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THE ACTION OF TRIETHYLCHOLINE ON THE BIOLOGICAL SYNTHESIS OF ACETYLCHOLINE

BY

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Triethylcholine causes a slowly developing failure of neuromuscular transmission in rapidly stimulated nerve-muscle preparations. At the time of maximal depression of the muscle twitches, the response to a close-arterial injection of acetylcholine is normal, suggesting that the site of the transmission failure is prejunctional (Bowman & Rand, 1961; Bowman, Hemsworth & Rand, 1962). In the isolated phrenic nerve-diaphragm preparation of the rat, concentrations of triethylcholine which interrupt neuromuscular transmission also cause a decrease in acetylcholine output from the stimulated nerve. The addition of choline restores both the contractions and the acetylcholine output towards normal (Bowman & Hemsworth, 1965). The action of triethylcholine therefore appears to resemble that of the hemicholiniums which are believed to interrupt cholinergic transmission by interfering with the synthesis of acetylcholine at the nerve endings (Schueler, 1960).

The experiments described in this paper were designed to provide direct evidence of any action of triethylcholine on acetylcholine synthesis in nervous tissue. Some of the results have been briefly reported elsewhere (Bull & Hemsworth, 1963).

METHODS

Enzyme. Particulate fractions of rabbit brain homogenates and fresh frozen sections of rabbit caudate nucleus were used as the source of choline acetyltransferase. The preparation of the subcellular fractions of rabbit brain was carried out as described by Hebb (1963), but using 0.44 M-sucrose (Løvtrup & Zelander, 1962). The preparations were stored at − 18° C until required. Before incubation the subcellular preparations were thawed, used undiluted or, before treatment with ether, diluted with sodium chloride solution to give a final salt concentration of 1%. An aliquot of diluted enzyme was stored at 4° C for 20 min, the remainder was activated by treating it with 0.25 ml. of ether per ml. of diluted enzyme and stored at 4° C, with occasional shaking (Hebb & Smallman, 1956) for the same period. The ether was removed before incubation by evaporation with oxygen-free nitrogen.

Fresh frozen sections of rabbit caudate nucleus were cut using a Pearse refrigerated microtome, put on to small pieces of cellophane and placed in small test-tubes, as described by Bull, Hebb & Ratković (1963); substrate solution was added and the mixture incubated for 1 hr at 39° C.

Acetylation system. A coupled enzyme system similar to that described by Hebb, Krnjević & Silver (1964) using phosphate acetyltransferase, co-enzyme A and acetyl phosphate as a source of acetyl co-enzyme A was used throughout. The incubation mixture contained (μmoles/ml.): potassium chloride, 218; acetyl phosphate, 12.5; magnesium chloride, 6.4; L-cysteine, 31.3; physostigmine sulphate,

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0.18; and sodium phosphate buffer (*p*H 6.9), 17.4; as well as phosphate acetyltransferase, 0.34 mg, and co-enzyme A, 30 to 40 units. Choline and triethylcholine in various amounts, but in a constant volume of 0.1 ml., were added to 0.4 ml. of this mixture which had been previously incubated for 10 min at 39° C. After adding an amount of enzyme equivalent to 20 mg of fresh tissue, the mixture was incubated for 1 hr at 39° C. The reaction was stopped by adding 0.25 ml. of N/3-hydrochloric acid and 3 drops of Universal (B.D.H.) indicator; the incubate was transferred to 50-ml. conical flasks with several additions of frog-Ringer solution and was boiled briefly; after cooling, 1.0 ml. of 0.5% disodium hydrogen phosphate was added and the volume of each sample made up to 20 ml. by adding more frog-Ringer solution. Controls for the assay procedure were obtained by incubating heat-inactivated enzyme (Feldberg, 1950).

Assay of acetylcholine. The acetylcholine synthesized during the incubation was assayed on the frog rectus abdominis muscle using acetylcholine chloride as standard. Except in a very high concentration (100 μg/ml.) when it exerted a weak curare-like action, triethylcholine was without effect on the frog rectus muscle and did not change the response of acetylcholine. High concentrations of choline (30 μg/ml.) slightly increased the sensitivity of the frog rectus to acetylcholine. However, errors due to these effects were controlled by adding to the acetylcholine standard a volume of control (heat-inactivated enzyme solution) containing amounts of choline and triethylcholine equivalent to the amount present in the test solution. The accuracy of the assays for successive comparisons were of the order $\pm 5\%$.

An additional control which showed that the substance synthesized was acetylcholine was that the activity of the test solution was destroyed by the addition of alkali. Sufficient N/3-sodium hydroxide was added to bring the solution to approximately *p*H 10; the sample was then allowed to stand for 15 min at room temperature, neutralized and then retested (Feldberg, 1950).

RESULTS

The choline acetyltransferase present in the large granule or " mitochondrial " fraction of rabbit brain homogenate was tested in the initial experiments, since, as Hebb & Smallman (1956) showed, 50 to 70% of rabbit brain enzyme is present in a " bound " form in this fraction.

Table 1 shows the inhibiting action of triethylcholine on the synthesis of acetylcholine by this fraction. When the amount of choline was optimal (0.8 mg) to give a maximal yield of acetylcholine, an equimolar concentration of triethylcholine (1.04 mg) inhibited the

TABLE 1

INHIBITING ACTION OF TRIETHYLCHOLINE ON THE SYNTHESIS OF ACETYLCHOLINE BY THE " MITOCHONDRIAL" FRACTION OF RABBIT BRAIN

Choline chloride added (mg)	Acetylcholine synthesized (μg/hr)	Inhibition (%) produced by triethylcholine		
		1·04 mg	2·08 mg	10·4 mg
0·2	2·2	21	32	56
0·4	3·0	15	29	53
0·8	3·9	11	20	40
1·6	3·8	7	12	21
3·2	3·5	2	5	3

synthesis of acetylcholine by 11%. Doubling the triethylcholine concentration almost doubled the inhibition and at the largest concentration of triethylcholine the inhibition was 40%. When the amount of choline added to the system was below optimal (0.4 mg) the inhibiting action of triethylcholine was more pronounced and, with the lowest amount of choline (0.2 mg), an even greater inhibition of acetylcholine synthesis was obtained, a 56% inhibition being produced when the triethylcholine : choline molar ratio was 40 : 1. On the other hand, when the amount of choline added to the system was increased, triethyl-

TABLE 2

INHIBITING ACTION OF TRIETHYLCHOLINE ON THE SYNTHESIS OF ACETYLCHOLINE BY ETHER-TREATED AND UNTREATED " MITOCHONDRIAL" FRACTION OF RABBIT BRAIN

Ratio of activities, ether-treated : untreated=8·5 : 2·7, or approximately 3 : 1

Preparation	Choline chloride added (mg)	Acetylcholine synthesized (μg/hr)	Inhibition (%) produced by triethylcholine			
			1·04 mg	2·08 mg	4·16 mg	10·4 mg
Ether-treated	0·4	8·5	6	10	10	15
Untreated	0·4	2·7	19	26	46	56

TABLE 3

INHIBITING ACTION OF TRIETHYLCHOLINE ON THE SYNTHESIS OF ACETYLCHOLINE BY THE SUPERNATANT FRACTION OF RABBIT BRAIN

Preparation	Choline chloride added (mg)	Acetylcholine synthesized (μg/hr)	Inhibition (%) produced by triethylcholine			
			1·04 mg	2·08 mg	4·16 mg	10·4 mg
Supernatant enzyme	0·4	8·8	5	8	9	14
Diluted supernatant enzyme	0·4	2·2	8	14	15	16

TABLE 4

ACTION OF TRIETHYLCHOLINE ON SYNTHESIS OF ACETYLCHOLINE BY FRESH FROZEN SECTIONS OF RABBIT BRAIN CAUDATE NUCLEUS

The amount of triethylcholine used was 10·4 mg. Acetylcholine values are in mg/hr/g of tissue

Choline chloride added (mg)	Acetylcholine synthesized		
	No triethylcholine (mg/hr/g)	With triethylcholine (mg/hr/g)	Change with triethylcholine (%)
0·4	5·17	2·67	−48
1·6	7·84	5·56	−28
3·2	9·41	10·00	+ 6

choline had less effect in inhibiting acetylcholine synthesis, and at a choline level four-times the optimal (3.2 mg), triethylcholine had no significant inhibiting effect on acetylcholine synthesis in amounts up to 10.4 mg.

The largest inhibition of acetylcholine synthesis by triethylcholine was 72% when the triethylcholine : choline molar ratio was 160 : 1.

Treatment with ether is known to activate choline acetyltransferase in brain homogenates, possibly by breaking down the lipid membranes enclosing the enzyme (Hebb & Smallman, 1956). The effect of triethylcholine on acetylcholine synthesis by ether-treated and untreated mitochondrial fractions was therefore tested. The results are shown in Table 2.

In agreement with the previous results (see Table 1), the synthesis of acetylcholine by the untreated " mitochondrial " fraction was inhibited by triethylcholine by 19 to 56%, depending on the amount of triethylcholine added. When the ether-treated fraction was used the inhibition of acetylcholine synthesis by triethylcholine was much smaller (6 to 15%).

In order to determine whether the inhibiting action of triethylcholine on the synthesis of acetylcholine by the untreated mitochondrial fraction could be related to a lower level of enzyme activity, two concentrations of the supernatant enzyme, which yielded approximately the same amount of acetylcholine as the untreated and the ether-treated mitochon-

drial fractions, were incubated with triethylcholine (Table 3). Under these conditions the percentage inhibition produced by triethylcholine on the synthesis of acetylcholine was similar using the ether-treated " mitochondrial " fraction and the two concentrations of supernatant enzyme (see Tables 2 and 3).

Triethylcholine also inhibited the synthesis of acetylcholine by fresh frozen sections of rabbit caudate nucleus. Table 4 shows that at low choline concentrations triethylcholine inhibited acetylcholine synthesis by 48%. When the choline concentration was increased fourfold there was less inhibition, 28%, but at the highest choline level there was a slight potentiation in acetylcholine synthesis.

DISCUSSION

These experiments have been designed to provide a situation where triethylcholine was present in an increasingly favourable position to compete with choline for interaction with the enzyme. The results show that triethylcholine inhibits the synthesis of acetylcholine by the large granule or " mitochondrial " fraction, which is the most active of the fractions separable by centrifugation of rabbit brain homogenate, and by fresh frozen sections of rabbit caudate nucleus. This inhibitory effect can be overcome by increasing the choline concentration.

Our results *in vitro* appear to bear a relation to the effects of triethylcholine *in vivo*. Bligh (1952) found the mean free plasma choline content varied markedly with species, cat plasma (range 0.5 to 0.8 μg/ml.) having a lower choline content than rabbit plasma (range 1.2 to 5.2 μg/ml.), and Bowman & Rand (1961) found that it was more difficult to produce a block in neuromuscular transmission with triethylcholine in the rabbit than in the cat. Previous work (Bowman & Rand, 1961; Bowman *et al.*, 1962) showed that 30 mg/kg of triethylcholine was sufficient to produce an 80% block in neuromuscular transmission in a 3 to 4 kg cat. Assuming the extracellular fluid to be 15% of the body weight, this amount of triethylcholine (30 mg/kg), if distributed evenly, would produce a triethylcholine : choline molar ratio of the order of 200 : 1, a ratio which produced more than 70% inhibition of acetylcholine synthesis *in vitro*. However, the triethylcholine : choline ratio produced *in vivo* at the site of action of triethylcholine is not known and may differ from that in the plasma.

Triethylcholine was shown to have a much greater inhibitory action on the untreated " mitochondrial " fraction, as opposed to the ether-treated " mitochondrial " and supernatant fractions (Tables 2 and 3). With the supernatant fraction, where the enzyme is fully activated without treatment with ether, using the same triethylcholine : enzyme concentration as was used with the " mitochondrial " fraction, triethylcholine had only a small inhibitory action on acetylcholine synthesis (compare Tables 2 and 3). Thus it seems that triethylcholine has a pronounced effect on the synthesis of acetylcholine *in vitro* only if the tissue binding of the choline acetyltransferase is not disturbed. These results, supported by the finding that increases in choline concentration will antagonize the inhibitory action of triethylcholine, suggest that triethylcholine is acting by competition with choline for access to the enzyme through some membrane structure. In this respect triethylcholine appears to be acting like hemicholinium (Gardiner, 1961; MacIntosh, Birks & Sastry, 1956).

The finding that triethylcholine has a small inhibitory action on acetylcholine synthesis by the fully activated supernatant enzyme fraction and by the ether-treated " mitochondrial " fraction shows that the compound has a slight direct inhibitory action on choline acetyltransferase. Hemsworth & Morris (1964) also showed that, with high concentrations, triethylcholine has a slight inhibitory action on fully activated choline acetyltransferase prepared from acetone-dried powders of rabbit brain. However, it is unlikely that this direct action is large enough to account for more than a small part of the neuromuscular blocking action. *In vivo* triethylcholine might be expected to prevent choline transport across the presynaptic nerve terminal membrane in addition to preventing access of choline to the subcellular particle containing choline acetyltransferase; so it may be that the whole of the effect of triethylcholine on neuromuscular transmission could be due to interference with choline transport through membrane structures.

The experiments with frozen sections of rabbit caudate nucleus also demonstrate the inhibition of acetylcholine synthesis by triethylcholine in the presence of low choline concentrations, when the tissue binding of the choline acetyltransferase is not disturbed. The potentiation of acetylcholine synthesis by triethylcholine observed at a high choline level is similar to, although less pronounced than, the effect of hemicholinium under similar conditions (Gardiner, 1961). Gardiner found hemicholinium to potentiate by up to 37% the synthesis of acetylcholine by the " mitochondrial " fraction and by the ether-treated cell free homogenate of guinea-pig brain, but in our experiments the potentiation of acetylcholine synthesis by triethylcholine occurred only with the crude enzyme preparation from fresh frozen sections of rabbit caudate nucleus.

The results reported here provide support for the hypothesis, advanced earlier by Bowman & Rand (1961), that triethylcholine affects neuromuscular transmission by interfering with the synthesis of acetylcholine at the nerve endings. These results suggest the effect of triethylcholine *in vivo* is achieved not by direct action on choline acetyltransferase but by depriving it of one of its required substrates.

SUMMARY

1. A study has been made of the effect of triethylcholine on the *in vitro* synthesis of acetylcholine by the large granule or " mitochondrial " fraction and the supernatant fraction of rabbit brain homogenates and by fresh frozen sections of rabbit caudate nucleus.

2. Triethylcholine inhibited the biological synthesis of acetylcholine by the " mitochondrial " fraction of brain and by fresh frozen sections of rabbit caudate nucleus.

3. The inhibitory action of triethylcholine was antagonized by increasing the choline concentration. Triethylcholine also had a small inhibitory action on the synthesis of acetylcholine by the supernatant fraction, which was about equivalent to its effect on the ether-treated " mitochondrial " fraction.

4. It is thought that the inhibitory effect of triethylcholine on the synthesis of acetylcholine in the untreated " mitochondrial " fraction depends for the most part upon its effect on choline transport and that a similar effect in the intact animal would interfere with acetylcholine synthesis sufficiently to affect neuromuscular transmission.

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