J. Physiol. (1956) 131, 329–340

THE INHIBITION OF ACETYLCHOLINE SYNTHESIS IN GUINEA-PIG BRAIN SLICES BY ESERINE AND NEOSTIGMINE

By HEATHER SHELLEY*

From the Department of Pharmacology, University of Oxford

(*Received* 1 *August* 1955)

In recent years evidence has accumulated that certain compounds, best known as powerful anticholinesterases, can also influence the action of acetylcholine (ACh) in other ways. Curare-like, atropine-like and quinidine-like properties of eserine and of neostigmine and other anticholinesterases possessing a quaternary nitrogen group have been described by a number of workers (for references see Paton & Perry, 1953; Tedeschi, 1954), and neostigmine has been observed to have a stimulating effect on skeletal muscle (Riker & Wescoe, 1946). These observations suggest that certain anticholinesterases have an affinity, not only for receptors on the cholinesterase molecule, but also for ACh receptors at other sites.

The work to be described was an attempt to show that eserine and neostigmine can influence ACh metabolism in yet another way, namely, by inhibiting ACh synthesis. There are several indications in the literature that eserine may have such an effect. Mann, Tennenbaum & Quastel (1938) infer that concentrations of eserine higher than $2{\cdot}25 \times 10^{-3}$M (eserine sulphate 1/1200) inhibit ACh synthesis in brain tissue, but they attribute this to a decreased respiration rate in the presence of oxidation products of eserine. Perry (1953) obtained evidence of a slower rate of ACh synthesis in the perfused eserinized superior cervical ganglion of the cat as compared with that in the non-eserinized ganglion. Briscoe & Trendelenburg (unpublished experiments) found that the ACh content of isolated rabbit atria which had been exposed to the action of $2{\cdot}7 \times 10^{-3}$M eserine was less than that of atria treated with lower concentrations of eserine.

In the present work the rate of ACh synthesis in guinea-pig brain slices respiring in a glucose-saline medium containing $5{\cdot}4 \times 10^{-4}$M eserine (to inhibit cholinesterase activity) has been compared with that of slices respiring in a

* Present address: The Nuffield Institute for Medical Research, Oxford.

similar medium containing higher concentrations of eserine or, in addition to eserine $5{\cdot}4 \times 10^{-4}$ M, high concentrations of neostigmine or di-isopropyl phosphorofluoridate (DFP). It was found that high concentrations of eserine or neostigmine inhibit ACh synthesis under these conditions, and that the addition of choline in similar concentrations prevented this inhibition. DFP did not inhibit ACh synthesis.

METHODS

Female albino guinea-pigs of 200–350 g weight were used throughout. In the early experiments the rate of ACh synthesis in brain slices from a control pig killed by decapitation was compared with that in brain slices from an animal of the same weight which had been killed by injecting eserine. Both the control pig and the eserinized pig received 20 mg atropine sulphate by intraperitoneal injection 20 min before death, so raising the minimum lethal dose of eserine sulphate administered by intraperitoneal injection to approximately 1 mg/g. This dose was sufficient to cause death due to respiratory failure within about 2 min of injection. In later experiments the atropine was omitted, and both the control and the experimental animals were killed by decapitation, high concentrations of anticholinesterases being added to the brain slices *in vitro*.

The brain was dissected from each guinea-pig within 2 min of death, severing the brain stem in the region of the corpora quadrigemina. Each brain was bisected down the mid-line and each half brain, consisting of one cerebral hemisphere and one olfactory lobe, was weighed. One half-brain was then transferred to a mortar for immediate extraction of ACh and the other was placed on the stage of a McIlwain-Buddle chopper (McIlwain & Buddle, 1953). While the first half-brain was minced with a pair of scissors, the second was chopped transversely into slices 120 μ thick which were transferred to a Warburg flask containing 0·25 ml./100 mg brain of the following incubation medium:

Sodium chloride	0·76 g	D-Glucose	0·18 g
Potassium chloride	0·20 g	Eserine sulphate	0·02 g
Calcium chloride	0·02 g (0·1 ml. 20% solution)	Water	100 ml.
Sodium bicarbonate	0·21 g		

The medium was freshly prepared for each experiment and was saturated with 95% oxygen and 5% carbon dioxide before the addition of the eserine. 0·25 ml. of incubation medium was also added to each 100 mg minced brain in the mortar. The Warburg apparatus was filled with 95% oxygen and 5% carbon dioxide and placed in a bath at 37·5° C, any additional anticholinesterase solution being added at this moment from the side bulb. Simultaneously, an equivalent amount of anticholinesterase was added to the minced brain in the mortar and this was immediately ground up finely with sand.

In some experiments this suspension was acidified immediately by the addition of 2·0 ml. N/3 hydrochloric acid, boiled, cooled and centrifuged at 3000 r.p.m. for 5 min. The residue was resuspended in 2·0 ml. incubation medium and recentrifuged, and the combined supernatants were stored at −15° C to await assay of their ACh content. In other experiments an attempt was made to determine whether eserine influenced the release of ACh from the brain tissue by separating the supernatant and residue by centrifugation (10 min at 3000 r.p.m.) before adding the acid. The supernatant was then transferred to a flask containing 2·0 ml. N/3 hydrochloric acid and the residue was resuspended in 2·0 ml. incubation medium and recentrifuged (5 min at 3000 r.p.m.). The combined supernatants were boiled, cooled and stored at −15° C. The residue was resuspended in 3·0 ml. incubation medium and transferred to a second flask containing 2·0 ml. N/3 hydrochloric acid, washing out the centrifuge tube with an additional 2·0 ml. incubation medium. This suspension was then boiled, cooled and stored at −15° C.

After 2 hr incubation at 37·5° C, during which manometer readings were taken at 10 min intervals, the brain slices and incubation medium were transferred to a mortar and treated in the same

way as the corresponding non-incubated half-brain. The ACh content of the brain slices and incubation medium, less that of the non-incubated half-brain, gave a measure of the ACh synthesized during incubation. All ACh estimations were made on the eserinized frog rectus abdominis by the method of Feldberg (1945) and the extracts were thawed, centrifuged when necessary, neutralized with N/3 sodium hydroxide solution and diluted to the equivalent of 50 mg brain tissue/ml. just before estimating their ACh content. All estimates of ACh content are expressed in terms of ACh chloride. Sodium ATP was prepared from a purified sample of Boots' barium ATP. Coenzyme A was kindly supplied by Prof. D. E. Green as a concentrate containing 0·6% pantothenic acid which was dissolved in water and neutralized with sodium hydroxide before use.

RESULTS

The half-brains varied in weight from 1·15 to 1·40 g. The initial ACh content of the control brains varied from 1·0 to 4·8 μg/g; 60–80% of this ACh was associated with the tissue debris and was not released into the extraction medium until the suspension was acidified. The control rate of ACh synthesis in presence of eserine $5{\cdot}4 \times 10^{-4}$ M varied considerably, from 15·0 to 32·6 μg/g/2 hr at 37·5° C. In order to minimize the effect of this natural variation, a control brain was incubated in every experiment and the guinea-pigs used in each experiment belonged to the same group and were of the same weight. After incubation, the ACh content of the tissue debris was three to four times the initial value but only about 40% of the total. Thus the greater part of the ACh was in the supernatant.

Inhibition of acetylcholine synthesis by high concentrations of eserine. Table 1 summarizes the results of twelve experiments to investigate the effect of increasing the eserine concentration above the control level. This was done by injecting eserine *in vivo* (section A), by injecting eserine *in vivo* and also increasing the amount of eserine added to the incubation medium *in vitro* (section B), or by omitting the injection and adding eserine *in vitro* only (section C). Methods A and B had the disadvantage that the final concentration of eserine was unknown. However, assuming that the injected eserine distributed itself evenly throughout the body, it was calculated that, since 1 g of brain tissue was suspended in 2·5 ml. incubation medium, the injection of 300 mg eserine sulphate into a 300 g guinea-pig would raise the final eserine concentration from $5{\cdot}4 \times 10^{-4}$ M to about $1{\cdot}6 \times 10^{-3}$ M. Therefore, in section B, the highest eserine concentration would be about $2{\cdot}9 \times 10^{-2}$ M. In section C, where the final eserine concentration could be calculated accurately, the maximum concentration was $8{\cdot}64 \times 10^{-3}$ M. Higher concentrations of eserine could not be used as they interfered with the ACh assay on the frog rectus abdominis.

When eserine was injected *in vivo* (sections A and B), the initial ACh content of the experimental brain (column 2) was always greater than that of the control brain (column 1), whereas when the injection was omitted (section C), there was no consistent difference. The injected eserine had no effect

upon the initial ACh content of the tissue debris, the increase in ACh being found only in the supernatant obtained before acidifying the suspension. After 2 hr at 37·5° C., the final ACh content of the experimental half-brain plus incubation medium (column 4) was less than that of the control (column 3) in all but Expt 2, where the final ACh content of both brains was the same. Whereas the difference was slight when the eserine concentration was raised by injection only (section A), it was greater when still larger amounts of eserine were added *in vitro* (sections B and C). Since the ACh content of both tissue

TABLE 1. The effect of high concentrations of eserine on acetylcholine synthesis in guinea-pig brain slices. I.P. = by intraperitoneal injection; I.V. = by intravenous injection

	Initial ACh content of brain (μg/g)		ACh content of brain after incubation (μg/g)		ACh synthesis (μg/g/2 hr)		
Extra eserine	Control (eserine 5·4 × 10^{-4} M) (1)	Control + extra eserine (2)	Control (eserine 5·4 × 10^{-4} M) (3)	Control + extra eserine (4)	Control (eserine 5·4 × 10^{-4} M) (5)	Control + extra eserine (6)	Percentage change in ACh synthesis (7)
A. *In vivo* only							
(1) 300 mg I.P.	4·0	4·8	24·0	20·0	20·0	15·2	−24·0
(2) 300 mg I.V.	3·5	6·4	23·0	23·0	19·5	16·6	−15·0
(3) 1·0 g I.P.	4·1	6·0	30·0	29·0	25·9	23·0	−11·0
B. 300 mg I.P. *in vivo* + *in vitro*							
(4) $2·7 \times 10^{-3}$ M	2·8	6·5	24·0	18·0	21·2	11·5	−46·0
(5) $2·7 \times 10^{-3}$ M	4·8	7·0	30·0	20·0	25·2	13·0	−48·5
(6) $2·7 \times 10^{-3}$ M	3·5	6·5	23·5	18·0	20·0	11·5	−42·5
(7) $2·7 \times 10^{-2}$ M	3·0	4·5	24·0	14·0	21·0	10·0	−52·5
C. *In vitro* only							
(8) $2·7 \times 10^{-3}$ M	4·0	3·0	22·0	15·0	18·0	12·0	−33·5
(9) $5·4 \times 10^{-3}$ M	2·5	3·0	22·5	12·5	20·0	9·5	−52·5
(10) $5·4 \times 10^{-3}$ M	2·5	2·0	23·5	16·0	21·0	14·0	−33·5
(11) $5·4 \times 10^{-3}$ M	2·5	2·0	17·5	7·5	15·0	5·5	−63·5
(12) $8·1 \times 10^{-3}$ M	4·0	1·0	22·0	10·0	18·0	9·0	−50·0
D. *In vitro* only, eserine oxidized							
(13) $5·4 \times 10^{-3}$ M	3·0	3·0	22·5	21·0	19·5	18·0	−7·5

debris and supernatant was reduced, there was no suggestion that eserine affected the release of ACh from the tissue. The rates of ACh synthesis in the control and experimental brains are compared in columns 5 and 6, and column 7 gives the percentage inhibition of ACh synthesis in each experiment.

The results suggest that under these conditions high concentrations of eserine inhibit ACh synthesis. Although in section A the inhibition of synthesis did not exceed 24% and could be attributed to natural variation or to the high initial ACh content of the brain slices, in sections B and C where the eserine concentration was raised *in vitro*, the inhibition was never less than 33·5%, and in Expt. 11 reached 63·5% with eserine $5·94 \times 10^{-3}$ M. It was clear that eserine added *in vitro* was as effective as eserine injected *in vivo*. A thirteenth experiment (section D) provided evidence that eserine itself, not

its oxidation products, was responsible for the inhibition of synthesis. A solution of eserine sulphate $5{\cdot}4 \times 10^{-2}$M was made alkaline with sodium hydroxide, heated to boiling, shaken with air until it was a bright red colour, cooled, and neutralized with hydrochloric acid. This solution, when added to the brain slices to give a final concentration equivalent to $5{\cdot}4 \times 10^{-3}$M eserine, had no effect on the rate of ACh synthesis.

Experiments to investigate the mechanism by which eserine inhibits acetylcholine synthesis. It was found (Shelley, unpublished experiments) that high concentrations of eserine inhibit tissue respiration. The oxygen uptake of rabbit brain slices, respiring in a phosphate-saline medium containing glucose with potassium hydroxide as carbon dioxide absorbent, was reduced by 27% in presence of $2{\cdot}7 \times 10^{-3}$M eserine, by 62% with $2{\cdot}7 \times 10^{-2}$M eserine, and by 89% with $2{\cdot}7 \times 10^{-1}$M eserine. Since in the present work no provision was made for the absorption of carbon dioxide, the oxygen consumption was not directly determined. The manometer readings taken during the 2 hr incubation period merely recorded the negative pressure due to oxygen consumption exceeding the appearance of respiratory carbon dioxide in the gas phase. It was assumed that this negative pressure, divided by the weight of tissue present, was proportional to the rate of tissue respiration. This assumption was justified by the observation that after the first 20–30 min of incubation a straight-line relationship was obtained when the results were plotted against time. In the presence of the high concentrations of eserine, the apparent rate of oxygen uptake was 10–20% less than that with eserine $5{\cdot}4 \times 10^{-4}$M.

According to Quastel, Tennenbaum & Wheatley (1936), ACh synthesis is dependent on tissue respiration. Thus high concentrations of eserine might inhibit ACh synthesis indirectly by inhibiting tissue respiration, thereby reducing the supply of such products of respiration as adenosine triphosphate (ATP) or acetyl-coenzyme A, which are essential for ACh synthesis. Accordingly, the effects of ATP, sodium acetate, ATP + sodium acetate, sodium citrate and coenzyme A were investigated. They were added to the incubation medium in the concentrations indicated in Table 2. The added substance always depressed the rate of ACh synthesis below the control level. In all but Expt. 5, raising the eserine concentration depressed the rate of synthesis still further (columns 3 and 4). The added substances did not affect the apparent respiration rate of the brain slices. Thus, with the possible exception of sodium citrate in high concentrations, none of these substances appeared to be able to prevent the inhibition of ACh synthesis by high concentrations of eserine.

Very different results were obtained when choline was added to the incubation medium. The results in Table 3, column 2, show that the addition of choline $6{\cdot}0 \times 10^{-3}$M—$4{\cdot}0 \times 10^{-2}$M increased the rate of ACh synthesis above the control level in column 1. When the eserine concentration was raised (column 3), the rate of ACh synthesis in the presence of $6{\cdot}0 \times 10^{-3}$M choline

was still depressed below the control level, but in the presence of $1{\cdot}2 \times 10^{-2}$M choline there was a slight increase in ACh synthesis. With choline $2{\cdot}0 \times 10^{-2}$M and $4{\cdot}0 \times 10^{-2}$M, there was a large increase in synthesis similar to that produced by these concentrations of choline in control brains. Thus, as the choline concentration increased, the inhibition produced by the extra eserine

TABLE 2. The effect of various metabolites and of eserine on acetylcholine synthesis in guinea-pig brain slices

Added metabolite	ACh synthesis (μg/g brain/2 hr): Control (eserine $5{\cdot}4 \times 10^{-4}$M)	Control+ metabolite	Control + extra eserine+ metabolite	Percentage change in ACh synthesis due to extra eserine in presence of metabolite
A	(1)	(2)	(3)	(4)
(1) Sodium ATP, $4{\cdot}2 \times 10^{-3}$M	22·9	19·0	12·9	−32·0
(2) Sodium acetate, $2{\cdot}85 \times 10^{-2}$M	27·0	23·7	11·0	−53·5
(3) Sodium ATP, $4{\cdot}2 \times 10^{-3}$M + sodium acetate $2{\cdot}85 \times 10^{-2}$M	24·3	12·8	5·0	−32·0
(4) Sodium citrate, $3{\cdot}4 \times 10^{-3}$M	26·3	19·3	10·0	−48·0
(5) Sodium citrate, $1{\cdot}7 \times 10^{-2}$M	24·7	14·9	15·9	+6·5
B				
(6) Coenzyme A, 20 units/ml.	15·0	12·0	7·3	−39·0

A: extra eserine = 300 mg eserine sulphate *in vivo* by intraperitoneal injection + $2{\cdot}7 \times 10^{-3}$M eserine *in vitro*.

B: extra eserine = $5{\cdot}4 \times 10^{-3}$M eserine *in vitro*.

TABLE 3. The effect of choline and of eserine on acetylcholine synthesis in guinea-pig brain slices

Choline concentration	ACh synthesis (μg/g brain/2 hr): Control (eserine $5{\cdot}4 \times 10^{-4}$M)	Control+ choline	Control+ extra eserine+ choline	Percentage change in ACh synthesis due to extra eserine in presence of choline
A	(1)	(2)	(3)	(4)
(1) $6{\cdot}0 \times 10^{-3}$M	26·6	31·6	21·2	−33·0
(2) $1{\cdot}2 \times 10^{-2}$M	18·3	20·7	18·5	−10·5
(3) $2{\cdot}0 \times 10^{-2}$M	27·0	42·7	40·0	−6·5
(4) $4{\cdot}0 \times 10^{-2}$M	32·6	46·2	48·5	+5·0
B				
(5) $1{\cdot}2 \times 10^{-2}$M	15·0	24·0	17·0	−29·0
(6) $2{\cdot}0 \times 10^{-2}$M	20·0	27·0	25·0	−7·5
(7) $4{\cdot}0 \times 10^{-2}$M	21·0	32·0	33·0	+3·0

A: extra eserine = 300 mg eserine sulphate *in vivo* by intraperitoneal injection + $2{\cdot}7 \times 10^{-3}$M eserine *in vitro*.

B: extra eserine = $5{\cdot}4 \times 10^{-3}$M eserine *in vitro*.

decreased (column 4) until with choline $2{\cdot}0 \times 10^{-2}$M it was negligible and with choline $4{\cdot}0 \times 10^{-2}$M it was entirely absent. The competitive nature of this effect is illustrated in Fig. 1, where the rate of ACh synthesis in the presence of each of two different high eserine concentrations is expressed as a percentage of the control level and is plotted against the concentration of added choline.

As the choline concentration increases, the inhibition of ACh synthesis decreases and therefore the ordinate approaches 100%. The two curves are almost parallel and, at a given choline concentration, the depression of ACh synthesis on curve *B* is greater than that on curve *A* where the eserine concentration is not so high.

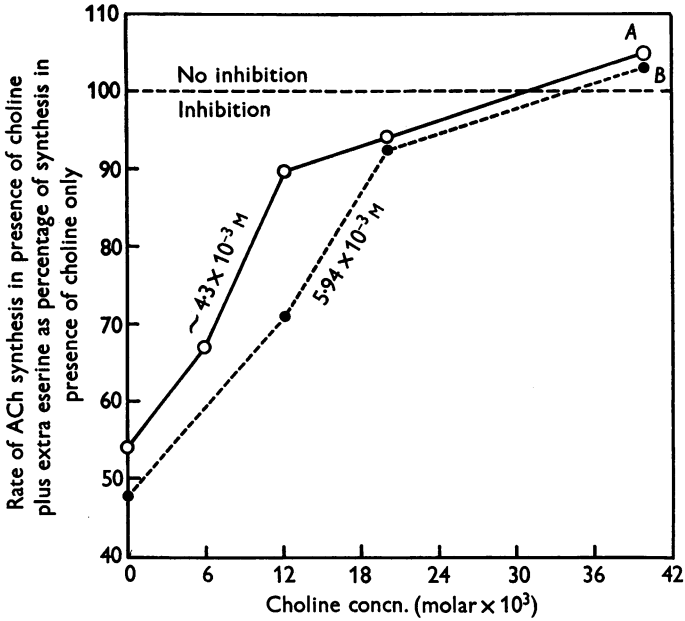

Fig. 1. The effect of choline on the inhibition by eserine of ACh synthesis in guinea-pig brain slices. Curve *A*: 300 mg eserine sulphate injected *in vivo*+eserine $5{\cdot}4\times10^{-4}$M+$2{\cdot}7\times10^{-3}$M added *in vitro*. Curve *B*: eserine $5{\cdot}4\times10^{-4}$M+$5{\cdot}4\times10^{-3}$M added *in vitro*. For explanation see text.

The effect of other anticholinesterases; DFP and neostigmine. When DFP 10^{-3}M was added to brain slices respiring in the presence of eserine $5{\cdot}4\times10^{-4}$M, a slight inhibition of ACh synthesis was observed (Table 4, Expt. 1), but DFP 10^{-2}M had no inhibitory effect (Expts. 2 and 3), although it reduced the apparent oxygen uptake to less than 50%. It was assumed that the effect observed in the presence of DFP 10^{-3}M was due to the natural variation in synthesis since in contrast to the results obtained with high concentrations of eserine and choline (Tables 1 and 3), the rates of ACh synthesis in the presence of DFP all lay within the normal range for control brains. The apparent increase in ACh synthesis in the presence of DFP 10^{-2}M was probably genuine, perhaps due to solubilizing properties of propylene glycol. The DFP was added as 0·3 ml. of a 10^{-1}M solution in propylene glycol, and the rate of ACh synthesis in a control brain to which had been added 0·3 ml. of the solvent was 28·0 μg/g/2 hr, an increase of 33% over the control value of 21·0 μg/g/2 hr.

In contrast, although neostigmine $2{\cdot}6 \times 10^{-4}$M and $1{\cdot}3 \times 10^{-3}$M had no inhibitory effect on the rate of ACh synthesis (Table 4, Expts. 4 and 5), neostigmine $2{\cdot}6 \times 10^{-3}$M and $6{\cdot}5 \times 10^{-3}$M depressed the rate of synthesis to just below the lower limit of the normal range (Expts. 6 and 7) and neostigmine $1{\cdot}3 \times 10^{-2}$M reduced the synthesis to less than half that of the control (Expt. 8). On the addition of choline 2×10^{-2}M (Table 5), the inhibitory effect of all these concentrations of neostigmine was abolished and, as in previous experiments with eserine, the rate of ACh synthesis was always greater than

TABLE 4. The effect of DFP and neostigmine on acetylcholine synthesis in guinea-pig brain slices

Concentration of added anticholinesterase	ACh synthesis (μg/g brain/2 hr): Control (eserine $5{\cdot}4 \times 10^{-4}$M)	ACh synthesis (μg/g brain/2 hr): Control + added anticholinesterase	Percentage change in ACh synthesis
	(1)	(2)	(3)
A. DFP			
(1) 10^{-3}M	20·0	17·3	−13·5
(2) 10^{-2}M	21·0	25·6	+22·0
(3) 10^{-2}M	16·5	23·5	+42·5
B. Neostigmine			
(4) $2{\cdot}6 \times 10^{-4}$M	18·0	22·5	+25·0
(5) $1{\cdot}3 \times 10^{-3}$M	16·5	15·5	−6·0
(6) $2{\cdot}6 \times 10^{-3}$M	16·5	14·25	−13·5
(7) $6{\cdot}5 \times 10^{-3}$M	19·5	14·0	−28·0
(8) $1{\cdot}3 \times 10^{-2}$M	19·5	7·5	−62·0

TABLE 5. The effect of choline and neostigmine on acetylcholine synthesis in guinea-pig brain slices

Concentration of neostigmine	ACh synthesis (μg/g brain/2 hr): Control (eserine $5{\cdot}4 \times 10^{-4}$M)	Control + choline 2×10^{-2}M	Control + neostigmine	Control + choline + neostigmine	Percentage change in ACh synthesis due to: Neostigmine	Neostigmine in presence of choline
	(1)	(2)	(3)	(4)	(5)	(6)
(1) $2{\cdot}6 \times 10^{-4}$M	18·0	28·5	22·5	32·0	+25·0	+12·5
(2) $2{\cdot}6 \times 10^{-3}$M	16·5	27·0	14·25	37·0	−13·5	+37·0
(3) $1{\cdot}3 \times 10^{-2}$M	19·5	38·0	7·5	36·0	−62·0	−5·5

the control rate in absence of added choline. The effect of neostigmine on ACh synthesis in the presence and absence of added choline is illustrated in Fig. 2. Curve 1 shows that, in the absence of added choline, there is a steady increase in the inhibition of ACh synthesis as the neostigmine concentration rises. Curve 2 shows that in the presence of 2×10^{-2}M choline, concentrations of neostigmine up to $1{\cdot}3 \times 10^{-2}$M do not inhibit ACh synthesis. These results suggest that, as had been demonstrated with eserine, there is competition between choline and neostigmine for some receptor which plays a vital part in ACh synthesis.

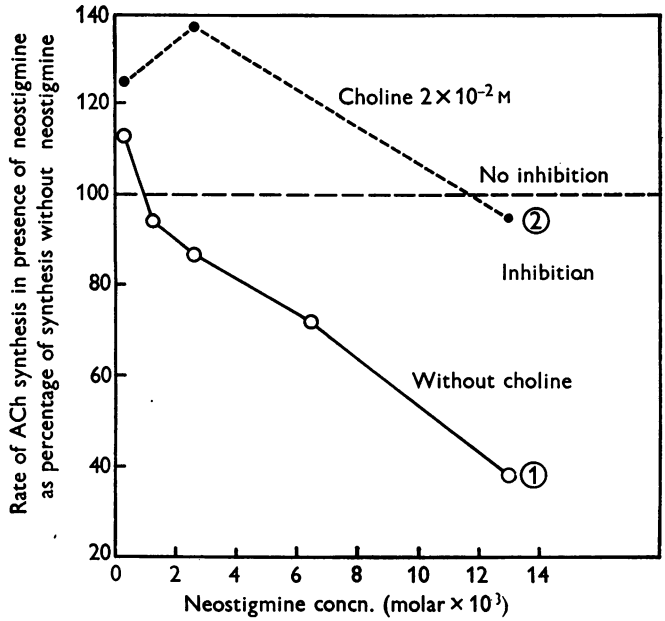

Fig. 2. The effect of neostigmine on acetylcholine (ACh) synthesis in guinea-pig brain slices in the presence and absence of added choline. Curve 1: in absence of added choline; curve 2: in presence of 2×10^{-2} M choline. For explanation see text.

DISCUSSION

The synthesis of ACh has been studied under many different conditions, in fresh or dried tissue or in cell-free extracts, in the presence or absence of added raw materials such as choline, acetate, citrate, ATP and coenzyme A. In the present work the conditions were made as physiological as possible by using fresh brain slices respiring in a buffered saline solution at pH 7·4 containing glucose as the only added substrate. This forced the tissue to use natural sources of choline and acetyl-coenzyme A for ACh synthesis. Similar methods were used by Quastel *et al.* (1936) with brain slices, and Mann *et al.* (1938) and Feldberg (1945) with minced brain tissue. They obtained values similar to those in the present work for both the initial ACh content of the brain tissue and the rate of ACh synthesis in the presence of eserine concentrations up to $5{\cdot}4 \times 10^{-4}$ M. Mann *et al.* and Feldberg also gave similar figures for the distribution of ACh between tissue debris and supernatant. Mann *et al.* concluded that whereas the supernatant contained mainly pharmacologically active, 'free' ACh (there was little increase in activity after acidifying the supernatant), the ACh associated with the tissue debris was in a pharmacologically inactive, 'bound' form which was converted to 'free' ACh by treatment

with acid. Feldberg suggested that ACh synthesis in brain tissue proceeds at a rate just sufficient to maintain this stock of 'bound' ACh at its normal resting level.

Although Mann *et al.* found that eserine concentrations up to $2{\cdot}25 \times 10^{-3}$ M (1/1200 eserine sulphate) had no effect on ACh synthesis in minced rat brain, in the present work eserine concentrations above $2{\cdot}7 \times 10^{-3}$ M had a significant inhibitory effect on ACh synthesis in guinea-pig brain slices. High concentrations of eserine did not affect the distribution of ACh between tissue debris and supernatant, and this confirms results obtained by Mann *et al.* with lower concentrations. Therefore the inhibition of ACh synthesis could not be attributed to retention of 'bound' ACh in the tissue. Mann *et al.* suggested that high concentrations of eserine might inhibit ACh synthesis because tissue respiration might be inhibited by oxidation products of eserine. However, a solution containing oxidation products of eserine equivalent to $5{\cdot}4 \times 10^{-3}$ M eserine had no inhibitory effect on ACh synthesis. Although tissue respiration was reduced by 10–20% when the eserine concentration was raised, the addition of acetate, citrate, ATP or coenzyme A did not prevent the inhibition of ACh synthesis. Thus the inhibition of ACh synthesis by eserine appeared to be independent of changes in tissue respiration, and this conclusion was supported by observations with DFP 10^{-2} M which inhibited tissue respiration by over 60% but did not affect the synthesis of ACh. Since DFP, like eserine, is a powerful anticholinesterase, this experiment also established that the power to inhibit ACh synthesis is not merely a consequence of cholinesterase inhibition.

Quastel *et al.* and Mann *et al.* observed that although the addition of acetate to respiring rat brain tissue had no effect on the rate of ACh synthesis, the addition of small amounts of choline increased the synthesis, particularly if glucose was also added. In the present work the addition of 10 to 100 times this amount of choline greatly increased the rate of ACh synthesis in guinea-pig brain slices and, if present in sufficiently high concentration, abolished the inhibition of synthesis by high concentrations of eserine. This suggests that the choline concentration is a rate-limiting factor for the synthesis of ACh, and that eserine inhibits ACh synthesis by competitively replacing choline in the acetylating system. The percentage inhibition of ACh synthesis by a given concentration of eserine would therefore depend on the amount of choline present. The eserine concentration necessary to inhibit synthesis in these experiments was very high, but in tissues where the choline concentration is less than that in brain it is possible that much lower eserine concentrations would inhibit ACh synthesis. It must also be emphasized that the inhibition observed here was the consequence of raising the eserine concentration above the already high level of $5{\cdot}4 \times 10^{-4}$ M. The effect of raising the eserine concentration from zero level was not investigated.

Perry (1953) observed that if the preganglionic fibres of the perfused

superior cervical ganglion of the cat were stimulated electrically when eserine $2{\cdot}7 \times 10^{-5}$M (1/100,000 eserine sulphate) was present in the perfusion fluid, the ACh output in a second period of stimulation was much less than in the first period of stimulation. In contrast, if the first period of stimulation had taken place in absence of eserine, there was no indication of decreased ACh output in a second period of stimulation in presence of eserine. Perry explained these results by suggesting that the ganglion tissue normally maintains its stock of ACh by re-acetylating choline from ACh, which on release has been hydrolysed immediately by cholinesterase. When eserine was present, this hydrolysis was prevented and the tissue was unable to replace the ACh released on stimulation. Perry also showed that choline was released into the perfusion fluid throughout the experiment. In the light of the present work, an alternative explanation is possible. The choline content of the ganglion might become so depleted that even this relatively low concentration of eserine would be able to inhibit ACh synthesis directly by the mechanism demonstrated in the present experiments. It would be interesting to see whether DFP, which did not inhibit ACh synthesis in the guinea-pig brain, would decrease the output of ACh from the superior cervical ganglion of the cat. Barnes & Duff (1954) did not observe any decrease in the ACh content of the blood of animals which had been injected repeatedly with paraoxon (E 600, diethyl-*p*-nitrophenyl phosphate), another phosphate anticholinesterase.

Although DFP was ineffective, neostigmine in concentrations above $2{\cdot}6 \times 10^{-3}$M resembled eserine in inhibiting ACh synthesis in guinea-pig brain slices. As with eserine, this inhibition could be prevented by adding large amounts of choline. This parallels the observation by Tedeschi (1954) that the atropine-like properties of anticholinesterases are confined to compounds containing nitrogen groups, and are not shared by phosphate anticholinesterases. Neostigmine resembles choline in possessing a quaternary methylated nitrogen group, eserine possesses a tertiary methylated nitrogen group but DFP does not possess any nitrogen group. Korey, de Braganza & Nachmansohn (1951) and Berman, Wilson & Nachmansohn (1953) have shown that choline acetylase, the enzyme responsible for the acetylation of choline by acetyl-coenzyme A, will also acetylate dimethyl- and monomethyl-aminoethanol, so establishing that the enzyme has an affinity not only for compounds containing a quaternary methylated nitrogen group but also for those with tertiary methylated groups. It is therefore concluded that the inhibition of ACh synthesis by high concentrations of eserine and neostigmine is associated with the presence of the tertiary and quaternary methylated nitrogen groups which enable the anticholinesterase to replace choline at the active centre of choline acetylase.

SUMMARY

1. The rate of acetylcholine synthesis in guinea-pig brain slices respiring in a glucose-saline medium at 37·5° C in presence of $5{\cdot}4 \times 10^{-4}$M eserine has been compared with that in presence of high concentrations of added eserine, DFP or neostigmine.

2. Eserine concentrations above $2{\cdot}7 \times 10^{-3}$M and neostigmine concentrations above $2{\cdot}6 \times 10^{-3}$M had a significant inhibitory effect on the rate of acetylcholine synthesis. DFP in concentrations up to 10^{-2}M did not inhibit acetylcholine synthesis.

3. The addition of ATP, acetate, ATP + acetate, citrate or coenzyme A did not prevent the inhibition of acetylcholine synthesis by high concentrations of eserine though this effect was sometimes obscured by the added substances themselves inhibiting acetylcholine synthesis.

4. The addition of choline to the respiring brain slices increased the rate of acetylcholine synthesis and, if given in high enough concentrations, prevented the inhibition of synthesis by high concentrations of eserine and neostigmine.

5. It is concluded that eserine and neostigmine compete with choline for a receptor at the active centre of choline acetylase and that this property is associated with the presence of a quaternary or tertiary nitrogen group.

I should like to thank Dr Edith Bülbring for suggesting that this work should be done, for her constant advice and encouragement, and Dr H. Blaschko for his helpful criticism. I should also like to acknowledge the technical assistance of Miss Roneen Hobbs and Miss Barbara Phillips.

REFERENCES

BARNES, J. M. & DUFF, J. I. (1954). Acetylcholine production in animals poisoned by diethyl-*p*-nitrophenyl phosphate (Paraoxon). *Brit. J. Pharmacol.* **9**, 153–158.

BERMAN, R., WILSON, I. B. & NACHMANSOHN, D. (1953). Choline acetylase specificity in relation to biological function. *Biochim. biophys. acta*, **12**, 315–324.

FELDBERG, W. (1945). Synthesis of acetylcholine by tissue of the central nervous system. *J. Physiol.* **103**, 367–402.

KOREY, S. R., DE BRAGANZA, B. & NACHMANSOHN, D. (1951). Choline acetylase. V. Esterifications and transacetylations. *J. biol. Chem.* **189**, 705–715.

MCILWAIN, H. & BUDDLE, H. L. (1953). Techniques in tissue metabolism. 1. A mechanical chopper. *Biochem. J.* **53**, 412–420.

MANN, P. J. G., TENNENBAUM, M. & QUASTEL, J. H. (1938). On the mechanism of acetylcholine formation in brain *in vitro*. *Biochem. J.* **32**, 243–261.

PATON, W. D. M. & PERRY, W. L. M. (1953). The relationship between depolarization and block in the cat's superior cervical ganglion. *J. Physiol.* **119**, 43–57.

PERRY, W. L. M. (1953). Acetylcholine release in the cat's superior cervical ganglion. *J. Physiol.* **119**, 439–454.

QUASTEL, J. H., TENNENBAUM, M. & WHEATLEY, A. H. M. (1936). Choline ester formation in, and choline esterase activities of, tissues *in vitro*. *Biochem. J.* **30**, 1668–1681.

RIKER, W. F., Jr. & WESCOE, W. C. (1946). The direct action of prostigmine on skeletal muscle; its relationship to the choline esters. *J. Pharmacol.* **88**, 58–66.

TEDESCHI, R. E. (1954). Atropine-like activity of some anticholinesterases on the rabbit atria. *Brit. J. Pharmacol.* **9**, 367–369.