Control of Synthesis and Release of Radioactive Acetylcholine in Brain Slices from the Rat

EFFECTS OF NEUROTROPIC DRUGS

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1. Studies of the synthesis and release of radioactive acetylcholine in rat brain-cortex slices incubated in Locke-bicarbonate-[U- 14 C]glucose media, containing paraoxon as cholinesterase inhibitor, revealed the following phenomena: (a) dependence of K^+ - or protoveratrine-stimulated acetylcholine synthesis and release on the presence of Na^+ and Ca^{2+} in the incubation medium, (b) enhanced release of radioactive acetylcholine by substances that promote depolarization at the nerve cell membrane (e.g. high K^+ , ouabain, protoveratrine, sodium L-glutamate, high concentration of acetylcholine), (c) failure of acetylcholine synthesis to keep pace with acetylcholine release under certain conditions (e.g. the presence of ouabain or lack of Na^+). 2. Stimulation by K^+ of radioactive acetylcholine synthesis was directly proportional to the external concentration of Na^+ , but some synthesis and release of radioactive acetylcholine occurred in the absence of Na^+ as well as in the absence of Ca^{2+} . 3. The Na^+ dependence of K^+ -stimulated acetylcholine synthesis was partly due to suppression of choline transport, as addition of small concentrations of choline partly neutralized the effect of Na^+ lack, and partly due to the suppression of the activity of the Na^+ pump. 4. Protoveratrine caused a greatly increased release of radioactive acetylcholine without stimulating total radioactive acetylcholine synthesis. Protoveratrine was ineffective in the absence of Ca^{2+} from the incubation medium. It completely blocked K^+ stimulation of acetylcholine synthesis and release. 5. Tetrodotoxin abolished the effects of protoveratrine on acetylcholine release. It had blocking effects (partial or complete) on the action of high K^+ , sodium L-glutamate and lack of Ca^{2+} on acetylcholine synthesis and release. 6. Unlabelled exogenous acetylcholine did not diminish the content of labelled tissue acetylcholine, derived from labelled glucose, suggesting that no exchange with vesicular acetylcholine took place. In the presence of 4mM-KCl it caused some increase in the release of labelled acetylcholine. 7. The barbiturates (Amytal, pentothal), whilst having no significant effects on labelled acetylcholine synthesis in unstimulated brain except at high concentration (1mM), diminished or abolished (at 0.25 or 0.5mM) the enhanced release of acetylcholine, due to high K^+ or lack of Ca^{2+} . The fall in tissue content of acetylcholine, due to lack of Ca^{2+} , was diminished or abolished by pentothal (0.25 or 0.5mM) or Amytal (0.25mM).

Although considerable early work on the factors controlling synthesis and release of acetylcholine in the nervous system was carried out with isolated brain preparations (Quastel *et al.*, 1936; Mann *et al.*, 1938, 1939a,b; McLennan & Elliott, 1950; Braganca & Quastel, 1952; Rowsell, 1954), much of the recent knowledge concerning acetylcholine release has been derived from studies of the neuromuscular junction (Fatt & Katz, 1952; Katz, 1962, 1971; Katz & Miledi, 1965, 1967a), or of isolated ganglia (Perry, 1953; Birks & MacIntosh, 1961; MacIntosh, 1963; Collier & MacIntosh, 1969) or of guinea-pig ileum (Paton *et al.*, 1971). Very useful reviews of the subject have appeared, e.g. Hebb & Krnjevic (1962), Hebb (1963), Hebb & Morris (1969), Whittaker (1969) and Potter (1970). The objects of the present work have been to observe whether the conclusions derived from

these studies also apply to results obtained with rat brain-cortex slices, especially in the light of new knowledge on subcellular distribution of acetylcholine (e.g. Chakrin & Whittaker, 1969), and to extend our knowledge of the nature of the factors controlling synthesis and release of acetylcholine in the brain. Methods similar to those of Browning & Schulman (1968) have been used.

Experimental

Materials

Animals. Adult rats (either sex) of the Wistar strain, weighing 200-250g, were used. They were obtained from the Vivarium, Department of Zoology, University of British Columbia, Vancouver, B.C., Canada.

Chemicals. All common laboratory chemicals were

of reagent grade and were used without further purification. Tetrodotoxin was obtained from Calbiochem, Los Angeles, Calif., U.S.A., protoveratrine from K and K Laboratories, Plainview, N.Y., U.S.A., sodium Amytal from E. Lilly and Co., Montreal, P.Q., Canada, and lidocaine from the Department of Pharmacology, University of British Columbia. [$U\text{-}^{14}\text{C}$]Glucose (2–3 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and EGTA [ethanedioxybis(ethylamine)tetraacetic acid] from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Paraoxon was a gift from Dr. A. Vardanis of the Agricultural Research Institute, London, Ont., Canada.

Methods

Preparation of brain-cortex slices. These were prepared as described by Shankar & Quastel (1972).

Incubation procedure. Slices were weighed at once on a torsion balance to the nearest milligram and suspended in a Locke–bicarbonate medium of the following composition: NaCl, 140 mM; KCl, 4 mM; CaCl_2 , 2 mM; NaHCO_3 , 25 mM; [$U\text{-}^{14}\text{C}$]glucose, 5 mM (specific radioactivity 53900 c.p.m./ μmol); paraoxon, 40 μM . The slices and medium were contained in vessels of the conventional Warburg manometric apparatus.

In media containing a high concentration of K^+ , the concentration of KCl was increased without decrease of NaCl. When Na^+ was replaced by Li^+ (in media containing a high concentration of K^+), NaCl was replaced by LiCl, NaHCO_3 by KHCO_3 , and KCl was added to the desired K^+ concentration.

The final volume was made up to 3 ml. After the addition of slices the medium was gassed for 15 min with $\text{O}_2 + \text{CO}_2$ (95:5) at 37°C and the incubation was carried out for another hour (total time 75 min).

Determination of acetylcholine. Labelled acetylcholine derived from labelled glucose was determined, after precipitation with ammonium reineckate, according to methods described by McCaman & Hunt (1965), Hemsworth & Morris (1964) and Morris & Grewaal (1971).

(a) Tissue. The slices from the manometric vessels were removed after incubation and given a quick wash in the Locke–bicarbonate medium containing paraoxon. The tissue was homogenized in 3 ml of water containing 40 μM -paraoxon and 64 μmol of unlabelled acetylcholine as carrier at pH 4.0. The protein was precipitated by addition of perchloric acid to 0.1 M. The suspension was cooled in ice and centrifuged for 5 min. The supernatant was transferred to another tube. The precipitate was washed twice with ice-cold 0.1 M-perchloric acid (1.5 ml portions), centrifuged, and the supernatants were combined. Excess of cold saturated ammonium reineckate at pH 2.0 (4.5 ml) was added to precipitate acetylcholine

and the precipitation was allowed to proceed overnight at 4°C. After centrifugation the precipitate was washed twice with cold 0.2 M-HCl (6 ml portions) and then with cold propan-1-ol (1.5 ml). The precipitate was dried by two successive washings with anhydrous diethyl ether.

The dried material was dissolved in ice-cold aq. 95% acetone (2.0 ml). The reineckate ions were precipitated with a slight excess of 0.1 M- AgNO_3 (in aq. 95% acetone), the suspension was centrifuged and the supernatant transferred to a graduated tube. The precipitate was washed twice with ice-cold aq. 95% acetone (1 ml portions) and the supernatants were combined. Excess of Ag^+ was precipitated with 0.1 M-HCl in aq. 95% acetone. The acetone was evaporated at 40°C with a stream of air. The volume was adjusted to 1 ml with water and the solution was centrifuged for 5 min to remove any AgCl ; 0.5 ml of this solution was used for determination of radioactivity.

(b) Medium. For determination of labelled acetylcholine, the medium was transferred from the Warburg manometric vessel to a centrifuge tube. The vessel was given one wash with 1.5 ml of water at pH 4.0. Unlabelled acetylcholine was added as carrier and the procedure given above was followed exactly except that the perchloric acid step was omitted.

Measurement of radioactivity. A sample (0.5 ml) of the solution was added to 10 ml of scintillant, consisting of equal volumes of dioxan, toluene and aq. 95% (v/v) ethanol containing 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.005%), 2,5-diphenyloxazole (0.5%) and naphthalene (8.0%).

Counting efficiency for ^{14}C was 78–80% and the recovery of acetylcholine by the above procedure was found to be over 90%.

Determination of acetylcholine. The amount of [^{14}C]acetylcholine formed was expressed as nmol of acetylcholine/g initial wet wt. of tissue. It was calculated by dividing the radioactivity of the tissue, or medium, acetylcholine (calculated for 1 g initial wet wt. of tissue), by one-third of the specific radioactivity of the radioactive glucose present in the incubation medium.

Control experiments. Control experiments were systematically carried out in which an incubation, in the absence of tissue, was allowed to proceed for the length of the experimental period. The control (or blank) values, obtained after following the procedure exactly as in the presence of tissue, were subtracted from the values found in the presence of the tissue. Control experiments were also carried out to observe the effects of the quick rinse in Locke–bicarbonate medium containing paraoxon on the acetylcholine content of the incubated brain tissue. The results showed that there was no loss of acetylcholine, within experimental error, from the tissue by this procedure.

Numbers of experiments. All experiments whose

results are recorded below were carried out at least four times. The means of these results $\pm$ S.D. from the mean are given.

Results

Determination of acetylcholine

Browning & Schulman (1968) have shown that the amounts of acetylcholine detected by bioassay after incubation of rat brain-cortex slices in the presence of [U- 14 C]glucose are approximately the same as those calculated from measurements of the amounts of labelled acetylcholine formed. This result indicated that all of the acetyl groups of the acetylcholine formed, during incubation, are derived from glucose, confirming previous conclusions (Quastel *et al.*, 1936; Mann *et al.*, 1938, 1939*a,b*) that glucose (or its breakdown product, pyruvate) is the major precursor of acetylcholine in adult brain. The equivalence of bioassays and radiochemical assays of acetylcholine formation in incubated rat brain slices are shown in Table 1. Bull & Oderfeld-Nowak (1971) have indicated good agreement between bioassay and radiochemical assay of acetylcholine by using labelled acetyl-CoA as precursor.

We have done all experiments, whose results are recorded below, with [U- 14 C]glucose as precursor of acetylcholine in incubated rat brain slices. The values obtained, whilst agreeing with those of Browning & Schulman (1968) (Table 1), are smaller than those recorded by Mann *et al.* (1939*a,b*). The reason for this disparity is that in the initial investigations of Mann *et al.* (1939*a,b*) slices of the entire cerebrum, rather than those of the brain cortex, were taken. These included parts of the caudate nuclei, which are now

known to be at least three or four times as active as brain cortex in synthesizing acetylcholine from glucose (Sattin, 1966; Michalek *et al.*, 1972).

Paraoxon (diethyl *p*-nitrophenylphosphate), the cholinesterase inhibitor used in these investigations, has been extensively used in studies of cholinesterase (and other esterases) inactivation (Hartley & Kilby, 1952; Aldridge, 1954; Myers, 1956; Kewitz & Nachmansohn, 1957; Welsh & Dettbarn, 1972) and for suppression of electrical activity in nerve fibres (Dettbarn & Davis, 1963; Dettbarn, 1967). Paraoxon was used in our experiments at a concentration of 40 μ M, which is ample for complete inhibition of the cholinesterase in brain tissue (Liang & Quastel, 1969*a*). We used paraoxon instead of eserine for two reasons. (a) Paraoxon, in contrast to eserine, does not block active uptake of acetylcholine into incubated brain slices from the incubation medium (Polak & Meeuws, 1966; Schuberth & Sundwall, 1967; Liang & Quastel, 1969*a,b*). The relation therefore between the amount of acetylcholine in the tissue and that in the medium may be more representative of events *in vivo*, where tissue uptake of acetylcholine is a possibility. (b) There seems to be present, in the samples of [U- 14 C]glucose that we used, a small quantity of a radioactive substance that combines with eserine but not with paraoxon, and the combination is precipitated by reineckate. Although the quantity is small, the radioactivity of the impurity amounting to one-tenthousandth of that of the glucose, it is sufficient to yield abnormally high values for acetylcholine in the medium (though not in the tissue).

The values for total radioactive acetylcholine (i.e. tissue concentration + medium concentration) that we have obtained with paraoxon as cholinesterase

Table 1. Comparison of values of the amounts of acetylcholine, formed by rat brain-cortex slices, incubated aerobically in Locke (or Ringer)-bicarbonate-glucose media for 1 h at 37°C

The glucose (10mM) was present either as unlabelled glucose or as [U- 14 C]glucose. Estimations were either by (a) bioassay or by (b) radiochemical techniques. The cholinesterase inhibitor present was either eserine or paraoxon.

Cholinesterase inhibitor present	Concn. of KCl present (mM)	Method of assay	Total acetylcholine found (nmol/g initial wet wt.)	Reference
Eserine	2	(a)	35*	Browning & Schulman (1968)
Eserine	2	(b)	39*	Browning & Schulman (1968)
Eserine	31	(b)	100	Browning & Schulman (1968)
Eserine	26	(a)	67	Sollenberg & Sörbo (1970)
Eserine	27	(a)	106	Michalek <i>et al.</i> (1972)
Paraoxon	4	(b)	57	Present work
Paraoxon	31	(b)	101	Present work

* In these experiments the incubation medium was Locke-phosphate-glucose in an atmosphere of O₂ for an incubation period of 3h.

Table 2. *Effects of changes of concentration of Na⁺ and K⁺, and of the presence of Li⁺, on [¹⁴C]acetylcholine synthesis and release*

Rat brain-cortex slices were incubated in 3 ml of Locke-bicarbonate-[U-¹⁴C]glucose media, as described in the text, for 1 h at 37°C in O₂+CO₂ (95:5). The concentrations of Na⁺ and K⁺ present in the media were as given below. When the amount of Na⁺ was decreased it was replaced by an equivalent quantity of Li⁺. Glucose concentration was 5 mM. Paraoxon (40 μM) was present at the commencement of all experiments.

Concn. of cation (mM)			[¹⁴ C]Acetylcholine formed (nmol/g)		
Na ⁺	K ⁺	Li ⁺	Tissue	Medium	Total
165	4	—	50.5 ± 4.9	7.3 ± 3.1	57.8
200	4	—	51.3 ± 3.9	11.0 ± 2.9	62.3
165	31	—	57.7 ± 6.0	44.0 ± 3.0	101.7
0	31	140	10.9 ± 1.2	19.3 ± 4.9	30.2
70	31	70	39.3 ± 6.7	29.8 ± 2.0	69.1
140	31	140	29.2 ± 2.0	27.9 ± 1.0	57.1
280	31	—	41.6 ± 1.1	25.4 ± 0.9	67.0

inhibitor approximate to the values of total acetylcholine obtained by bioassay with eserine as inhibitor (Table 1).

When paraoxon was added to the incubation medium at the end instead of at the beginning of the experiment, the most noticeable change was a relatively small fall (from 50.5 to 37.2 nmol/g) in the tissue concentration of acetylcholine and none, within experimental error, in the medium concentration of acetylcholine. This result would indicate that little acetylcholine was released into the medium, containing 4 mequiv. of K⁺/litre, during incubation in the presence of paraoxon. This was probably due to uptake of released acetylcholine, so that the considerable concentration gradient between tissue and medium acetylcholine was maintained (Liang & Quastel, 1969a; Bourdois & Szerb, 1972). When paraoxon was added at the end of the incubation period, some of the acetylcholine concentrated in the tissue leaked out into the incubation medium.

Effects of changes of concentration of Na⁺ on [¹⁴C]-acetylcholine synthesis and release in incubated rat brain slices

Increase in the concentration of Na⁺ to 200 mM caused no change, within experimental error, in the yields of acetylcholine in the tissue and medium (Table 2), showing that mild hyperosmotic conditions did not affect acetylcholine synthesis or release in brain-cortex slices.

With 31 mM-KCl in the incubation medium, there was a large increase in the concentration of acetylcholine in the incubation medium, with a relatively small change in concentration of the tissue acetylcholine (Table 2). This result is in agreement with that of previous workers (Mann *et al.*, 1939a; Browning & Schulman, 1968). When paraoxon was added at the

end of the experiment there was a considerable fall in the amount of acetylcholine appearing in the incubation medium but there was only a slight fall in the concentration of tissue acetylcholine. This result is in accord with the well-known conclusion that, in the presence of an increased concentration of K⁺, there is both an increased rate of formation and increased release of acetylcholine by incubated brain slices. The tissue content of acetylcholine, under the present experimental conditions, was little changed by the increased K⁺ concentration.

Replacement of Na⁺ by Li⁺ in the presence of 31 mM-KCl caused a large fall both in the rate of synthesis and in the amount of release of acetylcholine. Addition of paraoxon at the end, instead of at the beginning, of the experiment made very little difference to the contents of acetylcholine in tissue or medium. This result is in agreement with the conclusion of previous workers (Quastel, 1962; Birks, 1963) that acetylcholine synthesis (in autonomic ganglia) is a Na⁺-dependent process.

It is also evident from the results given in Table 2 that the concentration gradient between labelled acetylcholine in the tissue and the medium was greatly diminished by exclusion of Na⁺ from the incubation media, i.e. that the release of acetylcholine, in relation to its content in the tissue, was increased by deprivation of Na⁺. This result is in accord with the observation that acetylcholine release at neuromuscular junctions is increased by decreased Na⁺ in the bathing medium (Kelly, 1965; Gage & Quastel, 1966).

Results given in Fig. 1 show that the total quantity of labelled acetylcholine synthesized by incubated rat brain-cortex slices was directly proportional to the external concentration of Na⁺, under conditions where acetylcholine synthesis was stimulated by the presence of 31 mM-KCl. However, even in the absence

Fig. 1. Effect of Na^+ on [^{14}C]acetylcholine formation

The effects of various concentrations of Na^+ in the incubation medium on the formation of labelled acetylcholine from [$\text{U-}^{14}\text{C}$]glucose. Rat brain-cortex slices were incubated in $\text{O}_2 + \text{CO}_2$ (95:5) at 37°C in the presence of $40\text{ }\mu\text{M}$ -paraoxon and 31 mM -KCl.

Fig. 2. Effect of choline on [^{14}C]acetylcholine formation

The effects of various concentrations of choline in the incubation medium, from which Na^+ had been omitted, on the formation of labelled acetylcholine ($\circ$, in the tissue; $\bullet$, in the medium; $\blacktriangle$, total) from [$\text{U-}^{14}\text{C}$]glucose. Rat brain-cortex slices were incubated in $\text{O}_2 + \text{CO}_2$ (95:5) at 37°C in the presence of $40\text{ }\mu\text{M}$ -paraoxon and 31 mM -KCl.

of Na^+ from the incubation medium, there was some synthesis and release of acetylcholine (Table 2, Fig. 1). It may be noted, in this connexion, that transmitter release can occur at the neuromuscular junction in the absence of Na^+ (Katz & Miledi, 1967b).

In some experiments (Table 2, Fig. 1) Na^+ was replaced by Li^+ . Since Li^+ itself might affect acetylcholine synthesis and release (Bjegovic & Randic, 1971), experiments were done to observe the effects of the addition of Li^+ to Locke-bicarbonate media. Results (Table 2) showed that 140 mM-Li^+ caused some suppression of the synthesis of acetylcholine in a medium containing 140 mM-Na^+ . Thus a value for total labelled acetylcholine of 67 nmol/g , in the presence of 280 mM-Na^+ , was decreased to 57.1 nmol/g in the presence of $140\text{ mM-Na}^+ - 140\text{ mM-Li}^+$ (Table 2). Such suppression could not account for the large decrease in acetylcholine synthesis found when Na^+ was fully substituted by Li^+ (Table 2).

Effects of choline on [^{14}C]acetylcholine synthesis and release in brain slices in an Na^+ -free medium

Addition of 1 mM -choline to the medium caused a marked increase of synthesis of labelled acetylcholine, a value of 30.2 nmol/g found in the absence of

added choline (Table 2) being increased to 56.4 nmol/g in the presence of the choline (Fig. 2). Much of the additional labelled acetylcholine formed was in the incubation medium, the concentration of acetylcholine in the medium rising from 19.3 (Table 2) to 39.4 nmol/g (Fig. 2) in the presence of the choline. When paraoxon was added at the end of the experiment, the presence of 1 mM -choline in the incubation medium showed no change in tissue or medium [^{14}C]acetylcholine. When the choline concentration in the incubation medium was increased there was a further increase in the amount of [^{14}C]acetylcholine synthesized (Fig. 2), most of which was present in the incubation medium (Fig. 2). Even with 10 mM -choline, when the total amount of acetylcholine formed rose to 97.5 nmol/g , an amount almost identical with that found in the Locke-bicarbonate medium without added choline (Table 2), a large proportion of the labelled acetylcholine was found in the incubation medium.

These results indicated that the block in [^{14}C]acetylcholine synthesis caused by the omission of Na^+ from the incubation medium could be removed

to a considerable extent by the addition of choline to the incubation medium and moreover that, in the absence of Na^+ , the release process was stimulated so that much more [^{14}C]acetylcholine appeared in the incubation medium than in the presence of Na^+ .

Addition of 1 mM-choline to the usual Locke-bicarbonate medium caused no significant increase in the tissue content of labelled acetylcholine and in the medium an increase in labelled acetylcholine from 7.3 (Table 2) to 14.8 nmol/g. Thus the addition of 1 mM-choline to the Na^+ -containing medium enhanced [^{14}C]acetylcholine synthesis relatively slightly. Addition of 20 mM-choline had a somewhat larger effect, increasing the tissue content of [^{14}C]acetylcholine from 50.5 to 69.5 nmol/g and the [^{14}C]acetylcholine in the medium from 7.3 to 23.9 nmol/g.

Evidently the effect of Na^+ deprivation on labelled acetylcholine synthesis was much diminished by the addition of choline, indicating that the action of Na^+ was partly due to its promotion of choline transport against a concentration gradient. It is well known that choline is taken up by brain cells against a concentration gradient (Quastel, 1965; Schuberth *et al.*, 1966), the process being Na^+ -dependent (Schuberth *et al.*, 1967; Marchbanks, 1968), and it is to be expected that deprivation of Na^+ will lead to diminished choline uptake from the extracellular spaces. The stimulating effect of added choline on labelled acetylcholine synthesis in a Na^+ -free medium supports this conclusion. Nevertheless it is also evident, since the relief afforded by the presence of choline in the incubation medium was only partial, that Na^+ deprivation must cause an additional effect on labelled acetylcholine synthesis not alleviated by the addition of choline. The most obvious other effect was the suppression of the activity of the Na^+ pump. This would result in substances, including acetylcholine (Liang & Quastel, 1969a), that were concentrated in brain cells being released into the extracellular medium and also in the suppression of the K^+ stimulation of brain metabolism, including that of the

process of acetylcholine synthesis, as the following data indicate.

Effects of K^+ on [^{14}C]acetylcholine synthesis and release in brain slices

It is now well known that the presence of Na^+ is necessary for various phenomena in the brain cell caused by increased K^+ , e.g. stimulated brain respiration (Takagaki & Tsukada, 1957). Such K^+ -increased respiration, involving a greater rate of conversion of pyruvate into acetyl-CoA (Kini & Quastel, 1959; Nicklas *et al.*, 1971), might be considered as partly responsible for the increased rate of acetylcholine formation in the presence of 31 mM-KCl. Results (Table 4) showed that increase of the concentration of KCl in the incubation medium to 60 mM, with no equal decrease in Na^+ , considerably increased the amount of labelled acetylcholine formed, the major change being found in the incubation medium. The tissue [^{14}C]acetylcholine content was increased from 50.3 to 99.5 nmol/g and the [^{14}C]acetylcholine in the medium was increased from 7.3 to 147.4 nmol/g (Table 4). However, early results by Mann *et al.* (1939a) showed that when the concentration of KCl in the incubation medium was increased to 58 mM, with an equivalent diminution of the concentration of Na^+ to preserve iso-osmoticity, the total acetylcholine formed was diminished by 50%, and if the concentration of KCl in the incubation medium was raised to 100 mM, with a corresponding decrease in NaCl, the diminution amounted to 90%. Such results, taken together with those found with labelled acetylcholine (Table 4), showed that the K^+ stimulation of acetylcholine synthesis in incubated rat brain slices is a Na^+ -dependent phenomenon. The results (Table 4) also showed that in the presence of a high amount of K^+ the ratio of concentrations of [^{14}C]acetylcholine in the tissue and medium was diminished. The increased release of acetylcholine by a high concentration of K^+ was unlikely to be due to a

Table 3. *Effects of various concentrations of calcium chloride on [^{14}C]acetylcholine synthesis and release in rat brain-cortex slices*

Incubation conditions were as described in Table 2. Concentrations used were: [$\text{U-}^{14}\text{C}$]glucose, 5 mM; K^+ , 4 or 31 mM; Na^+ , 165 mM; paraoxon, 40 μM . When Ca^{2+} was omitted from the incubation medium, 1 mM-EGTA was added.

Concn. of CaCl_2 (mM)	Concn. of KCl (mM)	[^{14}C]Acetylcholine formed (nmol/g)		
		Tissue	Medium	Total
0	4	26.0 $\pm$ 2.3	25.4 $\pm$ 3.0	51.4
2	4	49.8 $\pm$ 4.9	9.6 $\pm$ 3.7	59.4
0	31	22.6 $\pm$ 3.5	15.2 $\pm$ 3.0	37.8
2	31	64.5 $\pm$ 3.0	44.2 $\pm$ 2.1	108.7
10	31	35.4 $\pm$ 1.2	14.6 $\pm$ 1.2	50.0

Table 4. *Effects of protoveratrine (10 μ M) on [14 C]acetylcholine synthesis and release in rat brain-cortex slices in the presence of various concentrations of K^+*

Incubation conditions were as described in Table 2. Concentrations used were: [U- 14 C]glucose, 5mM; Na^+ , 165mM; paraoxon, 40 μ M.

Concn. of K^+ in the incubation medium (mM)	[14 C]Acetylcholine formed (nmol/g)					
	Protoveratrine absent			Protoveratrine (10 μ M) present		
	Tissue	Medium	Total	Tissue	Medium	Total
0	53.8 $\pm$ 3.7	7.6 $\pm$ 1.7	61.4	33.2 $\pm$ 1.9	46.4 $\pm$ 11.2	79.6
4	50.3 $\pm$ 4.9	7.3 $\pm$ 3.1	57.6	19.0 $\pm$ 5.3	27.3 $\pm$ 6.7	46.3
31	61.5 $\pm$ 5.7	40.7 $\pm$ 1.7	102.2	17.7 $\pm$ 4.4	24.9 $\pm$ 3.3	42.6
60	99.5 $\pm$ 4.3	147.4 $\pm$ 10.4	246.9	18.7 $\pm$ 1.4	30.0 $\pm$ 3.5	48.7

changed concentration of ATP, as this only occurs to a marked extent with a very high K^+ concentration, e.g. with 100mM-KCl (Okamoto & Quastel, 1970a). It is more likely related to the depolarization effected by high K^+ (Eccles & Liley, 1959; Thesleff & Quastel, 1965) at the nerve-cell membrane.

This conclusion is supported by the following results obtained from studies of the action of Ca^{2+} and of protoveratrine, a substance known to produce marked changes in bioelectric phenomena and ion permeability in the nerve cell (Shanes, 1958a,b; Okamoto & Quastel, 1970b).

Effects of Ca^{2+} on the action of K^+ on [14 C]acetylcholine synthesis and release

The stimulating action of high K^+ concentrations on acetylcholine synthesis in rat brain slices incubated in Locke-bicarbonate-glucose media in the presence of paraoxon was dependent on the presence of small concentrations of Ca^{2+} , a phenomenon described by McLennan & Elliott (1950) that explains why acetylcholine synthesis proceeds at a lower rate in phosphate media than in bicarbonate media (Mann *et al.*, 1939a). The results (Table 3) show the marked effect of absence of Ca^{2+} on the formation of labelled acetylcholine in media containing high concentrations of K^+ (31mM). The synthesis of acetylcholine in such media was diminished by 65%. It was necessary, to demonstrate the effects of Ca^{2+} deprivation, to add EGTA to the incubation medium. Without EGTA variable results were often encountered. High concentrations of $CaCl_2$ (e.g. 10mM) suppressed the K^+ -stimulated synthesis of acetylcholine, an observation already made by Mann *et al.* (1939a).

The results indicated that although labelled acetylcholine synthesis and release can occur in brain slices, in the absence of Ca^{2+} from the incubation medium, Ca^{2+} was necessary for the stimulation by

high concentrations of K^+ of acetylcholine synthesis and release (Table 3).

Effects of protoveratrine on [14 C]acetylcholine synthesis and release in brain slices

Addition of protoveratrine (10 μ M) to brain-cortex slices incubated in a Locke-bicarbonate-glucose medium in the presence of paraoxon caused profound effects on acetylcholine synthesis and release (Table 4). In media containing either 4mM- K^+ , or in the absence of added K^+ , protoveratrine caused a greatly increased release of labelled acetylcholine without concomitant increase in labelled acetylcholine synthesis. The tissue content of labelled acetylcholine fell as the medium content of labelled acetylcholine rose. However, in media containing a high concentration of K^+ , protoveratrine blocked entirely the increased release and synthesis of labelled acetylcholine due to high concentrations of K^+ (31 or 60mM) (Table 4).

The increased release of acetylcholine from brain-cortex slices caused by protoveratrine was greater in a medium containing 2mM- $CaCl_2$ than in a Ca^{2+} -free medium. Thus the percentage increase due to protoveratrine was 274 in the presence of 2mM- $CaCl_2$ and 4mM- K^+ (Table 4), whereas it was only 19 in a medium containing no added $CaCl_2$, 1mM-EGTA and 4mM- K^+ . The lack of effect of protoveratrine, in a Ca^{2+} -free medium, indicated that the protoveratrine-evoked release of acetylcholine was a Ca^{2+} -dependent phenomenon.

The protoveratrine block of stimulation by K^+ of acetylcholine synthesis in slices was not due to inhibition of choline uptake, as addition of choline (1mM) to the incubation medium containing protoveratrine had no significant effect on either acetylcholine synthesis or release.

The fact that either Ca^{2+} deprivation or the presence of protoveratrine suppressed synthesis of

Table 5. *Effects of tetrodotoxin (10 μ M) on [14 C]acetylcholine synthesis and release in rat brain-cortex slices*

Incubation conditions were as described in Table 2. Concentrations used were: [U- 14 C]glucose, 5 mM; Na $^{+}$, 165 mM; paraoxon, 40 μ M.

Additions to the incubation medium	[14 C]Acetylcholine formed (nmol/g)					
	Tetrodotoxin absent			Tetrodotoxin present		
	Tissue	Medium	Total	Tissue	Medium	Total
Nil	49.8 $\pm$ 4.9	7.6 $\pm$ 3.7	57.4	43.2 $\pm$ 2.2	4.9 $\pm$ 3.6	48.1
Protoveratrine (10 μ M)	19.0 $\pm$ 5.3	27.3 $\pm$ 6.7	46.3	42.9 $\pm$ 3.9	5.8 $\pm$ 2.6	48.7
KCl (27 mM)	64.5 $\pm$ 6.0	44.2 $\pm$ 3.0	108.7	54.1 $\pm$ 14.0	23.7 $\pm$ 1.0	77.8
Ouabain (0.1 mM)	23.8 $\pm$ 3.1	37.1 $\pm$ 2.5	60.9	23.2 $\pm$ 2.5	31.4 $\pm$ 2.7	54.6
Sodium L-glutamate (5 mM)	49.0 $\pm$ 3.1	15.0 $\pm$ 1.4	64.0	43.7 $\pm$ 3.0	8.2 $\pm$ 1.2	51.9
EGTA (1 mM), no Ca $^{2+}$	26.6 $\pm$ 2.3	24.3 $\pm$ 3.0	50.9	32.2 $\pm$ 5.8	16.4 $\pm$ 3.6	48.6
NH $_4$ Cl (10 mM)	44.5 $\pm$ 5.6	28.9 $\pm$ 5.0	73.4	45.0 $\pm$ 5.2	25.5 $\pm$ 1.0	70.5

labelled acetylcholine suggested that the influx of Na $^{+}$ into the nerve cells, caused either by absence of Ca $^{2+}$ (Frankenhauser & Hodgkin, 1955) from the incubation medium or the presence of protoveratrine (Okamoto & Quastel, 1970b), might be a responsible factor. However, results shown in Table 2 indicated that when the medium concentration of Na $^{+}$ was raised to 200 mM there was little or no inhibition of acetylcholine synthesis. Joanny & Hillman (1964) showed that a rise in Na $^{+}$ concentration in the incubation medium leads to a corresponding rise in the tissue concentration of Na $^{+}$. Nevertheless, there was a possibility that the neuronal uptake of Na $^{+}$ might not be significantly elevated by an increase of Na $^{+}$ in the incubation medium.

The phenomenon in which a high concentration of K $^{+}$ greatly stimulates acetylcholine synthesis in brain slices was evidently linked with the integrity of the cell structure, as the stimulation was much suppressed or abolished in a rat brain homogenate. Protoveratrine (10 μ M) exerted only a small suppressing effect on labelled acetylcholine formation in a rat homogenate, a value of 40.5 nmol of acetylcholine formed/g in the presence of 60 mM-KCl being decreased to 32.5 nmol/g in the presence also of 10 μ M-protoveratrine.

These facts, indicating that high-K $^{+}$ stimulation of acetylcholine synthesis in incubated brain slices was blocked by the absence of Na $^{+}$ or Ca $^{2+}$ from the incubation medium, or by the presence of protoveratrine, and was suppressed by brain tissue homogenization, made it seem very unlikely that the K $^{+}$ stimulation could be primarily due to an acceleration of the rate of conversion of pyruvate into acetyl-CoA. Rather, it seemed more likely to be associated with the mechanisms connecting synthesis and storage of acetylcholine in the neuron.

Effects of substances promoting changes of cation permeability at the brain cell membrane on [14 C]acetylcholine synthesis and release

The following substances that promote influx of Na $^{+}$ into incubated brain slices were investigated: ouabain (Yoshida *et al.*, 1961; Okamoto & Quastel, 1970b), sodium L-glutamate (Bradford & McIlwain, 1966; Harvey & McIlwain, 1968; Okamoto & Quastel, 1970b) and ammonium chloride.

Ouabain. The addition of ouabain (0.1 mM) caused an enhanced release of labelled acetylcholine from rat brain-cortex slices incubated in a Locke-bicarbonate-glucose medium with little or no change in its synthesis (Table 5). This phenomenon might simply be a reflection of the fact that ouabain suppresses the active uptake of acetylcholine from the incubation medium (Liang & Quastel, 1969a) and hence promotes its release down its concentration gradient.

Sodium L-glutamate. Addition of sodium L-glutamate (5 mM) increased the release of labelled acetylcholine from incubated brain slices without changing the synthesis of labelled acetylcholine significantly (Table 5). The increased release was much smaller than that due to protoveratrine, ouabain or 31 mM-KCl (Table 5).

Ammonium chloride. Ammonium chloride (27 mM) causes diminished synthesis of acetylcholine in Locke-bicarbonate-tyrosine-glucose medium, but forms increased quantities of 'free' acetylcholine (Braganca *et al.*, 1953). Because 10 mM-NH $_4$ Cl causes a large influx of Na $^{+}$ into incubated rat brain-cortex slices (A. M. Benjamin & J. H. Quastel, unpublished work), the effects of 10 mM-NH $_4$ Cl on labelled acetylcholine synthesis and release were observed under the present experimental conditions. NH $_4$ Cl at 10 mM caused a much increased release of labelled acetylcholine, comparable with that due to protoveratrine (10 μ M),

and synthesis of labelled acetylcholine was somewhat enhanced (Table 5).

Effects of tetrodotoxin (10 μ M) on [14 C]acetylcholine synthesis and release

In the Locke–bicarbonate–paraoxon medium tetrodotoxin completely abolished the stimulating effects of protoveratrine (10 μ M) on labelled acetylcholine release, whereas it had relatively little effect on labelled acetylcholine release or synthesis in a normal medium (Table 5). The low tissue content of acetylcholine found in the presence of protoveratrine was increased by tetrodotoxin, so that the total amount of labelled acetylcholine formed in the presence of protoveratrine was unaffected by tetrodotoxin.

The profound action of protoveratrine on acetylcholine release, and its complete block by tetrodotoxin, support the conclusion that the site of acetylcholine release lies mainly in the neurons. The glia do not participate to a significant extent in the production of acetylcholine in the nervous system (Hebb, 1961), though it is possible that some neuroglia may do so.

The release of labelled acetylcholine due to a high concentration (31 mM) of KCl was partially inhibited by tetrodotoxin, which also diminished to some extent the tissue content of labelled acetylcholine. Evidently tetrodotoxin had a partial blocking effect on the stimulating action of high concentrations of K⁺ (31 mM) on both acetylcholine synthesis and release (Table 5).

Tetrodotoxin abolished the relatively small enhancing effect of sodium L-glutamate on acetylcholine release and there seemed to be some inhibition of acetylcholine synthesis (Table 5).

The large increase of labelled acetylcholine release caused by ouabain was somewhat diminished by tetrodotoxin (Table 5) and the effect on acetylcholine synthesis was not significant. This conclusion applied also to the release and synthesis of acetylcholine when the brain slices were incubated in a Ca²⁺-free medium in 1 mM-EGTA (Table 5).

Tetrodotoxin had no effect, within experimental error, on the release and synthesis of acetylcholine in the presence of 10 mM-NH₄Cl.

Effects of unlabelled acetylcholine on the synthesis and release of radioactive acetylcholine in rat brain-cortex slices

The addition of unlabelled acetylcholine, up to 100 mM, caused no fall in the content of labelled acetylcholine in the tissue (Table 6). This result indicated that there was no exchange between extra-cellular acetylcholine and the tissue acetylcholine. Had exchange taken place, the tissue content of labelled acetylcholine would have diminished and the labelled acetylcholine content in the incubation medium would have increased correspondingly. Even with 31 mM-KCl, addition of unlabelled acetylcholine caused no significant fall in the tissue content of labelled acetylcholine (Table 6). This agrees with the finding of Marchbanks (1968), who showed that

Table 6. *Effects of unlabelled acetylcholine on the synthesis and release of [14 C]acetylcholine in rat brain-cortex slices*

Conditions were as described in Table 2. Concentrations used were: [U- 14 C]glucose, 5 mM; Na⁺, 165 mM; paraoxon, 40 μ M. In experiments with 100 mM-acetylcholine 1 ml of medium was used for precipitation with ammonium reinekate. The precipitate was washed three times with water. No carrier was added.

Concn. of reagents in medium			[14 C]Acetylcholine formed (nmol/g)		
KCl (mM)	Unlabelled acetylcholine (mM)	Protoveratrine (μ M)			
4	0	0	50.5 $\pm$ 4.9	7.3 $\pm$ 3.7	57.8
4	1	0	50.8 $\pm$ 2.9	12.8 $\pm$ 2.2	63.6
4	5	0	56.2 $\pm$ 1.9	22.0 $\pm$ 2.1	78.2
4	100	0	70.6 $\pm$ 8.1	39.4 $\pm$ 3.2	110.0
31	0	0	57.7 $\pm$ 5.0	42.0 $\pm$ 1.8	99.7
31	1	0	50.7 $\pm$ 2.2	50.0 $\pm$ 9.0	100.7
31	5	0	45.5 $\pm$ 3.6	41.3 $\pm$ 7.9	86.8
31	100	0	52.8 $\pm$ 6.9	59.6 $\pm$ 10.0	112.4
4	0	10	19.0 $\pm$ 5.3	27.3 $\pm$ 6.7	46.3
4	1	10	16.7 $\pm$ 1.6	26.3 $\pm$ 1.8	43.0
31	0	10	17.7 $\pm$ 4.5	24.9 $\pm$ 3.4	42.6
31	1	10	13.7 $\pm$ 2.8	41.9 $\pm$ 6.2	55.6

when synaptosomes are loaded with labelled acetylcholine there is no exchange with the acetylcholine present in the vesicles.

It was evident, from results in Table 6, that addition of unlabelled acetylcholine to the incubation medium, containing 4 mM-K⁺, caused some increase in synthesis and release of labelled acetylcholine, particularly when 100 mM-acetylcholine was added to the incubation medium, as found by Collier & Katz (1970) with perfused ganglia. Possibly the phenomenon was due to the depolarizing action of acetylcholine (Armstrong & Ritchie, 1960; Koketsu & Nishi, 1968; Krnjevic *et al.*, 1971), a conclusion supported by the fact that unlabelled acetylcholine caused no significant change in labelled acetylcholine synthesis and release in the presence of 31 mM-KCl, which itself causes depolarization (Table 6).

Addition of 1 mM-acetylcholine caused no change in the tissue and medium contents of labelled acetylcholine in the presence of 10 μ M-protoveratrine, except for some enhancement of release in the additional presence of 31 mM-KCl.

According to Potter *et al.* (1968) acetylcholine is synthesized in the cytoplasm and is then incorporated into synaptic vesicles. If this is so, it is evident from results given in Table 6 that any unlabelled acetylcholine taken up from the incubation medium and penetrating the vesicles must be trapped there. However, it seems from the results of Marchbanks (1969) and of Ritchie & Goldberg (1970) that little exogenous acetylcholine penetrates the vesicles. It is also evident, from the results in Table 6, that there was no inhibition of labelled acetylcholine synthesis in the brain slices by high concentrations (up to 100 mM) of acetylcholine in the incubation medium. Potter *et al.* (1968) have calculated, from their studies of the properties of choline acetyltransferase isolated from rat brain, that free acetylcholine (of the order of 1–10 mM) might be in equilibrium with the high concentration (>100 mM) of acetylcholine in the vesicles. If the amount of synthesis of acetylcholine depends on the equilibrium position of the transferase with all four of its substrates (acetylcholine, choline, CoA and acetyl-CoA), it follows that the rise in concentration of acetylcholine should favour the back-reaction and lead to less synthesis of acetylcholine from labelled acetyl-CoA. This was clearly not the case (Table 6). Again, it appears that acetylcholine synthesis can be regulated by product inhibition, 100 mM-acetylcholine causing 45% inhibition (Kaita & Goldberg, 1969). Reisberg (1957) reported 50% inhibition of choline acetyltransferase by 50 mM-acetylcholine. Therefore if acetylcholine is synthesized in the cytoplasm and then transferred to the vesicles, 100 mM-acetylcholine in the cytoplasm should have produced some inhibition of labelled acetylcholine synthesis. The fact that this did not occur seems to indicate that much of the synthesis of labelled acetylcholine must have

occurred in a location that was not affected by exogenous acetylcholine, even at a concentration of 100 mM. A possible explanation is that acetylcholine was synthesized on, or in, the vesicular membrane (McCaman *et al.*, 1965; Saelens & Potter, 1966; De Robertis & de Lores Arnaiz, 1969; Ritchie & Goldberg, 1970), part of it being captured by a vesicle constituent (Whittaker, 1971, 1972; Matsuda *et al.*, 1971; Musick & Hubbard, 1972), the process continuing until no further storage by combination with the vesicle constituent was possible.

Effects of barbiturates on the synthesis and release of [¹⁴C]acetylcholine in rat brain-cortex slices

The results of experiments to observe the effects of two barbiturates, Amytal and pentothal, on the synthesis and release of acetylcholine in rat brain-cortex slices incubated aerobically in Locke-bicarbonate-[U-¹⁴C]glucose-paraoxon media under various conditions are recorded in Table 7 and may be summarized as follows.

1. Sodium Amytal (0.25 or 0.5 mM) had no significant effect on labelled acetylcholine synthesis in unstimulated brain but at 1.0 mM it exercised a very considerable inhibition (>50%). The release of labelled acetylcholine from the tissue into the medium tended to increase with increasing concentration of Amytal. Pentothal (0.25 or 0.5 mM) had no significant effect on labelled acetylcholine synthesis or release in unstimulated brain (Table 7).

2. Sodium Amytal [0.25 mM, the concentration in the blood of rats under Amytal anaesthesia (Reading & Wallwork, 1969), and at 0.5 mM] diminished the K⁺-stimulated release of labelled acetylcholine and also the enhanced release of labelled acetylcholine found in the absence of Ca²⁺ from the incubation medium. Pentothal (0.25 or 0.5 mM) was even more effective than Amytal at equivalent concentrations (Tables 3 and 7). Thus K⁺-evoked release of labelled acetylcholine amounted to 36.7 (i.e. 44.0–7.3) nmol/g (Table 2) and this was decreased to 26.6 (i.e. 39.2–12.6) nmol/g in the presence of 0.25 mM-Amytal, to 13.2 (i.e. 26.6–13.4) nmol/g with 0.5 mM-Amytal, to 7.5 (i.e. 20.1–12.6) nmol/g with 0.25 mM-pentothal, and to 1.9 (i.e. 16.8–14.9) nmol/g with 0.5 mM-pentothal (Table 7). The enhanced release of labelled acetylcholine, due to absence of Ca²⁺ from the incubation medium, amounting to 15.8 nmol/g (Table 3), was decreased to 3.2 (i.e. 15.8–12.6) nmol/g with 0.25 mM-Amytal, to 5.5 (i.e. 18.9–13.4) nmol/g with 0.5 mM-Amytal, and abolished by pentothal (0.25 or 0.5 mM) (Table 7).

3. The fall in the tissue content of labelled acetylcholine, caused by the absence of Ca²⁺ (Table 3), was abolished by the presence of 0.25 or 0.5 mM-pentothal, and decreased by 0.25 mM-Amytal although not by 0.5 mM-Amytal (Table 7).

Conditions were as described in Table 2. Concentrations used were: [U-¹⁴C]glucose, 40 μM; K⁺, 4 mM.

Addition to the incubation medium	$[^{14}\text{C}]\text{Acetylcholine formed (nmol/g)}$											
	Amytal						Pentothal					
	Concn. (mm)		0.25		0.5		1.0		0.25		0.5	
	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium
Nil	55.8 ± 9.0	12.6 ± 4.5	49.8 ± 7.4	13.4 ± 2.4	9.2 ± 1.4	16.3 ± 2.2	54.3 ± 11.4	12.6 ± 3.5	58.5 ± 10.5	14.9 ± 2.0		
KCl (27mm)	63.6 ± 6.0	39.2 ± 1.0	40.7 ± 2.4	26.6 ± 2.0	13.9 ± 2.1	12.9 ± 2.0	57.8 ± 3.6	20.1 ± 5.9	52.7 ± 5.9	16.8 ± 2.0		
EGTA (1mm), no Ca^{2+}	34.8 ± 1.0	15.8 ± 2.1	27.8 ± 1.0	18.9 ± 1.0			62.5 ± 4.4	12.0 ± 3.5	52.4 ± 3.3	13.4 ± 1.7		
Protopovratrine ($10\mu\text{M}$)	17.3 ± 1.3	24.5 ± 3.0	8.9 ± 2.3	18.6 ± 2.0			25.4 ± 2.8	26.0 ± 4.9	30.2 ± 5.7	24.6 ± 4.0		

Discussion

Results of these studies of [^{14}C]acetylcholine synthesis and release in rat brain-cortex slices, incubated aerobically in Locke-bicarbonate-glucose media containing paraoxon as cholinesterase inhibitor, show important similarities to those obtained in studies of the neuromuscular junction or of autonomic ganglia or guinea-pig ileum. Some of these are as follows.

1. Dependence of acetylcholine synthesis and release on the Na^+ content of the incubation medium (Table 2, Fig. 1).

2. Dependence of acetylcholine release and synthesis on the presence of Ca^{2+} in the incubation medium (Table 3) (cf. Harvey & MacIntosh, 1940; Katz & Miledi, 1969*a,b*, 1970). As the concentration of Ca^{2+} in the medium is increased, e.g. to 10 mM, the release of labelled acetylcholine is greatly diminished (Table 3).

3. Enhanced release of acetylcholine by substances that promote depolarization at the nerve cell membrane (cf. Del Castillo & Katz, 1954), e.g. high concentrations of K^+ , ouabain, protoveratrine, sodium L-glutamate, high concentration of acetylcholine (Tables 2, 3, 4, 5) (cf. Collier & Katz, 1970).

4. Failure of acetylcholine synthesis to keep pace with acetylcholine release in the presence of ouabain or Na^+ lack (cf. Paton *et al.*, 1971).

[¹⁴C]Acetylcholine synthesis and release obviously occurred in the absence of Ca²⁺ from the incubation medium, a fact to be correlated with the observation (Quastel *et al.*, 1971) that conditions exist (in mouse diaphragm) where transmitter release is independent of Ca²⁺, but the retention of labelled acetylcholine by the tissue was much diminished by the absence of Ca²⁺ from the incubation medium (Table 3).

Modes of action of Na⁺ and of protoveratrine

The Na^+ dependence of acetylcholine synthesis may be due partly to the suppression of choline transport, as the addition of choline diminished the effect of Na^+ lack, but part seemed to be independent of the presence of choline and appeared to be connected with the lack of activity of the Na^+ pump.

The considerable stimulation by a high concentration of K^+ of [^{14}C]acetylcholine synthesis and release in brain slices was abolished by protoveratrine ($10\mu M$). It is difficult to explain this phenomenon except on the grounds that depolarization by protoveratrine was so excessive that addition of K^+ could

have no further effect. Nevertheless, this point of view does not explain why there should be such disparity between the effects of protoveratrine and a high concentration of K^+ . The increased synthesis of acetylcholine that accompanies increased release, as shown with high concentrations of K^+ or with venoms (Braganca & Quastel, 1952), does not seem to apply to protoveratrine, or to lack of Ca^{2+} , or to the presence of ouabain or of sodium L-glutamate, with labelled acetylcholine formed in incubated rat brain-cortex slices. De Robertis (1969) pointed out that depletion of vesicles, which form the main store of acetylcholine, occurs in peripheral synapses stimulated at very high frequencies. Our results would be understandable if the synaptic vesicles, whose integrity is presumably essential for high rates of synthesis of acetylcholine, were substantially depleted by the large depolarization effected by protoveratrine and possibly other conditions. However, some of our results, concerning the failure of labelled acetylcholine synthesis to keep pace with acetylcholine release, as with ouabain or Na^+ lack, which resemble those of Paton *et al.* (1971) derived from studies of guinea-pig ileum, may be explained as due simply to suppression of the Na^+ pump, caused by block of Na^+ - K^+ -adenosine triphosphatase (as with ouabain) or by lack of Na^+ . Such suppression would account for diminished acetylcholine synthesis due to inhibition of choline transport and for the increased release of acetylcholine as this moves down its concentration gradient to the cell exterior (Liang & Quastel, 1969a).

Effects of tetrodotoxin

Tetrodotoxin, which completely blocked the enhanced release of labelled acetylcholine by protoveratrine, had smaller effects on the enhanced release due to high concentrations of K^+ , or ouabain, or L-glutamate or Ca^{2+} lack, and none on that due to NH_4^+ . Partial, or complete, reversing action of tetrodotoxin suggests that the release of labelled acetylcholine by incubated rat brain-cortex slices was at least in part dependent upon the activation of the Na^+ current system at the neuronal membrane known to be blocked by tetrodotoxin.

Effect of barbiturates

The barbiturates tested (Amytal and pentothal), at low concentrations, suppressed only evoked release of acetylcholine from brain slices. This fact may be correlated with the findings of Weakly (1969) that thiopentone and pentobarbitone decrease the amount of transmitter released by afferent impulses, or with the facts concerning the block of release of transmitter from nerve terminals by anaesthetics (Thesleff

& Quastel, 1965; Quastel *et al.*, 1972). Little interference with the biosynthesis of labelled acetylcholine in rat brain-cortex slices occurred with Amytal or pentothal except at relatively high concentrations. The action of the barbiturate, in reducing neuronal excitability, may be due to its suppression of mitochondrial metabolism resulting in release of bound Ca^{2+} within the neuron (Godfraind *et al.*, 1971; Shankaran & Quastel, 1972), this in turn affecting the fluxes of Na^+ and K^+ at the neuronal membrane. The permeability of the erythrocyte membrane to Na^+ and K^+ is regulated by internal Ca^{2+} (Romero & Whittam, 1971). This suggestion about the mode of action of barbiturates receives support from the demonstration (Table 7) that pentothal, and to some extent Amytal, at 0.25 mM reversed the fall in the acetylcholine content of the brain tissue that occurred in a Ca^{2+} -free incubation medium. The more powerful action of pentothal than of Amytal, at equivalent concentration, might be attributed to the known uncoupling activity on oxidative phosphorylation by thiobarbiturates (Aldridge & Parker, 1960). The suppression of K^+ -stimulated release of acetylcholine by the barbiturates might then be partly due to increased release of Ca^{2+} from the mitochondria.

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