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A CORRELATION BETWEEN CHOLINESTERASE INHIBITION AND INCREASE IN MUSCLE TONE IN RABBIT DUODENUM

BY

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Experiments with anticholinesterases on whole animals have suggested that symptoms of acetylcholine (ACh) accumulation do not appear until 50–80% of the true (aceto-) cholinesterase activity of the tissues has been inhibited, total inhibition of pseudo- (butyro-) cholinesterase being without effect (Gunter and Mendel, 1945; Hawkins and Gunter, 1946; Koelle and Gilman, 1946; Mazur and Bodanzky, 1946; Hawkins and Mendel, 1947; Nachmansohn and Feld, 1947; Riker and Wescoe, 1949). In contrast, experiments with isolated organs (Koelle, Koelle, and Friedenwald, 1950; Burn and Kottegoda, 1953) suggested that anticholinesterases modify function in much lower concentrations than are necessary to produce 50% inhibition of true cholinesterase and that inhibition of pseudo-cholinesterase alone can produce signs of accumulation of ACh.

The work to be described in this paper was designed to investigate the action of anticholinesterases on the isolated duodenum of the rabbit. The responses of preparations of intact duodenum and of the longitudinal muscle coat (prepared according to Ambache, 1954) to different concentrations of eserine and dyflos (di*iso*propyl phosphorofluoridate, DFP) have been recorded. The distribution of true and pseudo-cholinesterase in the intestinal wall has been investigated manometrically using different substrates, and the inhibition by eserine and DFP of cholinesterase activity in longitudinal muscle homogenates has been determined. In addition, estimations have been made of the residual cholinesterase activity in muscle strips exposed to the action of DFP before homogenizing the tissue.

METHODS

Rabbits were killed by a blow on the neck followed by bleeding from the severed carotid arteries.

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Isolated Intestine Preparations

Both whole intestine preparations and longitudinal muscle preparations were suspended in Krebs's bicarbonate solution oxygenated with O_2 containing 5% CO_2 at 34° C. The solution was made up as follows: NaCl 6.6 g., KCl 0.35 g., $CaCl_2$ 0.28 g. (1.41 ml. 20% soln.), KH_2PO_4 0.162 g. (1.62 ml. 10% soln.), $MgSO_4,7H_2O$ 0.294 g. (2.94 ml. 10% soln.), dextrose 2.08 g., $NaHCO_3$ 2.1 g., distilled water 1 litre. This was used in preference to Locke's or Tyrode's solution so as to avoid effects which may be due to loss of cholinesterase activity resulting from changes in *p*H (Bülbring, Kottegoda and Shelley, 1954). (In that paper errors occurred in the formula given for the modified Krebs's solution used; it contained NaH_2PO_4 0.825 g. in 5 l. and the $MgCl_2$ soln. was 1%.)

Intact Duodenum Preparations.—The duodenum was severed near the pyloric sphincter and about 9 in. was removed, washed thoroughly in cold Krebs's solution, and dissected free from fat and mesentery. This piece was then divided into 4 cm. lengths and each was numbered to indicate its original position with respect to the stomach. Each piece was suspended in turn with the lumen closed at both ends in a 30 ml. bath. Its movements were recorded on a smoked drum. Each piece was treated with a single dose of anticholinesterase for 30 min., and the response to a given dose of ACh before and after administration of the anticholinesterase was recorded. In comparing the responses to different doses of anticholinesterase, care was taken to ensure that the initial tension was the same for each successive piece of duodenum. Those pieces not in use were kept in Krebs's solution at room temperature, and the order in which they were used was changed in each experiment. On transferring them to Krebs's solution at 34° C., preparations up to 6 hr. old behaved normally, though the pendular movement was not always as vigorous as that of fresh preparations.

Longitudinal Muscle Strips.—These were prepared according to Ambache (1954). When free from the rest of the duodenum, the longitudinal muscle immediately contracted to about a third of its original length. 2.5 cm. lengths of this material were suspended in a 30 ml. bath and their movements were recorded.

The preparations took some time to relax, and pendular movement seldom appeared before 1 hr. after setting up. Several different concentrations of anticholinesterase were tested on each preparation and the responses to ACh were recorded as before. Active preparations were never obtained unless the duodenum had been allowed to cool to room temperature or slightly below before stripping off the muscle. The longitudinal muscle coat in the rabbit is only 2% by weight of the total intestinal wall, and it appears to be more resistant to the effects of stretching at low temperatures. A similar observation was made by Gunn and Underhill (1914) with circular muscle preparations from the cat intestine.

Anticholinesterases

A stock solution of DFP in propylene glycol and a freshly prepared solution of eserine sulphate in water were diluted just before use with 0.9% saline for isolated organ experiments or with distilled water for enzyme experiments.

Enzyme Preparations

Tissue homogenates were prepared from frozen tissue which was first chopped finely with the aid of a McIlwain chopper (McIlwain and Buddle, 1953) and then homogenized with dextrose-free Krebs's solution in a Potter-Elvehjem all-glass homogenizer. The homogenates, final concentration 100 mg./ml., were stored at −15° C. Both duodenum and ileum were used. Homogenates of intact intestine, intestine which had had the villi and muscularis mucosae removed by scraping the inner surface with a blunt scalpel, circular muscle coat, and longitudinal muscle coat, were prepared. The longitudinal and circular muscle coats could be separated from the rest of the intestinal wall more readily in tissue which had been frozen than in fresh tissue. Lengths of previously frozen intestine were pinned out flat in Krebs's solution with the inner surface exposed. On scraping this surface vigorously with a scalpel, the longitudinal muscle coat on the outer surface became partially detached and could easily be stripped away. Circular muscle could be obtained by stripping away the other layers, but it tended to stick to the submucosa. The nature of the different layers was confirmed by histological examination.

Manometric Technique

Warburg manometers with micro-flasks containing a total fluid volume of 0.8 ml. were used. The tissue was incubated in nitrogen containing 5% CO_2 at 37.5° C. Readings were taken at 5-min. intervals for at least 30 min. after the addition of substrate to enzyme, and cholinesterase activity was calculated from the initial slope of the curve of carbon dioxide production. Acetylcholine bromide (ACh), methacholine chloride (MCh), benzoylcholine chloride (BzCh), and butyrylcholine chloride (BuCh) were used as substrates in final concentrations of 0.025 M, 0.0375 M, 0.0075 M, and 0.025 M respectively. Inhibitors were incubated with the enzyme for 30 min. at room temperature plus 15 min. at 37.5° C. before adding the substrate.

Estimation of Residual Cholinesterase Activity in Muscle Strips Treated with DFP before Homogenizing

After 30 min. in the presence of DFP the tissue was washed in ice-cold Krebs's solution, dried on filter paper, weighed, frozen, and then chopped rapidly. The mince was homogenized in the maximum possible volume of ice-cold saline to give a homogenate containing 100 mg. tissue/ml. The whole procedure took not more than 15 min. and was repeated on a similar muscle strip which had not been treated with DFP. The cholinesterase activities of the two homogenates were compared using MCh and BuCh as substrates so as to obtain an estimate of the inhibition of true and pseudo-cholinesterase in the intact tissue.

RESULTS

The Action of Eserine and Dyflos on the Isolated Intact Duodenum of the Rabbit and on Preparations of the Longitudinal Muscle Coat

(*a*) *Intact Duodenum.*—The results obtained were qualitatively similar to those described by earlier workers. A shortening or "increase in tone" of the muscle occurred in response to eserine sulphate 2.7×10^{-8} M to 2.7×10^{-4} M and to DFP 10^{-8} to 10^{-5} M. The effect increased with eserine concentration from 2.7×10^{-8} M to 2.7×10^{-6} M; but in two experiments the effect was smaller in the presence of eserine 2.7×10^{-5} M; in a third experiment a smaller effect was only obtained with eserine 2.7×10^{-4} M. Similar results were obtained with DFP, a peak effect being observed at 10^{-5} M.

With low concentrations of anticholinesterases the increase in tone was achieved gradually and was sometimes accompanied by an increase in the amplitude of the pendular movements. As the anticholinesterase concentration increased, the increase in tone reached its maximum more rapidly and the pendular movement diminished. No effect was observed on the rate of the pendular movement until the peak concentrations were reached and the pendular movement ceased altogether. No difference in sensitivity to anticholinesterases was observed in the different regions of duodenum tested.

(*b*) *Longitudinal Muscle Strips.*—The results obtained with preparations of the longitudinal muscle coat were similar to those described for the intact duodenum. Pendular movement was irregular and not so vigorous as in the intact duodenum, but, once established, it could be maintained for long periods (at least 8 hr.).

Fig. 1 illustrates the responses to ACh and eserine of a typical preparation. This gave graded

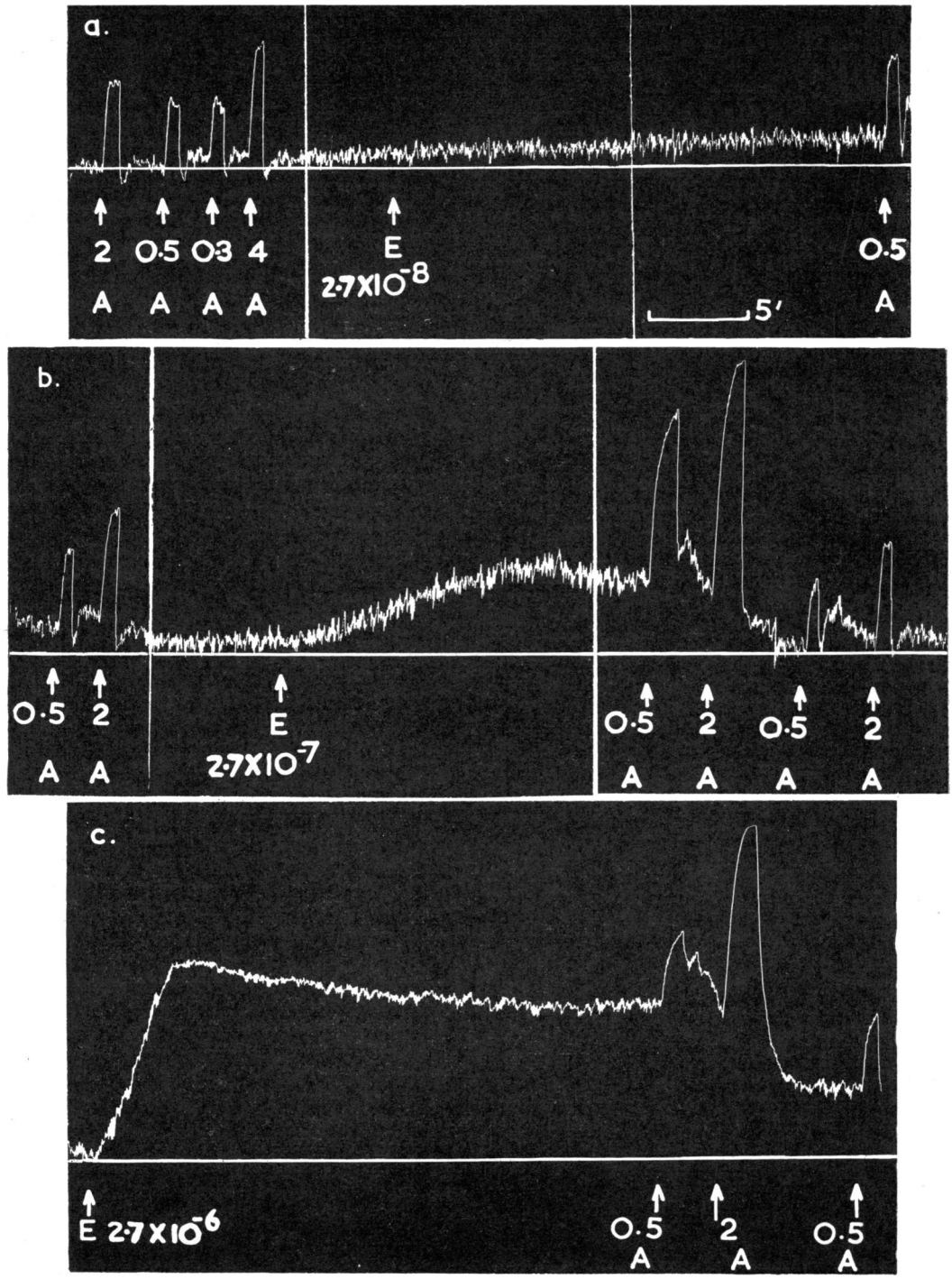

FIG. 1.—Rabbit duodenum longitudinal muscle layer: the effect of increasing molar concn. of eserine (E) on muscle tone and the response to acetylcholine (A, doses in μg.).

contractions in response to doses of ACh varying from 0.3 μg. to 4.0 μg. Eserine 2.7×10^{-8} M, 2.7×10^{-7} M, and 2.7×10^{-6} M (Fig. 1, a, b, and c) produced progressively greater increases in tone and potentiation of the response to ACh ; both the tone and the ACh response returned to normal after washing out the eserine. In two other experiments eserine 2.7×10^{-8} M had no effect, but similar responses were obtained with eserine 2.7×10^{-7} M and 2.7×10^{-6} M. As in experiments with intact duodenum preparations, eserine 2.7×10^{-5} M and 2.7×10^{-4} M produced smaller increases in tone, but in the two experiments in which eserine 2.7×10^{-4} M was tested the initial increase in tone was followed by relaxation of the muscle. In the presence of the higher concentrations of eserine the response to ACh disappeared, and in one experiment, the only one in which ACh was administered in the presence of eserine 2.7×10^{-4} M, 0.5 μg. ACh caused relaxation of the longitudinal muscle preparation. No such effect was ever observed with intact duodenum preparations. The close similarity between the response to eserine of the two types of preparation is shown in Fig. 2, where the changes in tone after various doses of eserine are represented graphically.

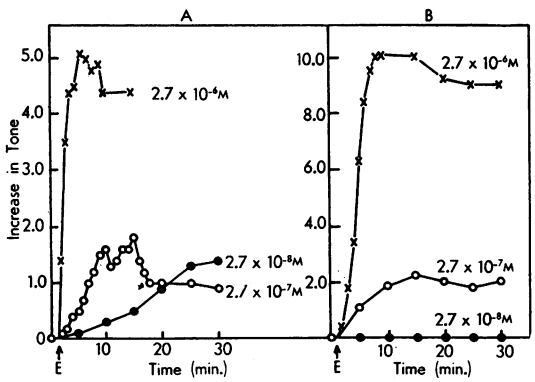

FIG. 2.—The effect of increasing concn. of eserine (E) on muscle tone in preparations of intact rabbit duodenum (A) and of a single longitudinal muscle strip (B). Ordinates: increase in tone, measured as change in level of record in cm. Abscissae: time in min.

Fig. 3 illustrates the effects of DFP in a typical experiment. Concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M produced progressively greater increases in tone, and potentiated the effects of ACh. Similar results were obtained in five other experiments. Unlike the effects of eserine, those produced by DFP persisted after washing. The potentiation of the response to ACh remained after washing out the DFP, and, although repeated washing sometimes restored the tone to its original level, a further period without washing usually resulted in a slow return to the level reached in the presence of DFP. As in the experiments with intact duodenum, DFP 10^{-4} M in four out of five experiments had a smaller effect than DFP 10^{-5} M, and therefore produced a decrease in tone. In two experiments DFP 10^{-3} M produced a large decrease in tone, to a level only slightly above normal. These effects are also illustrated in Fig. 3c, where DFP 10^{-4} M had no effect but DFP 10^{-3} M produced a decrease in tone. A reversal of the response to ACh was never observed in the presence of DFP. Fig. 4 shows the similarity between the response of longitudinal muscle and of intact duodenum preparations to various doses of DFP.

An attempt to summarize the effects of eserine and DFP on the tone of intact duodenum and longitudinal muscle preparations has been made in Table I. In every experiment the maximum change in level of the base line in response to

TABLE I

THE EFFECT OF ESERINE AND DFP ON THE TONE OF THE ISOLATED RABBIT DUODENUM AND OF ISOLATED LONGITUDINAL MUSCLE STRIPS

Eserine (Molar Concn.)	Increase in Tone as Mean % of Maximum		DFP (Molar Concn.)	Increase in Tone as Mean % of Maximum	
	Intact Duodenum	Longitudinal Muscle		Intact Duodenum	Longitudinal Muscle
2.7×10^{-8}	27.5	3.0	10^{-8}	—	0
2.7×10^{-7}	50.0	34.0	10^{-7}	18.5	18.5
2.7×10^{-6}	100.0	100.0	10^{-6}	87.5	71.0
2.7×10^{-5}	93.0	78.0	10^{-5}	100.0	100.0
2.7×10^{-4}	91.0	35.0	10^{-4}	—	73.0
No. of expts.	3	3	No. of expts.	2	6

each dose of anticholinesterase was expressed as a percentage of the peak value, observed with eserine 2.7×10^{-6} M and with DFP 10^{-5} M. For instance, in one experiment, concentrations of DFP 10^{-7}, 10^{-6}, and 10^{-5} M produced changes in level of the base line of 0.6, 3.3, and 4.5 cm.; the percentage changes were therefore expressed as 13.5, 74, and 100. As might be expected, there was some variation in different experiments, but the mean values (Table I) illustrate the similarity between the effects of eserine and DFP. Moreover, the effects of DFP are quantitatively similar on the two preparations.

An attempt to establish a quantitative relationship between the potentiation of the response to ACh and the concentration of anticholinesterase was unsuccessful. Although the degree of potentiation increased with rising anticholinesterase concentration, this was often obscured by the

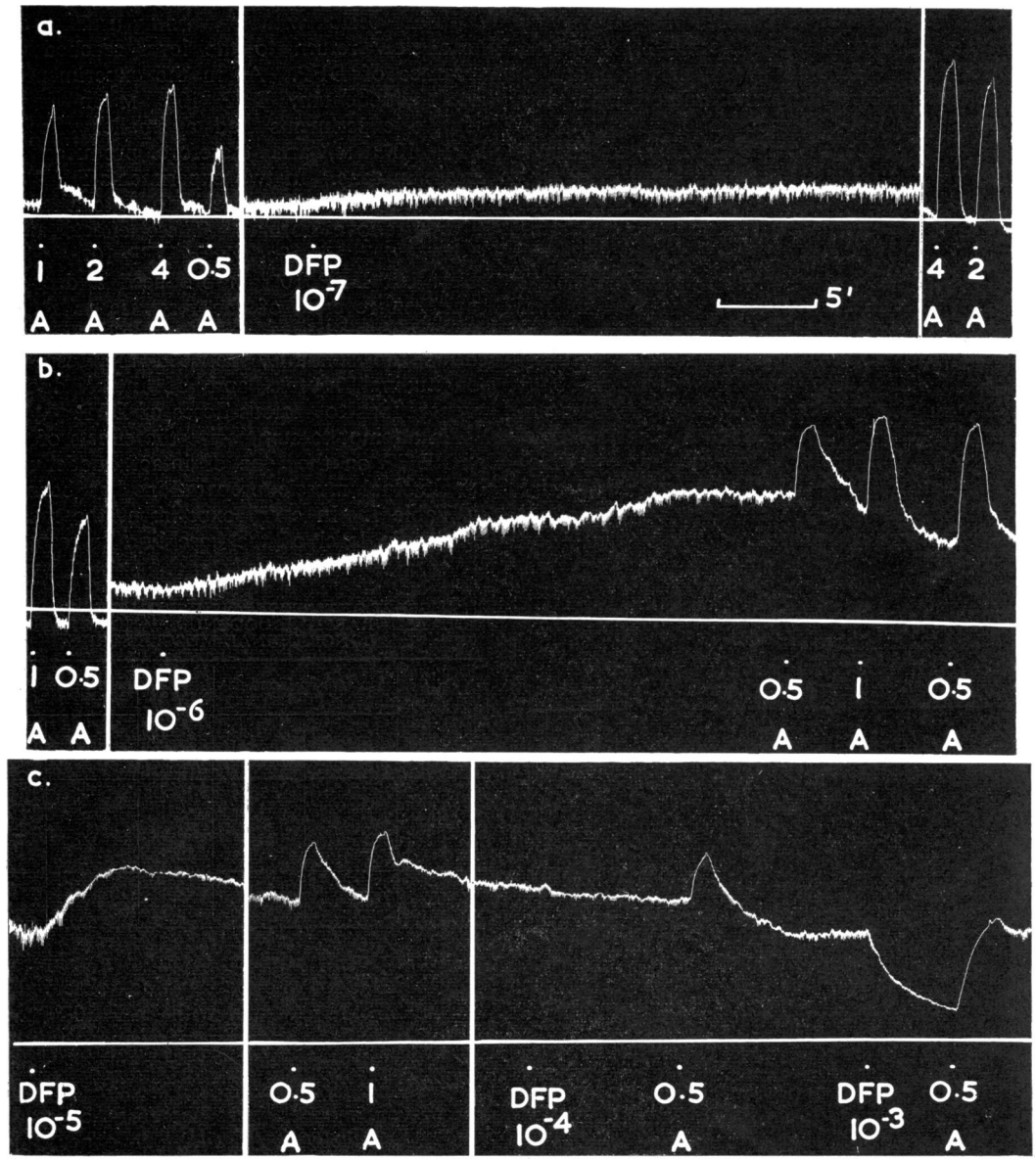

FIG. 3.—Rabbit duodenum longitudinal muscle layer: the effect of increasing molar concn. of DFP on muscle tone and the response to acetylcholine (A, doses in μg.).

change in tone of the preparation, particularly in the presence of high concentrations of anticholinesterase.

The Distribution of Cholinesterase Activity in the Intestinal Wall

The true and pseudo-cholinesterase activity of homogenates of intact intestine, as measured by the rates of hydrolysis of MCh and BzCh, were low and the homogenates were so viscous that the results were difficult to duplicate. Homogenates of the circular and longitudinal muscle layers were non-viscous and more active. The rates of hydrolysis of four different choline esters by the various homogenates are summarized in Table II.

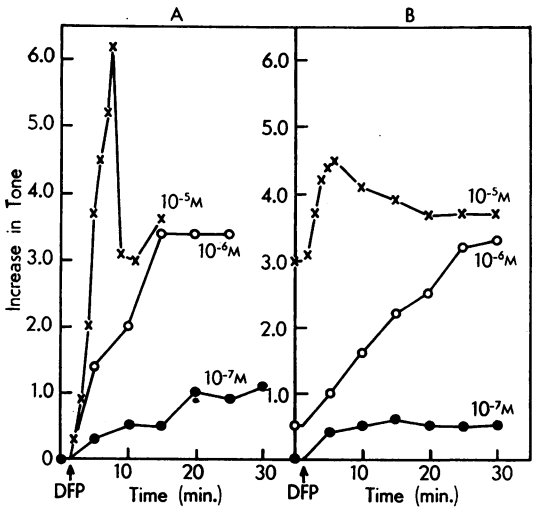

FIG. 4.—The effect of increasing concn. of DFP on muscle tone in preparations of intact rabbit duodenum (A) and of a single longitudinal muscle strip (B). Ordinates: increase in tone, measured as change in level of record in cm. Abscissae: time in min.

The results suggest that true and pseudo-cholinesterase are most highly concentrated in the longitudinal and circular muscle layers of the intestinal wall. Moreover, the longitudinal muscle layer has twice the true cholinesterase activity and nearly three times the pseudo-cholinesterase activity of the circular layer. It is known, and histological examination has confirmed, that most of the cells and fibres of Auerbach's plexus adhere to the longitudinal muscle layer, and this probably accounts for its greater cholinesterase activity.

Homogenates of scraped intestine had a lower pseudo-cholinesterase activity than homogenates of whole intestine, suggesting that the mucosa which had been removed is rich in pseudo-cholinesterase; it is probable that the muscle coats account for the residual pseudo-cholinesterase activity of the scraped intestine. In contrast, true cholinesterase activity was not diminished by removing the mucosa, and the activity of the muscle coats—which are not more than 5% of the weight of the intestinal wall—was insufficient to account for the total activity in whole and scraped intestine. These results suggest that pseudo-cholinesterase activity is concentrated in the muscle coats and in the mucosa, whereas true cholinesterase is present throughout the intestinal wall.

TABLE II

CHOLINESTERASE ACTIVITY IN THE DIFFERENT LAYERS OF THE RABBIT SMALL INTESTINE

Source of Enzyme	Expt.	Rate of Hydrolysis of Substrate μl. CO_2/g. Tissue/Hr.			
		ACh 0·025M	MCh 0·0375M	BzCh 0·0075M	BuCh 0·025M
Intact small intestine	1	1,040	405	200	
	2	1,530	500	250	
	Mean	1,285	453	225	
Scraped small intestine, i.e., longitudinal muscle+circular muscle+submucosa	3	1,160			
	4	1,400			
	5	980			
	6	1,280			
	7	785	560	50	
	8	1,330	560	0	
	9	1,370	450	110	
	Mean	1,186	523	53	
Circular muscle ..	10	2,000			
	11	3,200	845	310	
	Mean	2,600	845	310	
Longitudinal muscle	12	5,100			
	13	5,680	1,720	880	
	14	7,720	2,260	740	5,440
	15	6,560	2,640		4,920
	16		1,570		7,720
	17	5,080	1,540		5,840
	Mean	6,028	1,946	810	5,980

Expts. 2 and 9 were performed with tissue from the same rabbit. Expts. 10 and 12, 11 and 13 were performed with tissue from the same piece of intestine.

The Inhibition of True and Pseudo-cholinesterase in the Longitudinal Muscle Layer of Rabbit Intestine by Eserine and Dyflos

The results with eserine and DFP as inhibitors of the cholinesterase in homogenates of the longitudinal muscle layer are shown in Tables III and IV. The mean results for each substrate have been calculated from three experiments, excepting those for BzCh, which are from one experiment. Table III also contains results obtained with homogenates of scraped intestine.

Table IV includes estimates of the inhibition by DFP of true and pseudo-cholinesterase in the intact longitudinal muscle strip. These were

TABLE III

INHIBITION OF THE CHOLINESTERASES OF RABBIT SMALL INTESTINE HOMOGENATES BY ESERINE

Eserine Sulphate (Molar Concn.)	% Inhibition of Hydrolysis of Substrates Indicated				
	Scraped Intestine	Longitudinal Muscle Layer			
	ACh 0·025M	ACh 0·025M	MCh 0·0375M	BzCh 0·0075M	BuCh 0·025M
$2·7 \times 10^{-8}$	2·0	8·0	4·0	35·0	1·5
$1·35 \times 10^{-7}$	12·0	8·0	10·0	34·0	12·5
$2·7 \times 10^{-7}$	21·0	32·0	29·5	45·0	25·5
$1·35 \times 10^{-6}$	48·0	55·0	47·5	65·0	51·5
$2·7 \times 10^{-6}$	58·0	69·0	70·0	88·0	67·0
$1·35 \times 10^{-5}$	84·5	86·0	91·5	93·0	82·0
$2·7 \times 10^{-5}$	98·5	98·0	99·0	100·0	97·0
$1·35 \times 10^{-4}$	100·0	100·0	100·0	100·0	99·0
$2·7 \times 10^{-4}$	100·0	100·0	100·0	100·0	99·0
I 50	$1·9 \times 10^{-6}$	$1·35 \times 10^{-6}$	$1·5 \times 10^{-6}$	$3·05 \times 10^{-7}$	$1·6 \times 10^{-6}$

I 50 = molar concn. of eserine sulphate necessary for 50% inhibition of substrate hydrolysis.

TABLE IV

INHIBITION OF THE CHOLINESTERASES OF THE LONGITUDINAL MUSCLE OF RABBIT SMALL INTESTINE BY DFP ADDED TO INTACT MUSCLE STRIPS OR TO HOMOGENATES

DFP (Molar Concn.)	% Inhibition of Hydrolysis of Substrate Indicated				
	ACh 0·025M	MCh 0·0375M		BuCh 0·025M	
	Homogenate	Homogenate	Intact Muscle Strip	Homogenate	Intact Muscle Strip
10^{-9}	13·0			0·5	
5×10^{-9}	19·0			7·0	
10^{-8}	24·0	6·0	5·0	19·5	41·0
5×10^{-8}	35·0	10·5		58·0	
10^{-7}	54·0	19·0	10·0	88·0	74·5
5×10^{-7}	73·0	39·0		95·0	
10^{-6}	95·0	65·0	59·5	99·0	93·5
5×10^{-6}	100·0	94·0		100·0	
10^{-5}	100·0	100·0	100·0	100·0	99·5
I 50	$9\cdot5\times10^{-8}$	$7\cdot5\times10^{-7}$	$9\cdot0\times10^{-7}$	$4\cdot0\times10^{-8}$	$2\cdot5\times10^{-8}$

I 50 = molar concn. of DFP necessary for 50% inhibition of substrate hydrolysis.

obtained by comparing the residual cholinesterase activity after treating the intact strip with DFP for 30 min. with the activity of untreated control strips. Each estimate is the mean of two experiments, except that for DFP 10^{-5} M, which is the result of a single experiment. In most experiments the estimated inhibition of true and pseudo-cholinesterase obtained by treating the intact longitudinal muscle strip with DFP was similar to that obtained by adding DFP to the muscle homogenate.

Correlation of Increase in Tone in Longitudinal Muscle with Cholinesterase Inhibition

Figs. 5 and 6 represent an attempt to correlate the percentage inhibition of true and pseudo-cholinesterase with the corresponding increase in tone of longitudinal muscle preparations calculated

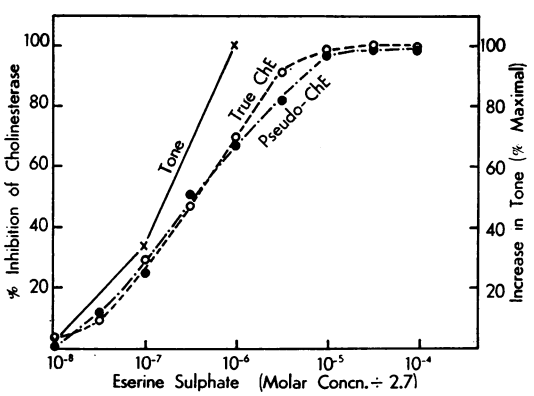

FIG. 5.—The effect of increasing concn. of eserine on cholinesterase (ChE) activity and tone in the longitudinal muscle of the rabbit small intestine.

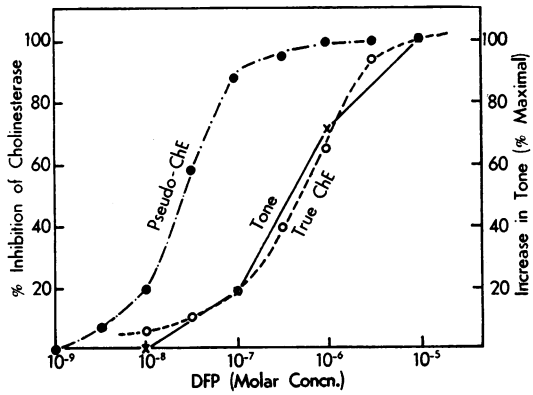

FIG. 6.—The effect of increasing concn. of DFP on cholinesterase (ChE) activity and tone in the longitudinal muscle of the rabbit small intestine.

as in Table I. In Fig. 5 the percentage increase in tone produced by a given concentration of eserine is always greater than the percentage inhibition of either true or pseudo-cholinesterase, but the curves are almost parallel, suggesting a relationship between increase in tone and inhibition of cholinesterase activity. As can be seen in Fig. 6, this correlation is more dramatic when DFP is the inhibitor. Since pseudo-cholinesterase is more readily inhibited than true cholinesterase by DFP, the inhibition curves for the two enzymes are quite distinct and the curve of increase in tone is exactly superimposed upon the inhibition curve for true cholinesterase.

DISCUSSION

The effects of eserine and DFP on the tone of isolated intestine preparations from several species have been investigated by a number of workers (see Heathcote, 1932; Feldberg and Solandt, 1942; Bacq, 1947; Feldberg and Lin, 1949; Koelle, Koelle, and Friedenwald, 1950), but little attempt has been made to relate the intensity of the effect to the concentration of anticholinesterase. Bacq found that isolated cat or rabbit intestine would not respond to a second dose of eserine or DFP, and Koelle *et al.* were unable to obtain a quantitative relationship between DFP concentration and the response of isolated cat intestine.

In the present work the effects of previous doses of anticholinesterase have been avoided, by testing each dose of eserine or DFP on a different piece of rabbit duodenum, and it was observed that the magnitude of the response increased with rising anticholinesterase concentration to a maximum in the presence of eserine 2.7×10^{-6} M or DFP 10^{-5} M. When the responses to lower concentrations of

anticholinesterase were expressed as percentages of the maximum response, there was quantitative agreement between different experiments. Similar results were obtained when successive doses of anticholinesterase were tested on the same longitudinal muscle preparation. As had been observed by Heathcote (1932), still higher concentrations of eserine produced smaller increases in tone and this was also true of DFP. These effects are probably due to actions of eserine and DFP distinct from their anticholinesterase action.

There has been no previous investigation of the rates of hydrolysis of choline esters by the different layers of the intestinal wall in the rabbit, but Koelle (1953) gives comparable figures for the hydrolysis of ACh, MCh, and BzCh by homogenates of whole intestine. Koelle investigated the distribution of true and pseudo-cholinesterase in the rabbit intestine by histochemical methods, and his results are similar to those obtained in the present work using manometric methods. He found that true cholinesterase occurred in nervous tissue throughout the intestinal wall, and in low concentrations in the interstitial cells of the muscle layers; pseudo-cholinesterase was mainly present in the interstitial cells and in the mucosa, but was also found in ganglion cells. Koelle also investigated the inhibition of rabbit cholinesterases by eserine and DFP, and his results are confirmed in this paper (see also Mazur and Bodanzky, 1946).

The aim of the present work was to correlate the increase in tone of the intestine preparations with the percentage inhibition of cholinesterase activity. As in all work of this kind, the chief difficulty was to determine the relationship between the inhibition of cholinesterase activity *in vitro* and the inhibition of ACh hydrolysis in the intact tissue.

This is especially difficult when a reversible inhibitor, such as eserine, is used. The addition of substrate to a cholinesterase preparation in the presence of eserine will partially reverse the eserine inhibition of the enzyme, and the extent of this reversal will depend both on the affinity of the enzyme for the substrate and on the concentration of added substrate. For this reason the measurement of cholinesterase inhibition by eserine *in vitro* in the presence of 0.025 M ACh will not give an accurate measure of the inhibition of ACh hydrolysis in the intact tissue, where the substrate concentration is unknown. In the intact tissue the ACh concentration is probably less than 0.025 M and therefore the inhibition of ACh hydrolysis will probably be greater than that measured *in vitro*. Similarly, the measurement of true or pseudo-cholinesterase inhibition by eserine using specific substrates such as MCh, BzCh, and BuCh will not give an accurate measure of the inhibition of ACh hydrolysis by these enzymes either *in vivo* or *in vitro*. This point may be illustrated by the results obtained for the inhibition by eserine of pseudo-cholinesterase in longitudinal muscle homogenates (Table III). When 0.0075 M-BzCh was used as substrate the apparent inhibition of pseudo-cholinesterase activity by a given concentration of eserine was always greater than the corresponding inhibition of ACh or MCh hydrolysis in agreement with earlier observations by Blaschko, Bülbring and Chou (1949). In contrast, when 0.025 M-BuCh was used, the apparent inhibition of pseudo-cholinesterase activity was similar to that of ACh or MCh hydrolysis (see also Fig. 5).

It is preferable, therefore, to use an irreversible inhibitor, such as DFP, in all work of this kind. Mackworth and Webb (1948) have shown that, in contrast to eserine, inhibition of cholinesterase activity by DFP is not reversed by the addition of high substrate concentrations. Therefore, comparison of the rates of hydrolysis of different substrates in the presence of DFP, added at a fixed time interval before the substrate, does give a true measure of the inhibition of the enzyme under investigation, and it can definitely be said that DFP inhibits pseudo-cholinesterase more readily than true cholinesterase. It may also be assumed that the inhibition produced by DFP in the intact tissue is no greater than that produced by adding DFP to the tissue homogenate, and this has been confirmed in the present work.

Another possible source of error is incomplete penetration of the intact tissue by the anticholinesterase. The longitudinal muscle layer of the rabbit intestine is very thin, however (about 120 μ), and penetration should be relatively easy, especially when preparations of isolated longitudinal muscle are used. It has been assumed that, after 30 min. in the presence of eserine or DFP, penetration of the anticholinesterase into this muscle layer is complete. The increase in tone of the longitudinal muscle usually reached a maximum within 30 min. of administering the anticholinesterase; there was also good agreement between the inhibition of cholinesterase by DFP added to longitudinal muscle homogenates and the cholinesterase inhibition in strips of longitudinal muscle treated for 30 min. with DFP before homogenizing them. Moreover, Koelle, Koelle, and Friedenwald (1950) examined suitably stained sections of cat intestine which, when intact, had been exposed to the action of DFP, and observed that the inhibition of the longitudinal muscle cholinesterase

corresponded to that determined manometrically with homogenates of whole intestine, though the inhibition in the deeper layers was often less.

Koelle *et al.* also made manometric determinations of the residual cholinesterase activity in these segments of cat intestine. They observed that concentrations of DFP which had produced large increases in tone had almost completely inhibited the pseudo-cholinesterase, whereas the true cholinesterase was hardly affected. Since, in addition, they had observed mainly pseudo-cholinesterase in the longitudinal muscle layer (see also Koelle, 1950; 1951), they concluded that there is a close relationship between pseudo-cholinesterase activity and intestinal motility. This conclusion was supported by Burn, Kordik, and Mole (1952), who, having observed diarrhoea in rats exposed to X-rays, tested the reaction of intestinal loops to ACh and found that they responded to much lower concentrations than usual. They observed that the pseudo-cholinesterase activity was reduced to less than half, while the true cholinesterase activity was unchanged.

The results obtained in the present work on the rabbit intestine suggest that true cholinesterase is at least as important as pseudo-cholinesterase. When the percentage increase in tone and the percentage inhibition of true cholinesterase by DFP were plotted against the concentration of DFP, the two curves were superimposed (Fig. 6). Maximum increase in tone coincided with 100% inhibition of true cholinesterase activity, whereas 100% inhibition of pseudo-cholinesterase occurred at a much lower concentration. In contrast, the percentage increase in tone produced by a given dose of eserine was always greater than the percentage inhibition of either cholinesterase (Fig. 5). However, since the inhibition of cholinesterase activity produced by eserine in the intact tissue was probably greater than that measured *in vitro*, it is possible that the increase in tone produced by both eserine and DFP corresponded with true cholinesterase inhibition in the intact tissue though not necessarily with inhibition as measured *in vitro*.

The results recorded here show that symptoms of ACh accumulation can occur when true cholinesterase activity is only partially inhibited. Previous work has suggested that there is a vast excess of true cholinesterase in the tissues, most of which can be inhibited without effect (Gunter and Mendel, 1945; Mazur and Bodanzky, 1946; Koelle and Gilman, 1946; Hawkins and Mendel, 1947; Nachmansohn and Feld, 1947; Hawkins and Gunter, 1949; Hawkins and Mendel, 1949; Riker and Wescoe, 1949), but in the present work an increase in tone in the rabbit intestine was regularly observed, not only with eserine 2.7×10^{-7} M, but also with DFP 10^{-7} M, a concentration sufficient to produce only 10–20% inhibition of true cholinesterase activity. Similarly, Burn and Kottegoda (1953) observed changes in the rate and amplitude of beat of isolated rabbit auricles in the presence of eserine 2.7×10^{-8} M to 2.7×10^{-7} M, sufficient to produce not more than 16% inhibition of ACh hydrolysis by auricle homogenates *in vitro* (Shelley, unpublished experiments); but again, since eserine is a reversible inhibitor, the inhibition of cholinesterase activity in the intact tissue was probably greater than that measured with the homogenates.

The ratio of cholinesterase activity to ACh content is not the same for all tissues, and it is possible that tissues with a low cholinesterase activity and a high rate of ACh production do not contain a large excess of cholinesterase. In the rabbit intestine cholinesterase activity is low compared with many tissues, and the rate of ACh production is high (Feldberg and Solandt, 1942; Feldberg and Lin, 1950). Moreover, the ACh content is high compared with other tissues (Chang and Gaddum, 1933), the longitudinal muscle layer containing 10–16 μg./g. (Welsh and Hyde, 1944), an exceptionally high ACh content for a mammalian tissue. The cholinesterase activity of rabbit auricles is also low (Bülbring, Kottegoda, and Shelley, 1954), and, though the rate of ACh synthesis is not high (Bülbring and Burn, 1949), the auricles contain up to 4.0 μg. ACh/g. (Briscoe and Trendelenburg, unpublished experiments). Under these conditions of low cholinesterase activity and high ACh content, it is conceivable that symptoms of ACh accumulation should appear when the degree of cholinesterase inhibition is comparatively slight, and should increase in intensity—as in the rabbit duodenum—until the cholinesterase activity has been reduced to zero.

Summary

1. The effect of eserine and dyflos (DFP) on tone in the isolated rabbit duodenum and in isolated strips of longitudinal muscle from the rabbit duodenum has been investigated.

2. An increase in tone first appeared with eserine 2.7×10^{-8} M or DFP 10^{-7} M. This effect increased, with rising anticholinesterase concentration, to a maximum in the presence of eserine 2.7×10^{-6} M or DFP 10^{-5} M. Above these concentrations the increase in tone declined with rising anticholinesterase concentration.

3. The distribution of true and pseudo-cholinesterase in the different layers of the intestinal wall was investigated manometrically using different substrates. Both enzymes were present in the longitudinal muscle layer, and the cholinesterase activity of this layer was higher than that of any other layer.

4. The inhibition of the true and pseudo-cholinesterase of the longitudinal muscle layer by eserine and DFP was investigated. Pseudo-cholinesterase was more readily inhibited than true cholinesterase by DFP, but, using 0.025 M-BuCh and 0.0375 M-MCh as substrates, both enzymes appeared to be equally inhibited by eserine.

5. An estimate of the inhibition of true and pseudo-cholinesterase activity by DFP in the intact longitudinal muscle strip was obtained by measuring the residual cholinesterase activity in longitudinal muscle strips which had been treated with DFP before homogenizing the tissue. These results were similar to those obtained by treating muscle homogenates with DFP.

6. The results suggest that increase in tone in the longitudinal muscle coat of the rabbit intestine first appears when less than 20% of the true cholinesterase is inhibited and reaches a maximum with total inhibition of true cholinesterase activity.

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