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POTENTIATION OF THE RESPONSE OF FROG RECTUS MUSCLE TO ACETYLCHOLINE BY *ISO*PROPYL METHYL PHOSPHONOFLUORIDATE AND ITS MODIFICATION BY PYRIDINE-2-ALDOXIME METHIODIDE

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Pyridine-2-aldoxime methiodide (P2AM) was used to study the relation between the recovery of cholinesterase activity of isolated frog rectus abdominis muscle and the change of isotonic response to acetylcholine after previous treatment with the anticholinesterase, *iso*propyl methyl phosphonofluoridate (sarin). Addition of P2AM to muscle which had been incubated with sarin produced an 88% decrease in potentiation to acetylcholine. This was accompanied by 71% and 35% recoveries of the cholinesterase activity of the intact and finely ground muscle respectively compared with controls from the contralateral muscle. Following pre-treatment with sarin, a two-hour rinsing with acetylcholine (3 μg./ml.) produced a 61% decrease in potentiation to acetylcholine accompanied by 24% and 4.5% recoveries of cholinesterase activity in intact and in ground muscle respectively. Since control experiments showed absence of uncombined sarin in the muscle after rinsing with acetylcholine solution, the results indicate a greater effectiveness of P2AM and acetylcholine in reactivating superficially situated cholinesterase of the frog rectus abdominis as compared with enzyme within the interior of the muscle.

Cohen and Posthumus (1955) observed a partial decrease in the response of the isolated frog rectus abdominis muscle to acetylcholine (ACh) upon prolonged washing following previous incubation with anticholinesterases. However, no corresponding reversal of the inactivation of the cholinesterase (ChE) activity could be demonstrated in finely ground preparations of these muscles referred to subsequently as "homogenates." In our laboratory, prolonged washing (up to 24 hr.) of sarin-incubated muscles with buffered frog Ringer did not yield any significant lowering of the potentiated response to ACh which could be ascribed to a true reversal. The partial lowering observed in some of our preparations appeared due to fatigue, or to some non-specific action of the anticholinesterase limiting the contraction of the muscle. This conclusion was based on the observation that muscles showing such a reduced response after washing with Ringer solution could not have their potentiated response to ACh restored by a second incubation with anticholinesterase. It was decided, therefore, to attempt a much greater degree of reversal of potentiation by treatment of the maximally potentiated muscle with pyridine-2-aldoxime methiodide (P2AM) shown by Wilson and Ginsberg (1955) and Childs, Davies, Green and Rutland (1955) to be a potent reactivator of inhibited ChE.

METHODS

*Iso*propyl methyl phosphonofluoridate (sarin) was obtained from the Field Toxicology Branch of Medical Research Directorate. The purity was 95% or better according to an analysis of acidity.

For other materials, and for the method of measuring isotonic contraction, see Fleisher, Howard, and Corrigan (1958).

Measurement of Cholinesterase Activity. — ChE activity was determined by a bioassay procedure using the isotonic responses of an eserine-treated rectus abdominis muscle from another frog as a measure of the ACh remaining in the mixture of substrate and the experimental muscle after various periods of incubation. The experimental muscle was blotted gently upon removal, weighed to the nearest mg., and placed in 1.9 ml. of frog Ringer at *p*H 7.2. At zero time, 0.1 ml. of substrate was added and the mixture shaken at 120 oscillations/min. in a water bath at

25°. Serial aliquots of 0.1 ml. each were removed at known time intervals and added to the bath containing the muscle. The contractures so elicited were compared alternately with those given by the addition of known standard amounts of ACh. The logarithms of the concentrations of ACh thus estimated were plotted against time. Since the concentration of ACh used in the incubation mixture was always 1.25×10^{-4} M or lower, first order kinetics could be applied to the calculation of enzyme activity (Augustinsson, 1950). From the linear plots thus obtained the time (in min.) for hydrolysis of one half the initial amount of substrate ($t_{1/2}$) was calculated. The first order rate constant was obtained by substitution in the formula: $k = \frac{0.693 \times 60_{hr.} - 1}{t_{1/2}}$ (Neilands and Stumpf, 1955) and ChE activity was expressed as k/mg. of tissue.

The ChE activity of finely ground muscle was determined in a final tissue concentration of 2.5 mg./ml. (unless otherwise noted) after grinding the entire muscle for 2 min. in a Potter-Elvehjem homogenizer powered by a cone-driven stirring motor. The substrate concentration was the same as that used for determining the ChE activity of intact muscle. Control studies, using the same amount of homogenate without added substrate, showed the absence of ACh when the homogenate was added to the eserine-treated assay muscle. Concurrent controls run with substrate in the absence of tissue showed no non-enzymatic hydrolysis under the conditions used (25° and *p*H 7.2).

RESULTS

Control Studies on the ChE Activity of Intact and Finely Ground Frog Rectus Abdominis Muscle

The need to refer changes in ChE activity of one rectus muscle following experimental treatment to the contralateral muscle required a preliminary control experiment. For this, the ChE activity of each intact muscle was determined followed by washing, grinding and measurement of the ChE activity of the homogenate. Results are given in Table I. It may be noted that, with the exception of the homogenates from frog No. 2, the ChE activity of one muscle of a pair, whether intact or as a homogenate, was within 10% of the other. In addition, the rate of hydrolysis by the intact muscle averaged less than 1/20 of that of an equal weight of finely ground tissue, pointing to the necessity of the sensitive bioassay technique used in the determination of ChE activity.

TABLE I

CHOLINESTERASE ACTIVITY OF INTACT AND GROUND-UP CONTRALATERAL FROG RECTUS ABDOMINIS MUSCLES

(Activity expressed as k/mg. of tissue fresh weight)

Frog No.	Intact Muscle			Ground Muscle		
	Left	Right	Difference %	Left	Right	Difference %
1	0·014	0·015	7	0·520	0·490	6
2	0·013	0·012	8	0·260	0·297	12
3	0·009	0·010	10	0·287	0·260	9
4	0·010	0·010	0	0·333	0·362	8
5	0·011	0·010	9	0·255	0·238	7
6	0·009	0·009	0	0·347	0·347	0
7	0·013	0·014	7	0·347	0·314	9
8	0·020	0·020	0	0·347	0·370	6
	Mean % Difference 5±1·4 (S.E.)			Mean % Difference 7±1·2 (S.E.)		

The Relation Between the Reduced Response of Sarin-treated Muscle and the Restoration of ChE Activity with P2AM

On the basis of the previous control study, the following experimental procedure was adopted. One muscle was used as a control for ChE activity, first as an intact muscle, then as a homogenate. Concurrently, the concentration of ACh required to elicit a given response in the experimental muscle was determined before and after incubation with 5.0×10^{-7} M sarin for 1 hr. The ratio of the two concentrations of ACh required was used to obtain the approximate potentiation ratio. The muscle was then incubated for 8 min. with 2.0×10^{-4} M P2AM and the concentration of ACh required to elicit the original response was again determined enabling the calculation of the change in the potentiation ratio. After washing with Ringer, the ChE activity of the intact experimental muscle, and of a homogenate prepared from that muscle, was determined under conditions identical with that of the control, with the exception that 5 mg. of tissue/ml. of incubation mixture was used in the determination of the ChE activity of the experimental homogenate to ensure sufficient activity for accurate measurement.

The first order rate constants obtained from the linear plots shown in Fig. 1 for both control and experimental preparations are given in Table II.

From Table II, the % decrease in the potentiation ratio following the administration of P2AM is calculated as:

$$\frac{\text{Potentiation ratio after sarin minus potentiation ratio after P2AM}}{\text{Potentiation ratio after sarin}} \times 100 = 88.$$

It may be noted that the observed recovery of 71% in the ChE activity of intact muscle is more closely related than that of the homogenate (35%) to the 88% decrease in the potentiation ratio following treatment with P2AM.

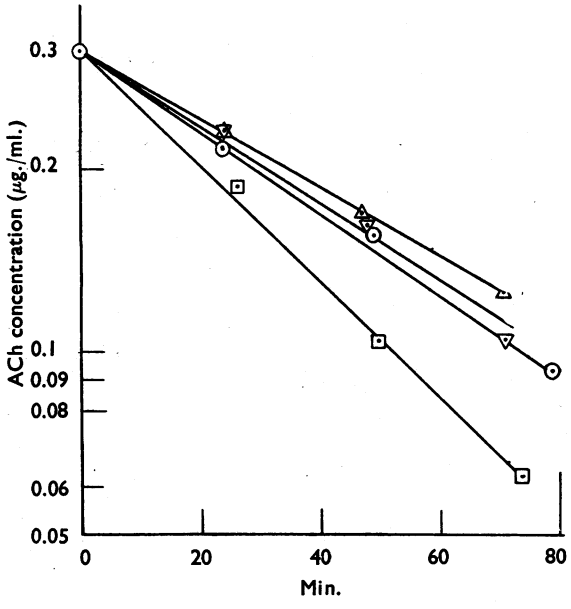

FIG. 1.—Cholinesterase activity of intact and homogenized frog rectus abdominis muscle (previously incubated with sarin) after treatment with P2AM. The lines from below upwards represent results with control homogenate (2·5 mg./ml.); initial activity of intact muscle; homogenate (5 mg./ml.) after P2AM; intact muscle after P2AM.

TABLE II

EFFECT OF P2AM ON CHOLINESTERASE ACTIVITY AND ON THE RESPONSE OF ISOLATED FROG RECTUS ABDOMINIS MUSCLE TO ACETYLCHOLINE FOLLOWING INCUBATION WITH SARIN

The approximate potentiation ratio=ACh required for initial response (μg./ml.)/ACh required following experimental treatment (μg./ml.); (a) negligible refers to a series of 4 inhibition controls obtained by measuring the ChE activity of sarin-incubated muscles (both as intact muscles and as ground preparations) which showed no significant ChE activity during the first 30 min.

Condition Studied	Muscular Response			Cholinesterase Activity	
	Rate of Contracture (mm./min.)	ACh Required (μg./ml.)	Approx. Potentiation Ratio	Intact Muscle k/mg.	Homogenate k/mg.
Before sarin	30·0	3·0	1·0	0·016	0·259
After sarin	30·0	0·2	15·0	Negligible (a)	
After P2AM	29·5	1·8	1·7	0·012	0·090

Effects of ACh upon the ChE Activity of Frog Rectus Muscle after Incubation with Sarin

To determine whether ACh by itself could produce any significant restoration of ChE in the muscle after incubation with sarin, the initial ChE activity of three muscles together was determined in 4 ml. of incubation mixture. The muscles were then washed with four portions of Ringer, and suspended in 5.0×10^{-7} M sarin for 1 hr. (sufficient to produce complete inactivation of ChE). After 12 washes over a 30 min. period with Ringer, the muscles were then reincubated with ACh as before and the ChE activity was determined.

The three muscles were again washed with four portions of Ringer, homogenized together, and the ChE activity of the homogenate determined in a final tissue concentration of 25 mg./ml. of incubation mixture, but with the same substrate concentration as before. The ChE activity of each preparation and that of a control homogenate made from the three corresponding contralateral muscles from the three frogs used to supply the experimental preparation is shown in Fig. 2. It

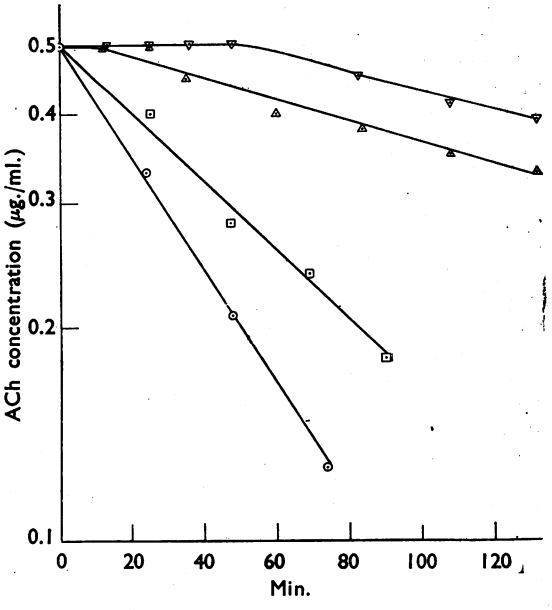

FIG. 2.—Cholinesterase activity of intact and homogenized frog rectus abdominis muscles after incubation with sarin. The lines from below upwards represent results with 3 intact muscles before sarin; control homogenate (2·5 mg./ml.); 3 intact muscles after sarin; experimental homogenate (25·0 mg./ml.). For further explanation, see text.

may be noted that during the first 30 min. of incubation with ACh following incubation with sarin, no significant ChE activity of the intact muscles could be demonstrated; however, after this time, the hydrolysis of ACh begins to take place at a rate equal to 24% of the initial activity. The measurements of recovery are based on the first order rate constants 0.007 $hr.^{-1}$ $mg.^{-1}$ and 0.002 $hr.^{-1}$ $mg.^{-1}$ found for the initial and experimental intact muscle activities respec-

tively; and 0.264 hr.$^{-1}$ mg.$^{-1}$ and 0.012 hr.$^{-1}$ mg.$^{-1}$ for the control and experimental activities of the ground muscle respectively. All values for $t_{1/2}$ were computed from the linear portions of the curves in Fig. 2. The homogenate from the experimental muscle required 80 min. of incubation with ACh before measurable ChE activity developed. Thereafter, the hydrolysis rate was equal to 4.5% of the corresponding control.

Evidence for Absence of Sarin in Frog Rectus Muscle Washed after Incubation with Sarin

This study was performed to determine whether the very low activity of the homogenate prepared from the experimental muscles in the previous study resulted from the presence of uncombined anticholinesterase.

Three experimental muscles were treated with sarin, followed by washing in a manner identical with the previous study. Then each muscle was divided into halves longitudinally. One half muscle from muscle 1 was grouped with one half from muscles 2 and 3 so that two corresponding sets of half muscles were obtained. The ChE activity of one set of halves was then determined after grinding up in a final tissue concentration of 25 mg./ml. of incubation mixture. The second group of three halves was homogenized in a control homogenate previously prepared from the three corresponding contralateral muscles, a procedure similar in principle to that used by Koelle and Steiner (1956). However, 10 times as much experimental tissue was taken as was present in the control homogenate, so that, if free inhibitor were present, a sufficient excess of it would be available during homogenization for complete inactivation of the ChE activity of the control. Results are given in Table III. Note that the ChE activity of the control, homogenized with 10 times as much washed sarin-treated muscle, is within 95% of its original activity indicating absence of significant amounts of uncombined anticholinesterase.

TABLE III

EVIDENCE FOR ABSENCE OF SARIN IN FROG RECTUS ABDOMINIS MUSCLES WASHED AFTER TREATMENT WITH SARIN

Condition Tested	Tissue Conc. (mg./ml.)	Cholinesterase Activity	
		Half Time (min.)	Rate Constant k/mg.
(1) Not treated with sarin	2·5	70	0·594
(2) Treated with sarin ..	25·0	98	0·424
(3) Combined homogenates	25·0+2 5	42	0·990
(3)–(2)			0·566

The Relation Between the Diminished Response to ACh of Sarin-treated Muscles and the Restoration of ChE Activity with ACh

It has been demonstrated that a partial return of ChE activity of intact muscle could be accomplished if sarin-treated muscles were incubated with ACh alone. When muscular responses were measured following incubation with ACh in a concentration of 20 μg./ml., a lowering of neuromuscular response was obtained, but the results were open to question because of possible injury to receptor sites in the muscle by this concentration of ACh.

It was, therefore, decided to wash the sarin-treated muscle with an ACh solution containing 3 μg. ACh/ml., a level which in control studies produced no injurious effect upon the neuromuscular response. The effects of a 2 hr. rinse with 1,000 ml. of an ACh solution containing 3 μg./ml. of ACh upon the responsiveness of sarin-treated muscle is shown in Table IV.

TABLE IV

EFFECT OF WASHING WITH ACh ON THE RESPONSE OF FROG RECTUS ABDOMINIS MUSCLE TO ACh AND ON CHOLINESTERASE ACTIVITY FOLLOWING PREVIOUS INCUBATION WITH SARIN

During the 2-hour wash with ACh no tension was placed upon the muscle. For cholinesterase determinations on sarin-treated muscle see Table II. In this study, determinations could be performed on the experimental muscle only after the wash with ACh.

Condition Studied	Muscular Response			Cholinesterase Activity	
	Rate of Contracture (mm./min.)	ACh Required (μg./ml.)	Potentiation Ratio	Intact Muscle k/mg.	Homogenates k/mg.
Before sarin ..	19·5	1·00	1·0	0·017	0·554
After sarin ..	19·5	0·08	12·5		
After washing with ACh ..	19·5	0·20	5·0	0·004	0·031

Studies of the ChE activity of this muscle following the ACh wash were done both on the intact muscle and on a homogenate prepared from it. The results were compared with the ChE activity of the contralateral muscle which had not been exposed to sarin (Table IV). It may be seen that a reduction in the potentiation ratio of 61% is accompanied by a recovery of 24% of the ChE activity of the intact muscle. The homogenate prepared from the experimental muscle showed only 5.6% of the ChE activity of the corresponding control.

DISCUSSION

The disparity between the ChE activity of intact muscle and that of homogenates following treatment with P2AM may result from the relatively

greater effectiveness of the quaternary amine in reactivating the more superficially situated ChE enzyme than that within the interior of the muscle. In consequence homogenization leading to a determination of the ChE of the whole tissue yields a value for the ChE activity which is affected by the lesser degree of reactivation of the internally situated enzyme which accounts for over 95% of the ChE activity (Tables I and II).

However, the closer association between recovery after sarin-induced poisoning of the ChE of the neuromuscular junction, where most of the surface ChE of the muscle is located (Couteaux and Nachmansohn, 1940), and the degree of return of the normal responsiveness to ACh after treatment with P2AM (Table II), suggests that it is the activity of the surface enzyme which is important. This view is supported by the work of Koelle (1957) with autonomic ganglia. After poisoning with an anticholinesterase most of the ChE of the ganglia is inactivated; treatment by intravenous injection of P2AM alters very little the total ChE of ganglia, but does induce a nearly complete regeneration of ChE activity at the periphery of the ganglion cell as evidenced by histochemical demonstration of ChE.

24% of the initial ChE activity of surface enzyme (intact muscle) returns if sarin-treated muscles are incubated with ACh alone, as compared with 4.5% of the corresponding activity in homogenates. This suggests that ACh itself may bring about a partial displacement of the anticholinesterases from the more important surface sites of muscle, but only negligible displacement from the bulk of internally situated ChE. The possibility that the very low activity of ChE in homogenates is due to a secondary inactivation by uncombined ChE inhibitor would appear to be excluded by the results recorded in Table III.

The latent period of 30 min. and 80 min. required for the sarin-treated intact muscle and homogenate respectively to develop measurable enzymatic activity in the presence of ACh (Fig. 2) may be explained in part by the lesser effectiveness of ACh compared to P2AM in reactivating the surface ChE of intact muscle. It is of interest that the 2-hour wash with an ACh solution containing 3 μg./ml. of ACh yielded a preparation whose activity followed first order kinetics immediately after the addition of substrate: that is, no latent period was required to develop measurable ChE activity. Of further interest was the finding that incubation with ACh in concentrations of 20 μg./ml. for 156 min. (Fig. 2) yielded approximately the same recovery of ChE as a 2-hour incubation with ACh in concentrations of 3 μg./ml. This observation suggests that only part of the available ChE activity of muscle may be reactivated with ACh under the experimental conditions. Yet the recovery of 24% of the surface ChE led to a lowering of 61% in the potentiated response of the muscle to ACh (Table IV). Since this relatively small recovery of ChE activity promoted a large degree of return toward normal function, one may interpret these observations as suggesting a possible mechanism for the spontaneous recovery of neuromuscular function observed by McNamara, Murtha, Bergner, Robinson, Bender, and Wills (1954) following administration of anticholinesterases.

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References

Augustinsson, K. B. (1950). In *The Enzymes*, ed. by Sumner, J. B., and Myrback, K., **1**, Pt. 1, p. 460. New York: Academic Press.

Childs, A. F., Davies, D. R., Green, A. L., and Rutland, J. P. (1955). *Brit. J. Pharmacol.*, **10**, 462.

Cohen, J. A., and Posthumus, C. H. (1955). *Acta Physiol. Pharmacol. Neerl.*, **4**, 17.

Couteaux, R., and Nachmansohn, D. (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 177.

Fleisher, J. H., Howard, J. W., and Corrigan, J. P. (1958). *Brit. J. Pharmacol.*, **13**, 288.

Koelle, G. B. (1957). *Science*, **125**, 1195.

—— and Steiner, E. C. (1956). *J. Pharmacol.*, **118**, 420.

McNamara, B. P., Murtha, E. F., Bergner, A. D., Robinson, E. M., Bender, C. W., and Wills, J. H. (1954). Ibid., **110**, 232.

Nielands, J. G., and Stumpf, P. K. (1955). *Outlines of Enzyme Chemistry*, p. 64. New York: Wiley.

Wilson, I. B., and Ginsberg, S. (1955). *Biochim. Biophys. Acta*, **18**, 168.