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THE ROLE OF CHOLINESTERASE AT THE MYONEURAL JUNCTION

BY

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This paper describes the changes in the behaviour of the isolated rat phrenic nerve diaphragm preparation after its exposure to the action of inhibitors of cholinesterase, and attempts to correlate these changes with the variations in cholinesterase activity that may be taking place in the preparation. The values for residual cholinesterase activity have been calculated from data provided by the work of Davison (1953), who has studied the action of some inhibitors on the true and pseudo cholinesterase of the rat. He has also studied the speed with which the inhibition of these enzymes is reversed.

For most of the work the inhibitor used was *p*-nitrophenyl diethyl phosphate—paraoxon—which has been studied in some detail by Aldridge (1950) and Davison (1953). These observations on the isolated diaphragm show that function is transitorily disturbed while the activity of cholinesterase is in the process of reduction to about 10% of that normally present, and that it is impossible to abolish the response to indirect stimulation even under conditions where virtually no activity of cholinesterase would be expected to remain.

Burgen, Keele, and Slome (1949) found that tetra ethyl pyrophosphate (TEPP), an inhibitor with properties very similar to those of paraoxon, first enhanced the response of the isolated diaphragm to indirect stimulation, but later a marked decrease occurred. Lovatt Evans (1951) and Burgen and Hobbiger (1951) have studied the effect of a number of inhibitors on the ability of the diaphragm to sustain a response to a tetanus and the times taken for this effect to be reversed after the inhibitors have been removed. The observations recorded here are substantially in agreement with those earlier reports.

METHODS

The preparation of the rat phrenic-nerve diaphragm was essentially that described by Bülbring (1946). A special holder was designed, and the preparation works successfully when the muscle and nerve are completely submerged in the bath.

The holder (Fig. 1) is made of perspex. A $\frac{1}{4}$-in. brass rod is threaded into a perspex block. This block is cemented to the back of the top plate of a $\frac{3}{16}$-in. perspex stem fitted at its foot with $\frac{1}{8}$-in. diam. perspex rod which forms the anchor for attaching the costal margin of the half diaphragm. This stem also carries a wire to the diaphragm anchorage which can be used if required to stimulate the muscle directly, the current returning to earth via the stainless steel ligature used to attach the diaphragm to the lever assembly.

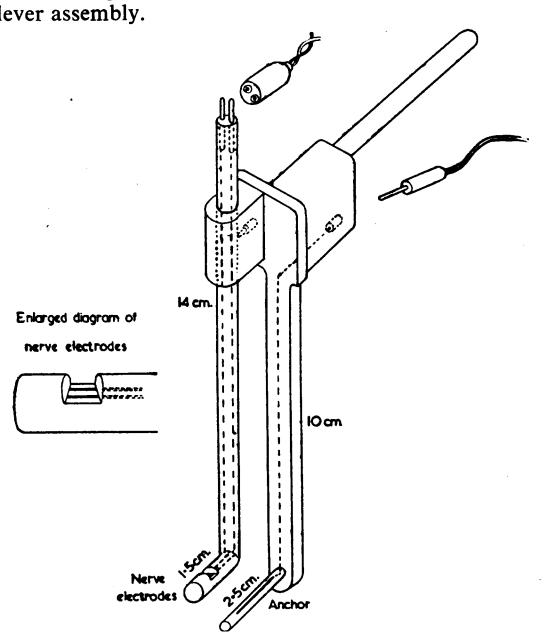

FIG. 1.—Diagram of perspex holder with electrodes for rat phrenic-nerve diaphragm preparation.

The top plate of the stem carries on its front face another smaller half-round shaped block which is drilled to take a $\frac{1}{4}$-in. diam. perspex rod. This rod carries wires which lie in slots along the sides of the rod and are held in place by cementing in strips

of perspex. These wires are four-stranded 39 S.W.G. stainless steel wires which are joined at the foot of the rod into short lengths of platinum wires which make contact with the nerve. A right-angled foot is made and a half section about 2 mm. is cut out forming a trough in which the nerve is laid and held in place by a rubber wedge. The platinum wires are laid across level with the floor of the trough. The rod carrying the nerve is adjustable vertically and can be rotated in its holder. The holder will eventually wear loose, and it is advisable to drill the holder block and insert a small spring-loaded plunger which bears gently on the rod, holding it in any required position.

The nerve was stimulated by rectangular impulses of 200 micro seconds duration and supramaximal intensity. They were normally applied at the rate of 8 per minute. Tetani at 50 per second were applied for 5 seconds.

The bath was 100-ml. capacity. Krebs solution at 37° C. aerated with $O_2 + 5\%$ CO_2 was used. Inhibitors of cholinesterase were added in ethanol in volumes of 0.1 ml. Acetylcholine was added in saline.

The names and inhibitory activity of the compounds used are given in Table I.

TABLE I

CHEMICAL NAMES AND ABBREVIATIONS OF THE INHIBITORS USED, TOGETHER WITH THEIR ACTIVITY AS INHIBITORS OF SHEEP RED CELL CHOLINESTERASE AND THEIR TOXICITY TO RATS

Chemical Name	Abbrev.	Molar Conc. Producing 50% Inhibition of Sheep RBC in 30 min.	Approx. Lethal Dose for Rats. mg./kg. Subcutaneously
Diethyl *p*-nitrophenyl phosphate	Paraoxon	$2{\cdot}1 \times 10^{-8}$	0·5
Tetraethyl pyrophosphate	TEPP	$1{\cdot}3 \times 10^{-8}$	0·3
Dimethyl *p*-nitrophenyl phosphate	Methyl paraoxon	$2{\cdot}5 \times 10^{-8}$	0·5
Di-isopropyl *p*-nitrophenyl phosphate	Isopropyl paraoxon	$4{\cdot}7 \times 10^{-7}$	5·0
Di-isopropyl fluorophosphonate	DFP	$4{\cdot}6 \times 10^{-7}$	2·0

RESULTS

The Effect of Adding Paraoxon to the Isolated Diaphragm

Stimulation at 8 per Minute.—If paraoxon is added to give a final concentration of 4×10^{-7} M in the bath containing the diaphragm there is a latent period of 2 minutes before the contractions begin to increase in size ; they then rapidly become from 50–200% greater in extent than the original ones.

The contractions remain at their maximum for only one or two responses and then rapidly diminish and return to their original size all within 10 minutes of adding the inhibitor (Fig. 2). During the period of enhanced contractions, fasciculations and irregular twitchings of the diaphragm can be seen, but these diminish as the contractions return to their original size.

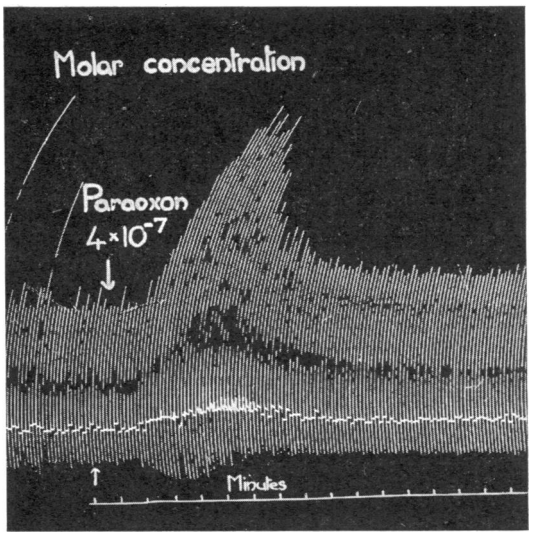

FIG. 2.—Rat phrenic-nerve diaphragm. Indirect stimulation 8 per min. Paraoxon (4×10^{-7}M) added. Marks of irregular twitchings can be seen at base line during the period of enhanced contractions.

After returning to its original level the response remains unchanged even though the diaphragm is left in the solution of inhibitor for several hours. Under these conditions no appreciable hydrolysis of paraoxon would take place.

Tetanus at 50 per Second for 5 Seconds.—The normal diaphragm responds by a sustained contraction. When paraoxon is added the ability to hold this response is lost during the period of the enhanced contractions and does not return as long as the inhibitor remains in the bath.

Sensitivity to Acetylcholine.—The contractions of a normal diaphragm stimulated at 8 per minute are reduced by 50% if acetylcholine is added to give a final concentration of $2\text{–}3 \times 10^{-3}$ M, and they are abolished at 5×10^{-3} M. On washing out the acetylcholine, the responses immediately return. They may be reduced or abolished by acetylcholine for periods of at least an hour, but will immediately return when it is removed.

After the addition of paraoxon (4×10^{-7} M) for 30 minutes the sensitivity of the diaphragm increases so that the response is now reduced by 50% in 5×10^{-6} M acetylcholine and abolished in 5×10^{-5} M (Fig. 3).

If acetylcholine is added to a suspension of red cells, it will protect the cholinesterase from inhi-

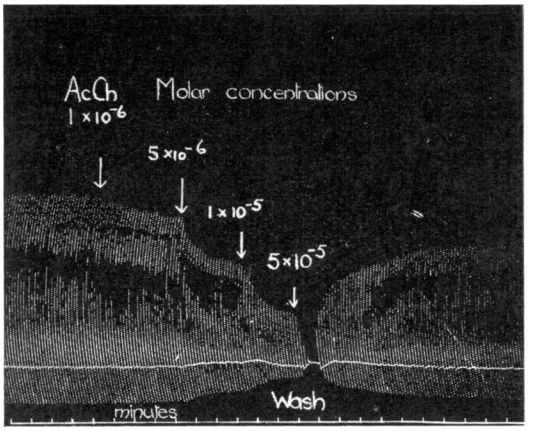

FIG. 3.—Rat phrenic-nerve diaphragm. Indirect stimulation 8 per min. Showing the effect of acetylcholine at different concentrations on size of response after previous incubation with 4×10^{-7}M paraoxon.

bition by paraoxon added subsequently. A concentration of 1×10^{-2} acetylcholine added to the diaphragm abolishes its response to stimulation and will also protect it from the full action of 1×10^{-7} M paraoxon, for, if both acetylcholine and inhibitor are removed after 1 hour, the diaphragm still retains its power to hold a tetanus. A concentration of 5×10^{-3} M acetylcholine will abolish the response of the diaphragm to stimulation, but will not protect it from the action of 4×10^{-7} M paraoxon, for the power to sustain a tetanus is lost when tested after removal of acetylcholine and inhibitor.

Effect of the Removal of Paraoxon.—In many preparations the removal of paraoxon by 3 washings after ½–1 hour had no effect on the size of the contractions at 8 per minute, but in a few there was some increase (10–20%). No other change was observed in periods of observation of up to 3 hours. The response to a tetanus, on the other hand, gradually changed, so that 30–40 minutes after removal of the inhibitor the preparation had regained its ability to hold a tetanus for 5 seconds. Over the next 3 hours the preparation gradually lost some of its sensitivity to acetylcholine, so that at the end of 3 hours a concentration of 1×10^{-4} M produced only a 33% reduction in the size of the contraction.

Effect of a Second Dose of Paraoxon.—The addition of paraoxon (4×10^{-7} M) one hour after the first dose had been removed had no effect on the response to regular stimuli. There was no enhancement, and no fasciculations or twitchings were seen. On the other hand, the ability to hold a tetanus was lost and the sensitivity to acetylcholine again increased. These changes were reversed by removing the inhibitor. This procedure has been repeated on as many as five separate occasions on one preparation in a day. The enhanced response was seen only after the first dose of paraoxon.

Effect of the Concentration of Added Paraoxon.—The changes in the response of the diaphragm to paraoxon and their reversal are presumably attributable to the progressive inhibition of cholinesterase and the reversal of this effect after removal of the inhibitor. The speed with which the changes in the response to the first dose of paraoxon take place depends upon the concentration of the inhibitor that is added, and some comparative figures are given in Table II.

TABLE II

THE EFFECT OF CONCENTRATION OF PARAOXON ON THE SPEED WITH WHICH CHANGES TAKE PLACE IN THE ISOLATED DIAPHRAGM

Concentration of Paraoxon, M.	Time (min.) between the Addition of the Inhibitor to the Bath and:		
	(*a*) 1st Enhanced Contraction	(*b*) Loss of Tetanus	(*c*) End of Enhancement
4×10^{-8}	13	39	
1×10^{-7}	6	18	20
4×10^{-7}	2	8	8
4×10^{-6}	0·5	1	1·75

In other experiments the response to tetanus was observed at 2-minute intervals from the time of adding the inhibitor until there was a complete failure to hold a sustained response. At concentrations of 2×10^{-8} M up to 1×10^{-7} M paraoxon this period was proportional to the concentration of inhibitor, but at concentrations of from 2 to 8 $\times 10^{-7}$ M the time taken for failure was not proportional to the concentration, and it appeared to take longer for the inhibitor to exert its effect. At concentrations greater than 8×10^{-7} M the interval was too short for measurement by this method. The disproportion between the concentration of inhibitor and the time taken for the production of an effect when this interval becomes very short may possibly be due to the time needed for the inhibitor to penetrate the diaphragm. This time only becomes significant when the action of the inhibitor is rapid.

The time taken for the reversal of one of the effects of the inhibitor, namely the return of the ability to hold tetanus, was also measured in some preparations. This was independent of the time during which the inhibitor had been in contact with the diaphragm, but was greater after exposure to higher concentrations of paraoxon.

If the return of function is due to a reversal of inhibited cholinesterase the time taken should be independent of the concentration of inhibitor used. An explanation of the discrepancy could be that additional time was needed for the high concentrations of inhibitor to diffuse out of the diaphragm into the bath.

It was important to find out whether paraoxon reacts with the cholinesterase in the rat's diaphragm in the same way that it does with other preparations of cholinesterase.

Diaphragms were exposed for 30 minutes to 2×10^{-8} M paraoxon in the bath. This was calculated to give 58% and 41% inhibition respectively of true and pseudo cholinesterase. After 30 minutes the diaphragms were removed, washed and homogenized, and the activity of the true and pseudo cholinesterase determined manometrically. Half of each diaphragm was not incubated in paraoxon and served as a control. In two experiments the activity of true cholinesterase had been inhibited by 53% and 57% (theoretical 58%) and that of pseudo cholinesterase inhibited by 31% and 53% (theoretical 41%). It therefore appears that inhibition of the cholinesterase of the rat diaphragm by paraoxon occurs at similar rates to the inhibition of cholinesterase in red cells or tissue homogenates.

The effect of increasing the concentration of paraoxon was to increase the speed with which the changes in the response of the diaphragm took place. If the concentration of paraoxon was increased to 4×10^{-4} M the preparation continued to respond quite satisfactorily to stimulation at 8 per minute. A preparation of red cell cholinesterase would be completely inhibited within a few seconds of the addition of paraoxon at this concentration.

Reaction of the Diaphragm to Other Inhibitors

As most of the work with paraoxon had been done in a concentration of 4×10^{-7} M, other inhibitors were added in a concentration that would give a comparable degree of inhibition. These figures were derived from the data in Table I on the activity against red cell cholinesterase.

With TEPP (2×10^{-7} M) the reactions of the diaphragm and pattern of reversal were indistinguishable from those with paraoxon.

With DFP (1×10^{-5} M) and isopropyl paraoxon (1×10^{-6} M) the initial response of the diaphragm was the same as with paraoxon, and the diaphragm continued to respond to regular stimuli for an indefinite period. On removing the inhibitor, however, there was no return of the power to hold a tetanus within a period of 4 hours. Sensitivity to acetylcholine diminished slightly after the inhibitor had been removed, but did not continue to do so over the next 3 hours.

After methyl paraoxon (1×10^{-6} M) the responses to the addition of the inhibitor are the same, but on removing it reversal occurs very rapidly and the ability to sustain a tetanus is regained within 3 minutes. If a second dose of methyl paraoxon is given 45–60 minutes after removing the first dose there is a period of enhanced contractions similar to that seen after the first dose. Aldridge (1953) has shown that methyl paraoxon produces an inhibition of true cholinesterase that is rapidly reversed.

Attempt to Obtain Complete Inhibition of Cholinesterase

Although very high concentrations of paraoxon (4×10^{-4} M) should produce virtually complete inhibition of cholinesterase there is an appreciable rate of reversal of cholinesterase inhibited by paraoxon. This means that an equilibrium must finally be established between inhibition and reversal at some stage before inhibition is absolutely complete. The level of cholinesterase activity at which such an equilibrium must finally be established could be further reduced by choosing an inhibitor from whose effects reversal was slower than paraoxon. A comparison was therefore made between the performance of diaphragms exposed to paraoxon (4×10^{-4} M) and to isopropyl paraoxon (1×10^{-4} M) for 1 hour. The preparations were stimulated for $\frac{1}{2}$ minute at different rates. Both gave a normal response to stimuli at 8, 16, 30, and 60 per minute. The diaphragm in paraoxon responded normally to stimuli at 2 and 5 per second, but that in isopropyl paraoxon failed to do so. At 10 per second the paraoxon diaphragm also failed. Thus under conditions where very small differences in residual cholinesterase activity must be present a difference in the performance of the preparation could be detected.

The Behaviour of Diaphragms Taken from Poisoned Rats

Rats were killed by the subcutaneous injection of 2 mg./kg. paraoxon. Death took place in 7–15 minutes and the chest was opened immediately and the diaphragm removed.

It took 15–60 minutes from the time of death to the first recording of a response of the diaphragm in the bath. Under these conditions the diaphragm would respond to single stimuli but failed to sustain a tetanus. The ability to hold a tetanus

returned gradually and was complete within 30–60 minutes. The diaphragm from a rat killed with isopropyl paraoxon showed no recovery of the ability to hold a tetanus.

Discussion

The addition of an inhibitor of cholinesterase to the isolated diaphragm produces a succession of changes to its response to indirect stimulation. The speed with which the changes take place is proportional to the concentration of inhibitor added, suggesting that they accompany the gradual changes in the activity of the cholinesterase in the preparation. Davison (1953) and Denz (1953) have shown that the diaphragm of the rat contains both true and pseudo cholinesterase, and it was found that the inhibition of these enzymes in the isolated preparation exposed to paraoxon occurred at a rate similar to that observed on other rat cholinesterase preparations.

Davison (1953) has been able to derive rate constants for the inhibition of both true and pseudo cholinesterase of the rat when acted on by paraoxon. It therefore seemed permissible to use these figures in order to calculate how much activity could be expected to remain in the diaphragm at different times after exposure.

The calculated rate constants for inhibition by paraoxon are as follows (Davison):

Rat true ChE .. 1.44×10^6 (min.$^{-1}$)
Rat pseudo ChE .. 8.7×10^5 (min.$^{-1}$)

and from these the activity of cholinesterase that would remain in the diaphragm at different times after adding the inhibitor (1×10^{-7} M paraoxon) can be calculated, and is as follows:

Time after Adding Paraoxon (min.)	Condition of Diaphragm	Calculated Activity %	
		True	Pseudo
6	Beginning of enhanced contraction	42	60
18	Failure of tetanus	8	22
20	End of enhanced contraction ..	5	17
60	50% inhibition by 5×10^{-6}M acetylcholine	0·1	0·5

It will be seen that when about half of the activity of cholinesterase is destroyed the response of the diaphragm to single stimuli increases and there is a period of fasciculation and irregular twitching, but as the inhibition progresses and nears completion these effects disappear, but the ability to hold a tetanus now fails. But this does not occur until less than 10% of the cholinesterase remains. However, a normal response to indirect single stimuli of 200 m.sec. duration is obtained after 1 hour in 1×10^{-7} M paraoxon when the cholinesterase activity must have become reduced to extremely low levels. Even a thousandfold increase in the concentration of inhibitor or the use of an inhibitor which produces an apparently completely irreversible inhibition (isopropyl paraoxon) will not abolish this response.

Davison has also determined the rate constants for the reversal of true and pseudo cholinesterase of the rat inhibited by paraoxon. His figures are as follows:

Reversal of true ChE after paraoxon ... 8×10^{-5} (min.$^{-1}$)
Reversal of pseudo ChE after paraoxon 2×10^{-3} (min.$^{-1}$)

The activity of true and pseudo cholinesterase that will have returned in the diaphragm after exposure to paraoxon may therefore be calculated, and is as follows:

Time after Removing Paraoxon (min.)	Condition of Diaphragm	Calculated Return of Activity %	
		True	Pseudo
30	Able to hold tetanus	—	3
60	50% inhibition by 6×10^{-5}M ACh	—	11
120	$1{\cdot}2 \times 10^{-4}$M ACh	—	21
180	2×10^{-4}M ACh	1·5	30

If the reversal of cholinesterase takes place at the same rate in the isolated whole diaphragm as it does in other cholinesterase preparations *in vitro*, it will be seen that very little activity is needed in order to allow the diaphragm to regain its ability to show a sustained response to a tetanus. There is a big difference in the speeds with which pseudo and true cholinesterase reverse after inhibition by paraoxon, and these calculations suggest either that pseudo cholinesterase might be able to carry out some function at the myoneural junction, or that only extremely small quantities of true cholinesterase are necessary. The possibility cannot be excluded that in the intact diaphragm the reversal of the inhibited enzymes is catalysed by some unknown factor not present in the homogenized tissue preparations from a study of which the rate constants for reversal were derived.

Interest seems to centre on two features of these observations. It is impossible to prevent conduction across the myoneural junction by inhibitors of cholinesterase. There is good evidence that they can reach the bulk of the cholinesterase, and histochemical observations show that in their presence in concentrations such as those used here no staining at all of cholinesterase takes place at the

myoneural junction (Denz, 1953). The conclusion therefore must be either that cholinesterase is not essential for the functioning of the myoneural junction under these conditions, or that some very small fraction of the enzyme at the myoneural junction is protected from the action of the inhibitor.

The second point of interest is that the maximum derangement of response of the muscle to indirect stimulation takes place when between 50% and 80% of the cholinesterase is inactivated, but after that the preparation again responds in an orderly fashion to single stimuli.

Furthermore, on removal of an inhibitor such as paraoxon which produces an inhibition that is only slowly reversible, the ability of the preparation to respond to tetanus is restored, but the fasciculations and enhanced response to single stimuli do not reappear. If an inhibitor producing a more rapidly reversible inhibition of cholinesterase (e.g., methyl paraoxon) is used, the addition of a second dose of inhibitor does result in enhanced responses.

A similar picture is seen in the whole animal poisoned with paraoxon and revived from the effects of a lethal dose by artificial respiration. The widespread fasciculations disappear and natural respiration returns, but the animal is not seriously disturbed by a second "lethal" dose of the inhibitor (Barnes, 1953).

The diaphragms from rats killed by paraoxon cannot sustain a tetanus when first set up in the bath, but it seems probable that failure of myoneural conduction is not responsible for the death of animals after TEPP (Douglas and Matthews, 1952). In any case such a diaphragm will still respond satisfactorily to single stimuli.

It seems possible that the widespread disturbances during the reduction of the cholinesterase if they occur in tissues other than muscle (e.g., the brain) may be responsible for death, but that, if this is averted by artificial respiration, then a more orderly function is restored once the level of cholinesterase has been reduced almost to zero. At this stage further doses of anticholinesterase will not interfere with function.

Summary

1. The action of inhibitors of cholinesterase upon the isolated rat phrenic-nerve diaphragm preparation has been studied.

2. The response to indirect stimulation goes through a series of changes as the enzyme is inhibited, but settles down to an apparently normal response once inhibition is complete.

3. It is impossible to prevent the response to single stimuli even by very high concentrations of inhibitor, but the response to a tetanus fails.

4. Both true and pseudo cholinesterase are present in the rat diaphragm, and they respond to inhibitors in the same way as cholinesterase in other tissues.

5. Calculations have been made which show that fasciculations and enhanced response to single stimuli take place while the cholinesterase activity is reduced from 50% down to 10% of normal, but then disappear.

6. The power to sustain a tetanus disappears when only about 10% of cholinesterase remains, but when reversal from complete inhibition is allowed to occur by removing the inhibitor, the power returns when calculations suggest that less than 5% of the pseudo and virtually no true cholinesterase has had time to be released from inhibition.

We wish to thank Mr. A. N. Davison for carrying out the cholinesterase determinations on the diaphragms exposed to paraoxon. Mr. C. R. Kennedy and Mr. H. McKinnon were responsible for the design and construction of the holder for the diaphragms.

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