

AD

**EDGEWOOD ARSENAL
SPECIAL PUBLICATION**

EASP 1100-8

**UTILIZATION OF [^{32}P] SOMAN FOR MEASUREMENT
OF ACETYLCHOLINESTERASE IN BRAIN TISSUES**

by

Norman C. Thomas

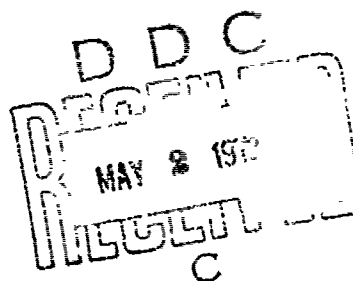
Joseph H. Fleisher

Larrel W. Harris

April 1972



Reproduced by
**NATIONAL TECHNICAL
INFORMATION SERVICE**
Springfield, Va 22151



**DEPARTMENT OF THE ARMY
EDGEWOOD ARSENAL
Somedical Laboratory
Edgewood Arsenal, Maryland 21010**

Distribution Statement

This document may be further distributed by the holder only with specific prior approval of the Commanding Officer, Edgewood Arsenal, ATTN: SMUEA-TS-R, Edgewood Arsenal, Maryland 21010.

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Disposition

Destroy this report when no longer needed. Do not return it to the originator.

ACCESSION FOR	
SPETI	WHITE SECTION ☒
DOC	BUFF SECTION ☐
UNANNOUNCED	☐
JUSTIFICATION	
BY	
DISTRIBUTION AVAILABILITY CODES	
DOC	ATL. USE OF SPECIAL
A	20

UNCLASSIFIED

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) CO, Edgewood Arsenal ATTN: SMUEA-BL-RBB Edgewood Arsenal, Maryland 21010		2a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	
		2b. GROUP NA	
3. REPORT TITLE UTILIZATION OF [³² P] SOMAN FOR MEASUREMENT OF ACETYLCHOLINESTERASE IN BRAIN TISSUES			
4. DESCRIPTIVE NOTES (Type of report and inclusive dates) This work was started in November 1967 and completed in July 1969.			
5. AUTHOR(S) (First name, middle initial, last name) Norman C. Thomas, Joseph H. Fleisher, Larrel W. Harris			
6. REPORT DATE April 1972		7a. TOTAL NO. OF PAGES 15	7b. NO. OF REFS. 19
8a. CONTRACT OR GRANT NO.		8b. ORIGINATOR'S REPORT NUMBER(S) EASP 1100-6	
8c. PROJECT NO.			
8d. TASK NO. 1W662710AD2502		8e. OTHER REPORT NUMBER (Any other number that may be assigned this report)	
9. DISTRIBUTION STATEMENT Approved for public release; distribution unlimited			
11. SUPPLEMENTARY NOTES Medical defense against chemical agents; prophylaxis and therapy for lethal agents		12. SPONSORING MILITARY ACTIVITY	
13. ABSTRACT Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) activity in brain tissues of the dog was measured before and after inhibition with ³² P-labeled pinacolyl methylphosphonofluoridate (soman). A high correlation was obtained between uninhibited enzyme activity and soman-derived radiophosphorus bound to the tissues as methyl [³² P] phosphonate. The methylphosphonate for 100% inhibition was taken equivalent to the catalytic sites. Turnover numbers calculated from the ratio of acetylcholine hydrolysis to catalytic sites approached values yielded by cell acetylcholinesterase.			
14. KEYWORDS ³² P-Soman Acetylcholinesterase Acetylcholinesterase inhibition Acetylcholinesterase turnover number Inhibition Dog brain Catalytic sites Estimation			

DD FORM 1472

REPLACES DD FORM 1472, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

UNCLASSIFIED

Security Classification

EDGEWOOD ARSENAL SPECIAL PUBLICATION

EASP 1100-6

UTILIZATION OF [^{32}P] SOMAN FOR MEASUREMENT
OF ACETYLCHOLINESTERASE IN BRAIN TISSUES

by

Norman C. Thomas
Joseph H. Fleisher
Larrel W. Harris

Medical Research Division

April 1972

Approved for public release; distribution unlimited.

Task 1W662710AD2502

DEPARTMENT OF THE ARMY
EDGEWOOD ARSENAL
Biomedical Laboratory
Edgewood Arsenal, Maryland 21010

FOREWORD

The work described in this report was authorized under Task 1W662710AD2502, Medical Defense Against Chemical Agents, Prophylaxis and Therapy for Lethal Agents. This work was started in November 1967 and completed in July 1969. The experimental data are contained in notebook MN-2135.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

This report is reprinted from Biochem. Biophys. Acta 235, 542-547 (1971) by permission of the copyright owner. The letter of permission to use the copyrighted material is on file in the Legal Office, Edgewood Arsenal, Maryland 21010. Reproduction of the copyrighted material in whole or in part is prohibited except with permission of the copyright owner; however, DDC and the National Technical Information Service are authorized to reproduce this document for United States Government purposes.

DIGEST

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) activity in brain tissues of the dog was measured before and after inhibition with ^{32}P -labeled pinacolyl methylphosphonofluoridate (soman). A high correlation was obtained between uninhibited enzyme activity and soman-derived radiophosphorus bound to the tissues as methyl [^{32}P] phosphonate. The methylphosphonate for 100% inhibition was taken equivalent to the catalytic sites. Turnover numbers calculated from the ratio of acetylcholine hydrolysis to catalytic sites approached values yielded by eel acetylcholinesterase.

Reprinted from

Biochimica et Biophysica Acta
Elsevier Publishing Company, Amsterdam • Printed in The Netherlands

BBA Report

BBA 61225

Utilization of [32 P]soman for measurement of acetylcholinesterase in brain tissues

NORMAN C. THOMAS, JOSEPH H. FLEISHER and LARREL W. HARRIS

Basic Medical Sciences Department, Medical Research Laboratory, Edgewood Arsenal, Md. 21010 (U.S.A.)

(Received April 6th, 1971)

SUMMARY

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) activity in brain tissues of the dog was measured before and after inhibition with 32 P-labeled pinacolyl methylphosphonofluoridate (soman). A high correlation was obtained between uninhibited enzyme activity and soman-derived radiophosphorus bound to the tissues as methyl [32 P] phosphonate. The methylphosphonate for 100% inhibition was taken equivalent to the catalytic sites. Turnover numbers calculated from the ratio of acetylcholine hydrolysis to catalytic sites approached values yielded by eel acetylcholinesterase.

It is well established that inhibition of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by organophosphorus compounds *in vitro* takes place through a phosphorylation of the enzyme¹⁻³. However, no correlation has been observed *in vivo* between the radiophosphorus bound to mammalian tissues following injection of 32 P-labeled diisopropylphosphorofluoridate (DFP) or isopropyl methylphosphonofluoridate (sarin) and the normal acetylcholinesterase content of such tissues⁴⁻⁶. The lack of correlation observed in these studies must be regarded as inconclusive since no distinction was made between phosphorus specifically bound to acetylcholinesterase and that bound non-specifically to other proteins^{3,7} and many tissues⁸. The distinction and measurement of specific binding of phosphorus in relation to inhibition of acetylcholinesterase *in vitro* and *in vivo* has been under study in our laboratory⁹⁻²⁰. We have shown that in rats given [32 P] sarin, that portion of brain acetylcholinesterase which could be reactivated *in vitro* by 2-pyridinium aldoxime methochloride approximated the percentage of phosphorus released as isopropyl methylphosphonate. The amount of phosphorus retained by the enzyme after incubation with 2-pyridinium aldoxime methochloride was present entirely

as methyl [^{32}P] phosphonate and paralleled the percentage of enzyme not reactivated ("aged" enzyme). The close correlation between the enzymatic and radioactivity measurements suggested that the method distinguished phosphorus bound to acetylcholinesterase from that bound to other sites⁹.

Pinacolyl methylphosphonofluoridate (soman) like DFP and sarin phosphorylates both acetylcholinesterase and non-specific sites⁶. It differs from DFP and sarin in producing an inhibited acetylcholinesterase which is only slightly reactivated by oximes¹¹. In explanation, Fleisher and Harris⁶ showed that the acetylcholinesterase phosphorylated by [^{32}P] soman *in vitro* undergoes a very rapid decrease in reactivatability in parallel with loss of a pinacolyl group (dealkylation). Rapid dealkylation of soman-inactivated erythrocyte acetylcholinesterase and eel acetylcholinesterase *in vitro* has also been shown by Coult *et al.*¹² and Michel *et al.*¹², respectively. In agreement with the low acetylcholinesterase activity of homogenates of rat liver and lung compared to that from brain¹⁴, the soman-derived phosphoryl residue in liver and lung tissue showed little dealkylation compared to that in brain tissue of rats given [^{32}P] soman⁶.

These findings suggested the possibility that the methylphosphonate content of a tissue might be related to the number of acetylcholinesterase sites following inactivation by soman. Experiments designed to test this possibility in brain tissue are described in this report.

Anaesthetized dogs were perfused with heparinized 0.9% saline until the brain was virtually free of blood. The brain was removed after sacrifice; and the caudate nucleus, thalamus, and portions of the medulla, hippocampus, cerebral and cerebellar cortex were excised, weighed and used for the following studies:

(A) *Rate of loss of reactivatability ("aging") in vitro*. Homogenates of each tissue (20%, w/v) were prepared in 0.01 M borate buffer at pH 8.8 and 0°. The preparations were incubated with $2 \cdot 10^{-8}$ M soman (final concentration) for 30 min at 0°. 30–90% inhibition with minimal aging resulted. The mixture was immediately equilibrated to 37°. Reactivatability by monoisonitrosacetone was measured before, and at suitable time intervals after, adjusting the pH to 7.4 to initiate aging as described previously⁶. The rate of loss of reactivatability by monoisonitrosacetone was first order with a half time of 6.04 ± 0.58 min ($P = 0.95$) for 18 preparations (3 for each brain tissue). This rate does not differ significantly from that reported earlier by us for dog erythrocyte acetylcholinesterase inhibited by soman¹⁵; and it is taken as approximating the rate of dealkylation⁶.

(B) *Ratio of hydrolysis of acetylcholine to acetyl- β -methylcholine in homogenates of dog brain tissues*. This study was performed in order that turnover numbers for acetyl- β -methylcholine in dog brain tissues could be converted to acetylcholine (Table I).

Acetylcholinesterase activity for both substrates was measured radiometrically according to Siakotos *et al.*¹⁶. The method is based upon the adsorption of unhydrolyzed substrates as their [$1\text{-}^{14}\text{C}$] acid choline esters on Amberlite CG-120 resin suspended in dioxane. The supernatant solution containing the product of hydrolysis, the free [$1\text{-}^{14}\text{C}$] acid, is counted in a liquid scintillation spectrometer. Details concerning the preparation of substrates, the resin, and the scintillation cocktail (fluor) are given by Siakotos *et al.*¹⁶.

TABLE I
ACETYLCHOLINESTERASE ACTIVITY AND SPECIFIC BINDING OF PHOSPHORUS FROM [32 P] IN SOME BRAIN TISSUES OF THE DOG

Tissue*	Control activity (moles of [14 C] acetyl- β -methylcholine per min per g of tissue wet wt. $\times 10^6$)	Methyl [32 P] phosphonate bound for 100% inhibition (μ g soman per g of tissue wet wt. $\times 10^3$)	Moles of active site per g*** $\times 10^{11}$	Turnover number (moles of substrate per mole of active site per min)
Caudate nucleus (9)	11.16 $\pm$ 1.16**	25.97 $\pm$ 1.34**	14.30	0.70
Cerebellar cortex (8)	2.02 $\pm$ 0.17	5.76 $\pm$ 0.73	3.17	0.61
Medulla (8)	1.43 $\pm$ 0.57	3.84 $\pm$ 0.29	2.11	0.68
Thalamus (8)	1.13 $\pm$ 0.10	3.23 $\pm$ 0.24	1.76	0.64
Hippocampus (9)	0.86 $\pm$ 0.05	2.05 $\pm$ 0.36	1.13	0.76
Cerebral cortex (8)	0.32 $\pm$ 0.02	1.28 $\pm$ 0.99	0.70	0.46
				[14 C] Acetyl- β -methylcholine ($\times 10^5$)
				4.06
				3.33
				3.54
				3.32
				4.04
				2.80

* Number of preparations in parentheses.

** Standard error of the mean.

*** A sample calculation to give the concentration of active sites is shown for the caudate nucleus.

$$\text{Active site in moles/g wet wt. of tissue} = \frac{25.97 \cdot 10^{-3}}{\text{mol.wt. soman in } \mu\text{g}} = \frac{25.97 \cdot 10^{-3}}{182 \cdot 10^6} = 0.143 \cdot 10^{-9} = 14.30 \cdot 10^{-11} \text{ moles}$$

10% homogenates of the brain tissues were prepared in water. The cerebral cortex homogenate was further diluted to 4% (w/v), that from caudate nucleus to 0.2%, and the remaining tissue homogenates to 1% with water. For assay, 0.1 ml of homogenate and 0.1 ml of 0.1 M sodium phosphate buffer at pH 7.4 containing 0.3 M NaCl and 1% Lubrol WX (I.C. I. Organics Inc., Stamford, Conn.) were put in each of two centrifuge tubes. 100 μ l of $3 \cdot 10^{-3}$ M [$1\text{-}^{14}\text{C}$] acetylcholine iodide (2.5 mC/mmole) was added to the first sample and the same volume of $3 \cdot 10^{-3}$ M [$1\text{-}^{14}\text{C}$] acetyl- β -methylcholine (4.16 mC/mmole) to the second sample of each homogenate. The contents were mixed immediately and incubated for 5 min at 37°. Preliminary studies showed substrate hydrolysis to be linear with time under these conditions, 5 ml of dioxane-resin suspension were then added to each tube. The mixture was then made to 10 ml with dioxane, mixed, centrifuged, and 5 ml of the supernatant was removed and counted. The readings were corrected for non-enzymatic hydrolysis of the labeled substrate with control solutions containing 0.1 ml of buffer medium and 0.1 ml of water in place of tissue. The readings (counts/min) were referred to a standard curve relating radioactivity (counts/min) to $\mu\text{moles} \cdot 10^{-3}$ of the labeled substrate. The total hydrolysis of substrate per g of tissue per min was calculated from the following formula:

$$\mu\text{moles} \cdot 10^{-3} = \frac{10 \text{ ml}}{5 \text{ ml}} \cdot \frac{1}{5 \text{ min}} \cdot \frac{1}{\text{mg of homogenate used}} \cdot \frac{1000 \text{ mg}}{\text{g}}$$

The values so obtained for the hydrolysis of [$1\text{-}^{14}\text{C}$] acetyl- β -methylcholine are given in Table 1. The ratio for acetylcholine/acetyl- β -methylcholine hydrolysis determined radiometrically on 18 tissue samples (6 brain tissues from each of 3 dogs) was 5.21 ± 0.23 ; $P = 0.95$. The ratio so obtained with 1.0 mM substrate concentrations agrees closely with one of 5.18 reported by Jackson and Aprison¹⁷ for 0.75 mM concentrations of the same substrates.

(C) *Inhibition and radiophosphorus binding following addition of [^{32}P]soman to brain homogenates.* [^{32}P] Soman in water was added in the proportion of 1:99 (v/v) to the diluted homogenates of each brain tissue so as to give final concentrations of $0.25 \cdot 10^{-9}$ M, $0.50 \cdot 10^{-9}$ M, $1.0 \cdot 10^{-9}$ M and $2.0 \cdot 10^{-9}$ M. The mixtures were incubated for 2–3 h at 37° so as to exceed 20 half-lives for "aging" (and presumably of dealkylation). Controls for each tissue incubated with water alone were performed concurrently. Aliquots (0.1 ml) were removed for assay of acetylcholinesterase activity using [$1\text{-}^{14}\text{C}$] acetyl- β -methylcholine as described above. Trichloroacetic acid was added to the remainder to 5% concentration. After 20 min in trichloroacetic acid solution, 20 mg of bovine albumin in 0.2 ml was added as a carrier. Controls containing albumin alone in the absence of tissue were also run. All samples were centrifuged at 2000 rev./min and supernatants discarded. The residues were washed twice with 5% trichloroacetic acid, once with ethanol-ether (3:7, v/v), and twice with ether, followed by centrifugation and removal of the supernatant after each wash. The protein-bound, soman-derived phosphorus was released by alkaline hydrolysis, and the ^{32}P -labeled pinacolyl methylphosphonate and methylphosphonate, respectively, were estimated by partition between isobutanol-benzene (1:1, v/v) and an aqueous medium as previously

reported⁸. The radioactivity contributed by methyl[³²P]phosphonate was obtained from $M = Z - F_A Z$ where M is the number of methyl[³²P]phosphonate counts bound to protein, Z is the total counts and $F_A Z$ is the number of counts contributed by pinacolyl methyl[³²P]phosphonate. Substitution for the value of F_A^* in $M = Z(1 - F_A)$, gives $M = Z[1 - 1.03(R - 0.053)/(R + 1)]$ where R is the ratio of radioactivity in the organic solvent phase to that in the aqueous phase. The counts were corrected for background and converted to μg by reference to the radioactivity of known quantities of pinacolyl methyl[³²P]phosphonate and methyl[³²P]phosphonate run concurrently as standards. From the known weight of the tissue, and use of the above formula, the amount of soman-derived pinacolyl methyl[³²P]phosphonate and methyl[³²P]phosphonate bound to protein could be calculated. At levels of inhibition below 50%, the contribution of pinacolyl methyl[³²P]phosphonate to the total radioactivity was usually less than 10%. Since sufficient time for complete dealkylation of soman-phosphorylated acetylcholinesterase had been taken, the pinacolyl methylphosphonate found was probably bound to sites other than acetylcholinesterase, and therefore was not used for calculation of the enzymatic sites. Instead, the radiophosphorus estimated as methyl[³²P]phosphonate for partial inhibition, was extrapolated to the values that would be expected for 100% inhibition (Table I).

A regression equation between the values for control enzymatic activity toward [¹⁴C]acetyl- β -methylcholine and methyl[³²P]phosphonate bound (Table I) was developed. By use of this equation, theoretical values for bound methyl[³²P]phosphonate were compared with the observed measurements. The correlation coefficient between the calculated and observed values for bound methylphosphonate was 0.99 with 95% confidence limits. This high correlation suggests that radioactivity measured as methylphosphonate under these conditions is specifically related to acetylcholinesterase activity. The turnover numbers obtained for acetylcholinesterase from the ratio of [¹⁴C]acetylcholine hydrolysis to catalytic sites measured directly as soman-derived methyl[³²P]phosphonate (Table I) ranged from $2.4 \cdot 10^5$ for cerebral cortex to $4.06 \cdot 10^5$ for caudate nucleus, comparable to those obtained by lengthier, more indirect procedures^{18,19}, and approaching values yielded by purified acetylcholinesterase from electric eel^{1,19}.

These findings further support the specificity of the measurement of catalytic sites in acetylcholinesterase with labeled soman under the conditions used in this report.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

REFERENCES

- 1 H.O. Michel and S. Krop, *J. Biol. Chem.*, 190 (1951) 119.
- 2 W.N. Aldridge and A.N. Davison, *Biochem. J.*, 55 (1953) 763.
- 3 R.J. Jandorf, H.O. Michel, N.K. Schaffer, R. Egan and W.H. Summerson, *Discussions Faraday Soc.*, 20 (1955) 134.

*The derivation of the formula for F_A is given in ref. 8.

- 4 B.J. Jandorf and B.P. McNamara, *J. Pharmacol. Exptl. Therap.*, 98 (1950) 77.
- 5 K.M. Wilson, *Porton Technical Paper*, 398 (1954).Unclassified.
- 6 R.L. Polak, *Acta Physiol. Pharmacol., Neerl.*, 12 (1963) 81.
- 7 K.A. Oosterbaan, M.G.P.J. Warringa, H.S. Jansz, F. Berends and J.A. Cohen, *Proc. 4th Intern. Congr. Biochem., Vienna, 1958*, Pergamon Press, New York, 1960, p.38.
- 8 J.H. Fleisher and L.W. Harris, *Biochem. Pharmacol.*, 14 (1965) 641.
- 9 L.W. Harris, J.H. Fleisher, J. Clark and W.J. Cliff, *Science*, 154 (1966) 404.
- 10 J.H. Fleisher, L.W. Harris and P.T. Berkowitz, *Biochem. Pharmacol.*, 19 (1970) 421.
- 11 T.A. Loomis and B. Salafsky, *Toxicol. Appl. Pharmacol.*, 5 (1963) 685.
- 12 D.B. Coulter, D.J. Marsh and G. Read, *Biochem. J.*, 98 (1966) 869.
- 12 H.O. Michel, B.E. Hackley, L.M. Berkowitz and E.B. Hackley, *Arch. Biochem. Biophys.*, 121 (1967) 29.
- 14 M.G. Ord and R.H.S. Thompson, *Biochem. J.*, 46 (1950) 346.
- 15 J.H. Fleisher, L.W. Harris and E.F. Murina, *J. Pharmacol. Exptl. Therap.*, 156 (1967) 345.
- 16 A.N. Siskos, M.G. Föbert and R. Hester, *Biochem. Med.*, 3 (1969) 1.
- 17 R.L. Jackson and M.H. Aprison, *J. Neurochem.*, 13 (1966) 1351.
- 18 J.A. Cohen and M.G.P.J. Warringa, *Biochim. Biophys. Acta*, 11 (1953) 52.
- 19 H.C. Lawler, *J. Biol. Chem.*, 235 (1961) 2296.

Biochim. Biophys. Acta, 235 (1971) 542-547