Biochemical Pharmacology, Vol. 26, pp. 609–615. Pergamon Press, 1977. Printed in Great Britain.

EFFECTS OF PENTOBARBITAL ON THE REGULATION OF ACETYLCHOLINE CONTENT AND RELEASE IN DIFFERENT REGIONS OF RAT BRAIN*

JUDITH A. RICHTER and MARSHALL B. WALLER

The Institute of Psychiatric Research and Department of Pharmacology, Indiana University School of Medicine, Indianapolis, IN 46202, U.S.A.

(*Received* 23 *June* 1976; *accepted* 27 *August* 1976)

Abstract—Pentobarbital administered to rats *in vivo* increased the levels of acetylcholine (ACh) in the cerebral cortex, striatum, hippocampus and pons-medulla but not in the midbrain or cerebellum. It has been suggested that the increased levels of ACh may be the result of an inhibition of release of the transmitter. However, when pentobarbital was tested *in vitro*, it inhibited potassium-stimulated ACh release from all regions. Release of ACh and tissue levels of ACh were compared directly in cerebral cortex and midbrain *in vitro*. Stimulation with 25 and 50 mM KCl enhanced ACh release from both regions but decreased tissue ACh only in the cerebral cortex. Pentobarbital inhibited potassium-stimulated ACh release from both regions but only increased tissue ACh in the cerebral cortex (stimulated with 25 mM KCl). These results suggest that the regulation of ACh synthesis and release is different in the two brain regions and that pentobarbital can inhibit ACh release without necessarily causing a concomitant rise in ACh tissue levels.

Barbiturates have long been known to increase brain levels of acetylcholine (ACh) when they are given *in vivo* [1–5]. In addition, these drugs have also been shown to decrease the release of ACh from the surface of the cerebral cortex *in vivo* [6–8], from the stimulated, perfused superior cervical ganglion [9], and from cerebral cortex slices incubated *in vitro* [10, 11]. In the case of the barbiturates, as well as with other classes of drugs and other treatments, it has been proposed that increased levels of ACh are the result of the inhibition of ACh release and a subsequent accumulation of the transmitter [12–15].

However, this proposal has not been tested directly for the barbiturates. Therefore, we have compared the effects of pentobarbital on tissue levels of ACh and the release of this transmitter from different regions of rat brain. The results suggest that the regulation of ACh levels in the tissue is different in the cortex and the midbrain and that an inhibition of ACh release by pentobarbital is not always associated with a rise in tissue ACh levels.

METHODS

Tissue preparation. To determine the effect of pentobarbital on brain ACh levels, male Wistar rats (200–300 g) were injected with 50 mg/kg of sodium pentobarbital (Sigma Chemical Co.) or 0.9% NaCl i.p. 30 min before they were killed by near-freezing in liquid nitrogen [16]. The brains were dissected in a cold box (−20°) into six regions. After a dorsal midline incision, the cerebral cortex was laid back and both hippocampi were removed. The striatum (caudate nucleus, putamen and pallidum) was removed bilaterally by dissecting it away from the cortex. The septum was included with the striatum. The cerebellum was removed by severing the cerebellar peduncles. After complete separation of the cerebral cortices (only one of which was used) a cut immediately caudal to the colliculi isolated the pons-medulla from the midbrain. The pons-medulla was separated from the spinal cord 3 mm caudal to the obex. The brain regions were individually wrapped in aluminum foil, frozen in liquid nitrogen, and stored at −70° until assayed for ACh.

To determine the amount of pentobarbital in each brain region, rats were injected i.p. with 50 mg/kg of sodium pentobarbital containing [^{14}C]-(ring)-pentobarbital (New England Nuclear), 0.025 mCi/m-mole, and killed 30 min later by near-freezing. The same brain regions described above were dissected at −20°, weighed, and homogenized at 0° in 1 ml of 0.5 M sodium acetate, pH 5.0. A 500-μl sample of the homogenate was counted in 12 ml Aquasol (New England Nuclear). Portions of some homogenates (300 μl) were extracted to separate unchanged pentobarbital from polar metabolites [17].

Rats were killed by decapitation for superfusion studies *in vitro*. The brain was removed and dissected on a petri dish on ice into the regions described above. Each region was weighed and then prisms were prepared by cutting at 0.4-mm intervals in two planes at a 45° angle with a Brinkman–McIlwain tissue chopper. The slices were suspended in 5 ml of incubation medium and the total suspension was loaded into a 25-mm Swinnex (Millipore Corp.) and superfused with medium at 37° at 0.5 ml/min as described previously [18]. The medium contained (mM): 120 NaCl, 0.75 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 glucose, either 5, 25 or 50 KCl and either 10^{-4} M eserine (physostigmine sul-

* A preliminary account of portions of this work was presented to the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Davis, Calif., August 1975.

fate, Sigma Chemical Co.) or 10^{-5} M paraoxon (diethyl-*p*-nitrophenylphosphate, Sigma Chemical Co.) and was continuously bubbled with 5% CO_2/95% O_2 (final pH, 7.5). When 25 or 50 mM KCl was used, no adjustments were made in the concentrations of other ions. Sodium pentobarbital was included in the medium as indicated in Results. Samples of superfusate (2.5 ml) were collected as timed fractions (5 min) with a fraction collector. Tissue was recovered by forcing excess medium out of the Swinnex with a syringe filled with air, and transferring the tissue with a spatula to a homogenizer containing 1 ml of the medium with which the tissue had last been perfused. The tissue was homogenized on ice with eight strokes of a pestle rotating at 840 rev/min.

In some experiments 0.25-g portions of sliced cerebrum (including the cerebral cortex, hippocampus, striatum and midbrain) were superfused with 5 mM KCl medium containing paraoxon and [^{14}C]pentobarbital (5×10^{-4} M, 0.02 μCi/μmole) or [^{14}C]pentobarbital and [^{3}H]inulin (1×10^{-7} M, 300 μCi/μmole, Amersham–Searle Corp.).

ACh assays. ACh in frozen brain regions was extracted and measured by the method of Goldberg and McCaman [19] as modified by Richter and Shea [20]. ACh in 1-ml superfusate samples and in 500 μl of the homogenized tissue slices was assayed using modifications described by Richter [18]. Blanks and standards were routinely prepared in incubation medium, since preliminary tests showed that the concentrations of KCl, pentobarbital, paraoxon and eserine used did not affect the assay. In some assays ATP–choline phosphotransferase (EC 2.7.1.32) from Sigma Chemical Co. was used. It was also found that ACS scintillation solution (Amersham–Searle Corp.) could be used as well as Aquasol (New England Nuclear).

Scintillation counting and calculations. Samples were counted in 12 ml of scintillation solution in a model 3375 Packard scintillation counter. Internal standards were used to determine counting efficiency, and when two isotopes were counted, the results were corrected for overlap. Results are expressed as the amount of ACh or pentobarbital in samples derived from 1 g (wet weight) of tissue ± S. E. M. Student's *t*-test was used to determine statistical significance ($P < 0.05$) of differences between means.

RESULTS

The amount of ACh in six regions of rat brain was measured 30 min after an i.p. injection of saline or 50 mg/kg of sodium pentobarbital. The barbiturate caused an increase in ACh in the hippocampus, cortex, striatum and pons-medulla but no increase in the midbrain or the cerebellum (Table 1). This differential effect was not the result of a regional concentration of the drug in the brain. Using [^{14}C]pentobarbital, we found the drug to be highest in the pons-medulla and lowest in the cerebral cortex. The small differences in drug distribution showed no correlation with the effect on ACh (Table 1). No more than 13 per cent of the radioactivity behaved as polar metabolites.

Preliminary experiments *in vitro* indicated that pentobarbital had a smaller effect on stimulated ACh release when it was introduced at the same time as 50 mM KCl compared to its effect when it was added to the 5 mM KCl medium for 50 min of superfusion before stimulation with 50 mM KCl. When [^{14}C]pentobarbital was used to determine the partition of pentobarbital between the tissue and the medium, it was found that the amount of radioactivity in the tissue reached a stable level between 45 and 60 min after superfusion began; the pattern of loss of [^{14}C]pentobarbital from the tissue when superfusion was switched to medium without the drug was a mirror image of the accumulation. In another experiment, the accumulation of inulin and pentobarbital was determined in six tissue samples 60–75 min after superfusion began. The ratio of substance/g of tissue (initial wet weight) to substance/ml of medium was 2.93 ± 0.11 for [^{14}C]pentobarbital and 0.57 ± 0.016 for [^{3}H]inulin. The higher value for the inulin space compared to reported values of 0.38 to 0.40 for incubated brain cortex slices [21, 22] could be explained in part by the adherence of some medium to the tissue and the effect of using aliquots of suspensions of cerebrum prisms on the accuracy of the estimate of the initial wet weight. In all further experiments, pentobarbital was added at the beginning of superfusion.

Table 1. Effects of pentobarbital *in vivo* on ACh in regions of rat brain*

Region	Tissue wt (mg)	ACh in control (nmoles/g)	ACh in PB-treated (nmoles/g)	Per cent change	PB (nmoles/g)
Cerebral cortex (one half)	403 ± 7.4	14.3 ± 0.64	22.1 ± 1.4	54†	152.0 ± 4.4
Hippocampus	110 ± 3.2	19.2 ± 0.70	28.2 ± 1.8	47†	160.0 ± 4.3
Striatum	148 ± 6.9	36.8 ± 2.4	46.3 ± 3.4	26†	172.0 ± 5.5
Pons-medulla	173 ± 10.9	19.6 ± 0.64	23.0 ± 1.0	17†	188.0 ± 2.0
Midbrain	332 ± 7.1	26.5 ± 0.76	24.6 ± 2.0	7	161.0 ± 6.2
Cerebellum	242 ± 5.7	4.18 ± 0.38	4.37 ± 0.23	4	156.0 ± 8.0

* Rats were given 50 mg/kg of sodium pentobarbital (PB), normal saline or 50 mg/kg of sodium [^{14}C]pentobarbital (0.025 mCi/m-mole) i.p. 30 min before killing by the near-freezing method. Brains were dissected at −20° and ACh or [^{14}C]pentobarbital was determined in the individual regions as described in Methods. The mean is given ± S. E. M.; N = 8–9 in each group.

† Indicates a significant change from control ($P < 0.05$). The amount of pentobarbital in the pons-medulla was significantly higher than in all other regions, and the amount in the striatum was significantly higher than that in the cerebral cortex.

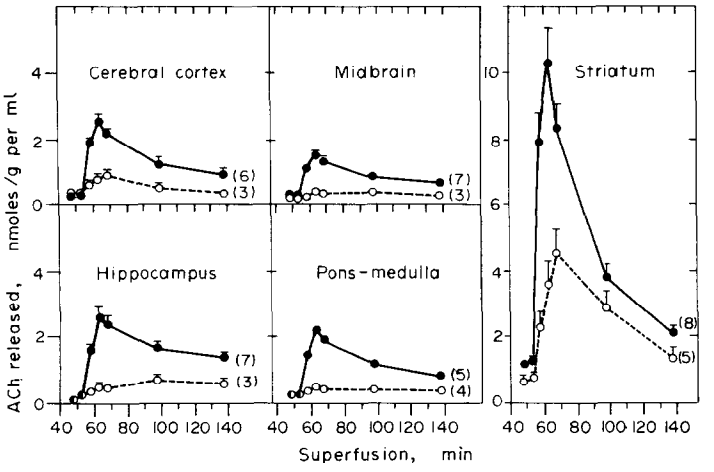

Fig. 1. Effect of pentobarbital on acetylcholine (ACh) release from superfused slices of different regions of rat brain. Rats were decapitated and the appropriate brain region was prepared for superfusion as described in Methods. The tissue was superfused at 0.5 ml/min with 5 mM KCl-eserinized medium with or without 5×10^{-4} M pentobarbital. After 50 min the superfusing medium was changed to one containing 50 mM KCl, eserine and 5×10^{-4} M pentobarbital where indicated. The effluents were collected in 2.5-ml fractions, and 1.0-ml portions were assayed for ACh as described in Methods. Results are means ± S. E. M. of the number of samples given in parentheses. Key: (——) control, (· · · · ·) pentobarbital.

and stimulation was begun at 50 min when the tissue and medium had reached equilibrium with respect to pentobarbital.

The effect of pentobarbital on ACh release from all regions (except the cerebellum) was examined by superfusing slices of these regions with medium containing 5×10^{-4} M pentobarbital and eserine as the cholinesterase inhibitor. As shown in Fig. 1, pentobarbital inhibited K^+-stimulated release (50 mM KCl medium) from all regions studied. Unstimulated ACh release (described by the 45–50 and 50–55 min collection points when the higher K^+ concentration has not yet affected the ACh released in the superfusate) was inhibited by pentobarbital only in the striatum. In the cerebral cortex and the striatum, other concentrations of pentobarbital were also studied and a dose-related effect of the drug on stimulated release was observed (Fig. 2). In the cerebral cortex 5×10^{-4} M pentobarbital had a large effect, and a smaller, but statistically significant inhibition occurred at 1×10^{-4} M pentobarbital. No statistically significant effect occurred at 2×10^{-5} M, although a tendency to increased release was seen. In the striatum, the inhibition by 5×10^{-4} M pentobarbital was less marked than in the cortex and 1×10^{-4} M had no effect. The effect of 5×10^{-4} M pentobarbital on the striatum was only observed at the time of peak ACh release (Figs. 1 and 2). In one experiment (not shown), 1×10^{-3} M pentobarbital inhibited the peak of K^+-stimulated ACh release from the striatum by more than 90 per cent.

These results suggested that the inhibition of ACh release may not be related to the increase in brain ACh levels, since the drug inhibited release in the

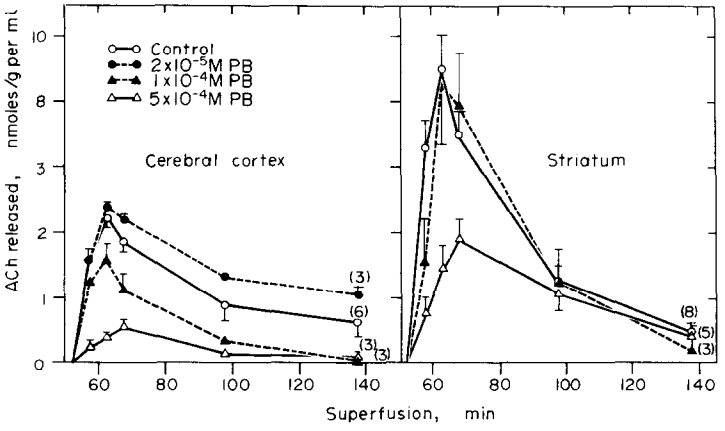

Fig. 2. Effect of different pentobarbital concentrations on K^+-stimulated ACh release from cerebral cortex and striatum. Details are as in Fig. 1 except that the concentrations of pentobarbital (PB) are noted on the figure. Note the different ordinate scales. In this figure also, the average value of the resting ACh release (45–50 and 50–55 min samples) was subtracted from the subsequent K^+-stimulated samples before the mean was computed.

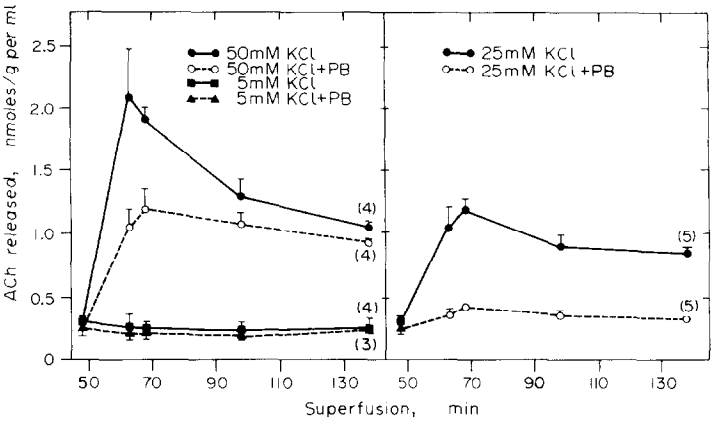

Fig. 3. Effect of pentobarbital on ACh release from superfused cerebral cortex slices. Slices of rat cerebral cortex were prepared and superfused as described in Methods in a medium containing 1×10^{-5} M paraoxon as the cholinesterase inhibitor. After 50 min of superfusion with 5 mM KCl medium with or without 5×10^{-4} M pentobarbital (PB), the superfusing medium was changed to a medium containing 25 or 50 mM KCl and 5×10^{-4} M pentobarbital as indicated. Samples of superfusate were collected and assayed for ACh as described in Methods. Results are means ± S. E. M. for the number of samples given in parentheses. The lines describing unstimulated release (5 mM KCl ± pentobarbital) can also be compared with the data in the right panel but were not reproduced there to avoid confusion. The statistical significance of differences between various treatments is indicated in the text.

midbrain but did not change ACh levels in this region after administration *in vivo*. However, ACh tissue levels and release were determined in quite different circumstances which it might not be valid to compare. In order to compare tissue levels and release in one system, we looked at the change in tissue ACh after stimulating ACh release *in vitro*. We did this using the organophosphate derivative paraoxon instead of eserine as the cholinesterase inhibitor, since no decrease in cerebral cortex ACh during K^+-stimulation occurred in the presence of another organophosphate inhibitor, soman, in contrast to eserine [23]. However, we found that when the tissue was continuously superfused in the presence of paraoxon, decreases in tissue ACh were seen in the cortex after K^+-stimulation but not in the midbrain (see Fig. 5).

When the cerebral cortex was superfused in the presence of paraoxon, 5×10^{-4} M pentobarbital inhibited the release of ACh by 50 mM KCl (Fig. 3). Stimulation with 25 mM KCl released less ACh than 50 mM KCl, and pentobarbital in combination with 25 mM KCl resulted in very little ACh release although the values at each time point were significantly different by the *t*-test from unstimulated release in the presence of pentobarbital (Fig. 3).

After 50 min of superfusion with medium containing 5 mM KCl there were 24.3 nmoles ACh/g in the cortex and this value did not change after an additional 90 min of superfusion (see Fig. 5). When 5×10^{-4} M pentobarbital was present in the 5 mM KCl medium, the cortical ACh decreased transiently (after 50 min superfusion) but returned to the control level after a further 90 min of superfusion. Stimulation with 50 mM KCl reduced the amount of ACh in the cortex to 10.6 nmoles/g, and the blockade of ACh release produced by pentobarbital in this situation (Fig. 3) corresponded to a small but statistically insignificant increase in tissue ACh (see Fig. 5). When cortex slices were stimulated with 25 mM KCl, the tissue ACh content decreased compared to control, but the decrease was not as great as that caused by 50 mM KCl. In this situation of lesser stimulation, in which the barbiturate inhibited release more extensively, the barbiturate significantly increased tissue ACh so that

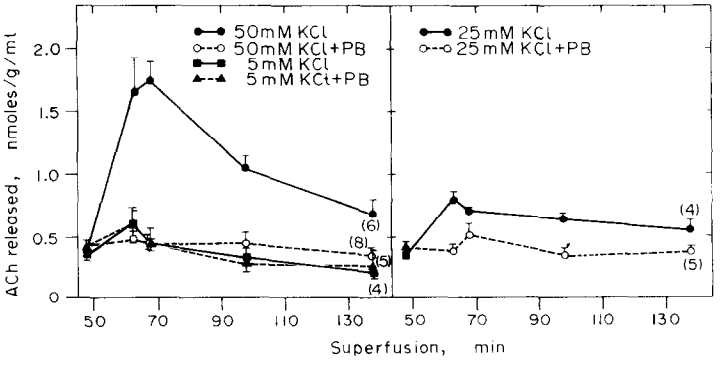

Fig. 4. Effect of pentobarbital on ACh release from superfused midbrain slices. Details are as in Fig. 3.

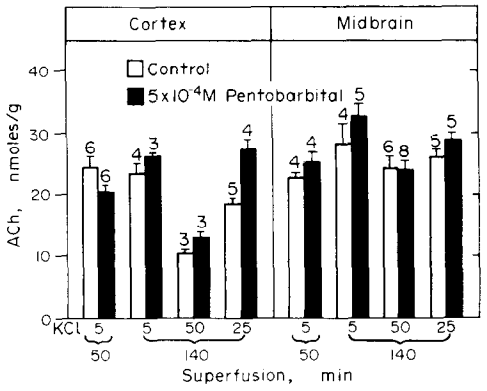

Fig. 5. Effect of pentobarbital and potassium stimulation on superfused cerebral cortex and midbrain slices. Slices were collected from the Swinnex after the indicated length and conditions of superfusion in paraoxon-containing medium and were assayed for ACh as described in Methods. Values are the means ± S. E. M. for the number of samples given above each bar. The statistical significance of differences between various treatments is indicated in the text.

it was equal to the level in control tissue. Thus, treatment of cortex with pentobarbital during stimulation with 25 mM KCl blocked ACh release and at the same time increased cortical ACh back to unstimulated levels.

In the midbrain superfused in the presence of paraoxon, 50 mM KCl caused a slightly smaller release of ACh compared to that seen in the cortex (compare Figs. 3 and 4). A similar difference was also seen when the stimulation was done in the presence of eserine (Fig. 1). A much greater difference in ACh release from the two brain regions was observed in response to 25 mM KCl which released very little ACh from the midbrain (Fig. 4, compare with Fig. 3). Pentobarbital completely blocked the 50 mM KCl-induced release of ACh from the midbrain as well as the small release caused by 25 mM KCl (Fig. 4).

In the midbrain after 140 min of superfusion in medium containing 5 mM KCl, there was no statistically significant increase in tissue ACh compared with 50 min superfusion (Fig. 5). At both 50 and 140 min, 5×10^{-4} M pentobarbital did not change the ACh content of unstimulated tissue. In contrast to the cortex, stimulation of ACh release from the midbrain with 50 mM KCl did not cause a large or statistically significant decrease in tissue ACh levels, nor was there a decrease in tissue ACh after treatment with 25 mM KCl. While pentobarbital blocked ACh release from the midbrain induced by 25 or 50 mM KCl there was no corresponding rise in tissue ACh levels such as was seen in the cerebral cortex when a 25 mM KCl-induced release was blocked by pentobarbital (Fig. 5).

DISCUSSION

Pentobarbital given *in vivo* increased ACh in the cerebral cortex, striatum and hippocampus, had a small but significant effect in the pons-medulla, and did not change ACh levels in the midbrain or cerebellum. These results are in general agreement with other reports in which the effects of barbiturates on ACh in different brain regions were compared [24–26]. In order to test the effect of pentobarbital on ACh release from these brain regions, we used a superfusion system *in vitro*. Potassium-stimulated release of ACh *in vitro* was inhibited by the drug in all regions.

It is recognized that the concentration of pentobarbital used *in vitro* was relatively high. A weighted average of the brain levels of pentobarbital 30 min after 50 mg/kg i.p. was 0.16 μmole/g or approximately 2×10^{-4} M. Goldstein and Aronow [27] found that at equilibrium pentobarbital *in vivo* achieved a ratio of 3 between brain tissue and plasma water and this figure agrees with our findings in our superfusion system *in vitro*. Thus, to mimic the situation *in vivo* during superfusion *in vitro* with salt solutions, 5×10^{-5} M pentobarbital would be more appropriate than 5×10^{-4} M. From Fig. 2 it can be seen that this former concentration would be ineffective in inhibiting K^+-stimulated ACh release in the striatum since 1×10^{-4} M did not inhibit release. In the cerebral cortex, however, 1×10^{-4} M significantly inhibits release and 5×10^{-5} M might also have an effect. The midbrain appeared to be more sensitive than the cortex (see Figs. 1, 3 and 4), such that 5×10^{-5} M might inhibit ACh release in this region. Differences between states *in vitro* and *in vivo*, however, make it difficult to compare effective concentrations exactly.

The cerebral cortex and the midbrain were studied further as examples of regions in which an increase and no increase in ACh levels occurred after administration *in vivo* of pentobarbital. While inhibiting ACh release from both regions *in vitro*, the barbiturate increased tissue ACh levels only in the cortex. These data suggest that, *in vivo* as well, there may be an inhibition of ACh release in midbrain regions after pentobarbital even though no change in tissue levels of ACh is seen. Therefore, there need not necessarily be a contradiction, suggested by Nordberg and Sundwall [26], between the lack of effect of barbiturates on brain stem ACh levels and the proposal that this is a primary site of action of these agents [28].

The data reported here also demonstrate a difference in the regulation of tissue levels of ACh in the cerebral cortex and the midbrain in several ways. While the levels of ACh found after near-freezing (and presumably occurring *in vivo*) were lower in the cerebral cortex than in the midbrain (see Table 1), after decapitation and superfusion for 50 min in physiological medium, the content of ACh in the two regions was nearly identical (see Fig. 5). Stimulation by 50 mM KCl released a similar amount of ACh from both regions, 36.3 nmoles/h/90 min from cortex and 32.4 nmoles/g/90 min from midbrain (calculated by the area under the curves, Figs. 3 and 4). However, this release was accompanied by a decrease in the cortex ACh content (12.8 nmoles/g or a 55 per cent decrease), whereas there was no change in ACh in the midbrain. Pentobarbital more effectively blocked ACh release from the midbrain than from the cortex (seen particularly during stimulation with 50 mM KCl), but increased tissue ACh only in the cortex (seen particularly during stimulation with 25 mM KCl).

These data on the cerebral cortex confirm the findings of Szerb *et al.* [29] that the tissue content of ACh and the release of ACh are inversely related in the cat cerebral cortex after various treatments including pentobarbital. However, the data presented here suggest that tissue ACh levels are more readily altered in the cerebral cortex than in the midbrain and that synthesis and release of ACh are not coupled in the same way in these two brain regions. Regional differences in the utilization of choline for phosphatidyl choline synthesis [30], and the turnover of ACh [31] also support the possibility of differences in the regulation of the cholinergic system in different brain regions that may not be solely related to the number of cholinergic terminals in these regions. While differences in the maximum velocity for high affinity choline uptake in different brain regions [32, 33] may be ascribed to a variation in the number of cholinergic nerve endings [33], alterations in the V_{max} by various treatments [34] suggest this may not be the only possible explanation.

Other regional differences in the effects of pentobarbital on the cholinergic system in addition to those described here have also been found. For example, while pentobarbital decreased turnover or utilization of ACh in whole brain [35–37], when different regions of the rat brain were examined, the barbiturate decreased ACh turnover in the cortex but not in the striatum [37]. An increase in striatal ACh content and a reduction in the conversion of choline to ACh in the striatum were observed, however. Pentobarbital administered *in vivo* has also been shown to inhibit sodium-dependent high affinity choline uptake (measured *in vitro*) in the hippocampus but not in the striatum even though pentobarbital increased ACh in both regions [37–39].

If there are differences in ACh regulation in different brain regions, several mechanisms might be considered. It is possible that there are intrinsic differences in cholinergic neurons of a qualitative or a quantitative nature. The differences we have found and others mentioned above may be manifestations (either separately or conjointly) of a regional specialization of cholinergic neurons. Alternatively, the differences may be the result of regional variations in the input the cholinergic neurons receive from neurons which utilize other transmitters. Among the known putative transmitters, norepinephrine has been shown to stimulate choline uptake [40] and choline acetyltransferase [41], and serotonin was found to inhibit acetylcholinesterase [42] and to stimulate ACh release in certain areas of the hypothalamus [43]. The amount of input from these or other specific neuron types onto the cholinergic neurons and, as a consequence, the degree of responsiveness of the cholinergic neurons to these other transmitter substances may differ in the cortex and the midbrain.

The regional differences found in the effect of pentobarbital on cholinergic function could be explained, therefore, by (1) intrinsic differences in cholinergic neurons; (2) differences in the modulation of cholinergic function in different regions by other neurotransmitters which change the response of cholinergic neurons to pentobarbital; and (3) an effect of pentobarbital on noncholinergic neurons which then results in an indirect effect of the drug on the cholinergic system dependent on the connections existing in different regions of the brain. These possibilities are not entirely exclusive. If, as Blaustein and Ector [44] have suggested, the barbiturates act by interfering with calcium uptake during the release process, they would be expected to inhibit the calcium-dependent release of all transmitters. This action might lead to both direct and indirect effects on cholinergic function.

Acknowledgements—This work was supported by USPHS Grant DA-60796-02 and a Postdoctoral Research Training Grant (TO1-MH-10695-10) to M. B. W. The excellent technical assistance of Mr. Kenneth Ramsey is gratefully acknowledged.

REFERENCES

1. J. M. Tobias, M. A. Lipton and A. Lepinat, *Proc. Soc. exp. Biol. Med.* **61**, 51 (1946).
2. D. Richter and J. Crossland, *Am. J. Physiol.* **159**, 247 (1949).
3. N. J. Gairman and G. Pepeu, *Br. J. Pharmac. Chemother.* **19**, 226 (1962).
4. J. Crossland and P. Slater, *Br. J. Pharmac. Chemother.* **33**, 42 (1968).
5. L. Beani, C. Bianchi, P. Megazzini, L. Ballotti and G. Bernardi, *Biochem. Pharmac.* **18**, 1315 (1969).
6. F. C. MacIntosh and P. E. Oborin, *Nineteenth Int. Physiol. Cong.* (abstr.), p. 580. (1953).
7. J. F. Mitchell, *J. Physiol., Lond.* **165**, 98 (1963).
8. G. Pepeu and A. Bartolini, *Eur. J. Pharmac.* **4**, 254 (1968).
9. E. K. Matthews and J. P. Quilliam, *Br. J. Pharmac. Chemother.* **22**, 415 (1964).
10. D. S. Grewaal and J. H. Quastel, *Biochem. J.* **132**, 1 (1973).
11. H. Kalant and W. Grose, *J. Pharmac. exp. Ther.* **158**, 386 (1967).
12. J. Crossland, *J. ment. Sci.* **99**, 247 (1953).
13. M. Sharkawi, *Br. J. Pharmac.* **40**, 86 (1970).
14. H. Rommelspacher, A. M. Goldberg and M. J. Kuhar, *Neuropharmacology* **13**, 1015 (1974).
15. J. A. Richter and A. Goldstein, *J. Pharmac. exp. Ther.* **175**, 685 (1970).
16. R. Takahashi and M. H. Aprison, *J. Neurochem.* **11**, 887 (1964).
17. R. Kuntzman, M. Ikeda, M. Jacobson and A. H. Conney, *J. Pharmac. exp. Ther.* **157**, 220 (1967).
18. J. A. Richter, *J. Neurochem.* **26**, 791 (1976).
19. A. M. Goldberg and R. E. McCaman, *J. Neurochem.* **20**, 1 (1973).
20. J. A. Richter and P. A. Shea, *J. Neurochem.* **23**, 1225 (1974).
21. J. P. Hamerstad and R. W. P. Cutler, *Brain Res.* **47**, 401 (1972).
22. H. Lund-Anderson, *Brain Res.* **65**, 239 (1974).
23. M. M. Bertels-Meeuws and R. L. Polak, *Br. J. Pharmac. Chemother.* **33**, 368 (1968).
24. A. Bartolini, R. Bartolini and E. F. Domino, *Neuropharmacology* **12**, 15 (1973).
25. R. Longoni, A. Mulas and G. Pepeu, *Br. J. Pharmac.* **52**, 429P (1974).
26. A. Nordberg and A. Sundwall, in *Cholinergic Mechanisms* (Ed. P. G. Waser), p. 229. Raven Press, New York (1975).
27. A. Goldstein and L. Aronow, *J. Pharmac. exp. Ther.* **128**, 1 (1960).
28. E. K. Killam, *Pharmac. Rev.* **14**, 175 (1962).
29. J. C. Szerb, H. Malik and E. G. Hunter, *Can. J. Physiol. Pharmac.* **48**, 780 (1970).
30. G. B. Ansell and S. Spanner, *Biochem. Pharmac.* **24**, 1719 (1975).

31. G. Racagni, D. L. Cheney, M. Trabucchi, C. Wang and E. Costa, *Life Sci.* **15,** 1961 (1975).
32. H. I. Yamamura and S. N. Snyder, *J. Neurochem.* **21,** 1355 (1973).
33. P. T. Carroll and G. G. Buterbaugh, *J. Neurochem.* **24,** 229 (1975).
34. J. R. Simon, S. Atweh and M. J. Kuhar, *J. Neurochem.* **26,** 909 (1976).
35. E. F. Domino and A. E. Wilson, *Psychopharmacologia* **25,** 291 (1972).
36. S.-M. Aquilonius, F. Flentge, J. Schuberth, B. Sparf and A. Sundwall, *J. Neurochem.* **20,** 1509 (1973).
37. M. Trabuci, D. L. Cheney, G. R. Racagni and E. Costa, *Pharmac. Res. Commun.* **7,** 81 (1975).
38. J. R. Simon and M. J. Kuhar, *Nature, Lond.* **255,** 162 (1975).
39. S. Atweh, J. R. Simon and M. J. Kuhar, *Life Sci.* **17,** 1535 (1976).
40. P. T. Carroll and G. G. Buterbaugh, *J. Neurochem.* **24,** 917 (1975).
41. A. K. S. Ho, G. Singer and S. Gershon, *Psychopharmacologia* **21,** 238 (1971).
42. M. H. Aprison, *Recent Adv. biol. Psychiat.* **4,** 133 (1962).
43. R. D. Myers and M. B. Waller, *Brain Res.* **84,** 47 (1975).
44. M. P. Blaustein and A. C. Ector, *Molec. Pharmac.* **11,** 369 (1975).