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MECHANISM OF THE ANTAGONISM BY PRALIDOXIME AND 1,1-TRIMETHYLENEBIS(4-HYDROXYIMINOMETHYLPYRIDINIUM) OF THE ACTION OF ECHOTHIOPHATE ON THE INTESTINE

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Pralidoxime chloride (pyridine-2-aldoxime methochloride ; Protopam Chloride) and 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) (TMB-4) antagonize the spasm of the isolated or intact small intestine of the rabbit caused by the anticholinesterase, echothiophate iodide (*S*-2-dimethylaminoethyl *OO*-diethyl phosphorothiolate methiodide ; Phospholine Iodide). *In vitro*, both oximes also antagonize the spasm caused by acetylcholine. The quantitative relationships have been studied in comparison with the activity of atropine against echothiophate and acetylcholine. Echothiophate-treated intestine which is subjected to a concentration of oxime sufficient to cause 100% restoration of function (but not cholinesterase reactivation) will go back into spasm on washing out both drugs. Strips treated with a high concentration of oxime, sufficient to cause 100% reactivation of cholinesterase, exhibit normal control tone and motility after washing. It is concluded that pralidoxime and 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) have an anticholinergic action as well as the ability to reactivate cholinesterase and that this action plays a significant part in the initial recovery of function under the conditions of these experiments.

A great deal of interest has been centred on certain oximes as reactivators of cholinesterase which has been "irreversibly" inhibited by organic phosphorus compounds. While there is now no doubt whatever that such reactivation does take place both *in vitro* and *in vivo* and that the therapeutic use of these oximes may contribute, under proper circumstances, to survival in animals or man, there is still no conclusive evidence that cholinesterase reactivation is the sole or even the principal mechanism by which they act (Grob, 1961) especially in the earliest phase of recovery. This investigation was undertaken to study this mechanism. For this purpose two of the most effective and widely known oximes, pralidoxime chloride (pyridine-2-aldoxime methochloride ; 2-hydroxyiminomethyl-1-methylpyridinium chloride ; Protopam Chloride) and 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) (TMB-4) were used. Echothiophate iodide (*S*-2-dimethylaminoethyl *OO*-diethyl phosphorothiolate methiodide ; Phospholine Iodide) was selected as an appropriate anticholinesterase, since these oximes are active antagonists to echothiophate in all tissues or species so far studied and act in the absence of atropine or any other antidote (Lehman, Fitch, Bloch, Jewell & Nicholls, 1960 ; Lehman & Nicholls, 1960 ; Tammelin, 1958).

METHODS

Intestinal motility in vivo. Male rabbits weighing 2 to 2.5 kg were anaesthetized by intraperitoneal injection of 65 mg/kg of allobarbitone (Dial). The abdominal cavity was opened, a loop of jejunum about 8 cm long was taken and small openings were made into the lumen at each end. A glass cannula 25 cm long and 4 mm inside diameter with a right-angle bend a few cm from the bottom was filled with peanut oil and the short limb was tied into the proximal end of the intestinal loop. The loop was flushed with oil, the distal end was tied and the skin was clamped over the loop. The cannula was supported in an upright position and the height of oil was adjusted to about 10 cm. The upper end was connected to a Sanborn electromanometer and recorder. The tracings shown were obtained at a sensitivity of 5 mm of mercury per cm. This is essentially a volume recording, since the system has a volume of 50 ml. and the pressure transducer operates with extremely small displacement. All drugs were given into a marginal ear vein.

Intestinal motility in vitro. Male rabbits of 2 to 2.5 kg weight were killed by a blow on the head and a length of intestine was excised from a point just distal to the ligament of Treitz to the caecum. This strip was placed in Ringer-Locke solution in a dish which was kept in an ice and water bath in the refrigerator and used as required. The Ringer-Locke solution had the following composition: NaCl 0.9%, $CaCl_2.2H_2O$ 0.034%, KCl 0.042%, $NaHCO_3$ 0.05% and glucose 0.1%. Strips of intestine about 2 cm long were mounted in an Anderson tissue bath (Metro Industries, Long Island City, N.Y.). The temperature was maintained at $35.5 \pm 0.2°$ C and oxygenation was at the rate of one bubble per sec. The upper end of the gut was connected by a thread to a lever which was rigidly attached to the rotor of a Bendix AY 31 synchronous converter. Tension on the tissue was obtained by a 3 g weight attached to the lever at the opposite end and at the same distance from the fulcrum as the thread bearing the tissue. The transducer was activated by and connected to a Sanborn strain-gauge amplifier and recorder. The sensitivity was adjusted so that 1.0 cm shortening of the intestinal strip caused a rise in the tracing of approximately 1.0 cm. The volume of the bath was 50 ml. and the drugs were added in a constant volume of 1.0 ml. in Ringer-Locke solution. In a few cases where the solubility of the second drug to be added was such that a volume of 1.0 ml. had to be exceeded, the stock solution of the second drug was adjusted by including the first drug so that the final concentration of the two drugs in the bath would be correct. Such solutions were always made up just before use because of the possibility of chemical interaction. Drug molarities refer to the final bath concentrations. Washing of tissue was carried out by draining the bath completely, refilling with fresh Ringer-Locke solution and allowing the tissue a 5 min rest with oxygenation. Whenever "washing" is referred to, this process was carried out 3 times.

Cholinesterase activity. Intestine was taken for these experiments by cutting strips of about equal size and weighing roughly 1.5 g from either end of the small intestine removed from the animal as in the preceding paragraph. In this way the effect of any gradient in cholinesterase activity from proximal to distal could be balanced out. Both ends of each piece were tied with thread so as to simulate conditions in the experiments in which intestinal motility was studied and prevent the bath solution from entering the lumen. The two tied strips were then immersed in 50 ml. of oxygenated Ringer-Locke solution at 35.5° C, drugs were added and washing procedures carried out exactly as in the motility experiments. The strip to be assayed was drained, blotted with filter-paper and the areas near where threads had been tied were cut off and discarded. The two pieces were then cut down as nearly as possible to a weight of 1.0 g each. Mucus was expressed gently from the lumen. The tissue was weighed, minced with scissors and homogenized in the micro cup of a Waring Blendor with 15 to 20 ml. of balanced salt solution having the composition: NaCl 0.9%, KCl 0.03%, $CaCl_2.2H_2O$ 0.034%, in water redistilled from glass. The entire Blendor cup and contents were thoroughly precooled in ice and water mixture and then driven for 2.5 min, recooled and driven another 2.5 min. In this way the tissue and homogenate were always kept well below room temperature. The whole operation was carried out as rapidly as possible and required not over 10 min from the end of the treatment period until dilution in the assay

vessel. Cholinesterase assays were carried out by the method of continuous automatic titration as previously described (Lehman *et al.*, 1960); temperature 37.5±0.1° C, *p*H 7.4± 0.05, substrate 0.012 M acetylcholine bromide. The homogenate was washed into the titration vessel with balanced salt solution and the volume adjusted to 100 ml. The titration was started and allowed to continue for about 15 min to establish the rate of the spontaneous, non-specific acid production for the tissue (Jensen-Holm, Lausen, Milthers & Møller, 1959). Concentrated substrate was then added in a volume of 1.0 ml. and the titration continued for another 15 min. The difference between the rate of alkali consumed before and after addition of substrate was further corrected for the spontaneous rate of hydrolysis of acetylcholine, and the result was expressed in m-mole of acetylcholine split per hr per g of sample. Each experiment consisted in a control, echothiophate-treated strip, echothiophate- and oxime-treated strip, and second control; the assays were run in this order. The entire group was discarded if the two controls failed to check within an arbitrary ±12.5% of the mean. The results were calculated as follows:

$$\%\ \text{cholinesterase reactivation} = \frac{(\text{echothiophate}+\text{oxime})-(\text{echothiophate})}{(\text{mean control})-(\text{echothiophate})} \times 100$$

where each value in parentheses signifies m-mole of acetylcholine hydrolysed per hr per g of intestine.

Experimental design. To the bath containing a single strip of intestine arranged for recording motility (or to two tied-off strips intended for cholinesterase determinations) was added sufficient echothiophate to make the concentration in the bath 10^{-5} M. After a period of 4 min to allow for maximum response, pralidoxime chloride, 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) or atropine was added. The fall in tonus was maximum in about 1 min, but a somewhat longer period was required for maximal recovery of rhythmic movements. Where the tissue was to be washed and homogenized or washed and observed, this operation was begun exactly 1 min after the addition of an oxime. In other experiments spasm was produced with 10^{-5} M acetylcholine. Here an oxime or atropine was added after 2 min, since the maximum effect of acetylcholine comes on much more rapidly than that of echothiophate.

RESULTS

Intestine in vivo. In doses of 0.01 to 0.1 mg/kg intravenously, echothiophate causes a rise in the base line of the intestinal motility recording (" tonus ") in the anaesthetized rabbit, and usually an increase in the amplitude and frequency of peaks. These changes are well sustained. Pralidoxime chloride and 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) reverse the effect at doses in the neighbourhood of 5 to 10 mg/kg. Typical records are shown in Fig. 1. These experiments demonstrate the antagonism of echothiophate by pralidoxime chloride, at least, *in vivo* at a dosage comparable to that used for treatment of organophosphate poisoning in man, and indicate that we are not concerned here with an effect which is demonstrable only with excessive doses on isolated tissue.

Isolated intestine. In Fig. 2 are shown typical effects on intestinal motility of pralidoxime chloride, 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) and atropine after additioin of echothiophate to the bath at 10^{-5} M. This concentration of echothiophate always leads to a marked shortening of the intestinal strip and reduction in amplitude of rhythmic movements and sometimes to complete spastic paralysis. The oximes or atropine cause relaxation of the spasm which begins instantly and reaches a maximum in a few minutes. At appropriate concentrations complete recovery of rhythmic movements and tone occurs in the continued presence of echothiophate. It will be noted that the antagonistic effect of the two

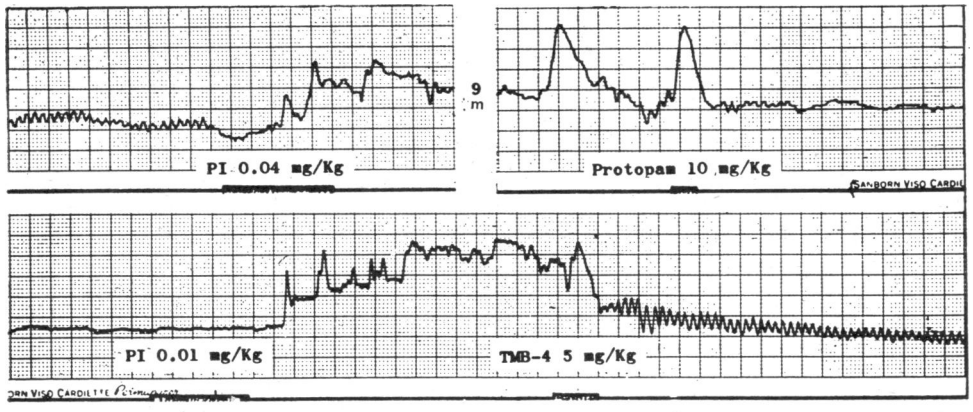

Fig. 1. Antagonistic effect of pralidoxime chloride (upper) and 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) (lower) on motility of the intestine of the rabbit, *in situ*, after stimulation by echothiophate. Time: 10 sec per large division in this and Figs. 2, 4, and 6. Sections of tracings are omitted from the illustrations for indicated periods in min.

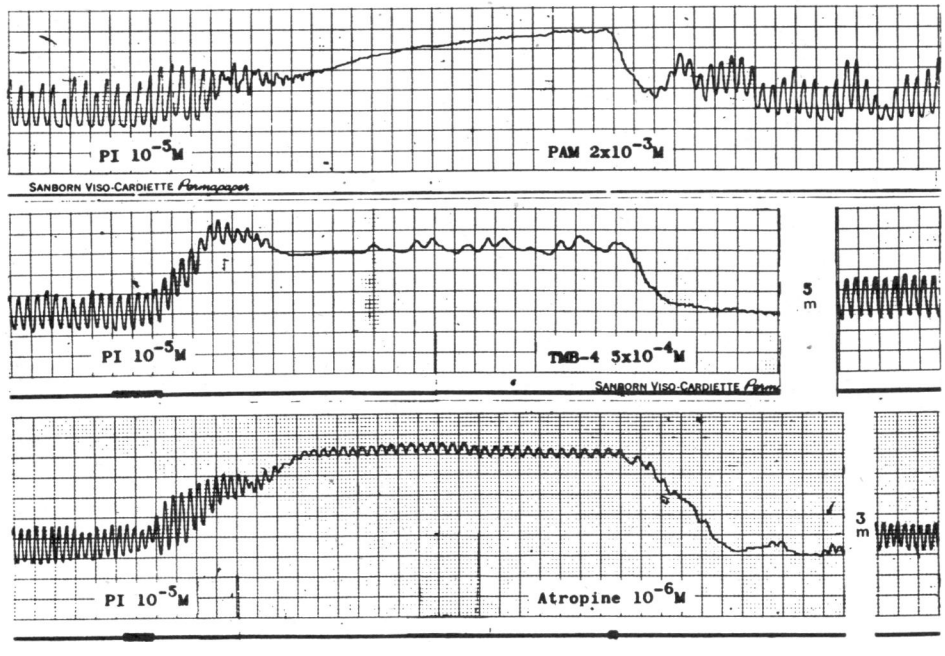

Fig. 2. Antagonistic effect of pralidoxime chloride (top), 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) (middle), and atropine (bottom) on the motility of the isolated intestine of the rabbit after stimulation by 10^{-5} M echothiophate. (m=minute.)

oximes and atropine is qualitatively similar. Motility was always recorded for 30 to 45 min after addition of the antagonist. The recovery of amplitude was measured in % of the control after the tracing became stabilized. The relationship between % recovery of amplitude of rhythmic movements and negative log molar concentration of antagonist is shown graphically in Fig. 3. As might be expected these curves appear to be sigmoid.

The action of the oximes *in vitro* is consistent with the *in vivo* observations above. If we assume a plasma volume of 38 ml./kg in the rabbit (Spector, 1956), then the instantaneous molarities for pralidoxime chloride and 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) that would be obtained on uniform distribution throughout the plasma would be 1×10^{-3} and 3×10^{-4} respectively, in rough agreement with the concentration necessary to cause 100% recovery of motility *in vitro* which may be estimated from Fig. 3 to be about 3×10^{-3} and 6×10^{-4} respectively.

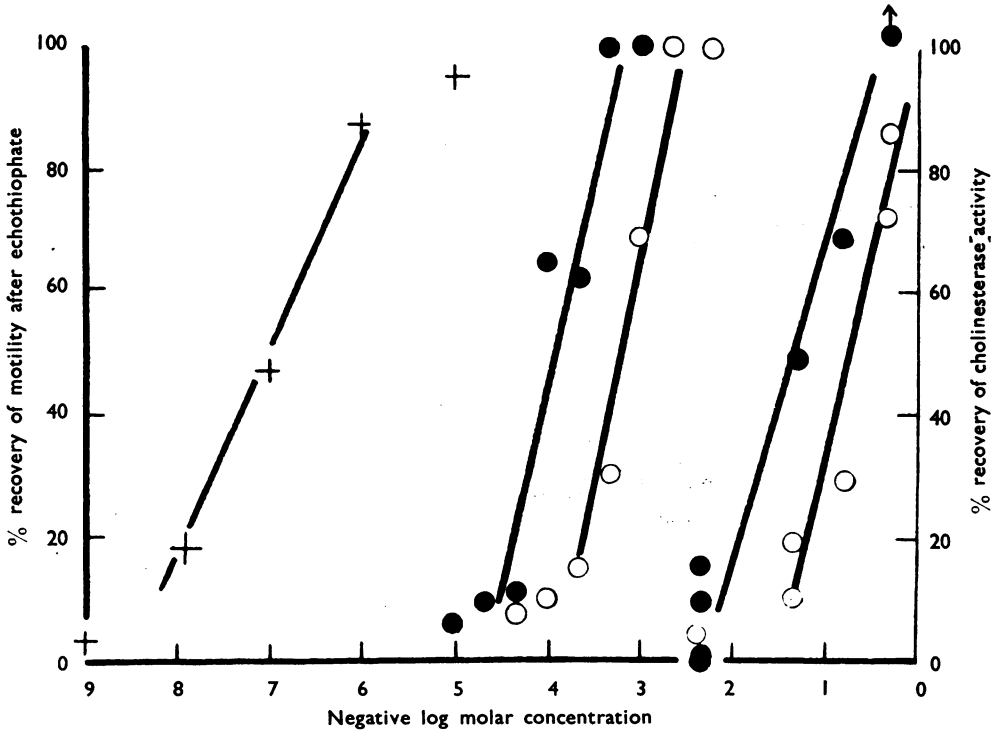

Fig. 3. *Three curves on the left:* Relationship between concentration of antagonist and recovery of amplitude of rhythmic movements of the isolated intestine of the rabbit. Antagonist was added to the bath after 4 min exposure to 10^{-5} M echothiophate and without washing. Each point represents the mean of 2 to 3 observations. *Two curves on the right:* Relationship between concentration of antagonist and recovery of cholinesterase activity of the isolated rabbit intestine. Antagonist was added to the bath after 4 min exposure to 10^{-5} M echothiophate, and washing was begun after 1 min exposure to the oxime. Points represent individual observations. (Atropine +——+; 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) ●——●; pralidoxime chloride ○——○).

Attention was then turned to the effect of pralidoxime chloride, 1,1′-trimethylene-bis(4-hydroxyiminomethylpyridinium bromide) and atropine against spasm due to acetylcholine, and the results are shown in Figs. 4 and 5. It will be clear that the oximes and atropine cause recovery of motility after acetylcholine and that the tracings of Fig. 4 are qualitatively similar to each other and to those of Fig. 2, except that spasm due to acetylcholine is almost instantaneous in onset as contrasted with that due to echothiophate which requires several minutes to reach a maximum. This latter observation is consistent with the view that echothiophate inhibits cholinesterase and causes its effects by gradual accumulation of acetylcholine. The question remains as to what extent the antagonistic action of the oximes can be explained simply as anticholinergic.

Figs. 3 and 5 were calculated from the concentrations of the antagonists necessary for 50% recovery of amplitude of rhythmic movements, and these data are given in Table 1. From inspection we see that none of the three drugs is equally effective against both echothiophate and acetylcholine. Thus, atropine and 1,1′-trimethylene-bis(4-hydroxyiminomethylpyridinium bromide) are respectively 37 and 3 times more effective against acetylcholine than against echothiophate, while pralidoxime chloride is 7.6 times more effective against echothiophate than against acetylcholine.

The next group of experiments were carried out in a tissue bath under conditions of time, temperature and manipulation identical to those used previously except that no motility recording was made. The intestine was treated with 10^{-5} M echothiophate, and this was followed by one of the oximes. The tissue was then

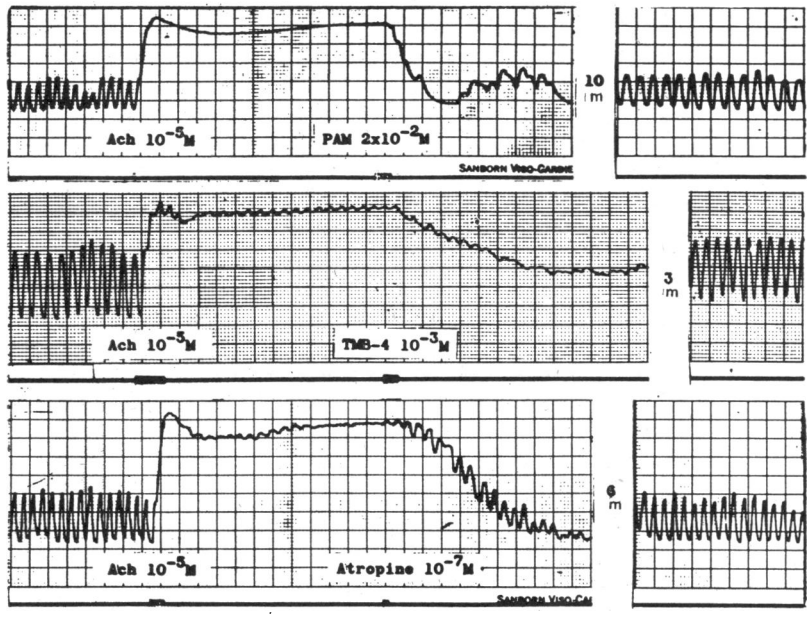

Fig. 4. Antagonistic effect of pralidoxime chloride (top), 1,1′-trimethylenebis(4-hydroxyimino-methylpyridinium bromide) (middle), and atropine (bottom) on the motility of the isolated intestine of the rabbit after stimulation by 10^{-5} M acetylcholine. (m=minutes.)

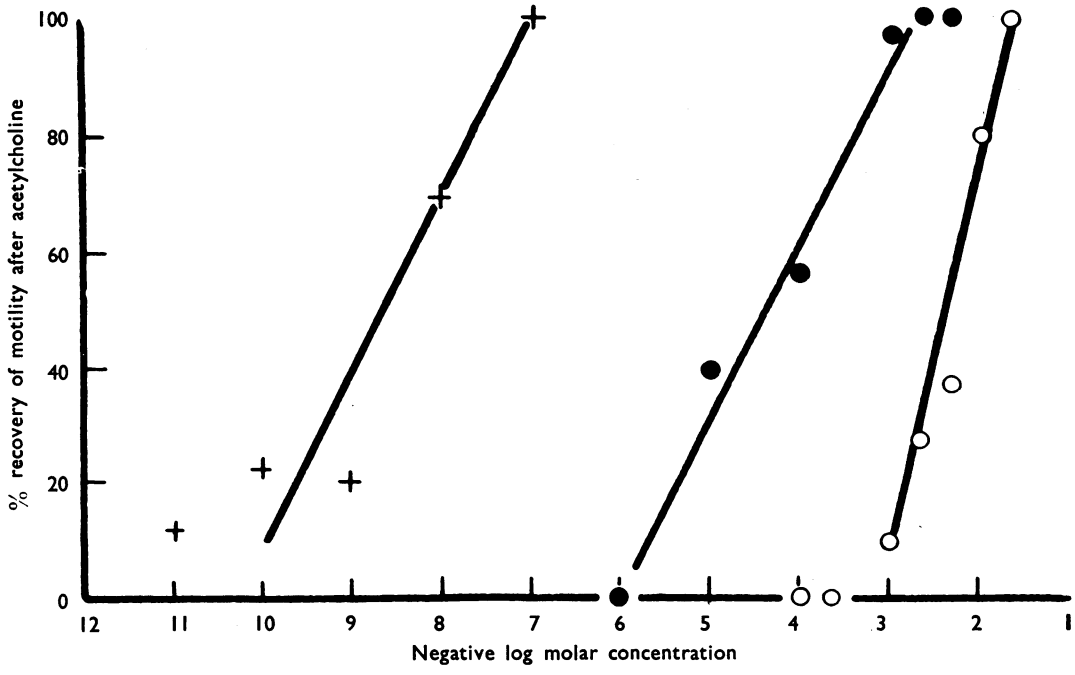

Fig. 5. Relationship between concentration of antagonist and recovery of amplitude of rhythmic movements of the isolated intestine of the rabbit. Antagonist was added to the bath after 2 min exposure to 10^{-5} M acetylcholine and without washing. Each point represents the mean of two to three observations. (Atropine + —— +; 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) ● —— ●; pralidoxime chloride ○ —— ○).

washed free of drugs using a standardized technique, homogenized and its cholinesterase activity determined. The results are expressed as % recovery of control activity, and this is plotted as a function of oxime concentration in Fig. 3. Concentrations necessary for 50% recovery of cholinesterase activity were read off from Fig. 3 and are given in Table 1. It will be apparent that roughly 300 times as high a concentration of pralidoxime chloride or 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) is needed to restore the cholinesterase activity of the tissue as is needed to cause relaxation of spasm and recovery of control motility.

TABLE 1

EFFECTIVE MICROMOLAR CONCENTRATIONS OF PRALIDOXIME, TMB-4 AND ATROPINE FOR VARIOUS ACTIVITIES AT THE 50% LEVEL

	Recovery of intestinal motility from spasm due to		Recovery of cholinesterase activity in gut treated with 10^{-5} M echothiophate
	10^{-5} M acetylcholine	10^{-5} M echothiophate	
Pralidoxime	4,700	620	170,000
TMB-4	40	125	40,000
Atropine	0·0025	0·093	

Whether or not this result was due to the oximes washing out of the tissue more readily than echothiophate with consequent reinhibition of the enzyme during homogenization was studied by adding the homogenate from echothiophate-treated and washed strips to an aliquot part of a control homogenate representing an equal amount of tissue (2 g). The data in Table 2 indicate that no significant inhibition of the cholinesterase activity of the control homogenate occurs under these circumstances.

TABLE 2

EFFECTIVENESS OF WASHING FOR REMOVAL OF UNCOMBINED ECHOTHIOPHATE

Cholinesterase activity in m-moles of acetylcholine hydrolysed per hr per g of tissue		
Control homogenate	Echothiophate treated and washed strip+ control homogenate	% of original activity in control homogenate
0·192	0·192	100
0·153	0·174	114
0·088	0·081	92
0·130	0·120	93

Tests showed that the cholinesterase activity of intact intestine is quite low as compared with homogenate, and this suggests the possibility that regeneration of functionally active enzyme might have occurred but that the magnitude was only a small % of the total and hence was not detectable with the methods used. An indirect approach was used to study this point. Intestine put into a firm spasm with 10^{-5} M echothiophate and washed shows no tendency to recover. A series of intestinal strips were treated with 10^{-5} M echothiophate and an oxime exactly as

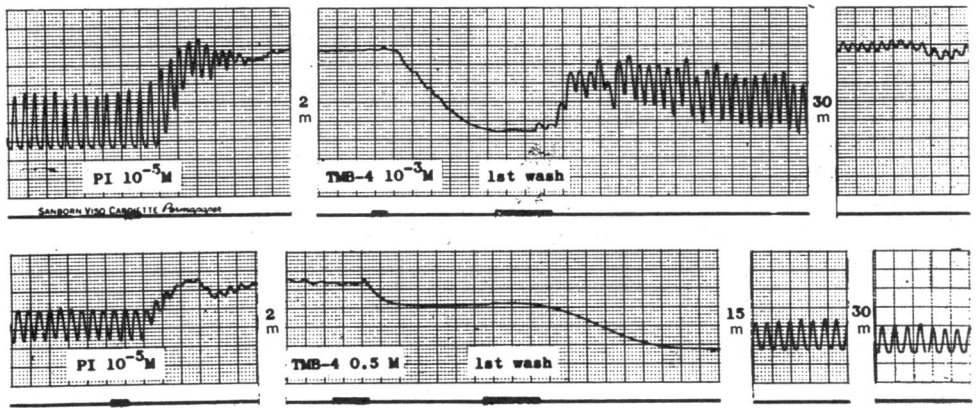

Fig. 6. Effect of washing on the motility of the isolated intestine of the rabbit after treatment with echothiophate and 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) successively. In the upper tracing, the concentration of the oxime is only sufficient to restore normal motility as in Fig. 2. In the lower tracing, the concentration of the oxime is sufficient to cause, also, reactivation of cholinesterase. Similar results were obtained with pralidoxime chloride. (m=minutes.)

before and then washed. The oxime concentrations were selected from Fig. 3 as those that would give respectively 100% restoration of motility and 80% to 100% recovery of cholinesterase activity. These concentrations were 0.001 M and 0.5 M for 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) and 0.004 M and 0.5 M for pralidoxime chloride. Typical tracings are shown in Fig. 6 for 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) and identical observations were obtained with pralidoxime chloride. The results were entirely consistent: at the lower concentration motility was restored temporarily, but after complete washing the tissue went back into spasm spontaneously ; after the higher concentration there was lasting restoration of normal tonus and motility. It is of interest that spasm in the first case came on slowly, probably due to the fact that accumulating acetylcholine was alone responsible without any primary action of echothiophate.

DISCUSSION

It was an unexpected finding that echothiophate causes complete cholinesterase inhibition of an intact intestinal strip which has been washed before homogenization, because echothiophate is a quaternary ammonium compound and does not readily cross the blood-brain barrier (Koelle & Steiner, 1956) or inhibit intraneuronal cholinesterase of the stellate or ciliary ganglia (McIsaac & Koelle, 1959). Hence it might be expected that it would not inhibit intracellular cholinesterase of the intestine. However, it does penetrate the conjunctiva and cornea (Leopold, Gold & Gold, 1957) and is readily absorbed on oral administration (Osserman, Cohen & Genkins, 1961), and hence shows a considerable degree of variation in its ability to cross membranes.

The data here reported indicate that both pralidoxime chloride and 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) have an anticholinergic action in the isolated rabbit intestine, and suggest that reactivation of cholinesterase may not be the most significant factor in the release of spasm that occurs within 1 min after oxime treatment under the conditions specified. Such a conclusion cannot at present be considered definitive for several reasons. In the motility experiments it would be expected that the oximes, if they are acting primarily as competitive acetylcholine antagonists, would have about the same relative activity against the two agonists as atropine, which is 37 times more active against acetylcholine than against echothiophate (see Table 1). The ratio found was much less than this, which suggests an additional mechanism of action. Nevertheless, the concentrations of oximes necessary to reactivate cholinesterase are well above those required for blocking acetylcholine (36 times for pralidoxime chloride and 1,000 times for 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) as well as those for blocking echothiophate. It must be noted, however, that echothiophate may have actions other than cholinesterase inhibition, and the oximes, besides causing reactivation, are cholinesterase inhibitors. In the experiments of Fig. 6 the possibility has not been ruled out that low concentrations of oximes reactivate cholinesterase which is superficially situated and that, after washing, echothiophate in deeper layers of the intestine reinhibits this cholinesterase, causing spasm. The methods used do not differentiate between acetylcholinesterase and butyrylcholinesterase, which

introduces a further complication, since the oximes are more effective in reactivating the former while the latter is probably more important in the control of intestinal motility. Further work will be required to settle these points, but it does seem conclusive that the anticholinergic action of the oximes must be taken into account in explaining their action.

The following published reports require consideration, since they bear directly on the nature of oxime action: chemical inactivation of echothiophate by pralidoxime has been ruled out by the slowness of this reaction (Lehman *et al.*, 1960). An atropine-like effect of pralidoxime iodide (P2AM) was denied by Kewitz, Wilson & Nachmansohn (1956) because of its failure to block the vasodepressor action of acetylcholine in the eviscerated cat. However, Bethe, Erdmann, Lendle & Schmidt (1957) have demonstrated such an action in the frog heart, rabbit intestine and guinea-pig auricle and at concentrations that are not inconsistent with those that might be obtained *in vivo*. Erdmann & Heye (1958) studied the action of paraoxon, parathion and systox in the isolated rabbit intestine, and reported that pralidoxime iodide caused an instantaneous relaxation of the phosphate ester stimulated gut, but that hypermotility returned on washing. However, repeated washing with Ringer solution containing pralidoxime ultimately restored activity to normal. They concluded that the initial relaxation was a true atropine-like action of the oxime which was not sustained on washing, while the lasting recovery after washing due to repeated treatment with oxime was the result of cholinesterase reactivation. Lindgren & Sundwall (1960) studied the effect of 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) on bradycardia due to stimulation of the vagus, and concluded that this oxime exerts a "vagolytic" effect due to atropine-like competitive inhibition. Atropine-like side-effects from pralidoxime iodide have been observed in man by Jager & Stagg (1958). Edery & Schatzberg-Porath (1959) found pralidoxime to have antidotal action against a phosphate ester which did not markedly inhibit brain or blood cholinesterase *in vivo*. Coleman, Little & Grant (1960) reported that pralidoxime was synergistic with other oximes as an antidote and felt that this could not be related to cholinesterase reactivation. Fleisher, Hansa, Killos & Harrison (1960) demonstrated that the partial recovery caused by 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) in the superficially situated cholinesterase of the diaphragm in sarin- or dyflos-treated animals was concomitant with recovery from neuromuscular block. These workers were impressed, however, with the slowness of recovery of enzyme activity as compared with recovery of function, and suggested a peripheral antagonism of anticholinesterases by 1,1′-trimethylenebis(4-hydroxyiminomethylpyridinium bromide) through a mechanism which is independent of cholinesterase reactivation. In a recent review of neuromuscular pharmacology, Grob (1961) summarized as follows: "It seems likely that the reversal by oximes of anticholinesterase neuromuscular block is not only attributable to the reversal of cholinesterase inhibition but also, and perhaps mainly, to inhibition of the action of acetylcholine."

As a working hypothesis, it is suggested that the quaternary oximes may have a blocking action which is relatively more effective against the acetylcholine which has accumulated due to inhibition of cholinesterase than against the packet of

acetylcholine which is liberated as the result of a nerve impulse. Otherwise, blocking by the oximes themselves would be a prominent feature of their pharmacology and the balance between doses of oxime and anticholinesterase would be critical, since too much of the oxime would readily increase the defect it was intended to relieve. This " anticholinergic action " may be the initial event which saves the life of a poisoned animal by restoring the activity of the respiratory muscles and providing the period of grace during which cholinesterase reactivation can occur as the result of oxime action, other mechanisms, or both.

When pralidoxime iodide was first proposed as an antidote for anticholinesterase poisoning (Wilson & Ginsburg, 1955; Childs, Davies, Green & Rutland, 1955), it appeared that its action was simply to reactivate alkyl phosphate-inhibited cholinesterase. It is now evident from the investigations of the last 6 years that this and other similar oximes have many actions. Further work will be necessary before these can be sifted to determine which are incidental and which are contributory to the antidotal effect.

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