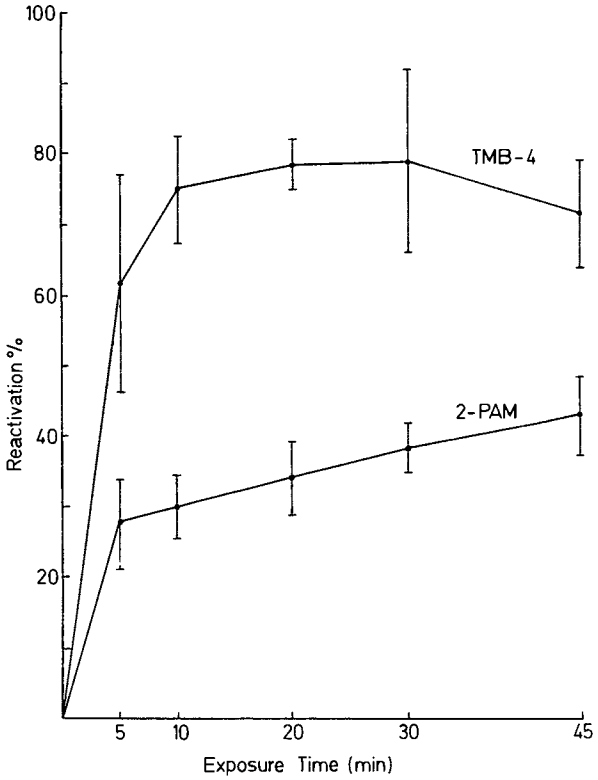


Fig. 6 Comparison of 2-PAM and TMB-4 as reactivators of DFP-inhibited acetylcholinesterase.
2-PAM and TMB-4 concentrations were 2×10^{-5} M. The experimental conditions were identical to those described in Figure 1.

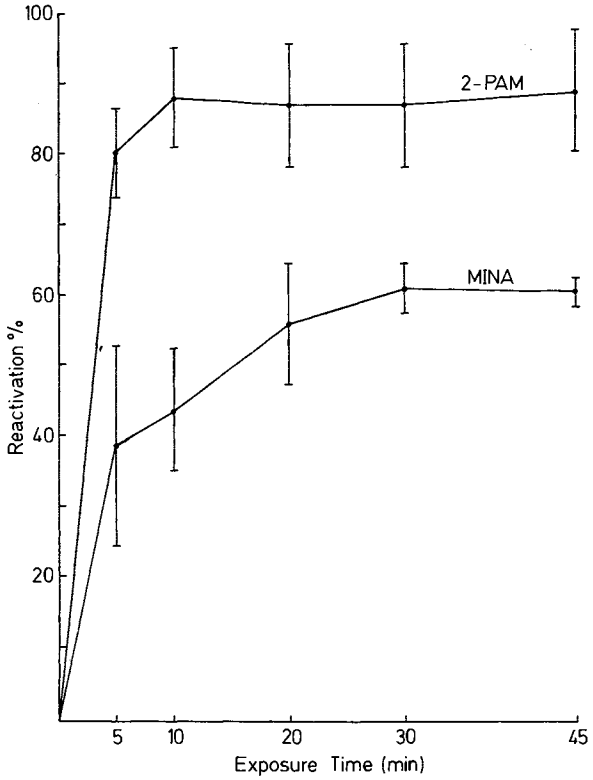


Fig. 7 Comparison of 2-PAM and MINA as reactivators of DFP-inhibited acetylcholinesterase.
2-PAM and MINA concentrations were 1.25×10^{-2} M. The experimental conditions were identical to those described in Figure 1.

Discussion

The AChE assay system developed in this study allowed for the independent and sequential determination of initial enzyme activity, enzyme inhibition, enzyme reactivation and determination of reactivated enzyme activity. By washing the immobilized AChE between the aforementioned steps one removes excess DFP, oxime, phosphorylated oxime, acetylthiocholine and DTNB thus eliminating undersirable interferences. Phosphorylated oxime resulting from the reactivation step is by conservative estimates diluted 10^{10}-fold by reactivator solution. Unlike soluble enzyme assays, the use of the immobilized enzyme system allowed for continuous monitoring at each step, required no aliquoting and eliminated substrate hydrolysis by regenerator as illustrated in Figure 3. This technique also allows for the comparison of reactivated AChE activity to actual initial enzyme activity. Since excess DFP was washed away, the titration of enzyme active sites was not required. Reactivation of inhibited enzyme was carried out in the presence of reactivator alone, thus eliminating interference due to competitive binding by substrate and reinhibition of the enzyme by phosphorylated oxime. Further, reversible inhibition due to reactivator binding was eliminated by a buffer wash prior to determining reactivated enzyme activity. A small disadvantage of this immobilized AChE assay lies in the requirement of approximately 50 mg of potential reactivator, however, the assay can be modified to eliminate the initial 5 minute single-pass reactivation cycle thus lowering the quantity of reactivator required.

The loss of enzyme activity due to enzyme degradation (1–2 %) was negligible during the course of a two-hour experiment. The extent of spontaneous hydrolysis of DFP-inhibited AChE and the amount of aging of inhibited enzyme were small and required no correction of experimentally determined data.

The assay was effective for the determination of the reactivating capabilities of quaternary pyridinium oximes (e.g., 2-PAM and TMB-4) as well as an uncharged oxime (MINA). The order of reactivating ability was TMB-4 > 2-PAM > MINA, which was consistent with earlier observations (see Table I). As shown in Figures 5–7 there was, at all concentrations of reactivators employed, an initial rapid recovery of enzyme activity which was followed by a slower drift to higher enzyme activity. This phenomenon, according to Schoene (12), was attributed to a rapid initial equilibrium followed by a slow drift to higher activity due to the breakdown of phosphorylated oxime. Since our system allowed for the removal of any phosphorylated oxime, we do not feel this is a valid explanation of our experimental results. Alternative explanations could be the heterogenous nature of the enzyme itself or the effects of immobilization (i.e., multiple covalent links) which could result in a variety of different enzyme forms each exhibiting their own sensitivity to inhibition and reactivation. Recently, we have observed similar reactivation kinetics of AChE in a mouse brain slice preparation pretreated with DFP (Andy Trammel, et al., unpublished data). A comprehensive explanation of this unusual kinetic behaviour will require further investigation.

Table I. Comparison of Reactivators Using Various Assay Methods.

Reactivator	Conc.	Time	% Reactivation	Method	Ref.
2-PAM	10^{-3}M	2 h	100	Ellman	8
2-PAM	2.5×10^{-3}M	45 min	80		*
2-PAM	10^{-5}M	45 min	75	CO_2equil	4
2-PAM	2×10^{-5}M	45 min	45		*
TMB-4	10^{-5}M	45 min	100	CO_2equil	3
TMB-4	2×10^{-5}M	45 min	70		*
TMB-4	10^{-4}M	45 min	60	Titrametric	7
MINA	10^{-2}M	45 min	15	Titrametric	5
MINA	10^{-2}M	45 min	60		*

*Measured in our laboratories using immobilized AChE and the Ellman technique.

Acknowledgement

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References

(1) Nishimura, T., Tamura, C., Uchida, Y. (1967) Nature 214, 706–708.
(2) Clement, J. G. (1979) Toxicol. Appl. Pharmacol. 14, 305–311.
(3) Reiner, E. (1965) Biochem. J. 97, 710–714.
(4) Hobbiger, F., Pitman, M. P., Sadler, P. W. (1960) Biochem. J. 75, 363–372.
(5) Childs, A. F., Davies, D. R., Green, A. L., Rutland, J. P. (1955) Brit. J. Pharmacol. 10, 462-465.
(6) Berends, F. (1964) Biochim. Biophys. Acta 81, 190–193.
(7) Schoene, K. (1972) Biochem. Pharmacol. 21, 163-170.

(8) Kenley, R. A., Howd, R. A., Mosher, C. W., Winterle, J. S. (1981) J. Med. Chem. 24, 1124–1133.
(9) Ashani, Y., Cohen, S. (1971) J. Med. Chem. 14, 621-626.
(10) Hagendorn, I., Stark, I., Lorenz, H. P. (1972) Angew. Chem. Intl. Ed. 11, 307–309
(11) Ellman, G. L., Courtney, K. D., Andres, V., Jr., Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88–95.
(12) Schoene, K. (1976) in Medical Protection Against Chemical-Warfare Agents (J.E.S. Stares, ed.), pp. 88–100, Alonqvist & Wiksell Int., Stockholm.
(13) Hackley, B. E., Jr., Steinberg, G. M., Lamb, J. C. (1959) Arch. Biochem. Biophys. 80, 211–214.
(14) Ngo, T. T., Laidler, K. J., Yam, C. F. (1979) Can. J. Biochem. 57, 1200-1204.
(15) Poziomek, E. J., Hackley, B. E., Steinberg, G. M. (1958) J. Org. Chem. 23, 714-717.
(16) deJong, L. P. A., Wolring, G. Z. (1978) Biochem. Pharmacol. 27, 2911–2917.

Soft Drugs VI. The Application of the Inactive Metabolite Approach for Design of Soft β-Blockers[1, 2]

Nicholas Bodor[1, 5], Yasuo Oshiro[1, 2], Thorsteinn Loftsson[1, 3], Michael Katovich[1], and William Caldwell[4]

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Abstract: The "inactive metabolite approach" was used to design β-blockers. The acidic inactive metabolite of metoprolol [4-(2-hydroxy-3-isopropylamino) propoxyphenylacetic acid] was used as the lead compound. Its esters (alkyl and cycloalkyl) were found active *in vivo* while reverting quantitatively to the same inactive metabolite in plasma. The cyclohexyl ester showed the best activity, which was cardioselective, similar to the parent compound metoprolol. Although most esters had a plasma half-life of approximately 1 min, their activity (antagonism of isoproterenol induced increase in heart rate) following intravenous administration lasted 45-90 minutes, and the maximum β-blockade was observed at 45–60 minutes in both rats and dogs.

The large number of β-adrenergic blocking agents are generally subject to facile, oxidative metabolic degradations. Many of these metabolites possess significant β-blocking activity, and some metabolites, e.g. of bufuralol (3), have longer biological half-lives than the parent drug. These pharmacokinetic properties make it difficult to optimize therapy in individual patients. It is therefore desirable to design β-blockers which are metabolized in a simple, predictable and controllable manner in one step to an inactive metabolite, regardless of the conditions of the patient and other drugs used. This would necessitate, however, avoiding oxidative metabolism. The general "soft-drug" design has these above objectives (4), and one of the most promising design concepts suggested, the "inactive metabolite approach" (4, 5), is eminently suited for the present problem, since many of the β-blockers have acidic, inactive metabolites, which can serve as the lead compounds (4). The principles of the "inactive metabolite approach" are: 1. select a known inactive metabolite of a drug; 2. modify the structure of the metabolite to resemble (isosterically and/or isoelectronically) the parent drug (activation process); 3. design the structure of the new derivatives in such a way that it metabolizes preferentially in one step to the starting inactive metabolite, without going through reactive, toxic intermediates (predictable metabolism); 4. control transport and binding properties, as well as the rate of metabolism and pharmacokinetics by chemical manipulations of the activated moiety.

The approach is applied in the present paper to the case of metoprolol, **1**, a selective β_1-adrenoreceptor antagonist. Its metabolism was extensively studied in rat, dog and man (6, 7), including patients with impaired renal function (8). As shown in Scheme 1, there are four metabolites resulting from the oxidation of metoprolol, among which the O-demethylmetoprolol **2** and α-hydroxymetoprolol **3** have selective β_1-blocker activity, but with 5 to 10 times lower potency than **1** (6, 9), while the acids **4** and **5** are inactive. The phenylacetic derivative **5** is the major metabolite found in the urine, and it can be the lead compound for our "inactive metabolite approach". Accordingly, a series of esters of **5** were prepared. They are expected to have β-blocking activities, and one hydrolytic (i.e., esterase) process deactivates the compounds yielding the starting inactive metabolite. The rate of hydrolysis deactivation could possibly be controlled by the structure of the esters. The β-adrenoreceptor antagonist activities of the esters were determined in rats and dogs, while relative esterase catalyzed cleavage rates were measured *in vitro* in human plasma.

Experimental

Chemistry. All melting points are uncorrected and were obtained with an electrothermal capillary melting point apparatus.

n-Propyl 4-(2-hydroxy-3-isopropylamino)propoxyphenylacetate **(11)**

A mixture of 4-hydroxyphenylacetic acid (9.12 g, 0.06 mol), n-propanol (40 ml) and $SOCl_2$ (2 ml, 0.028 mol) was refluxed for 3 hr and evaporated *in vacuo*. The residue was extracted with ethyl acetate (200 ml), washed with 10 % Na_2CO_3, then dried

[1] Department of Medicinal Chemistry, Box J-4, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, Florida 32610.
[2] On leave of absence from Otsuka Pharmaceutical Company, Osaka, Japan.
[3] On leave of absence from the University of Iceland, Reykjavik.
[4] Department of Pharmacology, College of Medicine University of Tennessee, Memphis, Tennessee 38163.
[5] To whom correspondence should be addressed.