AN ANALYSIS OF THE INFLUENCE OF ANTIMUSCARINIC AGENTS ON SYNTHESIS, STORAGE AND RELEASE OF ACETYLCHOLINE BY CORTICAL SLICES FROM RAT BRAIN

STELLINGEN

I

Via hetzelfde mechanisme als in dit proefschrift is gepostuleerd t.a.v. de stimulerende werking van atropine op de afgifte van acetylcholine in de hersenen, vergroot phenoxybenzamine de afgifte van noradrenaline uit de milt, de darm en de skeletspier. Dit effect van phenoxybenzamine komt misschien mede tot stand via blokkade van de wederopname van vrijgekomen noradrenaline, maar dan slechts voor een klein gedeelte.

S. M. Kirpekar & A. R. Wakade, Br. J. Pharmac. 39, 533, (1970)
J. Häggendahl, Bayer-Symposium II, 100, (1970)

II

De door Andén et al. en door Sanghvi & Gershon op proefdieren waargenomen effecten van 5-hydroxytryptophaan zouden kunnen worden verklaard uit desensibilisatie van centrale serotoninereceptoren door een overmaat aan vrije serotonine.

N. E. Andén, H. Corrodi, K. Fuxe & T. Hökfelt, Br. J. Pharmac. 34, 1, (1968)
I. Sanghvi & S. Gershon, Europ. J. Pharmac. 11, 125, (1970)

III

Bij zijn bespreking van de mogelijke hypothesen ter verklaring van de door oxotremorine opgewekte toename van het acetylcholinegehalte van de hersenen, heeft Holmstedt ten onrechte de mogelijkheid buiten beschouwing gelaten dat dit effect berust op een remming van de afgifte van acetylcholine uit de zenuwuiteinden.

B. Holmstedt, Actualités Pharmacologiques 23, 175, (1970)
J. Schuberth, B. Sparf & A. Sundwall, J. Neurochem. 16, 695, (1969)

IV

De bewering van Kirkpatrick & Lomax dat atropine na injectie in de area preoptica van de rat een hyperthermie veroorzaakt, wordt niet gestaafd door hun eigen observaties.

W. E. Kirkpatrick & P. Lomax, Life Sci 6, 2273, (1967)

V

De door Schuberth et al. beschreven methode om de turnover van acetylcholine in de hersenen te meten, is ondeugdelijk.

J. Schuberth, B. Sparf & A. Sundwall, J. Neurochem. 16, 695, (1969) en
J. Neurochem. 17, 461, (1970)

VI

Chiou & Long stellen dat het aangrijpingspunt van nicotine-achtige stoffen gelocaliseerd is op de cholinerge zenuwuiteinden van het geïsoleerde caviaileum; zij suggereren ten onrechte dat het antogonisme van triëthylcholine t.o.v. deze stoffen een argument is voor deze stelling.

C. Y. Chiou & J. P. Long, Arch. int. Pharmacodyn. 182, 269, (1969)

VII

Roberts & Thesleff hebben gevonden dat de hoeveelheid voor afgifte beschikbare acetylcholine in de motorische zenuwuiteinden van de met neostigmine behandelde rat is verminderd. Dit zou kunnen worden veroorzaakt doordat de hydrolyse van afgegeven acetylcholine door de neostigmine wordt geremd en dientengevolge minder choline in de synaptische spleet aanwezig is om door de zenuwuiteinden te worden opgenomen.

D. V. Roberts & S. Thesleff, Europ. J. Pharmac. 6, 281, (1969)

VIII

Het verdient aanbeveling aan epileptici die voortdurend anti-epileptica innemen, extra vitamine D voor te schrijven.

C. E. Dent, A. Richens, D. J. F. Rowe & T. C. B. Stamp, Brit. Med. J. IV, 69, (1970)
A. Richens & D. J. F. Rowe, Brit. Med. J. IV, 73, (1970)
R. Kruse, Monatschrift für Kinderheilkunde 116, 378, (1968)

IX

Een arts die zijn eigen normen aan zijn patiënten oplegt, functioneert op dat moment gebrekkig als arts.

X

Het aantrekken van gastarbeiders uit een sociaal-economisch onderontwikkeld land is slechts moreel verantwoord, indien het gastland deze arbeiders opleidt tot kader in een ontwikkelingsproject.

XI

Aangezien veel modern experimenteel onderzoek een produkt is van teamwork dient men als team te kunnen promoveren.

R. L. Polak

Leiden, 13 januari 1971.

AN ANALYSIS OF THE INFLUENCE OF ANTIMUSCARINIC AGENTS ON SYNTHESIS, STORAGE AND RELEASE OF ACETYLCHOLINE BY CORTICAL SLICES FROM RAT BRAIN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR C. SOETEMAN, HOOGLERAAR IN DE FACULTEIT DER LETTEREN, TEN OVERSTAAN VAN EEN COMMISSIE UIT DE SENAAT TE VERDEDIGEN OP WOENSDAG 13 JANUARI 1971 TE KLOKKE 16.15 UUR

DOOR

ROBERT LEOPOLD POLAK

GEBOREN TE AMSTERDAM IN 1925

1971

PRINTED BY DRUKKERIJ DE BIJ - AMSTERDAM

PROMOTOR: PROFESSOR DR E. M. COHEN

CONTENTS

INTRODUCTION

Acetylcholine release and central nervous activity. Certain forms of activation and inhibition of the cerebral cortex are probably mediated by acetylcholine (ACh). Variations in the amounts of ACh in the brain (Tobias, Lipton & Lepinat, 1946 ; Richter & Crossland, 1949 ; Elliott, Swank & Henderson, 1950) and in the cerebral cortex (Pepeu & Mantegazzini, 1964) are correlated with variations in central nervous activity. As a rule the amounts of "stored" or "bound" ACh in the brain are increased during anaesthesia or sleep as compared to those found during wakefulness, whereas a marked decrease occurs during convulsive seizures.

The amounts of ACh diffusing into a saline solution placed on the pial surface of the brain after topical inhibition of the cholinesterase (Elliott, Swank & Henderson, 1950 ; MacIntosh & Oborin, 1953) are correlated with the activity of the animal. In anaesthetized animals less ACh enters the solution when the depth of anaesthesia is increased (MacIntosh & Oborin, 1953 ; Mitchell, 1963) while ACh release rises to very high values during convulsions (Mitchell, 1963 ; Beleslin, Polak & Sproull, 1965 ; Celesia & Jasper, 1966). The release of ACh from the cerebral cortex of the waking animal decreases when the animal falls asleep (Celesia & Jasper, 1966) and increases when it explores its surroundings, eats, drinks or plays (Collier & Mitchell, 1967). Apparently increased cerebrocortical activity induces an augmentation of the release of ACh at the expense of the amounts of ACh stored in the tissue.

Possible pathways involved in ACh release from the cerebral cortex. Stimulation of a peripheral nerve in the anaesthetized cat, rabbit and sheep produces a widespread increase in the release of ACh from the surface of the cerebral cortex and a larger, but regionally restricted, release from the contralateral somatosensory receiving area of the cortex (Mitchell, 1963 ; Kanai & Szerb, 1965). Similarly, stimulation of the retinae by light or electrical stimulation of the lateral geniculate body in the anaesthized rabbit evokes an augmentation of the amounts of ACh released from the whole cerebral cortex and an additional increase from the visual cortex (Collier & Mitchell, 1966, 1967). Analogous results have been observed when the auditory pathways were stimulated (Neal, Hemsworth & Mitchell, 1968). Two ascending cholinergic pathways are held responsible for these effects.

The widespread release of ACh has been ascribed to activation of the non-specific reticulo-cortical system which may be concerned with the transition from sleep to wakefulness and with the maintenance of consciousness.

Electrical stimulation of the mesencephalic reticular formation leads to the "arousal response" of the electrocorticogram characterized by fast asynchronous low voltage activity normally associated with the waking state and with certain stages of sleep ("rapid eye movements sleep" or "REM sleep") accompanied by rapid eye movements, changes in muscle tone and variations in the frequencies of heart beat and respiration (Jouvet, 1961). Reticular stimulation also leads to an increase in the release of ACh from the convexity of the brain (Mitchell, 1963 ; Kanai & Szerb, 1965 ; Phillis & Chong, 1965 ; Celesia & Jasper, 1966). In general accordance with this is the work of Shute & Lewis (1963, 1967), who studied the histochemical distribution of acetylcholinesterase in the brain. They described presumably cholinergic pathways from the ventral tegmental area and substantia nigra to the subthalamus, hypothalamus, basal ganglia and cortex. This does not imply, of course, that ACh is the only "transmitter" or "modulator" substance released in association with the arousal response. In fact it has been reported that the liberation of glutamic acid is also increased when the mesencephalic reticular formation is stimulated (Jasper & Koyama, 1967, 1969).

The pathway concerned with the large augmentation of ACh release from the primary sensory areas of the cortex on stimulation of a peripheral nerve or primary sensory system probably originates in the specific thalamic nuclei. It may be the pathway responsible for the phenomena of repetitive afterdischarge and augmenting responses (Collier & Mitchell, 1966, 1967 ; Brownlee & Mitchell, 1968) as ACh tends to promote repetitive discharges of units in primary sensory cortical areas in response to single stimuli of specific thalamic nuclei (Krnjević & Phillis, 1963). It may serve to alter the local excitability of a cortical area after arrival of a primary afferent signal, which is probably not cholinergically transmitted (Collier & Mitchell, 1966 ; Krnjević, 1967). However, Phillis (1968) has offered an alternative explanation for the extra augmentation of ACh release from the primary cortical receiving areas observed by Mitchell and co-workers. He suggested that local intracortical cholinergic circuits might be activated by the primary afferent signal. In this connection it is relevant that histochemical investigations by Krnjević & Silver (1965) have revealed the presence of cholinesterase containing arcuate fibres in the cortex of the cat which link adjacent gyri and which probably originate from cells in the cortex. They also may give rise to ascending collaterals which end as parallel fibres lying in the most superficial layer of the cortex (Krnjević, 1967).

Influence of muscarinic, anticholinesterase and antimuscarinic substances on e.e.g. and behavior. Systemically injected muscarinic or anticholin-

esterase agents and antimuscarinic drugs profoundly influence electrocortical activity.

After injection of physostigmine or di*iso*propylphosphorofluoridate into the resting or sleeping animal the e.e.g. is activated and a pattern of fast asynchronous low voltage activity (arousal) occurs (Bradley & Elkes, 1953 ; Bradley, Cerquiglini & Elkes, 1953). As mentioned, this e.e.g. pattern normally is associated either with waking behavior or with REM sleep. According to Domino, Yamamoto & Dren (1968) the sleeping cat wakes up after an injection of pilocarpine or physostigmine, but after a certain interval a drowsy state appears which rapidly changes into REM sleep.

The arousal response of the e.e.g. evoked by cholinomimetic or anticholinesterase agents can be blocked by antimuscarinic drugs. Antimuscarinic drugs produce a pattern of the electrocorticogram characteristic for sleep showing high voltage slow waves interrupted by bursts of fast activity ("spindle bursts"). However, in the atropine injected animal this e.e.g. pattern is not necessarily associated with sleep (Wikler, 1952 ; Bradley & Elkes, 1953). Thus atropine disrupts the normal association between wakefulness and asynchronous fast low voltage activity of the e.e.g.. This "dissociation" (Wikler, 1952, 1968) of the electrocorticogram from the behavior has been the object of much controversy. Fink (1968) has even gone so far as to suggest that this "dissociation" may be due to limitations in techniques of observation and quantification, problems of terminology and species differences.

Influence of anticholinesterase and antimuscarinic agents on e.e.g. and ACh release. Celesia & Jasper (1966) studied the effect of neostigmine and atropine on the connections between electrocortical activity and ACh release from the cortex. It seems worth-while to present here their results in some detail. They irrigated the postcruciate area of the cortical surface of the unanaesthetized dozing cat with a neostigmine solution, measured the ACh release from this area, and recorded the electrical activity of the cortex inside and outside the perfusion chamber.

It was found that superfusion with neostigmine caused a gradual change in the electrical activity of the area of cortex beneath the irrigation chamber. Within forty to sixty minutes the slow waves and spindles of the drowsy or sleeping animal were replaced by low voltage, fast asynchronous activity simulating the arousal pattern, though the animal continued to be asleep, and the e.e.g. from other brain areas continued to show the sleep pattern. When the irrigation of the cortical surface was pursued for one to two hours local epileptiform discharge occured in five out of ten experiments. However, neither the localized cortical arousal nor the epileptiform discharge,

produced by neostigmine, significantly altered ACh output, provided the animal remained in a stable state of light sleep. This contrasted with the increased ACh release obtained when the e.e.g. was desynchronized by electrical stimulation of the mesencephalic reticular formation or when sustained seizure discharge was induced by the intravenous injection of leptazol. Although not explicitly stated by the authors these observations suggest that the localized activation of the cortex by neostigmine resulted from a direct stimulation of intracortical cholinoceptive — but not cholinergic — neurons, whereas the increased liberation of ACh under the influence of reticular stimulation or intravenous injection of leptazol was caused by the activation of afferent cholinergic axons probably impinging on these neurons.

Moreover, in the same experiments intravenous atropine administration readily blocked not only the local desynchronization of the electrocorticogram and the local epileptiform discharge induced by the neostigmine, but also the desynchronized activation resulting from reticular stimulation. This supports the idea of a cholinergic link in the genesis of these phenomena. Atropine, however, did not block the convulsions produced by leptazol which apparently induced a massive discharge of predominantly non-cholinergic neurons in the brain. The most striking finding was the fact that atropine strongly enhanced the release of ACh from the resting cortex as well as the increased release produced by electrical stimulation of the reticular formation or by injection of leptazol. Whereas all the other effects of atropine easily can be explained by blockade of postsynaptic ACh receptors, the enhancement of ACh release under the influence of atropine is more difficult to understand. Celesia & Jasper suggested that this atropine effect might result from interference with ACh binding mechanisms which they believed to account for a large part of ACh inactivation apart from its hydrolysis by cholinesterase. Finally, they reported the following very puzzling observation. Direct electrical stimulation of the cortex, sufficiently strong to set up a sustained epileptiform after-discharge of the e.e.g., markedly enhanced ACh release in animals which had received atropine, but did not influence ACh release in non-atropinized animals.

The finding that atropine enhances the release of ACh from the cerebral cortex is in agreement with results earlier obtained on the cat, the rabbit and the sheep by MacIntosh & Oborin (1953), Mitchell (1963) and Szerb (1964). The effect is not restricted to the cerebral cortex as atropine and hyoscine also stimulate the release of ACh from the caudate nucleus of the cat (Polak, 1965). A possible counterpart of this increased release of ACh in the brain has been reported by Giarman & Pepeu (1962, 1964). They found a decrease of the extractable ACh content of the cerebral cortex of the rat under

the influence of antimuscarinic drugs. These findings in combination with those presented in this thesis suggest that antimuscarinic drugs stimulate the turnover of ACh in the brain. In this context it is interesting to note that there may exist an analogy with catecholamine metabolism; substances which block either noradrenaline or dopamine receptors in the brain have been reported to stimulate the turnover of noradrenaline or dopamine, respectively (Carlsson, 1969; Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970).

The stimulating action of atropine on ACh release in the brain has been observed in different species. Since its mechanism was not known, I thought it worth-while to make an attempt at its elucidation. First I tried to reproduce the atropine effect *in vitro* in the expectation that this would facilitate its pharmacological analysis. We succeeded in demonstrating a stimulating effect of atropine on the release of ACh from cortical slices from rat brain resembling the atropine effect *in vivo*. A few possible explanations of the atropine effect (MacIntosh, 1963; Giarman & Pepeu, 1964; Szerb, 1964; Polak, 1965; Celesia & Jasper, 1966) were investigated and excluded as far as the effect of atropine on cortical slices was concerned. A new hypothesis assuming the existence of presynaptic muscarine receptors involved in a negative feed back mechanism regulating the release of ACh was formulated and the atropine effect was explained by blockade of these receptors. Some evidence in favour of this theory was adduced. This analysis has been published in a number of papers; five of these are collected in this thesis.

REFERENCES

ANDÉN, N. E., BUTCHER, S. G., CORRODI, H., FUXE, K. & UNGERSTEDT, U. (1970). Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Europ. J. Pharmac.* **11**, 303-314.

BELESLIN, D., POLAK, R. L. & SPROULL, D. H. (1965). The effect of leptazol and strychnine on the acetylcholine release from the cat brain. *J. Physiol., Lond.*, **181**, 308-316.

BRADLEY, P. B., CERQUIGLINI, C. & ELKES, J. (1953). Some effects of di*iso*propylfluorophosphate on the electrical activity of the brain in the cat. *J. Physiol., Lond.*, **121**, 51-52*P*.

BRADLEY, P. B. & ELKES, J. (1953). The effect of atropine, hyoscyamine, physostigmine and neostigmine on the electrical activity of the brain of the conscious cat *J. Physiol., Lond.*, 120, 14*P*.

BROWNLEE, W. C. & MITCHELL, J. F. (1968). The pharmacology of cortical repetitive after-discharges. *Br. J. Pharmac. Chemother.* **33**, 217-218*P*.

CARLSSON, A. (1969). Pharmacology of monoamine transmission. *Progress in brain research, mechanisms of synaptic transmission*, vol. **31**, pp. 53-59. Ed. by K. Akert & P. G. Waser, Amsterdam: Elsevier.

CELESIA, G. G. & JASPER, H. H. (1966). Acetylcholine released from cerebral cortex in relation to state of activation. *Neurology*, **16**, 1053-1063.

COLLIER, B. & MITCHELL, J. F. (1966). The central release of acetylcholine during stimulation of the visual pathway. *J. Physiol., Lond.*, **184**, 239-254.
COLLIER, B. & MITCHELL, J. F. (1967). The central release of acetylcholine during consciousness and after brain lesions. *J. Physiol., Lond.*, **188**, 83-98.
DOMINO, E. F., YAMAMOTO, K. & DREN, A. T. (1968). Role of cholinergic mechanisms in states of wakefulness and sleep. *Progress in brain research, anticholinergic drugs and brain functions in animals and man,* vol. **28**, pp. 113-133. Ed. by P. B. Bradley and M. Fink, Amsterdam: Elsevier.
ELLIOTT, K. A. C., SWANK, R. L. & HENDERSON, N. (1950). Effects of anaesthetics and convulsants on acetylcholine content of brain. *Am. J. Physiol.* **162**, 469-474.
FINK, M. (1968). Brain, behavior and anticholinergic drugs. *Progress in brain research, anticholinergic drugs and brain functions in animals and man,* vol. **28**, pp. XII-XVI. Ed. by P. B. Bradley & M. Fink, Amsterdam: Elsevier.
GIARMAN, N. J. & PEPEU, G. (1962). Drug-induced changes in brain acetylcholine. *Br. J. Pharmac. Chemother.* **19**, 226-234.
GIARMAN, N. J. & PEPEU, G. (1964). The influence of centrally acting cholinolytic drugs on brain acetylcholine levels. *Br. J. Pharmac. Chemother.* **23**, 123-130.
JASPER, H. H. & KOYAMA, I. (1967). Rate of release of acetylcholine and glutamic acid from the cerebral cortex during reticular activation. *Fed. Proc.* **26**, 373.
JASPER, H. H. & KOYAMA, I. (1969). Rate of release of amino acids from the cerebral cortex in the cat as affected by brainstem and thalamic stimulation. *Can. J. Physiol. Pharmac.* **47**, 889-905.
JOUVET, M. (1961). Telencephalic and rhombencephalic sleep in the cat. *Ciba Foundation Symposium on the nature of sleep,* pp. 188-208. Ed. G. E. W. Wolstenholme & W. O'Connor, London: J. & A. Churchill Ltd.
KANAI, T. & SZERB, J. C. (1965). Mesencephalic reticular activating system and cortical acetylcholine output. *Nature* **205**, 80-82.
KRNJEVIĆ, K. (1967). Chemical transmission and cortical arousal. *Anesthesiology* **28**, 100-105.
KRNJEVIĆ, K. (1969). Central cholinergic pathways. *Fed. Proc.* **28**, 113-120.
KRNJEVIĆ, K. & PHILLIS, J. W. (1963). Acetylcholine-sensitive cells in the cerebral cortex. *J. Physiol., Lond.*, **166**, 296-327.
KRNJEVIĆ, K. & SILVER, A. (1965). A histochemical study of cholinergic fibres in the cerebral cortex. *J. Anat.* **99**, 711-759.
MACINTOSH, F. C. (1963). Synthesis and storage of acetylcholine in nervous tissue. *Can. J. Biochem. Physiol.* **41**, 2555-2571.
MACINTOSH, F. C. & OBORIN, P. E. (1953). Release of acetylcholine from intact cerebral cortex. *Abstr. XIX Int. Physiol. Congr.*, pp. 580-581.
MITCHELL, J. F. (1963). The spontaneous and evoked release of acetylcholine from the cerebral cortex. *J. Physiol., Lond.*, **165**, 98-116.
NEAL, M. J., HEMSWORTH, B. A. & MITCHELL, J. F. (1968). The excitation of central cholinergic mechanisms by stimulation of the auditory pathway. *Life Sci.* **7**, 757-763.
PEPEU, G. & MANTEGAZZINI, P. (1964). Midbrain hemisection: effect on cortical acetylcholine in the cat. *Science* **145**, 1069-1070.
PHILLIS, J. W. (1968). Acetylcholine release from the cerebral cortex: its role in cortical arousal. *Brain Res.* **7**, 378-389.
PHILLIS, J. W. & CHONG, G. C. (1965). Acetylcholine release from the cerebral and cerebellar cortices: its role in cortical arousal. *Nature* **207**, 1253-1255.
POLAK, R. L. (1965). Effect of hyoscine on the output of acetylcholine into perfused cerebral ventricles of cats. *J. Physiol., Lond.*, **181**, 317-323.
RICHTER, D. &. CROSSLAND, J. (1949). Variation in acetylcholine content of the brain with physiological state. *Am. J. Physiol.* **159**, 247-259.
SHUTE, C. C. D. & LEWIS, P. R. (1963). Cholinesterase-containing systems of the brain of the rat. *Nature* **199**, 1160-1164.
SHUTE, C. C. D. & LEWIS, P. R. (1967). The ascending cholinergic reticular system: neocortical, olfactory and subcortical projections. *Brain* **90**, 497-520.
SZERB, J. C. (1964). The effect of tertiary and quaternary atropine on cortical acetylcholine output and on the electroencephalogram in cats. *Canad. J. Physiol. Pharmac.* **42**, 303-314.

TOBIAS, J. M., LIPTON, M. A. & LEPINAT, A. A. (1946). Effect of anaesthetics and convulsants on brain acetylcholine content. *Proc. Soc. exp. Biol., N.Y.*, **61**, 51-54.
WIKLER, A. (1952). Pharmacological dissociation of behavior and EEG "sleep patterns" in dogs: morphine, N-allylmorphine and atropine. *Proc. Soc. exp. Biol. N.Y.* **79**, 261-265.
WIKLER, A. (1968). Introduction. *Progress in brain research, anticholinergic drugs and brain functions in animals and man, vol.* **28**, pp. 1-2. Ed. by P. B. Bradley & M. Fink, Amsterdam: Elsevier.

Reprinted from: Biochemical Pharmacology (1966) **15**, 989-992.

THE INFLUENCE OF ATROPINE ON THE RELEASE AND UPTAKE OF ACETYLCHOLINE BY THE ISOLATED CEREBRAL CORTEX OF THE RAT

R. L. POLAK AND MARIA M. MEEUWS

INTRODUCTION

When a solution of a cholinesterase inhibitor is brought into contact with the cerebral cortex or with the ventricular surface of the caudate nucleus of cats, acetylcholine (ACh) is released into this fluid. Addition of atropine to this solution or its systemic administration produces a strong increase in this output of ACh (Mitchell, 1963; Szerb, 1964; Polak, 1965). It has been suggested that the effect of atropine might be explained by competition between atropine and ACh for certain sites of uptake in the CNS (Giarman & Pepeu, 1964; Szerb, 1964; Polak, 1965). In the present investigation the influence of atropine on the release and uptake of ACh by the rat brain cortex *in vitro* was studied. It was found that atropine enhanced the release of ACh from rat brain cortex slices into the medium in which they were incubated, provided that this medium contained 25 mM KCl. Two additional observations were made: (1) when ACh was added to the medium a considerable uptake of ACh into the slices took place; (2) this uptake could be inhibited by atropine, but the concentrations of atropine needed for this inhibitory action were more than hundred times larger than those in which atropine enhanced the release of endogenous ACh from the tissue. These observations do not seem to support the idea that atropine causes an increase in the output of ACh from the brain by impeding (re)absorption of released ACh.

METHODS

Portions of about 150 mg rat brain cortex slices, pretreated with the cholinesterase inhibitor soman (0·005 mM), were incubated during 1 hr at 37° in 2·5 ml medium saturated with 95% O_2 and 5% CO_2. The composition of this medium was as follows (mM): NaCl, 118·5; $NaHCO_3$, 24·9; KCl, 4·7 or 25; $CaCl_2$, 2·5; KH_2PO_4, 1·2; $MgSO_4$, 1·2; glucose, 10. Unless mentioned otherwise soman (0·005 mM) was present in the medium during the whole experiment. In some experiments atropine, ACh and/or eserine sulphate were added to the medium at the beginning of the incubation period. The brain

cortex slices were about 0·4 mm thick. After 1 hr the tissue was rinsed three times, and extracted with HCl by the method of Elliott, Swank & Henderson (1950). Other samples of tissue were extracted immediately after pretreatment with soman. The ACh activity of the extracts and of the incubating fluids was estimated by bioassay on the eserinised dorsal leech muscle against an ACh chloride standard solution and expressed in terms of this salt. Adequate dilutions of alkali treated and subsequently neutralized (Feldberg, 1945) extracts or dilutions of (modified) Krebs solution with or without atropine or eserine were added to the standard solutions to correct for substances other than ACh which might influence the sensitivity of the assay preparation. In some experiments smaller portions of tissue (about 75 mg) were incubated during a shorter time (30 min) in larger volumes (5 ml) of the medium containing 25 mM KCl.

RESULTS AND DISCUSSION

During incubation of the cerebral cortex slices ACh was set free into the medium. In a medium containing 25 mM KCl about five times as much ACh was released as in a 4·7 mM KCl medium. This confirms the results of Mann, Tennenbaum & Quastel (1939). The ACh content of the tissue did not change during incubation in either medium. Addition of 1 μg/ml atropine sulphate to the 25 mM KCl medium resulted in a further threefold enhancement of ACh release, as well as in a rise of the ACh content of the tissue (Table 1). In other experiments atropine sulphate in a concentration of 0·05 μg/ml also

TABLE 1. THE MEAN CONCENTRATIONS ± S.E. OF ACh IN RAT BRAIN CORTEX SLICES IMMEDIATELY AFTER PRE-INCUBATION WITH SOMAN AND AFTER 1 HR OF INCUBATION IN MEDIA OF DIFFERENT COMPOSITION AND THE MEAN AMOUNTS ± S.E. OF ACh RELEASED

Composition incubation medium	ACh content slices (μg/g)		ACh released into bath (μg/g/60 min)
	immediately after pre-incubation with soman	after 1 hr incubation	
Krebs with KCl 4·7 mM	6·9 ± 0·38 (8)	7·7 ± 0·28 (8)	0·83 ± 0·083 (8)
Krebs with KCl 25 mM		7·5 ± 0·50 (8)	4·3 ± 0·35 (8)
Krebs with 25 mM + atropine (1 μg/ml)		14·3 ± 1·08 (7)	14·1 ± 1·56 (7)

The numbers of experiments are given between brackets. In each experiment about 150 mg tissue was incubated in 2·5 ml medium.

increased the ACh output and 10 μg/ml did not produce a larger effect than 1 μg/ml. No significant atropine effect was observed in a medium with 4·7 mM KCl.

Uptake of added ACh was studied in experiments in which soman treated cerebral cortex slices were incubated in medium containing 4·7 mM KCl or 25 mM KCl or 25 mM KCl + 1 μg/ml atropine. ACh was added to the bath (final concentration 4 μg/ml) at the beginning of the incubation period. The ACh concentrations of the tissue and the medium after one hour were estimated and compared with controls in which rat cerebral cortex had been incubated in the same media but without the addition of ACh (Table 2, columns a, b and c). The results show that there was a considerable uptake of added ACh into the tissue against a concentration gradient and that atropine did not significantly inhibit this uptake in a concentration in which it maximally enhanced the output of endogenous ACh. As a consequence of the uptake the ACh concentration of the medium decreased. This decrease was particularly evident in the experiments in normal Krebs solution in which the endogenous ACh production was relatively small. It was less conspicuous in the medium with 25 mM KCl and least in the 25 mM KCl medium with atropine, in which there was a large endogenous ACh production.

In the columns (d), (e) and (f) of Table 2 the same results as in the columns (a), (b) and (c) respectively are expressed as total amounts of ACh found in the brain tissue and in the medium together. Since in the experiments to which column (f) refers, 10 μg ACh had been added to each incubation mixture, the figures found here should be 10 μg higher than the corresponding figures in column (e). Actually the results show that there was a reasonable recovery of the added ACh. It would appear that the added ACh was distributed in about the same way between the tissue and the medium in all three media, and that the differences in the results were brought about by superposition of this distribution on the changes in the concentration of endogenous ACh in the tissues and the media, produced by the addition of KCl and atropine to the medium.

The effect of atropine sulphate on the uptake of added ACh into slices of cerebral cortex was also studied in a series of experiments in which the concentration of the added ACh was kept practically constant during the time of incubation. This was achieved by incubating smaller amounts of tissue (about 75 mg) in larger volumes (5 ml) during a shorter time (30 min). This precaution facilitated the evaluation of the atropine effects. The experiments were performed in a medium containing 25 mM KCl. In a concentration of 10 μg/ml atropine sulphate inhibited the uptake of ACh into the slices by about 25 per cent and in a concentration as high as 100 μg/ml it

TABLE 2. ACh CONCENTRATIONS AND TOTAL AMOUNTS OF ACh IN SLICES IMMEDIATELY AFTER PRE-INCUBATION WITH SOMAN AND IN SLICES AND MEDIUM AFTER 1 HR INCUBATION OF ABOUT 150 mg RAT BRAIN CORTEX IN 2·5 ml KREBS OF DIFFERENT COMPOSITIONS

	Concentrations of ACh (μg/g and μg/ml)					Total amounts of ACh (μg)			Recovery added ACh
	Before incubation	After 1 hr incubation				Before incubation	After 1 hr incubation		
	(a)	No ACh added (b)		ACh (4μg/ml) added (c)		(d)	No ACh added (e)	10 μg ACh added (f)	(f) — (e)
	slices	slices	medium	slices	medium	slices	slices+medium	slices+medium	
Normal Krebs	6·1	8·5	0·065	31	2·3	0·9	1·3	10·0	8·7
	8·7	8·8	0 047	30	2·3	1·3	1·5	10·2	8·7
	7·0	8·3	0·034	28	2·0	1·0	1·4	9·5	8·1
	7·2	7·9	0·039	34	2·5	1·0	1·3	11·2	9·9
Mean	7·3	8 4	0·046	31	2·3	1·1	1·4	10·2	8·9
25 mM KCl Krebs		8·5	0·21	35	2·9		1·7	12·1	10·4
		9·2	0·22	29	2·5		1·9	10·9	9·0
		7·9	0·19	32	3·4		1·5	12·9	11·4
		7·8	0·20	29	2·8		1·5	11·2	9·7
Mean		8·4	0·21	31	2·9		1·7	11·8	10·1
25 mM KCl Krebs+ Atropine 1 μg/ml		15	1·2	40	3·3		5·2	13·9	8·7
		16	0·60	31	3·6		3·7	13·6	9·9
		14	0·62	40	4·1		3·7	15·7	12·0
		18	0·80	35	3·6		4·4	13·8	9·4
Mean		16	0·81	37	3·7		4·3	14·3	10·0

* Every figure is the result of one experiment.

caused an inhibition of about 70 per cent. This agrees with the findings of Creese and Taylor (1965) who demonstrated uptake of ^{3}H-carbachol into slices of rat brain cortex and inhibition of this uptake by atropine. The fact that in the experiments of these investigators 1 μg/ml of the atropine base, corresponding to 1·2 μg/ml of the sulphate, was about as effective as 10 μg/ml of the sulphate in our experiments might be related to the fact that the concentration of the ^{3}H-carbachol in their experiments was much smaller (0·15 μg/ml) than that of the ACh in our experiments (4 μg/ml).

Other experiments were performed with rat cerebral cortex in a medium containing eserine sulphate (0·4 mM) as the cholinesterase inhibitor, since this substance has been used by most other investigators (Mann *et al.*, 1939; Elliott *et al.*, 1950; Elliott & Henderson, 1951; Mitchell, 1963; Szerb, 1964). The results of these experiments were similar to those obtained with soman, as far as the influence of KCl and atropine on the output of ACh is concerned, but the uptake of added ACh was extremely small and during incubation of cortex tissue in an eserine sulphate medium containing 25 mM KCl with or without atropine the ACh concentration of the tissue fell to about 4 μg/g. In subsequent experiments on soman treated brain cortex slices it appeared that eserine sulphate (0·4 mM) strongly inhibits the uptake of ACh. This explains why Elliott and Henderson (1951) who 15 years ago demonstrated uptake of ACh into brain slices in an eserine sulphate medium, found only a comparatively small effect.

We gratefully acknowledge the many helpful discussions with Dr. E. M. Cohen.

REFERENCES

CREESE, R. & TAYLOR, D. B. (1965). Effect of atropine on the uptake of labeled carbachol by rat brain slices. *Life Sci.* **4**, 1545-1547.

ELLIOTT, K. A. C., SWANK, R. L. & HENDERSON, N. (1950). Effects of anaesthetics and convulsants on acetylcholine content of brain. *Am. J. Physiol.*, **162**, 469-474.

ELLIOTT, K. A. C. & HENDERSON, N. (1951). Factors affecting acetylcholine found in excised rat brain. *Am. J. Physiol.*, **165**, 365-374.

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

GIARMAN, N. J. & PEPEU, G. (1964). The influence of centrally acting cholinolytic drugs on brain acetylcholine levels. *Br. J. Pharmac. Chemother*, **23**, 123-130.

MANN, P. J. G., TENNENBAUM, M. & QUASTEL, J. H. (1939).CIII. Acetylcholine metabolism in the central nervous system; the effects of potassium and other cations on acetylcholine liberation. *Biochem. J.* **33**, 822-835.

MITCHELL, J. F. (1963). The spontaneous and evoked release of acetylcholine from the cerebral cortex. *J. Physiol., Lond.*, **165**, 98-116.

POLAK, R. L. (1965). Effect of hyoscine on the output of acetylcholine into perfused cerebral ventricles of cats. *J. Physiol., Lond.*, **181**, 317-323.

SZERB, J. C. (1964). The effect of tertiary and quarternary atropine on cortical acetylcholine output and on the electroencephalogram in cats. *Can. J. Physiol. Pharmac.*, **42**, 303-314.

Reprinted from: British Journal of Pharmacology and Chemotherapy (1968) **33**, 368-380.

INFLUENCE OF ANTIMUSCARINIC SUBSTANCES ON *IN VITRO* SYNTHESIS OF ACETYLCHOLINE BY RAT CEREBRAL CORTEX

MARIA M. BERTELS-MEEUWS and R. L. POLAK

INTRODUCTION

When a solution of a cholinesterase inhibitor is brought into contact with the exposed cerebral cortex or the ventricular surface of the caudate nucleus of a living animal, acetylcholine (ACh) is liberated into this fluid. There is an increase in the amount of ACh released when atropine is added to the solution or injected intravenously (Mitchell, 1963; Szerb, 1964; Polak, 1965).

In the following experiments the influence of atropine and some atropine-like drugs was studied on the release and synthesis of ACh by slices of cortex from rat brain, treated with a cholinesterase (ChE) inhibitor. In certain conditions atropine was found to enhance the release of ACh from the tissue into the incubation medium and this increased release was associated with an increased synthesis of ACh. Some other antimuscarinic compounds also stimulated ACh output.

Preliminary reports of some of the results of this investigation have been published elsewhere (Polak & Meeuws, 1966 ; Polak, 1967).

METHODS

Female albino rats (160-190 g) were lightly anaesthetized with ether and decapitated. The brains were immediately removed from the skulls and placed in ice-cold oxygenated medium. In the cold room one slice in the dorsal plane was prepared from both hemispheres by means of a Stadie-Riggs microtome and weighed on a torsion balance. The thickness of the slices was about 0·5 mm. Portions of 71-208 mg of tissue were pre-incubated for 60 or 90 min at 37° C in 25 ml vessels containing 2·5 ml of medium to which soman (5×10^{-6}M) had been added in order to inactivate the ChE. Six or seven vessels with slices were incubated simultaneously. In the experiments in which pre-incubation lasted 90 min, the medium was replaced by freshly prepared medium containing soman at the end of each half hour and the tissue was rinsed once before the experiment started. In the latter type of experiments the drug under investigation was present in the medium during the last 30 min of pre-incubation.

At the start of the experiment the pre-incubation medium was replaced by 2·5 or 5 ml of medium to which extra KCl and drugs had or had not been added. At the end of the incubation period, which lasted either 60 or 30 min, the media were collected in graduated centrifuge tubes. The slices were rinsed once and then extracted with HCl for the determination of their ACh ("total extractable ACh") content according to the method of Elliott, Swank & Henderson (1950). The washings were added to the incubation media. In many experiments one portion of tissue was extracted at the beginning of the experiment, that is immediately after pre-incubation, in order to determine the ACh content at zero-time. The extracts and media were frozen after adjusting the pH to 4 and stored until assayed for ACh.

The composition of the medium was as follows (mM): NaCl, 118·5 ; $NaHCO_3$, 24·9 ; KCl, 4·7 ; $CaCl_2$, 2·5 ; KH_2PO_4, 1·2 ; $MgSO_4$, 1·2 ; glucose, 10. Except when stated otherwise, soman (5×10^{-6}M) was present in the medium during both pre-incubation and incubation. In some experiments physostigmine sulphate was used instead of, or in addition to, soman. The medium was kept in equilibrium with an atmosphere of 95% oxygen and 5% carbon dioxide and the vessels were shaken continuously during pre-incubation and incubation. When extra KCl was added no osmotic compensations were made.

The ACh-like activity of the extracts and of the incubation media was estimated by bioassay on the dorsal leech muscle, treated with physostigmine, against an ACh chloride standard solution and expressed in terms of μg of this salt per gram of tissue (wet weight). No exhaustive attempts to confirm the identity of ACh were performed, but suitable dilutions of either alkali treated (pH near 14 during 120 min) and subsequently neutralized samples (Feldberg, 1945) or of the experimental medium (containing drugs which might be destroyed by alkali treatment) were added to the standard solution in all assays in order to correct for substances other than ACh, which might influence the sensitivity of the assay preparation. In several experiments known amounts of ACh were added to an extract after its ACh content had been determined, and a determination of the ACh content of the mixture was performed in order to detect possible sensitizing or desensitizing substances which might have appeared or disappeared as a result of the treatment with alkali. There was no indication of the presence of such substances. In a few experiments *d*-tubocurarine was found to antagonize the effects of an extract to the same extent as those of equipotent doses of the standard solution.

Presentation of the results and statistical evaluation. All results are presented as means of at least four observations $\pm$ S.E.M. ; the numbers of

observations are given in the figures. Either Student's t test or Wilcoxon's rank sum test at the 5% level of significance was used to decide whether differences were significant.

The activities of the optical stereo-isomers of hyoscyamine, 3-quinuclidinyl benzylate and (—)-hyoscine in stimulating ACh release from slices of cerebral cortex were compared by plotting the log concentrations against the differences between the amounts of ACh released from samples of slices incubated in the presence and in the absence of the drug under investigation. Four experiments were made for each drug concentration, and in each experiment three test samples containing the drug and three control samples were set up together. The ACh content of the samples was assayed separately, and each test sample was paired with a different control sample, chosen at random. Potency ratios with 99% fiducial limits were determined according to Finney (1952).

Materials used. Commercially obtained ACh chloride, atropine sulphate, lysergic acid diethylamide (LSD), cocaine hydrochloride, physostigmine sulphate, phenobarbital sodium, tropine and tropic acid were used. Because the chemical purity of tropine was considered doubtful, thin-layer chromatography on silica gel was performed and the presence of about 0·1% hyoscine was detected. Soman (3,3-dimethyl 2-butyl methylphosphonofluoridate) and Ro 2-3308 (3-quinuclidinyl benzylate) were synthesized in the Chemical Laboratory RVO-TNO, where the sulphates of (—)- and (+)-hyoscyamine were also prepared. The $[\alpha]_{589}^{20}$ of the (—)-hyoscyamine was —27·5°, that of the (+)-hyoscyamine +25·7°. If it is assumed that the optical purity of the (—)-isomer was 100% (compare Werner & Miltenberger, 1959, and Rosenblum & Taylor, 1954) it can be calculated that the (+)-hyoscamine was contaminated with 3% (—)-hyoscyamine.

For the sake of comparison of the activities of different drugs the concentrations of the sulphates of atropine, (—)- and (+)-hyoscyamine and physostigmine are expressed in the text as molar concentrations of the alkaloid moieties, that is M should be read as g-equiv/l.

RESULTS

In the experiments in which the slices were pre-incubated for 60 min (Figs. 1, 5 and 6 and Table 1) ACh production per gram of tissue during incubation in a 25 mM KCl medium was consistently greater than that in the experiments, in which the pre-incubation period was 90 min (Figs. 2, 3 and 4). In these, however, the medium was replaced at the end of each 30 min during the pre-incubation. This may have induced a loss of tissue fragments or constituents which would explain the difference in the results.

Medium with 4·7 mM KCl. When, after pre-incubation for 1 hr with soman, the slices of rat cerebral cortex were incubated in a medium containing 4·7 mM KCl, ACh was released from the tissue into the bath fluid. Because the amounts of ACh extracted from the slices at the end of the incubation were about the same as or slightly larger than immediately after pre-incubation, synthesis of ACh must have occurred. This is evident from the experiments of Fig. 1 in which the ACh extractable from the slices rose from $6{\cdot}7 \pm 0{\cdot}3$ μg/g to $7{\cdot}3 \pm 0{\cdot}5$ μg/g, and in which $0{\cdot}66 \pm 0{\cdot}07$ μg of ACh per gram of tissue was in addition released into the bath fluid.

Increased KCl concentrations. In confirmation of the results obtained by Mann, Tennenbaum & Quastel (1939), release and synthesis of ACh were found to be enhanced on raising the KCl concentration in the medium (Fig. 1). After incubation for 1 hr in a medium containing 25 mM KCl the ACh of the tissue amounted to $7{\cdot}6 \pm 0{\cdot}6$ μg/g and $4{\cdot}2 \pm 0{\cdot}2$ μg/g was released into the medium. This is about six times as much as during incubation in a 4·7 mM KCl medium. When the KCl concentration was raised to 50 mM, release and synthesis of ACh were stimulated even more; the ACh concentration of the tissue reached $13{\cdot}9 \pm 1{\cdot}4$ μg/g; in addition ACh $19{\cdot}4 \pm 1{\cdot}4$ μg/g was released into the medium.

Atropine. As shown in Fig. 1, atropine in a concentration of 3×10^{-6}M had no effect on the synthesis of ACh when the concentration of KCl in the medium was 4·7 mM, but enhanced synthesis when the concentration was 25 or 50 mM. With 25 mM KCl in the medium, the amounts of ACh released increased from $4{\cdot}2 \pm 0{\cdot}2$ to $13{\cdot}5 \pm 1{\cdot}5$ μg/g during 1 hr incubation, and at the end of this period the ACh content of the tissue slices had risen from $7{\cdot}6 \pm 0{\cdot}6$ to $13{\cdot}2 \pm 0{\cdot}9$ μg/g. With 50 mM KCl in the medium the release increased from $19{\cdot}4 \pm 1{\cdot}4$ to $24{\cdot}7 \pm 1{\cdot}3$ μg/g, but the ACh content of the tissue slices had risen insignificantly only, from $13{\cdot}9 \pm 1{\cdot}4$ to $15{\cdot}6 \pm 1{\cdot}1$ μg/g.

Fig. 2 illustrates the effects of different concentrations of atropine on ACh synthesis in slices of cerebral cortex incubated in a medium containing 25 mM KCl. Atropine was effective in a concentration as low as 6×10^{-9}M.

Stereo-isomers of atropine. The effect of the stereo-isomers of atropine on ACh release was investigated on incubation in a medium containing 25 mM KCl. As shown in Fig. 3, the log dose-response curves of the two stereo-isomers were linear and parallel, but (—)-hyoscyamine was 16 (99% fiducial limits 11-25) times as potent as (+)-hyoscyamine. On the assumption that the (+)-stereo-isomer was contaminated with about 3% of its optical antipode (see METHODS), the actual potency ration between the two isomers would be nearer to 30.

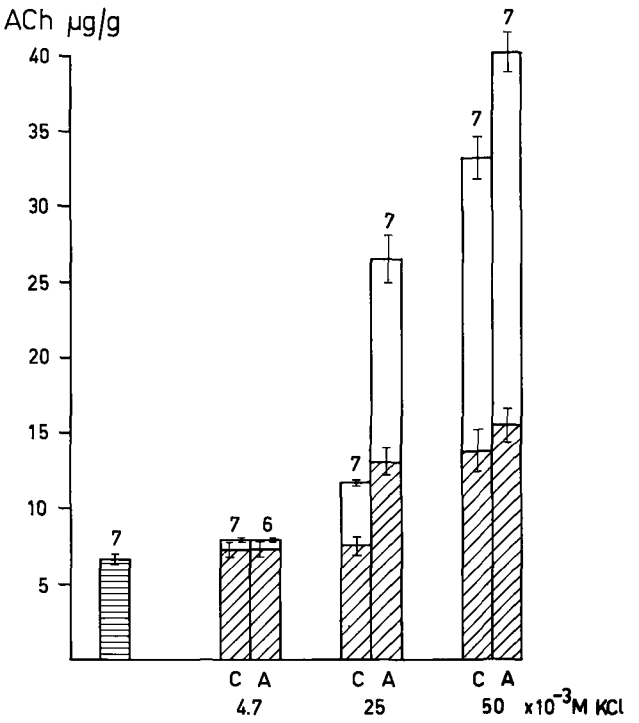

FIG. 1. Influence of atropine on synthesis and release of ACh by slices of cerebral cortex. Portions of 89-154 mg of tissue. Pre-incubation (60 min in 2.5 ml of medium with 4·7 mM KCl) and incubation (60 min in 2.5 ml of medium with different KCl concentrations) in the presence of 0·005 mM soman. Incubation with (A) and without (C) 3 x 10^{-6}M atropine. Mean amounts of ACh extracted ±S.E.M. and released ±S.E.M. (µg/g). In this and the following figures the numbers of observations are indicated above or beside each column. ▤, ACh content at 0 min ; ▨, ACh content at 60 min ; □, ACh released during 60 min. In all figures vertical lines indicate 2 x S.E.M.

Ro 2-3308 and (—)-hyoscine. The antimuscarinic compound Ro 2-3308, which has strong hallucinogenic properties (Bell & Gershon, 1964) as well as (—)-hyoscine stimulated ACh release in a 25 mM KCl medium. The regression lines obtained with these substances were linear and parallel to those of hyoscyamine. Ro 2-3308, the effect of which is shown in Fig. 3, was 4·6 (99% fiducial limits 3·0-7·4) times as effective as (—)-hyoscyamine and stimulated ACh release in a concentration as low as 1·2 x 10^{-9}M. The potency of (—)-hyoscine was 0·56 (99% fiducial limits 0·30-1·00) times that of (—)-hyoscyamine. Its regression line is not presented in Fig. 3 because of its vicinity to that of (—)-hyoscyamine, but its effect is shown in the block diagram of Fig. 4.

Other drugs. Relatively high concentrations of LSD and phenobarbital did

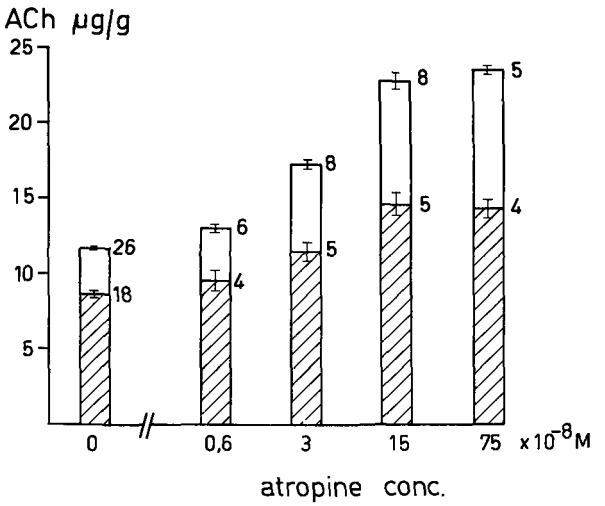

FIG. 2. Influence of atropine on synthesis and release of ACh by slices of cerebral cortex. Portions of 124-172 mg of tissue. Pre-incubation (90 min in 2.5 ml of medium with 4·7 mM KCl) and incubation (60 min in 2.5 ml of medium with 25 mM KCl) in the presence of soman 0·005 mM. Mean quantities of ACh extracted ±S.E.M. and released ±S.E.M. (µg/g). □, ACh released during 60 min; ▨, ACh content slices after 60 min.

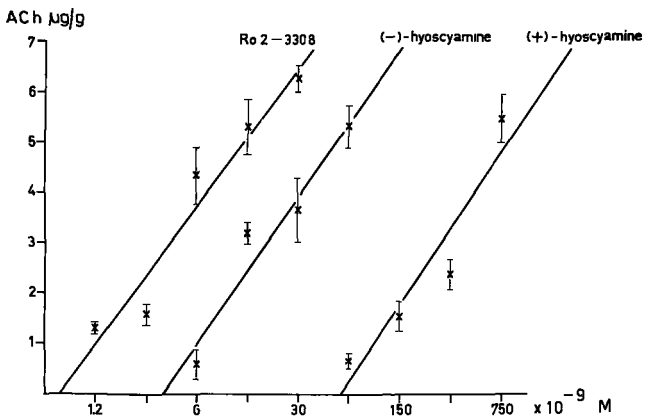

FIG. 3. Influence of (—)- and (+)-hyoscyamine and of Ro 2-3308 on release of ACh from slices of cerebral cortex. Portions of 103-163 mg of tissue. Pre-incubation and incubation as in Fig. 2. Abscissa: drug concentration. Ordinate: ACh release in the presence of the drug minus that in a randomized paired control. Presented are means of four observations ± S.E.M. and the calculated least square regression line of each drug.

not influence ACh release from the isolated cerebral cortex incubated in a 25 mM KCl medium. Cocaine and tropic acid, two drugs chemically related to atropine but without its antimuscarinic activity, were also ineffective. They

were tested in a concentration of about 10^{-6}M. The result is shown in the block diagram of Fig. 4, which also illustrates the effect of tropine. This breakdown product of atropine did stimulate ACh output when applied in high concentrations, but chromatographic analysis showed it to be contaminated with about 0·1% hyoscine. This may explain its stimulating effect.

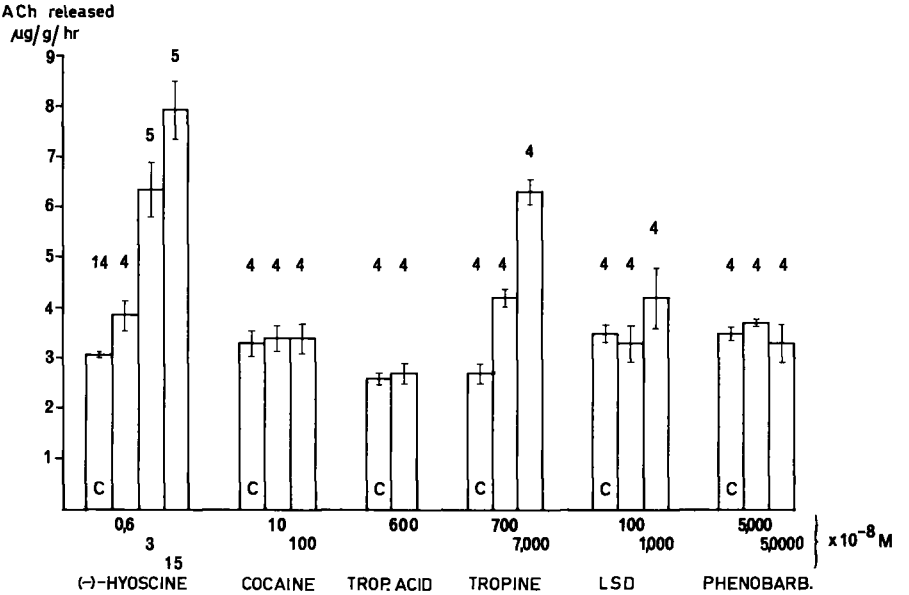

FIG. 4. Influenced of several drugs on release of ACh from slices of cerebral cortex. Portions of tissue of 96-150 mg. Pre-incubation and incubation as in Fig. 2. Mean amounts of ACh released ± S.E.M. (µg/g). C, Control; □, in presence of drug.

Acetylcholine depleting effect of atropine in strong concentrations. In the presence of 25 and 50 mM KCl, atropine in concentrations of 6 x 10^{-9} to 3 x 10^{-6}M was shown to increase not only the release of ACh but also the ACh content of the tissue slices. On raising the atropine concentration to 3 x 10^{-5}M, however, this increase of ACh in the tissue slices became smaller, and with a concentration of 3 x 10^{-4}M the ACh content actually decreased. In Fig. 5 the effects of atropine 3 x 10^{-4} and 3 x 10^{-6}M are compared at different KCl concentrations of the medium. The initial ACh content of the tissue slices was 6·6 ± 0·2 µg/g. It fell to 5·5 ± 0·3 and 5·3 ± 0·2 µg/g on incubation with atropine 3 x 10^{-4}M at a KCl concentration of either 25 or 50 mM, whereas at these KCl concentrations it rose to 13·7 ± 0·7 and 17·0 ± 0·7 µg/g on incubation with atropine 3 x 10^{-6}M. In the presence of 4·7 mM

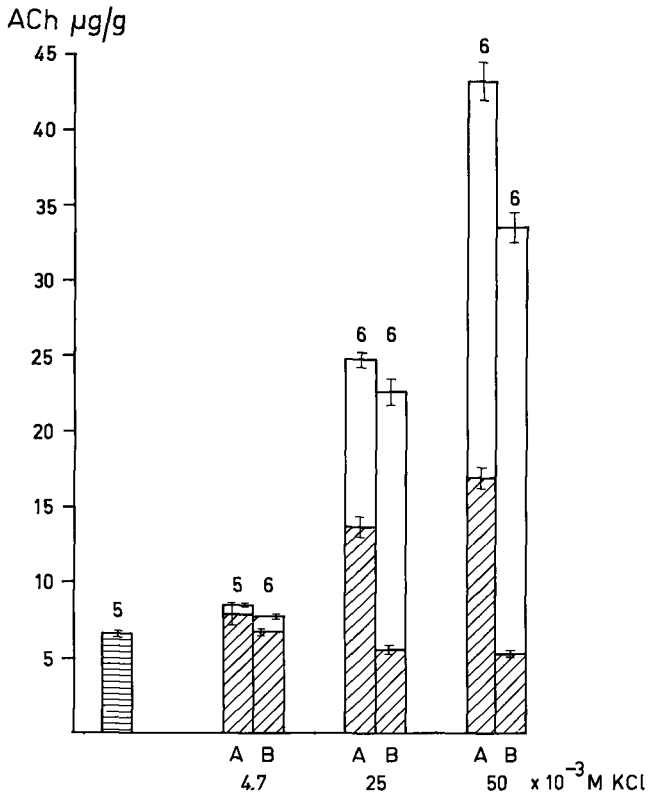

FIG. 5. Influence of high concentrations of atropine on ACh extractable and released from slices of cerebral cortex. Portions of 108-167 mg of tissue. Pre-incubation and incubation as in Fig. 1, except that incubation was with atropine 3 x 10^{-6}M (A) or 3 x 10^{-4}M (B). ▤, ACh content slices at 0 min; ▨, ACh content slices at 60 min: □, ACh released during 60 min.

KCl, atropine in either concentration had scarcely any effect on the ACh content of the tissue slices.

In the experiments with 25 mM KCl the sum of the amounts of ACh released into the medium and extracted from the tissue after incubation with 3 x 10^{-4}M atropine was about the same as after incubation with 3 x 10^{-6}M and much larger than in the absence of atropine (compare Fig. 1). Thus in this condition both atropine concentrations stimulated the synthesis of ACh to the same extent. On the other hand, in a medium containing 50 mM KCl the combined amount of ACh found in medium and tissue slices was smaller on incubation with 3 x 10^{-4}M atropine than with 3 x 10^{-6}M and equal to that found without atropine (compare Fig. 1).

Acetylcholine depleting effect of physostigmine. Physostigmine reduced the ACh content of the tissue slices incubated in a 25 or 50 mM KCl medium without stimulating synthesis of ACh. The effect was obtained whether soman was present in the medium or not. As in the experiments with atropine, depletion did not occur in a 4·7 mM KCl medium.

Table 1 illustrates the depletion produced with different concentrations of physostigmine in the presence of soman and with a 25 mM KCl concentration in the medium. Physostigmine was effective in concentrations lower than or similar to those used conventionally to inactivate the cholinesterase of brain tissue in *in vitro* experiments. The reduction in the ACh content of the tissue slices was associated with a corresponding increase in the amount of ACh released; there was thus no evidence for increased synthesis.

In Fig. 6 the effect of 8 x 10^{-4}M physostigmine is compared with that of 5 x 10^{-6}M soman. The experiments with the two inhibitors of cholinesterase were not performed simultaneously so no significance can be attached to small differences observed. Pre-incubation was either with physostigmine or with soman, and with 4·7 mM KCl in both conditions. The mean ACh con-

TABLE 1. EFFECT OF PHYSOSTIGMINE ON ACh EXTRACTED FROM SLICES OF CEREBRAL CORTEX AND RELEASED INTO THE MEDIUM

Physostigmine concentration (M)	ACh (μg) per gram of tissue		
	In slices	Released into bath	Total
5 x 10^{-5}	5·1	3·9	9·0
	5·0	4·4	9·4
1 x 10^{-4}	3·8	5·6	9·4
2 x 10^{-4}	4·0	7·3	11·3
4 x 10^{-4}	3·9	7·3	11·2
	5·4	5·3	10·7
8 x 10^{-4}	4·5	5·4	9·9
1 x 10^{-3}	4·8	6·4	11·2
	4·7	5·9	10·6
2 x 10^{-3}	6·4	4·8	11·2
	5·6	4·4	10·0
	5·5	4·4	9·9
Mean	4·9	5·4	10·3
No physostigmine	7·4	3·5	10·9
	6·0	2·9	8·9
	6·5	2·8	9·3
	8·6	2·5	11·1
	9·2	2·6	11·8
	6·9	2·6	9·5
	8·4	3·1	11·5
	7·7	3·4	11·1
Mean	7·6	2·9	10·5

Portions of 71-208 mg of tissue. Pre-incubation (60 min in 2·5 ml of medium containing 4·7 mM KCl) and incubation (30 min in 5 ml of medium containing 25 mM KCl) in the presence of soman (0·005 mM). Each figure represents one observation.

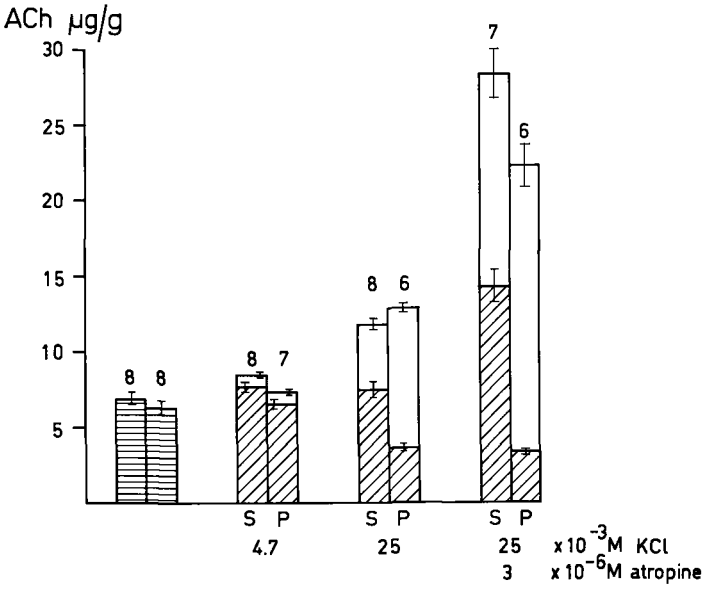

FIG. 6. Influence of potassium and atropine on ACh content of slices of cerebral cortex and on release of ACh into the medium during incubation with either soman or physostigmine. Portions of 136-171 mg of tissue. Pre-incubation and incubation as in Fig. 1, except that either 5 x 10^{-6}M soman (S) or 8 x 10^{-4}M physostigmine (P) was present in the medium. ▤, ACh content slices at 0 min; ▨, ACh content slices at 60 min; □, ACh released during 60 min.

tent of the tissue slices after the pre-incubation period was $6{\cdot}3 \pm 0{\cdot}4$ µg/g in the presence of physostigmine and $6{\cdot}9 \pm 0{\cdot}4$ µg/g in the presence of soman. When incubation was continued for 1 hr the ACh content of the tissue slices incubated with physostigmine remained practically unchanged ($6{\cdot}5 \pm 0{\cdot}2$ µg/g) whereas it rose to $7{\cdot}7 \pm 0{\cdot}3$ µg/g in the slices incubated with soman. On increasing the KCl concentration to 25 mM, the ACh content of the tissue decreased during the incubation period to $3{\cdot}7 \pm 0{\cdot}2$ µg/g in the physostigmine experiments, but in the soman experiments it increased to about the same extent as during incubation with 4·7 mM KCl ($7{\cdot}5 \pm 0{\cdot}5$ µg/g). Whether incubation was with physostigmine or soman, however, synthesis of ACh was about equal because the ACh depletion of the tissue slices in the presence of physostigmine was associated with a corresponding increase in the release of ACh into the medium.

The last two columns of the block diagram of Fig. 6 show the effect of atropine added to the incubation medium as well. Synthesis of ACh was stimulated, but whereas the ACh content rose to $14{\cdot}3 \pm 1{\cdot}1$ µg/g in the tissue slices incubated with soman it fell to $3{\cdot}3 \pm 0{\cdot}2$ µg/g in those incubated with

physostigmine. Although this depletion was associated with a larger release of ACh into the medium ($19{\cdot}0 \pm 1{\cdot}4$ μg/g) than in the corresponding soman experiments ($14{\cdot}1 \pm 1{\cdot}6$ μg/g), the total amounts of ACh synthesized were smaller.

DISCUSSION

The ability of atropine to increase the release of ACh from brain tissue has so far been demonstrated only in *in vivo* experiments. The present experiments show that atropine exerts this action also in incubated slices of rat cerebral cortex, but only on incubation in a high potassium medium. This accords with previous findings which indicate that isolated cerebral tissue more nearly approximates that in the living animal if the potassium concentration of the medium is raised (see Quastel, 1965).

The effect of atropine was obtained with a concentration as low as 6×10^{-9}M, suggesting that it is linked to the antimuscarinic property. This view is supported by the finding that (—)-hyoscyamine was about 30 times as potent as (+)-hyoscyamine, that other antimuscarinic drugs shared with atropine this ability to stimulate ACh release, and that a number of substances without antimuscarinic activity, such as cocaine, phenobarbital, tropic acid, tropine and LSD, had no appreciable effect on the release of ACh. The results obtained with phenobarbital differ, for reasons unknown, from those obtained by McLennan & Elliott (1951). They incubated rat brain slices in a 27 mM KCl medium and found that synthesis of ACh was stimulated by low and inhibited by high concentrations of phenobarbital.

The effect of atropine is dependent on the KCl concentration of the medium, but KCl itself increases the release of ACh from incubated slices of brain tissue (Mann *et al.*, 1939 ; Welsh & Hyde, 1944 ; McLennan & Elliott, 1950). According to the previous findings the effect is maximal at a concentration of 25 mM KCl ; an increase to 50 mM KCl actually resulted in a diminution of ACh release (Mann *et al.*, 1939). In the present experiments the effect of KCl in increasing ACh release was confirmed, but more ACh was produced when the medium contained 50 instead of 25 mM KCl. Differences in technique seem to be responsible for the discrepancy in these results. One factor may be the NaCl concentration of the medium which was reduced in the previous experiments in order to compensate for the increased KCl concentration. This was not done in the present experiments. Bhatnagar & MacIntosh (1967) pointed out that the inhibition of ACh synthesis obtained by Mann *et al.* with excessively high KCl concentrations may have been the result of the removal of the Na from the medium, and they produced evidence in favour of this view.

The increased release of ACh brought about by raised potassium concentrations is probably a direct consequence of the depolarization of nerve endings produced by this cation, analogous to the effect of depolarization of motor nerve endings in the isolated diaphragm by potassium or by an electrical current which results in an immediate increase in the resting output of ACh (Liley, 1956). Depolarization of cell membranes in slices of cerebral cortex either by potassium or by electrical pulses increases oxygen consumption, aerobic glycolysis and lactate formation (see McIlwain, 1966). It also leads to an accelerated conversion of pyruvate to acetyl-coenzyme A (Kini & Quastel, 1959), which may be directly linked to the stimulation of ACh synthesis by high potassium concentrations. The potassium induced increase in the metabolism is probably associated with energy requiring recovery processes involved in re-establishing the disturbed ionic gradients across the cell membrane.

It is known that the increase in metabolism induced by electrical pulses or potassium is much more sensitive to drugs and to changes in substrate than the "resting" metabolism (Quastel, 1965 ; McIlwain, 1966). Atropine and other antimuscarinic substances which were studied on this increased metabolism inhibited the oxygen consumption and glycolysis of slices of cerebral cortex stimulated by electrical impulses (McIlwain, 1951 ; O'Neill, Simon & Cummins, 1963). On the other hand McIlwain did not obtain inhibition of the oxygen consumption and glycolysis stimulated by increased potassium concentration, but O'Neill *et al.* observed the inhibiting effect of antimuscarinic substances also in this condition. The concentration of the antimuscarinic substances required to inhibit respiration augmented either by electrical impulses or by increased potassium concentration was of the order of 10^{-4}M. This is at least 1,000 times stronger than the concentration which, in the present experiments, produced maximal increase in the release and synthesis of ACh. The two phenomena therefore seem to be unrelated.

It has been postulated that atropine increases the release of ACh from the brain in the living animal by blocking its reabsorption or by displacing it from its stores, either of which would lead to a decreased ACh content of the tissue (Giarman & Pepeu, 1964 ; Szerb, 1964 ; Polak, 1965). In the present experiments, however, not only the release but also the synthesis of ACh was stimulated by low concentrations of atropine. This action of atropine probably is dependent on intact cellular structures, because in cell-free biochemical systems prepared from rat brain atropine or hyoscine were found not to influence the synthesis of ACh and acetyl-coenzyme A (Giarman & Pepeu, 1964 ; Schuberth, 1965).

Because the same extremely low concentrations of atropine increased both

synthesis and release of ACh the two effects appear to be coupled. A regulating negative feed-back mechanism between the ACh concentration in the tissue and the rate of ACh formation has been postulated by Mann *et al.* (1939) on the basis of the stimulating effect of potassium, but a second negative feed-back mechanism may exist between the ACh concentration in the environment of a store of ACh and the rate of its release. In favour of such a mechanism are the recent findings that the release of ACh from minced brain (Bhatnagar & MacIntosh, 1967) and from the synaptosome fraction of a brain homogenate (Hosein, Levy & Ko, 1967) is inhibited by the addition of ACh to the medium. If ACh receptors were involved in this negative feed-back system, their sensitivity might be reduced by atropine. Atropine would then stimulate the release and synthesis of ACh by blocking the inhibiting effect produced by the released ACh.

If reduction of the ACh content of the stores in the tissue slices were to act as the appropriate stimulus for synthesis of ACh one would expect increased synthesis of ACh to be always associated with a decreased ACh content of the tissue. Often, however, the reverse was found—for instance, when incubation was in a soman containing medium with a high KCl concentration or when antimuscarinic substances were added to such a medium. To reconcile these results with the theory that reduction of the ACh stores acts as the stimulus for synthesis, it is necessary to postulate that the ACh is stored in two compartments and that by determination of the ACh content of the slices a reduction in one compartment may not be revealed when accumulation in the other has occurred. The results obtained with physostigmine suggest that two such compartments exist and that reduction takes place in one.

When physostigmine was added to a medium containing a high KCl concentration and soman the ACh content of the tissue slices was found to decrease, but to a certain extent only. No further decrease occurred even when the physostigmine concentration used was increased manyfold. This result is in favour of the hypothesis of two ACh holding compartments, one being physostigmine-sensitive, the other physostigmine-resistant. Only the physostigmine-sensitive compartment would be depleted by physostigmine. Further, the finding that physostigmine produced no or only a slight decrease in the ACh content of the tissue slices incubated in a low potassium medium may indicate that in this condition nearly all ACh was present in the physostigmine-resistant compartment. On the other hand the rise which occurred in the ACh content of the tissue slices incubated with soman instead of physostigmine in conditions in which synthesis and release of ACh were stimulated — that is, in a high potassium medium, with or without atropine — must

have taken place in the physostigmine-sensitive compartment, because it was not observed when in otherwise similar conditions soman was replaced by physostigmine.

Relatively high concentrations of atropine, in addition to their stimulating effect on ACh synthesis, appear to empty the physostigmine-sensitive compartment in the same way as physostigmine. This would explain why the rise in the ACh content of the slices incubated with 25 mM KCl and 3 x 10^{-6}M atropine was changed into a fall when the atropine concentration was raised to 3 x 10^{-4}M.

SUMMARY

1. A study was made of the influence of some antimuscarinic drugs on the release and synthesis of acetylcholine (ACh) by slices of cerebral cortex from rats after pre-incubation with the irreversible cholinesterase inhibitor soman.

2. During aerobic incubation at 37° C in a medium containing glucose and soman with 4·7 mM KCl, ACh was released into the bath and synthesized by the tissue. The release and synthesis increased when the potassium concentration of the medium was raised to 25 or 50 mM.

3. Atropine induced an additional increase in the release and synthesis of ACh on incubation in a high-potassium medium. This effect was obtained with atropine in a concentration as low as 6 x 10^{-9}M. On incubation in a low-potassium medium (4·7 mM) atropine did not have this effect.

4. The stimulation of release and synthesis of ACh by atropine seems to be linked to its anti-muscarinic properties. (—)-Hyoscyamine, which is known to have a much stronger antimuscarinic activity than (+)-hyoscyamine, was 30 times as potent in stimulating ACh release as its (+)-stereo-isomer. Hyoscine and Ro 2-3308, a hallucinogenic antimuscarinic drug, also stimulated the release of ACh. Ro 2-3308 was effective in a concentration as low as 2 x 10^{-9}M.

5. Drugs without antimuscarinic activity, such as cocaine, tropic acid, lysergic acid diethylamide and phenobarbital did not increase release and synthesis of ACh.

6. High concentrations of tropine stimulated ACh release but this effect was probably caused by contamination with hyoscine.

7. The increased release and synthesis of ACh which occurred with and without atropine in the presence of soman and a high potassium concentration in the medium were associated with an increase in the ACh content of the tissue slices. When physostigmine was added as well to the medium or replaced the soman the ACh content of the tissue slices decreased although

the stimulating effect of potassium and atropine on release and synthesis of ACh still occurred.

8. Atropine in a high concentration (10^{-4}M) also decreased the ACh content of the tissue slices, yet release and synthesis of ACh were stimulated in the same way as with atropine in weak concentrations.

9. The results are discussed on the assumption that the ACh is stored in the tissue in two compartments, one being physostigmine-sensitive and the other physostigmine-resistant. Only the physostigmine-sensitive compartment is depleted by physostigmine or high concentrations of atropine.

We are greatly indebted to the Chemical Laboratory RVO-TNO for the supply of soman, 3-quinuclidinyl benzylate and (—)- and (+)-hyoscyamine, to Dr. W. F. Stevens for the chromatographic analysis of the tropine, to Dr. M. Wijnans for the statistical evaluation of the results and to Dr E. M. Cohen and Dr. E. Meeter for their stimulating interest and valuable criticism.

REFERENCES

BHATNAGAR, S. P. & MACINTOSH, F. C. (1967). Effects of quaternary bases and inorganic cations on acetylcholine synthesis in nervous tissue. *Can. J. Physiol. Pharmac.*, **45**, 249-268.

BELL, C. & GERSHON, S. (1964). Experimental anticholinergic psychotomimetics: Antagonism of yohimbine and tacrine (THA). *Med. Exp.* **10**, 15-21.

ELLIOTT, K. A. C., SWANK, R. L. & HENDERSON, N. (1950). Effects of anaesthetics and convulsants on acetylcholine content of brain. *Am. J. Physiol.*, **162**, 469-474.

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

FINNEY, D. J. (1952). *Statistical Method in Biological Assay*. London: Charles Griffin.

GIARMAN, N. J. & PEPEU, G. (1964). The influence of centrally acting cholinolytic drugs on brain acetylcholine levels. *Br. J. Pharmac. Chemother.*, **23**, 123-130.

HOSEIN, E. A., LEVY, R. & KO, G. K. W. (1967). Failure of acetylcholine to provoke release of material with acetylcholine-like activity from rat brain subcellular particles. *Exp. Neurol.*, **17**, 420-424.

KINI, M. M. & QUASTEL, J. H. (1959). Carbohydrate-amino-acid interrelations in brain cortex *in vitro*. *Nature, Lond.*, **184**, 252-256.

LILEY, A. W. (1956). The effects of presynaptic polarization on the spontaneous activity at the mammalian neuromuscular junction. *J. Physiol., Lond.*, **133**, 571-587.

MANN, P. J. G., TENNENBAUM, M. & QUASTEL, J. H. (1939). C111. Acetylcholine metabolism in the central nervous system; the effects of potassium and other cations on acetylcholine liberation. *Biochem. J.*, **33**, 822-835.

MCILWAIN, H. (1951). Atropine and related compounds on the metabolism of electrically stimulated sections of mammalian cerebral cortex. *Br. J. Pharmac. Chemother.*, **6**, 531-539.

MCILWAIN, H. (1966). *Biochemistry and the Nervous System*, 3rd ed., pp. 55-76. London: Churchill.

MCLENNAN, H. & ELLIOTT, K. A. C. (1950). Factors affecting the synthesis of acetylcholine by brain slices. *Am. J. Physiol.*, **163**, 605-613.

MCLENNAN, H. & ELLIOTT, K. A. C. (1951). Effect of convulsant and narcotic drugs on acetylcholine synthesis. *J. Pharmac. exp. Ther.* **103**, 35-43.

MITCHELL, J. F. (1963). The spontaneous and evoked release of acetylcholine from the cerebral cortex. *J. Physiol., Lond.*, **165**, 98-116.

O'NEILL, J. J., SIMON, S. H. & CUMMINS, J. T. (1963). Inhibition of stimulated cerebral cortex respiration and glycolysis by cholinolytic drugs. *Biochem. Pharmac.*, **12**, 809-820.

POLAK, R. L. (1965). Effect of hyoscine on the output of acetylcholine into perfused cerebral ventricles of cats. *J. Physiol., Lond.*, **181**, 317-323.
POLAK, R. L. (1967). The influence of antimuscarinic drugs on synthesis and release of acetylcholine by the isolated cerebral cortex of the rat. *J. Physiol., Lond.*, **191**, 34-35*P*.
POLAK, R. L. & MEEUWS, M. M. (1966). The influence of atropine on the release and uptake of acetylcholine by the isolated cerebral cortex of the rat. *Biochem. Pharmac.*, **15**, 989-992.
QUASTEL, J. H. (1965). Effects of drugs on metabolism of the brain *in vitro*. *Br. Med. Bull.*, **21**, 49-56.
ROSENBLUM, E. I. & TAYLOR, W. S. (1954). Preferential crystallisation of one stereo-isomer from solutions of atropine sulphate in ethanol. *J. Pharm. Pharmac.*, **6**, 256-258.
SCHUBERTH, J. (1965). On the biosynthesis of acetyl coenzyme A in the brain. 1. The enzymic formation of acetyl coenzyme A from acetate, adenosine triphosphate and coenzyme A. *Biochim. biophys. Acta*, **98**, 1-7.
SZERB, J. C. (1964). The effect of tertiary and quaternary atropine on cortical acetylcholine output and on the electroencephalogram in cats. *Can. J. Physiol. Pharmac.*, **42**, 303-314.
WELSH, J. H. & HYDE, J. E. (1944). The effects of potassium on the synthesis of acetylcholine in brain. *Am. J. Physiol.*, **142**, 512-518.
WERNER, G. & MILTENBERGER, K. (1960). Zur Trennung der optischen Antipoden von Homatropin und Atropin ; Synthese von L(+)- und D(—)-Homatropin-Sulfat. *Justus Liebigs Annln Chem.*, **631**, 163-168.

Reprinted from: British Journal of Pharmacology (1969) **36**, 144-152

THE INFLUENCE OF DRUGS ON THE UPTAKE OF ACETYLCHOLINE BY SLICES OF RAT CEREBRAL CORTEX

R. L. POLAK

INTRODUCTION

In the course of an investigation of the release and synthesis of acetylcholine (ACh) from cortical slices of rat brain, treated with the irreversible cholinesterase inhibitor 3,3-dimethyl 2-butyl methylphosphonofluoridate (soman), ACh added to the medium was found to accumulate in the tissue. The amounts of ACh taken up by the tissue were much larger than those found in earlier experiments by Elliott & Henderson (1951). These authors used physostigmine to inhibit the cholinesterase, which may explain the lower uptake of ACh, because physostigmine has a strong inhibitory effect on its accumulation in slices of cerebral cortex (Polak & Meeuws, 1966).

The present experiments deal with the influence of several conditions on the accumulation of ACh in slices of rat cerebral cortex. Some of the present results have been communicated to the Nederlandse Vereniging voor Fysiologie en Farmacologie (Polak, 1967).

METHODS

Female albino rats (160-190 g) were lightly anaesthetized with ether and decapitated. The brains were immediately removed from the skulls and placed in ice-cold oxygenated medium. As a rule a slice in the dorsal plane with a thickness of about 0.5 mm was prepared from the cortex of both hemispheres in the cold room by means of a Stadie-Riggs microtome and weighed on a torsion balance. In experiments in which ACh uptake was studied in anaerobic conditions, slices thinner than 0.4 mm were used. They were prepared with a recessed glass guide according to the method of McIlwain & Rodnight (1962).

Incubation procedure. A pre-incubation period of 60 or 90 min was followed by an incubation period of 30 min. Six to eight 25 ml vessels containing about 75 mg of tissue in 2·5 ml of medium to which soman (5 x 10^{-6}M) had been added to inactivate the cholinesterase were set up simultaneously for pre-incubation. During the incubation period the vessels con-

tained 5 ml of medium to some of which ACh (4 μg/ml) had been added unless otherwise stated in the text. At the end of the 30 min incubation period, the media were transferred to graduated centrifuge tubes. The slices were rinsed three times with fresh incubation medium and the washings were added to the incubation media in the centrifuge tubes. The tissue ACh was extracted according to the method of Elliott, Swank & Henderson (1950). Each sample of tissue was homogenized in 3 ml of acidified medium (100 ml of medium + 15 ml of 0·3 N HCl) for 2 min and then left at room temperature for 60 min. Thereafter it was centrifuged, the precipitate was resuspended in fresh acidified medium and re-centrifuged. The supernatants from both centrifugations were then amalgamated. The extracts and media were frozen after adjustment of the pH to 4 and stored until assayed for ACh.

The composition of the medium used in the pre-incubation period was as follows (mM): NaCl, 118·5 ; $NaHCO_3$, 24·9 ; KCl, 4·7 ; $CaCl_2$, 2·5 ; KH_2PO_4, 1·2 ; $MgSO_4$, 1·2 ; glucose, 10 ; soman 5 x 10^{-8}. The same medium was used for the 30 min incubation period except that the KCl concentration was 25 mM in most experiments, so that they should be identical with previous experiments on the effect of atropine on the synthesis and release of endogenous ACh by cortical slices (Bertels-Meeuws & Polak, 1968). The medium was kept in equilibrium with an atmosphere of 95% oxygen and 5% carbon dioxide. For anaerobic incubation, a mixture of 95% nitrogen and 5% carbon dioxide was used. Unless otherwise stated the temperature was 37° C.

The ACh content of the extracts and incubating media was determined by bio-assay on the dorsal muscle of the leech treated with physostigmine and was expressed as μg/g of tissue (wet weight). Suitable dilutions of alkali treated (pH near 14 for 120 min) and subsequently neutralized extracts (Feldberg, 1945) or of the experimental medium (containing drugs which might be destroyed by alkali treatment) were added to the standard solution in all assays in order to correct for substances other than ACh, which might influence the sensitivity of the assay preparation.

Presentation of the results and statistical evaluation. Results are expressed as means $\pm$ standard error of the mean with the numbers of observations in parenthesis. Welch's t test (Hald, 1952) was used to determine the significance of differences at the 5% level. When the number of observations in a group was less than 6 the rank sum test (Wilcoxon, 1945) was used instead. It was possible to calculate linear and parallel regression lines from the data of Table 1. Potency ratios relative to the effect of choline with the 95% fiducial limits were determined according to Hald (1952).

Autoradiographic experiments. Slices were pre-incubated for 60 min in

the usual medium with 5 x 10^{-6}M soman. Thereafter incubation was carried out for 30 min in the same medium to which (^{3}H-acetyl) ACh chloride (4 μg/ml) had been added. In a few experiments a different procedure was followed. With the intention of displacing the labelled ACh from aspecific sites in the tissue, a 15 min exposure to (^{3}H-acetyl) ACh chloride was followed by exposure to non-radioactive ACh (4 μg/ml) for 45 min. In all cases, the slices were rinsed three times with fresh medium at the end of the incubation period. Subsequently they were immersed in isopentane cooled in liquid nitrogen.

Autoradiographic preparations were made by the methods of Appleton (1964), designed to prevent displacement of labelled water soluble materials during the preparation of histological sections. Kodak AR 10 stripping film was applied, with its emulsion layer facing upwards, to a cover glass which was mounted by Sellotape on a microscopical slide. Cryostat sections (6 μ) of the labelled tissue were cut in the dark room. The sections were taken off the knife by briefly placing the emulsion layer against the section. After exposure for 1-3 weeks at —20° to —30° C the slices were allowed to attain room temperature and to dry by ventilation. The sections were fixed for 1 min in 5% acetic acid-ethanol followed by 10 min in 80% ethanol, and rinsed in distilled water. Development and fixation of the film were carried out immediately afterwards. Following staining with haemotoxylin-eosin, the cover glass supporting the film and the section was detached from the slide and mounted with the section downwards on a new slide.

Materials. All drugs used were obtained commercially with the exception of 3,3-dimethyl 2-butyl methylphosphonofluoridate (soman), ethyl N,N-dimethyl phosphonoamidocyanate (tabun) and O-ethyl S-diethylaminoethyl ethylphosphonothiolate (Am 1), which were synthetized in the Chemical Laboratory RVO-TNO.

Oligomycin was dissolved in ethanol. Five mg of the dry compound was dissolved in 0·2 ml and 0·01 ml of this ethanol solution was made up to 250 ml with the incubation medium. On dilution, a fine precipitate appeared which remained in suspension. This may have resulted in the oligomycin concentration being lower than that mentioned in the text.

ACh concentrations were always expressed in terms of ACh chloride, although in a number of experiments, the perchlorate was used. The specific activity of the (^{3}H-acetyl) ACh chloride was 1·37 mCi/mg; it was used without the addition of carrier.

RESULTS

When slices of cerebral cortex were incubated in a medium to which ACh

had been added, some of the ACh accumulated in the tissue. After 30 min incubation in a medium containing 25 mM KCl and ACh (4 μg/ml) the ACh concentration in the slices amounted to $24{\cdot}01 \pm 0{\cdot}37$ μg/g (49 observations), whereas it was only $7{\cdot}17 \pm 0{\cdot}28$ μg/g (14 observations) after 30 min incubation in the same medium without added ACh. Practically the same value, $7{\cdot}6 \pm 0{\cdot}6$ μg/g (seven observations), was obtained in previous experiments without ACh (Bertels-Meeuws & Polak, 1968).

Recovery of added ACh. The ratio between the amount of tissue and the volume of incubation medium was chosen in such a way that the amounts of ACh taken up by the slices were too small to influence the ACh concentration in the medium perceptibly during the 30 min of incubation. In sixty-one experiments using an ACh concentration in the medium of 4 μg/ml an ACh concentration of $3{\cdot}88 \pm 0{\cdot}04$ μg/ml was found in the medium at the end of the incubation period. When the recovery of added ACh (20μg/5 ml of medium) was calculated it amounted to $19{\cdot}92 \pm 0{\cdot}20$ μg or 99·6%. The calculation was made by determining the difference between the total ACh present both in slices and medium at the end of the incubation period in these experiments and in sixty-one control experiments, set up simultaneously without the addition of ACh.

Influence of the KCl concentration on the uptake of ACh. The uptake of ACh by cortical slices was dependent on the concentration of potassium. It was maximal when the KCl was omitted from the medium and decreased with increasing potassium concentration (Fig. 1). The relationship between the log potassium concentration of the medium and the ACh concentration in the tissue after 30 min incubation in the presence of ACh (4 μg/ml) was found to be linear.

Substances which act on cholinergic transmission. Hemicholinium-3, Am 1, physostigmine, atropine and choline inhibited the accumulation of ACh in slices of cerebral cortex in a medium containing ACh (4 μg/ml) and 25 mM KCl. This is shown in Table 1. From these data linear and parallel regression lines representing the ACh concentration in the slices after incubation as a function of the log concentration of the drug were calculated (Fig. 2). In the experiments with hemicholinium-3, Am 1 and choline, the drugs were present in the medium during the last 30 min of the 90 min period of pre-incubation as well as during the following 30 min incubation period, whereas the physostigmine and the atropine were only allowed to act upon the tissue during the 30 min incubation period following a 60 min period of pre-incubation. Disregarding this difference in method hemicholinium-3, Am 1, physostigmine and atropine were found to be 27 (95% fiducial limits

38-19), 8·8 (12·2-6·4), 6·8 (9·8-4·7) and 1·6 (2·3-1·1) times as potent as choline respectively in inhibiting ACh uptake.

The effect of physostigmine on the uptake of ACh by slices of cerebral cortex when studied in a medium containing 4·7 mM KCl was found not to be significantly different from that obtained with 25 mM KCl in the medium according to the criteria for identity given by Hald (1952).

In contrast to the blocking effect of the cholinesterase inhibitors physostigmine and Am 1 on the accumulation of ACh in cortical slices high concentrations of soman or tabun, which also have potent anticholinesterase actions, did not influence the uptake of ACh (Table 1).

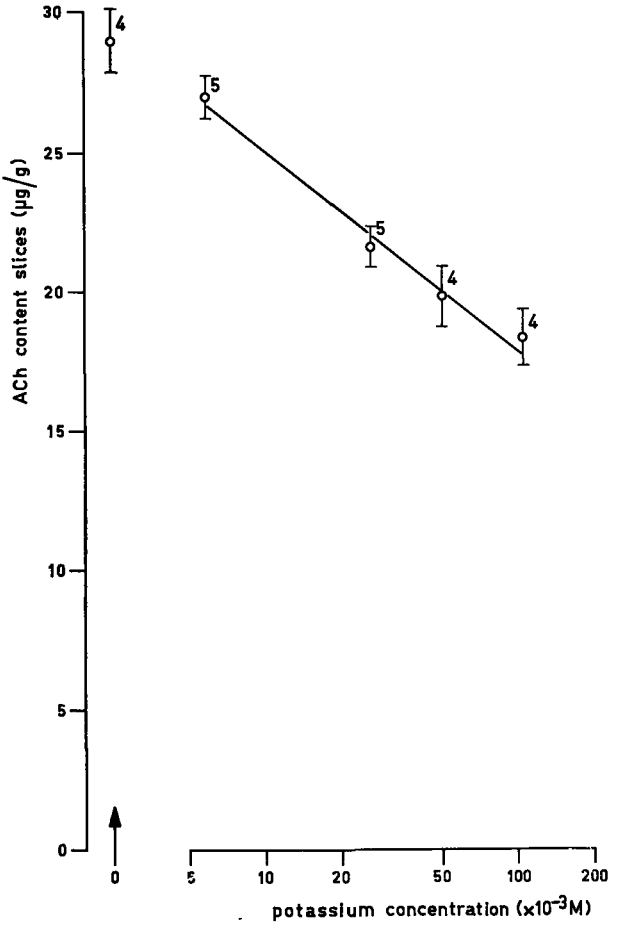

FIG. 1. Influence of potassium on the accumulation of ACh in cortical slices. Mean values of four or five observations (indicated by the numbers) ± S.E.M. and the calculated least square regression line.

TABLE 1. INFLUENCE OF DRUGS ON THE UPTAKE OF ACh BY CORTICAL SLICES.

Drug	Conc. (M)	ACh conc. slices (μg ± S.E.M.)
Soman	0	22·6 ± 0·5 (6)
	10^{-4}	22·4 ± 0·5 (6)
Tabun	0	25·4 ± 0·8 (5)
	10^{-4}	25·2 ± 1·2 (6)
HC-3	0	25·3 ± 0·7 (11)
	10^{-6}	19·7 ± 0·7 (4)
	10^{-5}	12·6 ± 0·5 (5)
	3×10^{-5}	8 8 ± 0·2 (6)
Am 1	0	23·6 ± 0·8 (18)
	10^{-6}	22·8 ± 0·1 (3)
	10^{-5}	16·2 ± 0·9 (6)
	3×10^{-5}	12·8 ± 0·5 (6)
	10^{-4}	7·6 ± 0·4 (3)
Choline	0	23·3 ± 0·7 (9)
	10^{-5}	21·9 ± 0·9 (5)
	10^{-4}	15·5 ± 0·5 (4)
	10^{-3}	9·1 ± 0·3 (5)
Atropine	0	23·3 ± 0·9 (8)
	$2{\cdot}9 \times 10^{-6}$	24·0 ± 0·7 (4)
	$2{\cdot}9 \times 10^{-5}$	18·5 ± 0·9 (4)
	$8{\cdot}6 \times 10^{-5}$	14·8 ± 0·6 (4)
	$2{\cdot}9 \times 10^{-4}$	9·8 ± 0·3 (4)
Physostigmine	0	25 4 ± 0 6 (5)
	3×10^{-6}	22
	6×10^{-6}	16, 16
	$1{\cdot}2 \times 10^{-5}$	17, 18
	$2{\cdot}4 \times 10^{-5}$	15, 13
	5×10^{-5}	11, 13
	1×10^{-4}	9·2
	2×10^{-4}	7·3

For details of pre-incubation, see text. Incubation was for 30 min in a medium containing ACh 4 μg/ml, 25 mM KCl, 5×10^{-3} mM soman and one of the substances mentioned in the table. The number of experiments is given in parenthesis; otherwise individual observations are recorded.

Metabolic poisons. The accumulation of ACh in the tissue takes place against a concentration gradient. This suggests that the process requires energy. To test this the effect of a few metabolic poisons on ACh uptake was studied. They were included in the medium for the last 30 min of the pre-incubation period and during incubation. It appeared that 2,4-dinitrophenol inhibited the accumulation of ACh in the slices, as shown in Table 2. Its log concentration-effect curve was not linear. It was very steep between

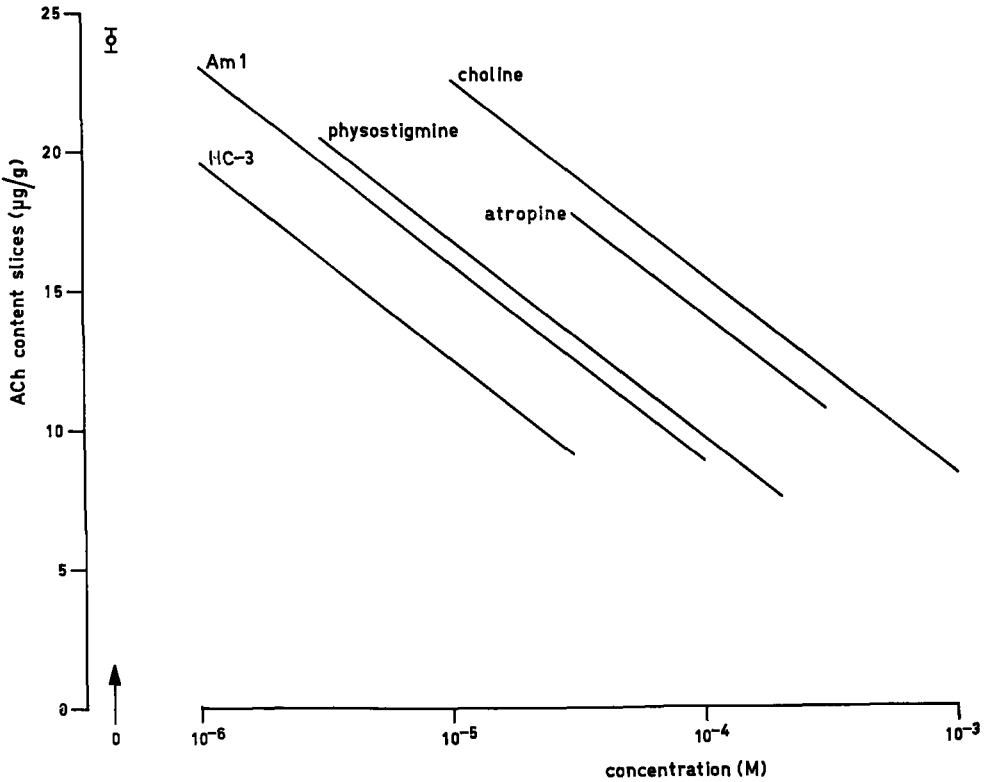

FIG. 2. Influence of drugs on the accumulation of ACh in cortical slices. Mean slope of the regression lines calculated from the data in Table 1.

1 x 10^{-4}M and 2 x 10^{-4}M. Oligomycin, sodium azide, amylobarbitone sodium and *p*-chloromercuribenzoate also inhibited ACh uptake (Table 2). The effect of ouabain, which is known to inhibit cation transport across the cell membrane and to be antagonized by potassium (see Skou, 1965), was tested in media containing either 4·7 or 25 mM KCl. In both conditions it inhibited ACh uptake only slightly.

Anaerobic incubation. The uptake of ACh by slices incubated anaerobically was compared with that in aerobic conditions in a medium containing 4·7 mM KCl.

According to McIlwain & Rodnight (1962) oxygenation is incomplete in slices of cerebral cortex thicker than 0·4 mm. The present experiments were therefore performed with thinner slices, prepared by the method proposed by these authors. As shown in Table 3, the uptake of ACh during aerobic incubation appeared not to be significantly different from that found in the

experiments reported above in which slices with a thickness of about 0.5 mm were used. During incubation in an atmosphere of 95% nitrogen and 5% carbon dioxide the accumulation of ACh was strongly inhibited. The results summarized in Table 3 further demonstrate that during anaerobic incubation part of the endogenous ACh was lost from the tissue. It apparently was released into the bath for there was a corresponding increase in the amounts recovered from the medium.

Temperature. The ACh uptake was temperature-dependent. The ACh concentration in the tissue after incubation for 30 min in a 25 mM KCl medium containing ACh 4 μg/ml was $21{\cdot}7 \pm 0{\cdot}6$ μg/g (11 observations) at 37° C, $16.3 \pm 0{\cdot}5$ μg/g (eight observations) at 27° C and $10{\cdot}8 \pm 0{\cdot}4$ μg/g (six observations) at 17° C. Since the ACh concentration in the slices immediately after pre-incubation (at 37° C) is approximately 7 μg/g about 15, 9 and 4 μg/g had accumulated in the tissue at 37°, 27° and 17° C respectively. The Q_{10} of the uptake process therefore was about 2.

Autoradiography. Autoradiographs from sections of slices which had been incubated with (^{3}H-acetyl) ACh showed a diffuse and uniform labell-

TABLE 2. INFLUENCE OF SOME METABOLIC POISONS ON THE UPTAKE OF ACh BY CORTICAL SLICES.

Drug or condition	Conc. (M)	ACh conc. slices (μg/g) ± S.E.M.
2,4-dinitrophenol	0	$22{\cdot}6 \pm 0{\cdot}9$ (13)
	$2{\cdot}5 \times 10^{-5}$	$17{\cdot}4 \pm 1{\cdot}0$ (4)
	5×10^{-5}	$13{\cdot}5 \pm 0{\cdot}4$ (4)
	1×10^{-4}	$12{\cdot}4 \pm 0{\cdot}3$ (8)
	2×10^{-4}	$6{\cdot}3 \pm 0{\cdot}1$ (10)
Oligomycin	0	$21{\cdot}6 \pm 1{\cdot}4$ (4)
	10^{-6} g/ml	$14{\cdot}9 \pm 0{\cdot}3$ (4)
Sodium azide	0	$21{\cdot}5 \pm 0{\cdot}6$ (8)
	2×10^{-4}	$21{\cdot}3 \pm 1{\cdot}3$ (3)
	5×10^{-4}	$18{\cdot}9 \pm 0{\cdot}4$ (4)
	1×10^{-3}	$15{\cdot}0 \pm 1{\cdot}0$ (4)
	2×10^{-3}	$12{\cdot}0 \pm 1{\cdot}5$ (4)
Amylobarbitone sodium	0	$26{\cdot}2 \pm 0{\cdot}7$ (6)
	2×10^{-3}	$10{\cdot}1 \pm 0{\cdot}4$ (6)
p-Chloromercuribenzoate	0	$23{\cdot}9 \pm 1{\cdot}0$ (10)
	$2{\cdot}5 \times 10^{-4}$	$15{\cdot}1 \pm 0{\cdot}6$ (3)
	1×10^{-3}	$10{\cdot}5 \pm 0{\cdot}5$ (6)
Ouabain	0	$22{\cdot}2 \pm 0{\cdot}5$ (8)
	10^{-4}	$22{\cdot}5 \pm 0{\cdot}9$ (3)
	10^{-3}	$18{\cdot}8 \pm 0{\cdot}3$ (6)

Details as in Table 1.

TABLE 3. INFLUENCE OF ANAEROBIC INCUBATION ON THE ENDOGENOUS ACh CONTENT OF CEREBRAL CORTEX AND ON THE UPTAKE OF ADDED ACh.

	ACh content of slices and amounts of ACh released into medium (μg/g of tissue) after incubation for 30 min in a medium:			
	Without added ACh		Containing ACh 4 μg/ml	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Slices	6·4 ±0·1	5·1±0·3	23·8±0·7	7·9±0·3
Medium	0·26±0·01	1·4±0·2		
Total	6·7 ±0·1	6·5±0·2		

The KCl concentration of the medium was 4·7 mM. The figures are the means of seven observations ± S.E.M.

ing of the cytoplasm of all cells. The cell nuclei appeared not to be labelled. The radioactivity showed no visible preference for certain cells or cell structures. It made no difference whether incubation with (^{3}H-acetyl) ACh was followed by incubation with non-radioactive ACh or not.

DISCUSSION

When slices of rat cerebral cortex were incubated in a medium containing ACh after their cholinesterase activity had been inhibited by soman ACh accumulated in the tissue up to a concentration about six times as high as that in the surrounding medium. This suggests that the accumulation of ACh is an energy requiring process. The suggestion is supported by the observation that the uptake of ACh did not take place in anaerobic condition, and that it was partially or totally blocked by the inhibitors of oxidative phosphorylation 2,4-dinitrophenol, oligomycin and amylobarbitone sodium, and by *p*-chloromercuribenzoate, an inhibitor of glycolysis.

A common mechanism may underlie the inhibitory action of hemicholinium-3, Am 1, physostigmine, atropine and choline on the uptake of ACh, because the log concentration-effect curves of these substances were found to be linear and parallel. This mechanism may be linked to the affinity of these substances for sites with which ACh also combines. Schuberth & Sundwall (1967), who also observed the inhibiting effects of some of these compounds, postulated a competitive mechanism of action on the basis of the kinetics of their inhibiting action. It is remarkable that the cholinesterase inhibitors physostigmine and Am 1 inhibited ACh uptake, whereas the cholinesterase inhibitors soman and tabun did not. In older unpublished experiments DFP was also found to have no effect on ACh uptake. Cholinesterase inhibitors such as soman, tabun and DFP are believed to react with an ester binding group ("esteratic site") on the enzyme molecule ; cholinesterase inhibitors

such as physostigmine and Am 1, which possess a positively charged group, combine with a negative group ("anionic site") in addition (Cohen & Oosterbaan, 1963). Since soman, tabun and DFP do not inhibit the uptake of ACh by cortical slices, the inhibitory effect on ACh uptake is apparently not linked to the cholinesterase blocking property. It may, however, be related to the affinity for anionic sites. All drugs without a known effect on energy metabolism, which were found to inhibit ACh uptake, possess a positively charged group at physiological pH (7·4) and therefore are able to combine with anionic sites. These drugs are hemicholinium-3, Am 1, physostigmine, atropine, choline, oxotremorine, morphine and ditran (a mixture of 70% 1-ethyl-2-pyrrolidylmethyl phenyl-cyclopentylglycolate and 30% 1-ethyl-3-piperidyl phenyl-cyclopentylglycolate, Abood and Biel, (1962). Oxotremorine, morphine and ditran were reported by Schuberth & Sundwall (1967) to inhibit ACh uptake by cortical slices of mouse brain.

Autoradiographs from sections of cerebral cortex in which (^{3}H-acetyl) ACh had accumulated showed that the radioactive material had penetrated into the cytoplasm of the cells. No preference of the (^{3}H-acetyl) ACh for certain sites in the tissue was seen. This and the finding that the accumulation of ACh is blocked in anaerobic conditions raise the question as to the physiological significance of the ACh uptake by tissue slices.

SUMMARY

1. The ACh content of cortical slices from rat brain was determined after incubation for 30 min in a medium containing ACh (4 μg/ml). The cholinesterase activity of the slices had been inhibited by pretreatment with 3,3-dimethyl 2-butyl methylphosphonofluoridate (soman).

2. ACh accumulated in the tissue slices, up to a concentration of about six times that in the medium.

3. The uptake of ACh was partly inhibited by potassium in high concentrations.

4. Hemicholinium-3, O-ethyl S-diethylaminoethyl ethylphosphonothiolate, physostigmine, atropine and choline, in that order of potency, inhibited the accumulation of ACh in the cortical slices, but soman and ethyl N,N-dimethyl phosphonoamidocyanate (tabun) had no effect on the uptake of ACh.

5. Substances interfering with energy metabolism, such as 2,4-dinitrophenol, oligomycin, sodium azide, amylobarbitone sodium and *p*-chloromercuribenzoate inhibited the uptake of ACh. Ouabain had little inhibitory effect.

6. In anaerobic conditions the accumulation of ACh in the tissue slices was nearly blocked.

7. The uptake of ACh in the tissue slices was dependent on temperature. The Q_{10} was about 2.

8. Autoradiography of sections from slices in which (^{3}H-acetyl) choline had accumulated showed a diffuse distribution of radioactivity in the cytoplasm of all cells. There was no visible preference for certain cells or cell structures.

I am greatly indebted to Mrs. M. M. Bertels-Meeuws and Mrs. A. P. Thiessen-Clay for their technical assistance, to Dr. D. Bootsma for the performance of the autoradiography, to Dr. M. Wijnans for the statistical analyses and to Professor Dr. E. M. Cohen and Dr. E. Meeter for their helpful criticism of the manuscript.

REFERENCES

ABOOD, L. G. & BIEL, J. H. (1962). Anticholinergic psychotomimetic agents. *Int. Rev. Neurobiol.*, **4**, 217-273.

APPLETON, T. C. (1964). Autoradiography of soluble labelled compounds. Part III. *J. R. microsc. Soc.*, **83**, 277-281.

BERTELS-MEEUWS, M. M. & Polak, R. L. (1968). Influence of antimuscarinic substances on *in vitro* synthesis of acetylcholine by rat cerebral cortex. *Br. J. Pharmac. Chemother.*, **33**, 368-380.

COHEN, J. A. & OOSTERBAAN, R. A. (1963). The active site of acetylcholinesterase and related esterases and its reactivity towards substrates and inhibitors. In *Anticholinesterase Agents*, ed. Koelle, G. B. *Handbuch exp. Pharmak.*, suppl. 15, pp. 299-373. Berlin: Springer-Verlag.

ELLIOTT, K. A. C., SWANK, R. L. & HENDERSON, N. (1950). Effects of anaesthetics and convulsants on acetylcholine content of brain. *Am. J. Physiol.*, **162**, 469-474.

ELLIOTT, K. A. C. & HENDERSON, N. (1951). Factors affecting acetylcholine found in excised rat brain. *Am. J. Physiol*, **165**, 364-374.

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

HALD, A. (1952). *Statistical Theory with Engineering Applications*, pp. 573, 579. New York: John Wiley and Sons Inc.

MCILWAIN, H. & RODNIGHT, R. (1962). *Practical Neurochemistry*, pp. 112-120. London: J. and A. Churchill Ltd.

POLAK, R. L. (1967). Uptake of acetylcholine by the isolated cerebral cortex of the rat. *Acta physiol. pharmac. neerl.*, **14**, 505.

POLAK, R. L. & MEEUWS, M. M. (1966). The influence of atropine on the release and uptake of acetylcholine by the isolated cerebral cortex of the rat. *Biochem. Pharmac.*, **15**, 989-992.

SCHUBERTH, J. & SUNDWALL, A. (1967). Effects of some drugs on the uptake of acetylcholine in cortex slices of mouse brain. *J. Neurochem.*, **14**, 807-812.

SKOU, J. C. (1965). Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. *Physiol. Rev.*, **45**, 596-617.

WILCOXON, F. (1945). Individual comparisons by ranking methods. *Biometr. Bull.*, **1**, 80-83.

Reprinted from: British Journal of Pharmacology (1970) **40**, 406-417.

STIMULATION BY ATROPINE OF ACETYLCHOLINE RELEASE AND SYNTHESIS IN CORTICAL SLICES FROM RAT BRAIN

P. C. MOLENAAR AND R. L. POLAK

INTRODUCTION

Atropine and hyoscine were shown to enhance the release of acetylcholine (ACh) from the surface of the exposed cerebral cortex of the cat, rabbit, and sheep, and from the superfused caudate nucleus of the cat when hydrolysis of ACh was prevented by a cholinesterase inhibitor (Mitchell, 1963; Szerb, 1964; Polak, 1965). A similar effect was observed in cortical slices from rat brain incubated with a cholinesterase inhibitor provided the incubation medium contained a high concentration of potassium which in itself caused a great enhancement of the release and synthesis of ACh. This was demonstrated as early as 1939 by Mann, Tennenbaum & Quastel. Atropine or other antimuscarinic agents added to such a medium in low concentration further greatly increased the release and synthesis of ACh (Polak & Meeuws, 1966; Bertels-Meeuws & Polak, 1968).

The experiments described in the present paper were carried out to obtain information about the mechanism of the atropine effect. For this purpose, the action of tetrodotoxin (TTX), botulinum type A toxin, physostigmine, and of changes in the calcium and magnesium concentration of the medium were examined on the synthesis and release of ACh in incubated cortical slices from rat brain. Some of the results have been communicated elsewhere (Polak, 1970).

METHODS

Female albino rats (160-190 g) from two different strains, Brofo and Glaxo, were lightly anaesthetized with ether and decapitated. The brains were immediately removed from the skull. From each hemisphere one cortical slice thinner than 0·4 mm was cut at room temperature with a recessed glass guide and weighed on a torsion balance after removal of excess of adherent fluid. The cortical slices were collected and stored for 20-60 min in an oxygenated phosphate buffered (pH 7·4) medium of the following composition (mM): NaCl, 118·5 ; KCl, 4·7 ; $CaCl_2$, 0·8 ; $MgSO_4$, 1·2 ; Na_2HPO_4, 9·2 ; glu-

cose, 10 ; at room temperature (20°-25° C). The method is that described by McIlwain & Rodnight (1962).

A pre-incubation of 60 min preceded an incubation period of the same duration except when stated otherwise. In most experiments eight 25 ml vessels, each containing 56-201 mg of tissue and 2·5 ml of the pre-incubation medium, were simultaneously set up. After pre-incubation, the cortical slices were rinsed once with 2·5 ml of the medium used for incubation and then incubated in 2·5 ml.

The composition of the pre-incubation medium was (mM): NaCl, 118·5 ; $NaHCO_3$, 24·9 ; KCl, 4·7 ; $CaCl_2$, 2·5 ; $MgSO_4$, 1·2 ; KH_2PO_4, 1·2 ; glucose, 10 ; and the cholinesterase inhibitor soman, 0·005. The composition of the incubation medium differed with regard to the KCl, $CaCl_2$ and $MgSO_4$ concentration. That of KCl was always 25 mM and that of the other two salts varied in different experiments as described in the text. No osmotic compensations were made for these changes. The incubation medium contained in addition the drugs whose effects were to be examined. Pre-incubation and incubation were carried out at 37° C under continuous shaking in an atmosphere of 95% oxygen and 5% carbon dioxide.

After incubation the fluid in which the slices had been incubated was collected in calibrated tubes, the slices were washed three times with 1·0 ml of fluid of the same composition as that of the pre-incubation medium, and the washings were added to the tubes. In order to determine the "total extractable" ACh content the slices were extracted by homogenization in an HCl-acidified pre-incubation medium according to the method of Elliot, Swank & Henderson (1950). In some experiments the cortical slices of one or two of the vessels were extracted immediately after pre-incubation to determine the ACh content of the tissue at zero time.

Extracts and media were frozen after adjustment of the volumes to 10 ml and of the pH to 4 and then stored at —12° C until assayed for ACh.

The ACh-like activity of the extracts and media was assayed against an ACh perchlorate standard solution on the dorsal leech muscle treated with physostigmine. The values are expressed in terms of μg of the chloride per gram of tissue (initial wet weight). Suitable dilutions of alkali treated (pH near 14 for 120 min) and subsequently reneutralized extracts or of the media used for incubation were added to the standard solutions in order to correct as far as possible for substances other than ACh, which might influence the sensitivity of the assay preparation.

Experiments with botulinum type A toxin. To examine the effect of the toxin the procedure was slightly modified. For each experiment, thirty-two slices derived from sixteen rats (Glaxo) were trimmed so that the initial wet

weight of each slice was between 30 and 35 mg. The slices were randomly distributed over two 100 ml Erlenmeyer vessels so that each vessel received sixteen slices. The vessels contained 10 ml of the pre-incubation medium with soman 0·005 mM. To one of them botulinum type A toxin was added, and to the other only the solvent of the toxin. Pre-incubation was carried out for 2 or 4 hr under continuous shaking in an atmosphere of 95% oxygen and 5% carbon dioxide at 37° C. The following precautions against the toxin were taken during and after pre-incubation. The flow of humid 95% oxygen-5% carbon dioxide which was led through the vessels passed first a trap filled with cotton wool and then a trap filled with a solution of $Ca(OCl)_2$ (8%) before reaching the air of the fume hood in which pre-incubation was carried out. After pre-incubation the slices were rinsed five times with the phosphate buffered medium used for the preparation of the slices before they were randomly distributed over eight 25 ml vessels containing four slices per vessel. Subsequently they were rinsed once with the medium used for incubation and incubated in 2·5 ml for one hour. During the subsequent extraction the acidified tissue homogenates, instead of being stored at room temperature, were heated to between 90° and 100° C for 5 min so as to inactivate any botulinum toxin which might have been present. For the same reason the incubation media were similarly heated during 5 min after acidification to pH 4. In two experiments intraperitoneal injections into mice of either 0·5 ml pooled incubate or 0·5 ml pooled extract were found not to be lethal. For calculating the ACh per gram of tissue the mean initial wet weight of the slices was used.

Drugs used. Atropine sulphate (Brocades), acetylcholine perchlorate (BDH), physostigmine sulphate (NBCo), soman (3,3-dimethyl 2-butyl methylphosphonofluoridate). The soman was synthesized by the Chemical Laboratory RVO-TNO, Rijswijk Z.H., The Netherlands. Crude botulinum type A toxin was kindly supplied to us by Dr. J. Keppie from the Microbiological Research Establishment, Porton Down, England. A stock solution was prepared in a medium containing 100 mM NaCl, 75 mM Na-acetate and 0·2% gelatin (pH 6·2) and stored at +4° C. The solution was not homogeneous. Dilutions were freshly made for each experiment. To obtain an indication of the potency of the toxin it was injected intraperitoneally into mice to determine the 96 hr LD50 according to the method of Schantz (1964). The LD50 was found to be about 3 ng. Tetrodotoxin (Sankyo) was tested for its potency on several indirectly stimulated phrenic nerve-diaphragm preparations from rats. It produced total paralysis within 12 min in a concentration of 0·1 μg/ml and within 3 min in a concentration of 0·3 μg/ml.

Statistical evaluation. The statistical significance at the 5% level was

established by Welch's t test. When the number of observations was smaller than six, Wilcoxon's rank sum test was used. For quotients and differences between results obtained in the presence and in the absence of atropine each observation in the presence of atropine was paired at random with one observation in its absence, and the means $\pm$S.E.M. of these quotients and differences were calculated. Bonferoni's t statistics were used for all multiple comparisons (Miller, 1966).

RESULTS

Under comparable conditions, the amounts of ACh released and synthesized by slices from Glaxo rats were often greater than those from Brofo rats, whereas the relative magnitude of the stimulating effect of atropine on the release and synthesis of ACh was somewhat smaller. In previous experiments from this laboratory cortical slices from Brofo rats were used; however, all the effects described were also observed with Glaxo rats. The results illustrated in Table 1 and Fig. 3 were obtained from Glaxo, those in the other figures from Brofo rats.

Tetrodotoxin. According to Dudar & Szerb (1969), tetrodotoxin (TTX) reduces the release of ACh from the exposed cerebral cortex of the anaesthetized cat and abolishes the stimulating action of atropine on this release. When the toxin was examined on the cortical slices from rat brain it was found not to have these effects. This is illustrated in Fig. 1.

The first column shows the ACh content of cortical slices before incubation, and the other columns give the ACh in the slices and incubation media after 1 hr incubation. The results shown in the second and third column were obtained without atropine. Approximately 5 μg/g ACh was synthesized during the 1 hr incubation whether TTX had been added to the medium (third column) or not (second column). Thus TTX appears to have no effect on synthesis and release of ACh in incubated cortical slices from rat brain. The results represented by the fourth and fifth column were obtained in the presence of atropine; they show that synthesis and release of ACh were greatly enhanced but that this effect of atropine was not inhibited by TTX. Thus TTX does not abolish the effect of atropine on synthesis and release of ACh in incubated cortical slices from rat brain.

The concentration of TTX used in the experiment of Fig. 1 was 0·8 μg/ml. Since the concentration of TTX topically applied by Dudar & Szerb to the surface of the cerebral cortex of the cat was much higher — 10 μg/ml — this concentration was used for an experiment similar to that of Fig. 1. Again synthesis and release of ACh were not affected by the toxin whether atropine was present in the incubation medium or not.

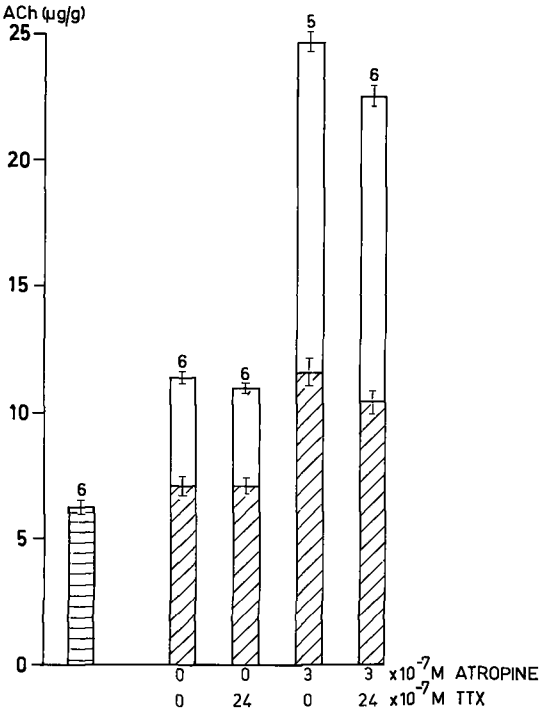

FIG. 1. Influence of TTX on release and synthesis of ACh by cortical slices from rat brain. The incubation period was 60 min, the medium contained 25 mM KCl and 0·005 mM soman. The concentrations of atropine and TTX are given below the columns. ▤, Extractable ACh content of the slices before incubation; ▨, extractable ACh content of the slices after incubation; □, ACh released during incubation. The released ACh ± S.E.M. is presented on top of and is separate from the extractable ACh ± S.E.M. after incubation. The numbers of experiments are indicated by the figures above the columns.

Botulinum type A toxin. Pre-incubation of cortical slices for 2 hr in a medium containing 20,000 mouse LD50/ml of botulinum type A toxin resulted in a depression of synthesis and release of ACh during incubation. The depression was not enhanced by prolongation of the pre-incubation to 4 hr or by raising the botulinum toxin concentration to 250,000 mouse LD50/ml, but reducing the toxin concentration to 3,000 mouse LD50/ml led to a significantly smaller depression. Statistical analysis demonstrated that it was permissible to pool the results obtained with 20,000 LD50/ml (pre-incubation 2 or 4 hr) and 250,000 LD50/ml (pre-incubation 2 hr since they were not signifcantly different. Table 1 gives the pooled results obtained under these conditions.

The upper half of Table 1 summarizes results obtained without botulinum

toxin and shows that atropine increased the ACh in the incubation medium as well as in the cortical slices. The total yield of ACh from both sources was increased from 15·9 to 25·5 μg/g, emphasizing the strong stimulating effect of atropine both on release and synthesis. The lower half of Table 1 shows that after pre-incubation with botulinum toxin the amounts of ACh released into the incubation medium were greatly reduced. In the absence of atropine 3·1 instead of 6·1 μg/g and in its presence 4·8 instead of 12·5 μg/g were released into the medium during the 1 hr incubation. So the release of ACh was greatly depressed by the pretreatment with toxin. Pretreatment with the toxin had no significant effect on the ACh content of the cortical slices when the subsequent incubation was without atropine; with atropine, the increase in the ACh content no longer occurred. After incubation with atropine the ACh content was only 9·4 μg/g as compared with 9·2 μg/g, whereas the corresponding values for the untreated slices were 13·0 and 9·8 μg/g. From the total yield obtained from slices plus media, particularly when incubation was with atropine, it is evident that the toxin had a depressant effect not only on release but also on synthesis of ACh.

TABLE 1. INFLUENCE OF PRETREATMENT WITH BOTULINUM TYPE A TOXIN ON THE RELEASE AND SYNTHESIS OF ACh BY CORTICAL SLICES INCUBATED FOR 1 HR IN A 25 mM KCl CONTAINING MEDIUM WITH OR WITHOUT 3 x 10^{-7}M ATROPINE (MEAN VALUES ± S.E.M.)

		number of observations	ACh in μg/g		
			in incubation medium	in slices	in medium plus slices
No botulinum toxin	no atropine	8	6.1 ± 0.23	9.8 ± 0.50	15.9 ± 0.57
	atropine	8	12.5 ± 0.42	13.0 ± 0.69	25.5 ± 0.72
Botulinum toxin	no atropine	7	3.1 ± 0.17	9.2 ± 0.43	12.3 ± 0.59
	atropine	7	4.8 ± 0.25	9.4 ± 0.52	14.2 ± 0.71

Ca ions. The $CaCl_2$ concentration of the pre-incubation medium was 0·8 mM instead of the usual 2·5 mM and that of the incubation medium was either 0, 0·8, 2.5 or 5·5 mM. The results obtained under these conditions are summarized in Fig 2. They show that synthesis and release of ACh were greatest when the incubation medium contained 2·5 mM $CaCl_2$, and lowering its concentration to 0·8 mM resulted in a decrease of synthesis and release; there was a further decrease when the incubation medium contained no calcium. The effect was more pronounced in the presence than in the absence of atropine. Potassium and also calcium are therefore required to bring out the full stimulating effect of atropine. Raising the calcium concentration from 2·5 to 5·5 mM also decreased the synthesis and release of ACh but

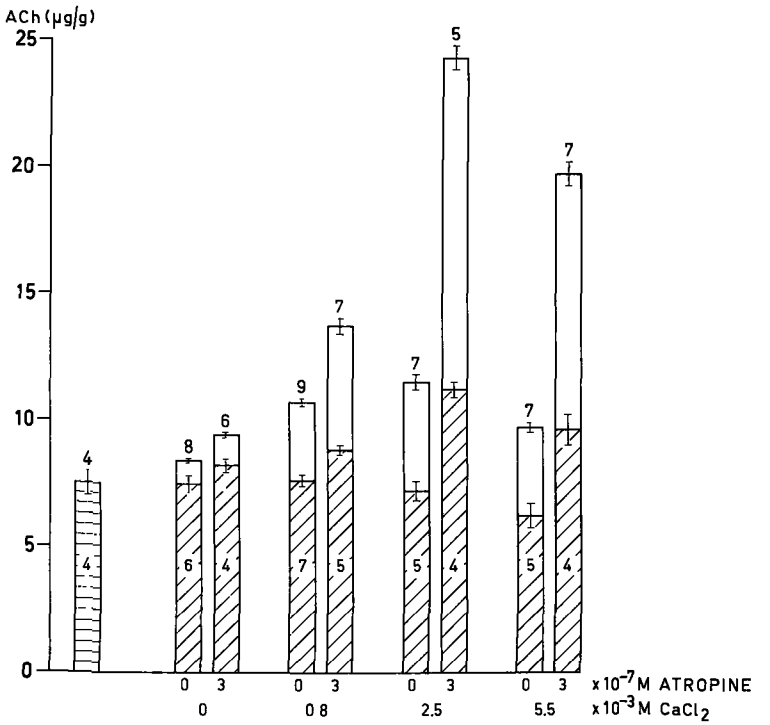

FIG. 2. Influence of the calcium concentration of the medium on the release and synthesis of ACh by cortical slices. The results are presented in the same way as in Fig. 1. The numbers of experiments for the released ACh are indicated by the figures above the columns and for the ACh content of the slices by the figures inside the columns. The concentrations of atropine and $CaCl_2$ are given below the columns.

the stimulating action of atropine was little affected. At a 2·5 mM $CaCl_2$ concentration atropine increased the total yield of ACh from 11·5 to 24·3 µg/g (211%) and at a 5·5 mM concentration from 9·7 to 19·7 µg/g (203%). If the release into the incubation medium alone is taken into account it will be seen that atropine multiplied the release differently at different calcium concentrations. In media containing 0, 0·8, 2·5 and 5·5 mM $CaCl_2$ the multiplication factors were $1{\cdot}3 \pm 0{\cdot}10$ (six observations), $1{\cdot}8 \pm 0{\cdot}12$ (seven observations), $3{\cdot}2 \pm 0{\cdot}18$ (five observations) and $3{\cdot}0 \pm 0{\cdot}16$ (seven observations), respectively. All multiplication factors, except those obtained in 2·5 and 5·5 mM $CaCl_2$, were significantly different from one another ($P_2 < 0{\cdot}05$).

When the incubation medium contained physostigmine in addition to soman the omission of calcium from the medium also reduced synthesis and release of ACh, and the stimulating effect of atropine was not only reduced but abolished. This is evident from the results shown in Fig. 3. They also show that

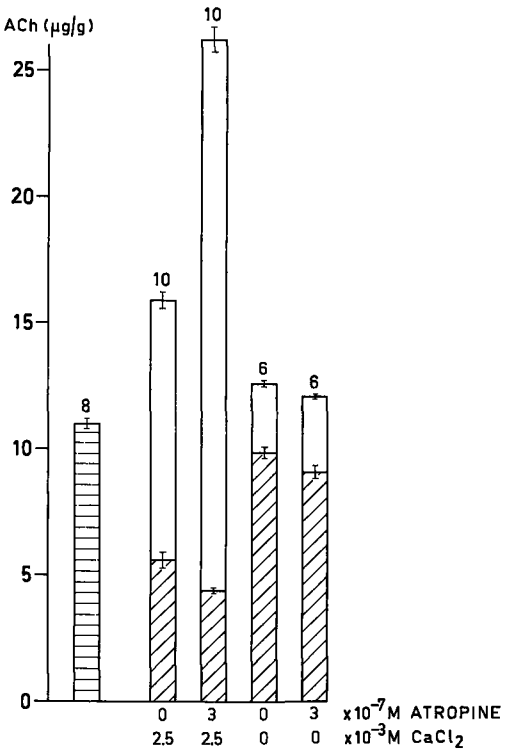

FIG. 3. Inverse relationship between the final extractable ACh content of the slices and the rate of ACh release. The results are presented in the same way as in Figs. 1 and 2. Physostigmine (8 x 10^{-4} M) present in medium during incubation.

the ACh content of the cortical slices was affected differently on incubation with physostigmine. In contrast to the experiments of Fig. 2 the ACh content of the cortical slices became greatly reduced on incubation in the presence of 2·5 mM $CaCl_2$. The content decreased from $11{\cdot}1 \pm 0{\cdot}21$ µg/g to $5{\cdot}6 \pm 0{\cdot}29$ µg/g and, when atropine was added to the incubation medium, even further to $4{\cdot}4 \pm 0{\cdot}11$ µg/g. In previous experiments with cortical slices from Brofo rats a similar decrease of the ACh content had been obtained but the enhancement of this effect by atropine was not noted (Polak & Meeuws, 1966; Bertels-Meeuws & Polak, 1968). A reduction of the ACh content of the cortical slices was also present when calcium had been omitted from the incubation medium, but the reduction was much smaller. When the ACh released is considered in connection with the ACh content of the slices an inverse relationship is evident: the greater the release the lower the content.

Mg ions. The effect of $MgSO_4$ in the incubation medium was examined in the presence of 2·5 mM $CaCl_2$. As seen from the results given in Fig. 4

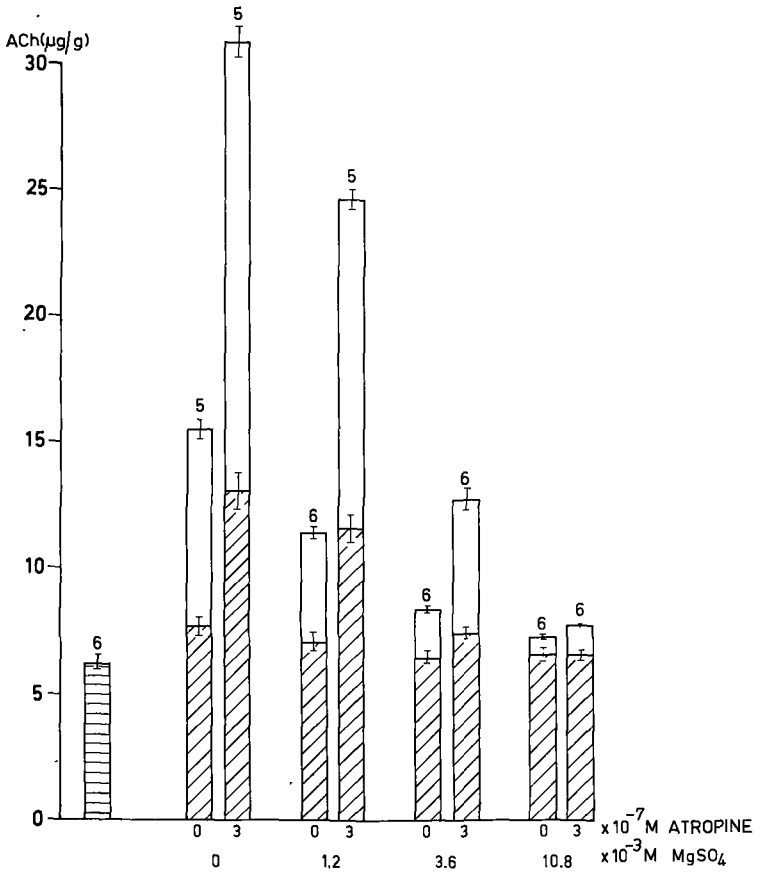

FIG. 4. Influence of the magnesium concentration of the medium on the release and synthesis of ACh by cortical slices. The results are presented in the same way as in Fig. 1. The concentrations of atropine and $MgSO_4$ are given below the columns.

raising the Mg concentration progressively reduced synthesis and release of ACh. This occurred in the absence and in the presence of atropine, but up to a Mg concentration of 3·6 mM the stimulating action of atropine on the ACh release was little affected. The factor by which the atropine multiplied the release was $2·3 \pm 0·18$ (five observations) in the absence of Mg and $3·2 \pm 0·22$ (five observations) and $2·8 \pm 0·11$ (six observations) in the presence of 1·2 and 3·6 mM $MgSO_4$ respectively. These multiplication factors were not significantly different from one another. Synthesis and release of ACh were further reduced when the Mg concentration was raised to 10·8

mM, but the stimulating action of atropine on the ACh release was still present although it was now smaller ; the factor by which the atropine multiplied the release was only $1{\cdot}7 \pm 0{\cdot}11$ (six observations), which is significantly smaller than the multiplication factors of 3·2 and 2·8 obtained in the presence of lower concentrations of $MgSO_4$, but not significantly different from the 2·3 obtained in its absence.

DISCUSSION

The previous finding that atropine increases the release of ACh from and its synthesis by cortical slices from rat brain incubated in a Krebs-Ringer solution containing a high potassium concentration (Polak & Meeuws, 1966 ; Bertels-Meeuws & Polak, 1968) has been confirmed. This stimulating effect of atropine could not have resulted from an anti-cholinesterase action of atropine because the cholinesterase activity of the cortical slices was fully inhibited by the use of soman. Nor can it easily be explained by interference with the re-uptake of released ACh, a suggestion originally made to explain the increased release of ACh from the surface of the cerebral cortex or from the caudate nucleus obtained in *in vivo* experiments (Szerb, 1964 ; Polak, 1965). When this suggestion was made the possibility of increased synthesis of ACh by atropine was not taken into consideration since these *in vivo* experiments dealt only with release of ACh. In the cortical slices, however, atropine not only stimulates release but also synthesis of ACh, since the total yield of ACh — that is, the ACh present in the slices and released into the incubation medium — was increased after incubation with atropine. To explain this increased yield without increased synthesis, one would have to assume that in the absence of atropine the released ACh is at once taken up by the tissue and metabolized, and that atropine prevents this metabolism. When this possibility was investigated it was found that although cortical slices treated with soman or other organophosphorus anticholinesterases have the ability to take up large amounts of ACh added to the medium, this ACh was not metabolized in significant amounts and atropine in low concentration did not affect the uptake (Polak & Meeuws, 1966 ; Schuberth & Sundwall, 1967; Liang & Quastel, 1969a, b ; Polak, 1969). Thus the increased yield signifies increased synthesis of ACh.

Another suggestion was made by MacIntosh (1963) to explain the finding that atropine stimulates the release of ACh from the exposed cerebral cortex of the living animal. According to this suggestion atropine would block cholinergic synapses which form a part of inhibitory neuronal circuits controlling the activity of the intra-cortical neurones from which the ACh is released. Recent findings by Dudar & Szerb (1969) appeared to support this hypo-

thesis since they found that TTX, a compound which blocks nervous conduction (Kao, 1966), applied to the exposed cerebral cortex of anaesthetized cats abolished the stimulating action of atropine. The same result was obtained when the TTX was applied in a solution containing 50 mM KCl. These results are different from those obtained in the present experiments with cortical slices from rat brain, in which TTX did not inhibit the stimulating action of atropine on the release and synthesis of ACh. This difference between the effect of TTX on the cat brain *in vivo* and on cortical slices from rat brain is at present unexplained. But, as far as cortical slices from rat brain are concerned, the inability of TTX to influence the stimulating effect of atropine restricts its site of action to the nerve endings from which the ACh presumably originates.

To attribute to atropine a stimulating action not only on release but also on synthesis of ACh may appear to be in contradiction to the finding that atropine has no influence on the synthesis of ACh by cell-free choline acetylase systems (Giarman & Pepeu, 1964 ; Schuberth, 1965). However, in cortical slices in which the cellular structures are more or less intact increased synthesis may be brought about indirectly by the increased release of ACh. This possibility was tested experimentally by investigating the effect of procedures which diminish the release, such as a reduction of the calcium concentration or an increase of the magnesium concentration of the incubation medium, or pre-incubation of the slices with botulinum type A toxin. If, under these conditions, atropine were to produce an increased ACh content of the cortical slices, it would be evidence for a direct stimulating action of atropine on the synthesis. It was found, however, that under all three conditions the stimulating effect of atropine on both the release and the synthesis of ACh was greatly reduced. This suggests that it is primarily the release which is enhanced by atropine and that its stimulating action on the synthesis results from the increased release. This suggestion is supported by results obtained with physostigmine.

It has been mentioned that ACh added to the incubation medium is taken up in large amounts by the cortical slices. This uptake was observed in the presence of soman with low and with high KCl concentrations and in the absence and in the presence of atropine in low concentrations. The uptake of added ACh, however, was strongly inhibited by physostigmine (Polak, 1969). Yet physostigmine appears to inhibit also the uptake of endogenous ACh released from cortical slices. Evidence for this effect of physostigmine is based on earlier observations (Polak & Meeuws, 1966 ; Bertels-Meeuws & Polak, 1968) which showed that the addition of physostigmine to incubation media containing a high KCl concentration resulted in a decrease

of the total extractable ACh content of the slices, whereas the amounts of ACh recovered from the incubation medium were increased by the same amount. Consequently, the sum of released ACh and extracted ACh was not changed. Moreover, physostigmine did not influence the stimulating effect of atropine on the synthesis of ACh. These results are readily explained on the assumption that the ACh released from the slices is partly taken up by the tissue and that this re-uptake is inhibited by physostigmine. It has now been found that the decrease in the extractable ACh of the slices during incubation in a medium containing a high KCl concentration and physostigmine was inhibited when the release and synthesis of ACh were decreased by omission of the calcium ions from the medium. In contrast, the reduction of the ACh content in the slices was definitely enhanced by atropine. If atropine were to stimulate the synthesis primarily, one would expect the ACh content of the slices to increase and certainly not to decrease under its influence. However, if the primary effect were on the release, a reduction could be expected. This reduction was actually found, but only in the presence of physostigmine, because in its absence it was probably masked by the re-uptake of released ACh.

The release of ACh from cortical slices resembles in many respects the spontaneous release of ACh at the neuromuscular junction which gives rise to miniature endplate potentials (MEPP's). Like the MEPP frequency (Elmquist & Feldman, 1965 ; Katz & Miledi, 1967) it is not affected by TTX. However, whereas botulinum toxin *in vitro* completely abolishes the MEPP frequency at the neuromuscular junction (Brooks, 1956), it only partly blocks the release of ACh from cortical slices. On the other hand, raised potassium concentrations in the medium, which strongly increase the MEPP frequency (Del Castillo & Katz, 1954 ; Liley, 1956 ; Hubbard, 1961) also stimulate the release of ACh from cortical slices, whereas reduction of the calcium or elevation of the magnesium concentration results in a decrease of the ACh release from slices in a way similar to the effects of these ions on the MEPP frequency (Hubbard, 1961 ; Gage & Quastel, 1966). Finally the release of ACh from the exposed cerebral cortex of anaesthetized cats has been shown to be affected by the calcium and magnesium concentration of the topically applied salt solutions (Randič & Padjen, 1967; Hemsworth & Mitchell, 1969) in about the same way as these ions affected the release from cortical slices in the present experiments.

SUMMARY

1. Cortical slices from rat brain were incubated in media containing the

irreversible cholinesterase inhibitor soman and a high KCl concentration, and the release and synthesis of acetylcholine (ACh) were determined.

2. Atropine enhanced the release and synthesis of ACh.

3. Tetrodotoxin, a substance which blocks nervous conduction, did not influence the release and synthesis of ACh, in the absence or in the presence of atropine. Therefore the nerve endings are probably the site at which atropine acts when stimulating the release and synthesis of ACh.

4. Pretreatment of the slices with botulinum type A toxin partially blocked the release and synthesis of ACh and reduced the extra amounts of ACh released and synthesized under the influence of atropine.

5. Lowering the calcium or raising the magnesium concentration in the incubation medium reduced the release and synthesis of ACh and their enhancement by atropine.

6. Physostigmine decreased the total extractable ACh content of the slices during incubation in a 25 mM KCl containing medium. This decrease was nearly prevented when the release and synthesis of ACh were inhibited by omission of the calcium ions from the medium, but was enhanced by atropine.

7. The observations made with pretreatment by botulinum type A toxin, with changes in the calcium and magnesium concentration as well as with physostigmine, all support the theory that it is primarily the release of ACh which is enhanced by atropine and that its stimulating action on the synthesis results from the increased release.

The authors are greatly indebted to Mrs. Anne Thiessen for skilful technical assistance, to Dr. M. Wijnans for statistical calculations, to Dr. J. Keppie for the botulinum type A toxin, and to Dr. E. Meeter and Professor Dr. E. M. Cohen for stimulating discussions.

REFERENCES

BERTELS-MEEUWS, M. M. & POLAK, R. L. (1968). Influence of antimuscarinic substances on *in vitro* synthesis of acetylcholine by rat cerebral cortex. *Br. J. Pharmac. Chemother.*, **33**, 368-380.

BROOKS, V. B. (1956). An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature endplate potentials. *J. Physiol., Lond.*, **134**, 264-277.

DEL CASTILLO, J. & KATZ, B. (1954). Changes in end-plate activity produced by pre-synaptic polarization. *J. Physiol., Lond.*, **124**, 586-604.

DUDAR, J. D. & SZERB, J. C. (1969). The effect of topically applied atropine on resting and evoked cortical acetylcholine release. *J. Physiol., Lond.*, **203**, 741-762.

ELLIOTT, K. A. C., SWANK, R. L. & HENDERSON, N. (1950). Effect of anaesthetics and convulsants on acetylcholine content of brain. *Am. J. Physiol.*, **162**, 469-474.

ELMQUIST, D. & FELDMAN, D. S. (1965). Spontaneous activity at a mammalian neuromuscular junction in tetrodotoxin. *Acta physiol. scand.*, **64**, 475-476.

GAGE, P. W. & QUASTEL, D. M. J. (1966). Competition between sodium and calcium ions in transmitter release at mammalian neuromuscular junctions. *J. Physiol., Lond.*, **185**, 95-123.

GIARMAN, N. J. & PEPEU, G. (1964). The influence of centrally acting cholinolytic drugs on brain acetylcholine levels. *Br. J. Pharmac. Chemother.*, **23**, 123-130.

HEMSWORTH, B. A. & MITCHELL, J. F. (1969). The characteristics of acetylcholine release mechanisms in the auditory cortex. *Br. J. Pharmac.*, **36**, 161-170.
HUBBARD, J. I. (1961). The effect of calcium and magnesium on the spontaneous release of transmitter from mammalian motor nerve endings. *J. Physiol., Lond.*, **159**, 507-517.
KAO, C. Y. (1966). Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmac. Rev.*, **18**, 997-1049.
KATZ, B. & MILEDI, R. (1967). Tetrodotoxin and neuromuscular transmission. *Proc. Roy. Soc. B*, **167**, 8-22.
LIANG, C. C. & QUASTEL, J. H. (1969a). Uptake of acetylcholine in rat brain cortex slices. *Biochem. Pharmac.*, **18**, 1169-1185.
LIANG, C. C. & QUASTEL, J. H. (1969b). Effects of drugs on the uptake of acetylcholine in rat brain cortex slices. *Biochem. Pharmac.*, **18**, 1187-1194.
LILEY, A. W. (1956). The effects of presynaptic polarization on the spontaneous activity of the mammalian neuromuscular junction. *J. Physiol., Lond.*, **134**, 427-443.
MACINTOSH, F. C. (1963). Synthesis and storage of acetylcholine in nervous tissue. *Can. J. Biochem. Physiol.*, **41**, 2555-2571.
MANN, P. J. G., TENNENBAUM, M. & QUASTEL, J. H. (1939). CIII. Acetylcholine metabolism in the central nervous system; the effects of potassium and other cations on acetylcholine liberation. *Biochem. J.*, **33**, 822-835.
MCILWAIN, H. & RODNIGHT, R. (1962). *Practical Neurochemistry*, pp. 113-120. London: J. and A. Churchill Ltd.
MILLER, R. G. (1966). *Simultaneous Statistical Inference*. London-New York: McGraw-Hill Book Co.
MITCHELL, J. F. (1963). The spontaneous and evoked release of acetylcholine from the cerebral cortex. *J. Physiol., Lond.*, **165**, 98-116.
POLAK, R. L. (1965). Effect of hyoscine on the output of acetylcholine into perfused cerebral ventricles of cats. *J. Physiol., Lond.*, **181**, 317-323.
POLAK, R. L. (1969). The influence of drugs on the uptake of acetylcholine by slices of rat cerebral cortex. *Br. J. Pharmac.*, **36**, 144-152.
POLAK, R. L. (1970). An analysis of the stimulating action of atropine on release and synthesis of acetylcholine in cortical slices from rat brain. *Proceedings of the International Symposium on the Effect of Drugs on Cholinergic Mechanisms in the CNS*, Skokloster, Sweden, in the Press.
POLAK, R. L. & MEEUWS, M. M. (1966). The influence of atropine on the release and uptake of acetylcholine by the isolated cerebral cortex of the rat. *Biochem. Pharmac.*, **15**, 989-992.
RANDIČ, M. & PADJEN, A. (1967). Effect of calcium ions on the release of acetylcholine from the cerebral cortex. *Nature, Lond.*, **215**, 990.
SCHANTZ, E. J. (1964). Botulism, Proceedings of a symposium. Publ. No. 999-FP-1, p. 92. Cincinnati, Ohio: U.S. Department of Health, Education and Welfare, Public Health Service.
SCHUBERTH, J. (1965). On the biosynthesis of acetyl coenzyme A in the brain. 1. The enzymic formation of acetyl coenzyme A from acetate, adenosine triphosphate and coenzyme A. *Biochim. biophys. Acta*, **98**, 1-7.
SCHUBERTH, J. & SUNDWALL, A. (1967). Effects of some drugs on the uptake of acetylcholine in cortex slices of mouse brain. *J. Neurochem.*, **14**, 807-812.
SZERB, J. C. (1964). The effect of tertiary and quaternary atropine on cortical acetylcholine output and on the electroencephalogram in cats. *Can. J. Physiol. Pharmac.*, **42**, 303-314.

To be published in: British Journal of Pharmacology (1971)

THE STIMULATING ACTION OF ATROPINE ON THE RELEASE OF ACETYLCHOLINE BY RAT CEREBRAL CORTEX *IN VITRO*

R. L. POLAK

INTRODUCTION

Atropine increases the release of acetylcholine (ACh) from incubated tissue slices of rat cerebral cortex. As the increased release was shown not to be secondary to an increased synthesis of ACh, the atropine must affect the release itself (Molenaar & Polak, 1970). In the present paper two possible explanations for this action of atropine are examined.

Atropine might act by replacing ACh at the storage sites of the nerve endings. In that case, the release should come to an end as soon as these sites are occupied by atropine. This possibility was examined by studying the release during prolonged incubation of the tissue slices. According to another possibility, suggested earlier (Polak, 1967) the released ACh inhibits further release of ACh and this inhibition is antagonized by atropine. Its action would therefore be a kind of 'disinhibition'. In that case, the addition of sufficient ACh to the incubation medium should inhibit the action of atropine. This would be difficult to demonstrate as the added ACh would interfere with the assay of the released ACh on the physostigmine treated leech preparation used for this purpose. The difficulty was overcome by using, instead of ACh, oxotremorine or acetyl β-methylcholine (methacholine), two strong muscarinic drugs which do not contract the leech muscle.

METHODS

Two strains of female albino rats weighing between 160 and 190 g were used, Glaxo rats for the experiments on prolonged incubation and Brofo rats for the experiments with oxotremorine and methacholine. The two strains were the same as in previous experiments from this laboratory (Molenaar & Polak, 1970).

The method for preparing, pre-incubating and incubating the cortical slices from the brain were the same as described previously (Molenaar & Polak, 1970). Incubation was in a medium containing a high KCl (25 mM) concentration and the cholinesterase inhibitor soman (0·005 mM). Incubation was preceded by a pre-incubation period of 60 min in a medium which dif-

fered from the incubation medium in having a lower KCl (4·7 mM) concentration and in not containing the drugs to be investigated.

For the prolonged incubation experiments in which the incubation medium was withdrawn and replaced every half hour the Erlenmeyer flasks containing the slices with the incubation medium had an opening at the bottom which was closed by a polyethylene stopper through which a hollow needle had been passed. The outer protruding end of the needle was connected by polyethylene tubing to a syringe so as to allow withdrawal and replacement of the medium without interrupting the incubation. A special device was needed to prevent plugging of the needle by pieces of tissue. A small cylindrical cage consisting of a frame of stainless steel covered with nylon gauze was placed vertically above the opening of the needle and fixed to the stopper.

The tissue slices were extracted according to the method of Elliott, Swank & Henderson (1950) and the ACh of the extracts and of the incubation media was assayed against ACh perchlorate on the dorsal leech muscle pretreated with physostigmine. The details of the procedure were the same as described previously (Molenaar & Polak, 1970) but the ACh values are expressed in nmoles per gram of wet tissue.

Drugs. Atropine sulphate (Brocades); choline chloride (B.D.H.); methacholine chloride (Brocades); physostigmine sulphate (NBCo); soman synthesized by the Chemical Laboratory RVO-TNO; oxotremorine, the free base, kindly provided by Dr. B. C. Barrass from the Chemical Defense Establishment, Porton Down, England.

The concentrations of the sulphates of atropine and physostigmine are expressed as molar concentrations of the alkaloid moieties, that is M should be read as g-equiv/l.

RESULTS

Effect of atropine during prolonged incubation. The results of an experiment in which incubation with and without atropine was extended to 3.5 hr are shown in Fig. 1. On incubation without atropine the amounts of ACh released in the successive half hour samples did not differ much though there was a slight tendency of a decrease from $16 \pm 0{\cdot}3$ nmoles/g in the first, to $14 \pm 0{\cdot}7$ nmoles/g in the seventh half hour period. On incubation with atropine, however, there was a definite and progressive decrease from $44 \pm 2{\cdot}4$ nmoles/g in the first to $23 \pm 1{\cdot}2$ nmoles/g in the seventh half hour sample, and if only the stimulating effect of atropine, i.e. the increase in the release, is taken into account this increase became progressively smaller as seen from the results shown in Table 1. The increase amounted to 28 nmoles/g during the first 30 min period but to only 9 nmoles/g during the seventh 30 min period.

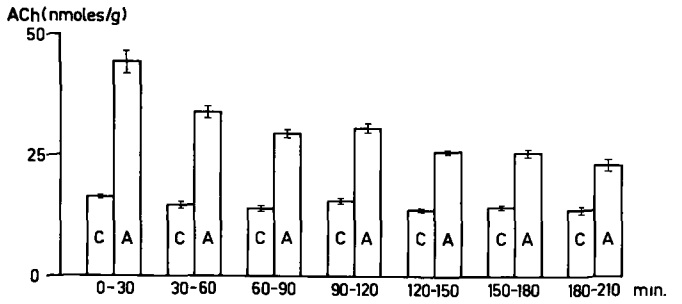

FIG. 1. ACh release (expressed in nmoles per gram initial weight of wet tissue) from cortical slices in seven successive 30 min periods obtained from eight portions of slices incubated simultaneously in separate vessels, four without (C) and four with (A) atropine 3 x 10^{-6}M. Each column represents the mean value from four vessels and shows the standard error of the mean.

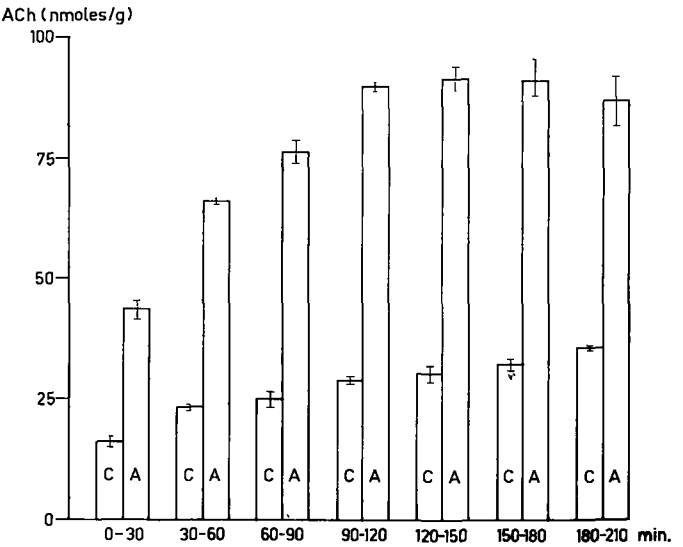

FIG. 2. Similar experiment as that of Fig. 1 but with the addition of choline 3 x 10^{-5}M to the incubation medium in each vessel.

This progressive reduction of the stimulating action of atropine on ACh release could be attributed to choline lack since it did not occur when extra choline was added to the incubation medium. This is illustrated in Fig. 2. Prolonged incubation under this condition actually led to an increase in ACh release both in the presence and in the absence of atropine. If only the increase produced by atropine is taken into account it became greater with time. As shown in Table 1 it amounted to 27 nmoles/g in the first 30 min period and rose to 61 nmoles/g in the fifth 30 min period and then fell slightly to

51 nmoles/g in the seventh 30 min, which is still nearly twice as great as that during the first 30 min period.

TABLE 1. THE CHANGE IN THE INCREASE IN ACh (nmoles/g) RELEASE PRODUCED BY ATROPINE IN SEVEN SUCCESSIVE 30 MIN SAMPLES ON INCUBATION WITHOUT (a) AND WITH (b) THE ADDITION OF CHOLINE TO THE INCUBATION MEDIUM.

min	a	b
0—30	28	27
30—60	19	42
60—90	15	51
90—120	15	61
120—150	12	61
150—180	11	58
180—210	9	51

The increase is the difference between the release in the presence and absence of atropine. Same experiments as those of Fig. 1 and 2.

Effect of oxotremorine and methacholine. With oxotremorine two effects were observed. On incubation of the cortical slices without atropine the amount of ACh extracted from the slices was reduced and the amount in the incubation medium increased but the sum of both was not significantly changed by the oxotremorine. On incubation in the presence of atropine its stimulating effect on the release was inhibited.

The effect of oxotremorine on the ACh content of the tissue in the absence of atropine is seen from a comparison of the first two columns of the experiment b of Fig. 3. A similar effect was previously observed when physostigmine was added to the incubation medium containing soman, although physostigmine did not prevent the stimulating action of atropine (Bertels-Meeuws & Polak, 1968).

The antagonistic effect of oxotremorine on the increased ACh release produced by atropine is shown for two atropine concentrations in the experiments a and b of Fig. 3. The results of these experiments suggest that the antagonism may be of a competitive nature because the effect of $0{\cdot}3 \times 10^{-7}$M atropine was abolished by 10×10^{-6}M oxotremorine (exp. a), whereas the stronger effect of 3×10^{-7}M atropine was only reduced by 20×10^{-6}M but abolished by 100×10^{-6}M oxotremorine (exp. b).

With methacholine the same two effects as with oxotremorine were obtained. On incubation for one hour without atropine a concentration of 10^{-4}M metacholine reduced the amount of ACh extracted from the tissue slices and increased by nearly the same amount the ACh in the medium.

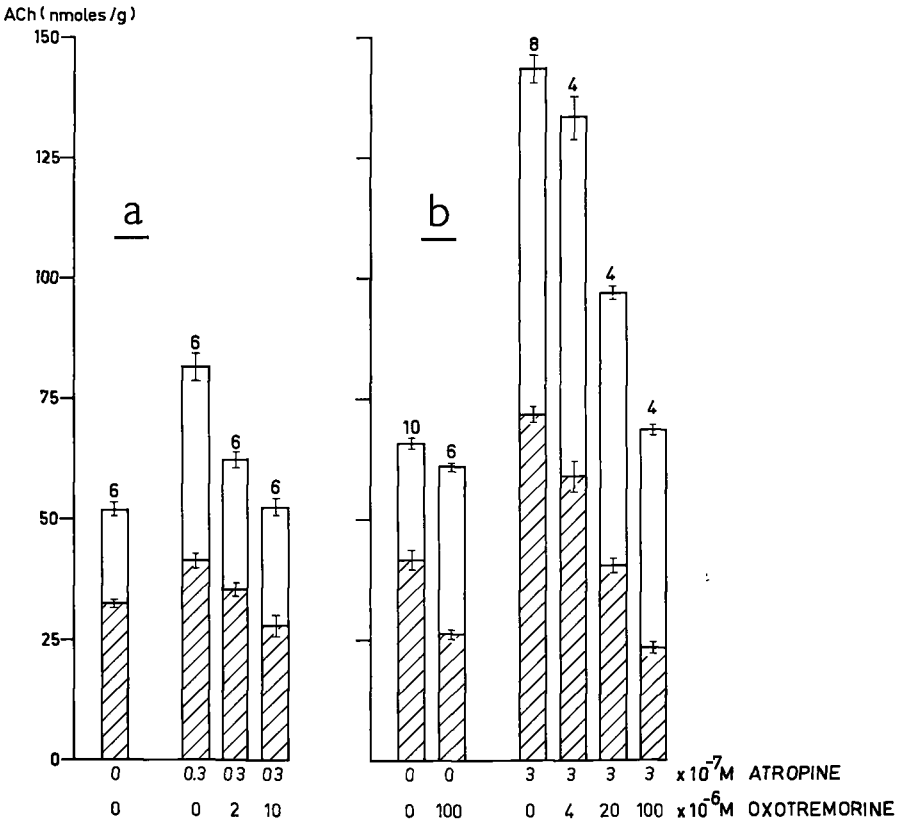

FIG. 3. ACh (expressed as nmoles per gram initial weight of wet tissue) of cortical slices and incubation media after 1 hr incubation with and without atropine and oxotremorine. For each column the ACh extracted from the tissue slices is represented by the hatched, and the ACh in the incubation medium by the blanc area. The figures on top of the columns give the number of observations from which the mean values were obtained.

In order to study the antagonism between methacholine and atropine without interference by the reduction in ACh content of the slices and the corresponding increase in the medium the experiments were carried out in the presence of physostigmine (in addition to soman). Under this condition these changes are produced by the physostigmine and therefore occur also in the controls i.e. in the absence of methacholine. Such an experiment is illustrated in Fig. 4.

As seen from the first four columns the methacholine had little effect in the absence of atropine since physostigmine was present in the media; there was no significant reduction in the ACh content of the tissue slices but the release was somewhat inhibited. On the other hand the stimulat-

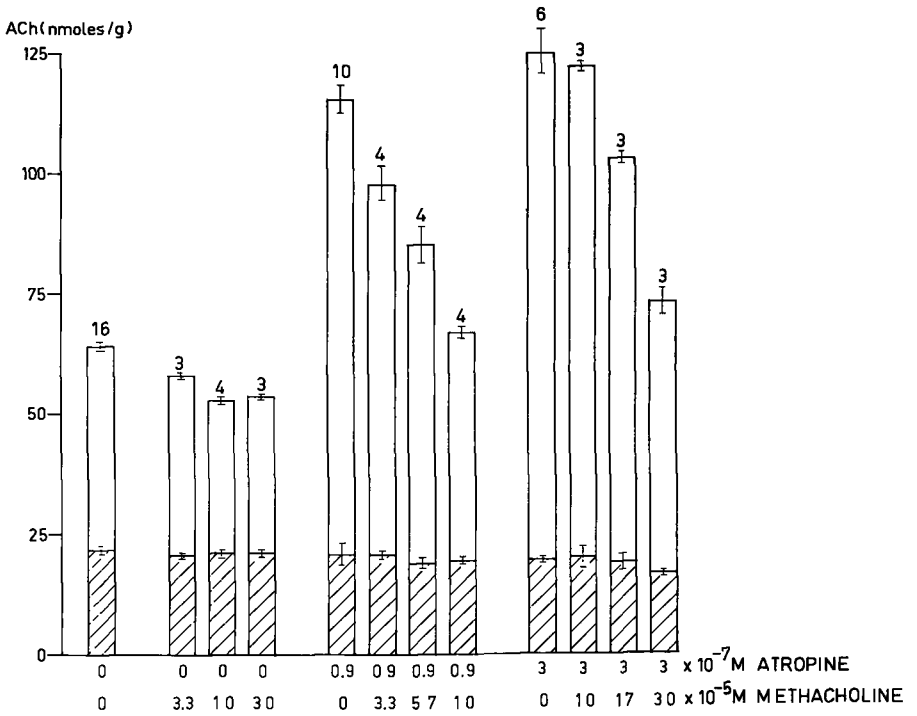

FIG. 4. ACh (expressed as nmoles per gram initial weight of wet tissue) of cortical slices and incubation media after 1 hr incubation with and without atropine and methacholine. Physostigmine sulphate (10^{-4}M) present in incubation medium. Other details same as Fig. 3.

ing effect of atropine on the release of ACh was greatly inhibited. Again this effect seemed to be of a competitive nature, since, as seen from the last eight columns, it required weaker concentrations of methacholine to inhibit and nearly abolish the stimulating effect by atropine in a concentration of 0·9 x 10^{-7}M than in a concentration 3 x 10^{-7}M.

DISCUSSION

Two possibilities were considered to explain the stimulating action of atropine on the release of ACh from incubated tissue slices. Atropine was thought either to act by replacing ACh at the storage sites of the nerve endings, or to act by a kind of disinhibition, i.e. by inhibiting an inhibitory effect of the released ACh on further release.

The results obtained on prolonged incubation would be difficult to explain on the assumption that atropine expels and replaces the ACh at its storage sites, because in that case, the effect of atropine should come to an end

after a certain incubation period when the sites have become occupied by the atropine. A reduction in the amount of ACh released by the atropine was obtained on prolonged incubation but it was fully accounted for by lack of choline and no longer occurred when choline was added to the incubation medium. In this condition, the amounts of ACh released by the atropine actually increased during the first two and a half hours of incubation and then decreased, but slightly only, so that even after three and a half hours they were still nearly twice those released during the first half hour. Choline not only increased the stimulating effect of atropine on the release, but also augmented the release which occurred without atropine. This suggests that during prolonged incubation of cortical slices the choline supplied by them is not sufficient to bring about optimal conditions for synthesis and release; and the effect of this deficiency would naturally become even more pronounced when the release is stimulated by atropine.

On the other hand the assumption that the action of atropine can be looked upon as a kind of disinhibition, counteracting an inhibitory effect of released ACh on the further release is supported by the results obtained with oxotremorine and methacholine. The stimulating effect of atropine on the release and synthesis of ACh is linked to the antimuscarinic property (Bertels-Meeuws & Polak, 1968). If atropine enhances ACh release from the nerve endings, it is to be expected that endogenous ACh has the opposite action. This implies the existence of a negative feed back mechanism in which released ACh by acting on presynaptic muscarinic receptors inhibits the release of more ACh from the nerve endings (Polak, 1967). Due to technical limitations the effect of sufficiently high concentrations of added ACh could not be tested, but the atropine effect was antagonized by oxotremorine and by methacholine, two strong muscarinic agents which do not interfere with the bio-assay of ACh on the leech muscle. The effective concentrations of oxotremorine and methacholine, however, were very high in comparison to the low concentrations in which atropine enhanced the release and synthesis of ACh but this may merely imply a very high concentration of the released ACh at the presynaptic membrane.

Both oxotremorine and methacholine not only antagonized the action of atropine but in addition caused, in the absence of atropine from the incubation medium, a decrease in the ACh content of the cortical slices with a nearly corresponding increase in release. A similar effect was previously obtained with physostigmine which does not prevent the stimulating action of atropine on the release (Bertels-Meeuws & Polak, 1968). Since all three drugs inhibit ACh uptake by cortical slices, as shown for physostigmine by Polak & Meeuws (1966) and later by Polak (1969), for oxotremorine by

Schuberth & Sundwall (1967) and for methacholine by Liang & Quastel (1969), this effect is readily explained by inhibition of re-uptake of released ACh, and it is not linked to the atropine antagonism which is not obtained with physostigmine.

SUMMARY

1. In cortical slices from rat brain incubated in a medium containing the irreversible cholinesterase inhibitor soman (0·005 mM) and a high concentration of KCl (25 mM) atropine exerts a stimulating action on the release of acetylcholine (ACh).

2. Two possibilities were examined to explain this action. Atropine might expel ACh from the nerve endings by occupying its storage sites or it might prevent an inhibitory action of the released ACh on its further release by occupying muscarinic receptors at the presynaptic endings; its action would then be a kind of "disinhibition".

3. The stimulating action of atropine on ACh release persisted during prolonged incubation (up to 3.5 hr) provided choline was added to the medium. This finding would be difficult to explain if atropine were to act by expelling ACh from its storage sites.

4. The stimulating action of atropine on ACh release was inhibited by oxotremorine and by methacholine, added to the medium in high concentrations. This finding is readily explained if atropine acts by "disinhibition".

I wish to thank Mrs. Anne Thiessen for her excellent technical assistance, Dr. M. Wijnans for the statistical calculations, Professor Dr. E. M. Cohen for stimulating discussions and Dr. B. C. Barrass for the oxotremorine.

REFERENCES

BERTELS-MEEUWS, M. M. & POLAK, R. L. (1968). Influence of antimuscarinic substances on *in vitro* synthesis of acetylcholine by rat cerebral cortex. *Br. J. Pharmac., Chemother.*, **33**, 368-380.

ELLIOTT, K. A. C., SWANK, R. L. & HENDERSON, N. (1950). Effect of anaesthetics and convulsants on acetylcholine content of brain. *Am. J. Physiol.* **162**, 469-474.

LIANG, C. C. & QUASTEL, J. H. (1969). Effects of drugs on the uptake of acetylcholine in rat brain cortex slices. *Biochem. Pharmac.*, **18**, 1187-1194.

MOLENAAR, P. C. & POLAK, R. L. (1970). The influence of atropine on the production of acetylcholine by rat cerebral cortex *in vitro*. *Br. J. Pharmac.*, **40**, 406-417.

POLAK, R. L. (1967). The influence of antimuscarinic drugs on synthesis and release of acetylcholine by the isolated cerebral cortex of the rat. *J. Physiol., Lond.*, **191**, 34-35 *P*.

POLAK, R. L. (1969). The influence of drugs on the uptake of acetylcholine by slices of rat cerebral cortex. *Br. J. Pharmac.*, **36**, 144-152.

POLAK, R. L. & MEEUWS, M. M. (1966). The influence of atropine on the release and uptake of acetylcholine by the isolated cerebral cortex of the rat. *Biochem. Pharmac.*, **15**, 989-992.

SCHUBERTH, J. & SUNDWALL, A. (1967). Effects of some drugs on the uptake of acetylcholine in cortex slices of mouse brain. *J. Neurochem.*, **14**, 807-812.

SUMMARY AND CONCLUSIONS

There is a number of observations which indicate that atropine and hyoscine have the ability to increase the release of acetylcholine (ACh) from the central nervous system in the living animal (MacIntosh & Oborin, 1953 ; Mitchell, 1963 ; Szerb, 1964 ; Polak, 1965). The present thesis deals with experiments on cortical slices from rat brain. They show that atropine-like substances also stimulate the release of ACh from rat cerebral cortex *in vitro* and were carried out in order to analyse this effect.

The method used was as follows. Cortical slices were prepared from each hemisphere of the rat brain, incubated in a bicarbonate buffered Krebs-Ringer solution containing the cholinesterase inhibitor, soman, and the ACh content of the medium and of the cortical slices after extraction with hydrochloric acid was assayed on the leech preparation sensitized for ACh with physostigmine. The actual period of incubation during which synthesis and release of ACh were investigated was preceded by a pre-incubation period in the same medium in order to allow the soman to act.

Under these conditions ACh is released into the medium and new ACh is synthetized by the slices. Furthermore the ACh production was found to be increased if the KCl concentration in the medium is raised. In high potassium media atropine produces an additional increase in the release as well as in the synthesis of ACh (Polak & Meeuws, 1966; Bertels-Meeuws & Polak, 1968). Media containing 25 mM KCl were routinely used for incubation because the stimulating effect of atropine appeared to be dependent on the potassium concentration of the medium and is optimal at about 25 mM. It was found that the stimulating action of atropine must be linked to its antimuscarinic activity since it depends in the same way as the antimuscarinic activity upon the steric configuraton of atropine and since other antimuscarinic substances than atropine also stimulate the release of ACh from cortical slices. Moreover, these agents are effective in remarkably low concentrations (Bertels-Meeuws & Polak, 1968).

As these results were obtained in the presence of soman, one explanation of the stimulating action of atropine is excluded. That is, it cannot result from a possible anticholinesterase activity of atropine, because the cholinesterase was already completely inactivated by the soman. Moreover, atropine is practically devoid of anticholinesterase activity. Another possibility to be considered was that the released ACh is, at least in part, taken up by the tissue and that this re-uptake is prevented by atropine. This possibility had been suggested by several authors (Giarman & Pepeu, 1964 ; Szerb, 1964 ; Polak, 1965 ; Celesia & Jasper, 1966). Such a mechanism is, how-

ever, unlikely to account for the very great increase in the total yield of ACh produced by the atropine, unless one were to assume that any ACh taken up is at once metabolized. This possibility was investigated and it was found that, although ACh added to the incubation medium is taken up in large amounts by the cortical slices, this ACh is not or not significantly metabolized (Polak & Meeuws, 1966 ; Schuberth & Sundwall, 1967 ; Polak, 1969 ; Liang & Quastel, 1969a, b). Moreover, the uptake is not prevented by atropine in concentrations which produce its stimulating effect on release (Polak & Meeuws, 1966 ; Polak, 1969). Consequently, the stimulating action of atropine cannot be attributed to inhibition of re-uptake of ACh.

The observation that cortical slices have the ability to accumulate large amounts of added ACh (Polak & Meeuws, 1966) has been confirmed by several authors (Schuberth & Sundwall, 1967 ; Liang & Quastel, 1969a). Anoxia, metabolic inhibitors, and substances with a positively charged group in the molecule were found to inhibit the uptake (Polak, 1967 ; Schuberth & Sundwall, 1967 ; Polak, 1969 ; Liang & Quastel, 1969a, b). The physiological significance of the phenomenon, however, has remained an object of speculation.

It has been reported that atropine influences neither the synthesis of ACh in cell-free choline acetylase systems (Giarman & Pepeu, 1964) nor that of acetyl Co A by brain extracts (Schuberth, 1965). These findings, of course, do not exclude a stimulating effect of atropine on the synthesis of ACh in more or less intact cellular structures, like cortical slices. In fact, our results (Polak & Meeuws, 1966 ; Bertels-Meeuws & Polak, 1968) demonstrate that the synthesis of ACh was stimulated by the atropine since the sum total of released and extracted ACh was greatly increased under its influence. However, the stimulation of the synthesis might have been brought about indirectly by the increased release of ACh. This possibility was tested experimentally by investigating the effect of procedures which interfere with the release mechanism, such as a reduction of the Ca concentration or an increase of the Mg concentration of the incubation medium, or a pre-incubation of the slices with botulinum type A toxin. If, under these conditions, atropine were to produce an increased ACh content of the cortical slices, this would be evidence for a direct stimulating action of atropine on the synthesis. We found, however, that under all three conditions the stimulating effect of atropine was greatly reduced (Molenaar & Polak, 1970). This suggests that it is primarily the release which is enhanced by atropine and that its stimulating action on the synthesis results from this increased release.

Results obtained with physostigmine support this idea. Mention has been made that ACh added to the incubation medium is taken up in large amounts

by the cortical slices. This uptake was obtained in the presence of soman with low and with high KCl concentrations and in the absence or in the presence of atropine (in low concentrations). However, when studying the effect of adding ACh to a medium which contained physostigmine as well as soman, we found that physostigmine inhibited the uptake of ACh (Polak & Meeuws, 1966 ; Polak, 1969). Other experiments (Bertels-Meeuws & Polak, 1968) showed that physostigmine apparently has a corresponding action with regard to released ACh. After incubation of cortical slices in 25 mM KCl containing media with and without physostigmine the sum total of released and extracted ACh was practically the same. However, after incubation in the presence of physostigmine the mean ACh content of the slices was only 4·9 μg/g as compared to 7·6 μg/g in its absence, whereas the mean values for the released ACh under these two conditions were 5·4 and 2·9 μg/g. It thus appears that nearly half of the released ACh was taken up by the slices incubated without physostigmine and that on incubation with physostigmine the prevention of this uptake revealed a reduction of the ACh content of the slices below its initial value which would have been about 7 μg/g. Such reductions were obtained on incubation with 25 mM KCl, i.e. under a condition in which the release of ACh was stimulated. When the release of ACh was not or less strongly stimulated, for instance on incubation with a low (4·7 mM) KCl concentration (Bertels-Meeuws & Polak, 1968) or in media containing a high (25 mM) KCl concentration but no $CaCl_2$ (Molenaar & Polak, 1970) the reduction of the ACh content of the slices unmasked by physostigmine was much smaller. On the other hand, when the stimulation of the release of ACh by 25 mM KCl in the presence of $CaCl_2$ was enhanced by atropine this enhancement was associated with a definite further reduction in the ACh content of the slices (Molenaar & Polak, 1970). These findings support the view that the stimulating action of atropine on the synthesis of ACh results from the increased release. If atropine were to stimulate synthesis primarily one would expect the ACh content of the slices to increase under its influence or at least to remain constant, but certainly not to decrease. However, if the primary effect were on the release a reduction could be expected, and this reduction was actually found, but only in the presence of physostigmine because in its absence it was masked by the re-uptake of the released ACh.

Since atropine primarily stimulates the release of ACh we considered the following two possible mechanisms:

(1) an expulsion of ACh from its stores by replacement with atropine and
(2) an interruption of a neuronal negative feed back circuit.

Ad (1). If the increased release were to result from expulsion of ACh from its stores by their occupation by atropine one would expect that during prolonged incubation the effect should gradually decrease and come to an end. This, however, did not happen. Provided the medium contained a sufficient amount of choline, the atropine continued to stimulate ACh release in seven successive 30 min samples (Polak, 1971).

Ad (2). MacIntosh (1963) postulated that atropine stimulates the release of ACh from the exposed cerebral cortex *in vivo* by blocking synapses which form a part of inhibitory neuronal circuits controlling the activity of the intracortical neurones from which the ACh is released. Recent findings by Dudar & Szerb (1969) appeared to support this hypothesis. They found for instance that topical application of tetrodotoxin, a compound which blocks nervous conduction (Kao, 1966), to the exposed cerebral cortex of anaesthetized cats abolished the stimulating action of atropine on the release of ACh. We have not been able to confirm this inhibitory effect of tetrodotoxin on the stimulating action of atropine in cortical slices from rat brain (Molenaar & Polak, 1970). Therefore, as far as this preparation is concerned, an interruption of a neuronal negative feed back circuit which requires nervous conduction cannot be involved in the stimulating action of atropine.

Yet the idea that atropine interferes with a negative feed back mechanism, might still be valid in our preparation. For instance the ACh released might act on the nerve endings thereby inhibiting further release of ACh from these nerve endings, and this inhibiting action could be prevented by atropine. Theoretically the best method for testing this hypothesis would have been to examine the effect of added ACh on the stimulating action of atropine. Since the amounts of ACh which would have to be added to the incubation medium in order to exert this effect would probably be far in excess of those released and synthesized by the slices it would be difficult to assay the ACh released and synthesized during the incubation. This difficulty was overcome by adding instead of ACh other similarly acting substances which do not interfere with the ACh assay on the leech muscle preparation. Two such substances were used for this purpose: oxotremorine and methacholine. Oxotremorine in a concentration of 10^{-5}M prevented the stimulating effect of atropine 3 x 10^{-8}M, but a concentration of 10^{-4}M was required to prevent the stimulating effect of atropine 3 x 10^{-7}M. Methacholine was about three times less effective. However, as with oxotremorine, it was possible to antagonize the stimulating action of increasing concentrations of atropine by raising the concentration of methacholine (Polak, 1971).

The findings suggest that the antagonism between atropine and the two muscarinic agents is of a competitive nature. They support the idea that

(a) the ACh released acts on muscarinic receptors in the nerve endings and thereby inhibits its further release and

(b) that the stimulating effect of atropine is to be explained by its competition with the ACh for these muscarinic receptors, thereby removing the brake on release.

REFERENCES

BERTELS-MEEUWS, M. M. & POLAK, R. L. (1968). Influence of antimuscarinic substances on *in vitro* synthesis of acetylcholine by rat cerebral cortex. *Br. J. Pharmac.* **33**, 368-380.

CELESIA, G. G. & JASPER, H. H. (1966). Acetylcholine released from cerebral cortex in relation to state of activation. *Neurology*, **16**, 1053-1063.

DUDAR, J. D. & SZERB, J. C. (1969). The effect of topically applied atropine on resting and evoked cortical acetylcholine release. *J. Physiol., Lond.*, **203**, 741-762.

GIARMAN, N. J. & PEPEU, G. (1964). The influence of centrally acting cholinolytic drugs on brain acetylcholine levels. *Br. J. Pharmac.*, **23**, 123-130.

KAO, C. Y. (1966). Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmac. Rev.*, **18**, 997-1049.

LIANG, C. C. & QUASTEL, J. H. (1969a). Uptake of acetylcholine in rat brain cortex slices. *Biochem. Pharmac.*, **18**, 1169-1185.

LIANG, C. C. & QUASTEL, J. H. (1969b). Effects of drugs on the uptake of acetylcholine in rat brain cortex slices. *Biochem. Pharmac.*, **18**, 1187-1194.

MCILWAIN, H. & RODNIGHT, R. (1962). *Practical Neurochemistry*, pp. 113-120. London: J. and A. Churchill Ltd.

MACINTOSH, F. C. (1963). Synthesis and storage of acetylcholine in nervous tissue. *Can. J. Biochem. Physiol.*, **41**, 2555-2571.

MACINTOSH, F. C. & OBORIN, P. E. (1953). Release of acetylcholine from intact cerebral cortex. *Abstr. XIX Physiol. Congr.*, 580-581.

MITCHELL, J. F. (1963). The spontaneous and evoked release of acetylcholine from the cerebral cortex. *J. Physiol., Lond.*, **165**, 98-116.

MOLENAAR, P. C. & POLAK, R. L. (1970). Stimulation by atropine of acetylcholine release and synthesis in cortical slices from rat brain. *Br. J. Pharmac.*, **40**, 406-417.

POLAK, R. L. (1965). Effect of hyoscine on the output of acetylcholine into perfused cerebral ventricles of cats. *J. Physiol., Lond.*, **181**, 317-323.

POLAK, R. L. (1967). Uptake of acetylcholine by the isolated cerebral cortex of the rat. *Acta Physiol. Pharmac. Neerl.*, **14**, 505.

POLAK, R. L. (1969). The influence of drugs on the uptake of acetylcholine by slices of rat cerebral cortex. *Br. J. Pharmac.*, **36**, 144-152.

POLAK, R. L. (1971). The stimulating action of atropine on the release of acetylcholine by rat cerebral cortex *in vitro*. *Br. J. Pharmac.*, in press.

POLAK, R. L. & MEEUWS, M. M. (1966). The influence of atropine on the release and uptake of acetylcholine by the isolated cerebral cortex of the rat. *Biochem. Pharmac.*, **15**, 989-992.

SCHUBERTH, J. (1965). On the biosynthesis of acetyl coenzyme A in the brain. I. The enzymic formation of acetyl coenzyme A from acetate, adenosine triphosphate and coenzyme A. *Biochim. Biophys. Acta*, **98**, 1-7.

SCHUBERTH, J. & SUNDWALL, A. (1967). Effects of some drugs on the uptake of acetylcholine in cortex slices of mouse brain. *J. Neurochem.*, **14**, 807-812.

SZERB, J. C. (1964). The effect of tertiary and quaternary atropine on cortical acetylcholine output and on the electroencephalogram in cats. *Can. J. Physiol. Pharmac.*, **42**, 303-314.

SAMENVATTING

Hersenschorscoupes van de rat werden aëroob geïncubeerd in media met de irreversibele cholinesteraseremmer soman. Onder deze omstandigheden gaven zij acetylcholine (ACh) af aan het medium en synthetiseerden nieuwe ACh. Afgifte en aanmaak werden sterk gestimuleerd door de KCl-concentratie van het medium te verhogen. In een medium met 25 mM KCl veroorzaakten atropine en andere antimuscarine-achtige stoffen een verdere sterke toename van de afgifte en aanmaak van ACh.

Met soman behandelde hersenschorscoupes bleken grote hoeveelheden aan het medium toegevoegde ACh in zich op te nemen zonder deze ACh om te zetten. Ook een gedeelte van de door het weefsel zelf afgegeven ACh bleek te worden geresorbeerd. Lage concentraties atropine remden deze opname niet. Hieruit werd geconcludeerd dat het stimulerende effect van atropine niet op een remming van de wederopname van vrijgekomen ACh berust.

Aannemelijk werd gemaakt dat de toename van de synthese van ACh onder invloed van atropine een gevolg is van de stimulering van de afgifte: het stimulerende effect op de synthese werd veel kleiner indien de afgifte werd geremd door vermindering van de calciumconcentratie of vermeerdering van de magnesiumconcentratie in het medium of door voorbehandeling van de hersenschorscoupes met botulinum-toxine.

De wederopname van afgegeven ACh bleek te kunnen worden geremd door middel van physostigmine. In aanwezigheid van deze stof gingen de onder invloed van atropine toegenomen afgifte en synthese van ACh gepaard met een daling van het ACh-gehalte van de coupes. Deze waarneming ondersteunt de opvatting dat atropine primair de afgifte van ACh vergroot en dat de stimulerende werking op de synthese van ACh het gevolg is van de versterkte afgifte.

De toename van de afgifte van ACh onder invloed van atropine berust niet op een bezetting van de opslagplaatsen van ACh door atropine en een daaruit voortvloeiende verdrijving van ACh uit deze opslagplaatsen, want het stimulerende effect van atropine hield uren aan indien het incubatiemedium voldoende choline bevatte om de aanmaak van de benodigde hoeveelheden ACh te onderhouden.

Door enkele onderzoekers is de mogelijkheid geopperd dat atropine de afgifte van ACh uit de blootgelegde hersenschors aan een daarmee in contact gebracht oplossing van een cholinesteraseremmer bij het intacte dier zou stimuleren door cholinerge synapsen te blokkeren die deel uitmaken van intra- of sub-corticale reflexbogen die de neuronen waaruit de ACh af-

komstig is zouden remmen. Atropine zou dan dus ontremmend werken. Deze hypothese werd verdedigd op basis van de waarneming dat de stimulerende werking van atropine werd onderdrukt door tetrodotoxine, een stof die de prikkelgeleiding in zenuwvezels blokkeert. In hersenschorscoupes van de rat evenwel bleek het stimulerende effect van atropine op de aanmaak en afgifte van ACh niet door tetrodotoxine te worden geremd. Hieruit werd geconcludeerd dat atropine — althans in hersenschorscoupes — het vrijkomen van ACh stimuleert via een directe invloed op de zenuwuiteinden zelf.

De mogelijkheid werd geopperd dat vrijgekomen ACh via muscarine-receptoren op de presynaptische membraan verdere afgifte van ACh afremt en dat atropine door blokkade van deze receptoren de remmende invloed van ACh op de afgifte van meer ACh zou opheffen. Deze hypothese, waarin eveneens een ontremmende werking van atropine wordt gepostuleerd, wordt ondersteund door de waarneming dat het stimulerende effect van atropine op de afgifte van ACh kon worden tegengegaan door oxotremorine of methacholine, twee stoffen met een sterke muscarine-achtige werking.

NAWOORD

Na het eindexamen Gymnasium β aan het Amsterdams Lyceum te Amsterdam te hebben afgelegd, begon ik in 1947 met de studie in de medicijnen aan de Universiteit van Amsterdam. In 1952 legde ik het kandidaatsexamen af en in 1955 het doctoraalexamen. In 1957 volgde het artsexamen.

Voor het vervullen van de militaire dienstplicht werd ik op het Medisch Biologisch Laboratorium van de Rijksverdedigingsorganisatie TNO te Rijswijk gedetacheerd. Hierna zette ik mijn werk op het Medisch Biologisch Laboratorium voort, in dienst van de Rijksverdedigingsorganisatie TNO. In 1963 ontving ik een NAVO-beurs, die mij in staat stelde van september 1963 tot januari 1965 farmacologisch onderzoek te verrichten in het National Institute for Medical Research te Mill Hill, London N.W.7, onder leiding van Prof. Dr. W. Feldberg. Na mijn terugkeer in Nederland voerde ik de onderzoekingen uit die in dit proefschrift zijn beschreven.

Gaarne betuig ik dank aan mijn promotor, Prof. Dr. E. M. Cohen, directeur van het Medisch Biologisch Laboratorium, voor de diepgaande kritische belangstelling die hij voor dit onderzoek heeft getoond en voor de prettige sfeer waarin ik heb kunnen werken.

Zeer erkentelijk ben ik Prof. Dr. W. Feldberg voor zijn belangrijke bijdrage aan mijn wetenschappelijke vorming en voor zijn bereidheid mij te allen tijde met raad en daad bij te staan. Ook zijn hulp bij de uiteindelijke redactie van de manuscripten was mij uiterst welkom.

Mijn dank gaat verder uit naar Dr M. Wijnans voor de statistische bewerking van de resultaten, naar Dr E. Meeter voor zijn actieve en inspirerende belangstelling en naar Mevr. M. M. Bertels-Meeuws en Mevr. A. P. Thiessen-Clay voor de enthousiaste en bekwame wijze waarop zij mij gedurende jaren hebben geassisteerd. Dr D. Bootsma, Drs P. C. Molenaar en Mej. M. R. J. van Benthem dank ik voor de uitvoering van enkele proeven en de heer G. R. Ike voor zijn grote hulpvaardigheid als bibliothecaris.

De heren H. E. Groot Bramel, M. J. M. Boermans en J. J. Weber ben ik zeer erkentelijk voor de uitstekende verzorging van de figuren en Mevr. G. C. S. Heijdt, Mej. G. Landwier en Mej. L. M. T. J. Roelofs voor het typen van diverse manuscripten. Veel dank ben ik verschuldigd aan de heer M. Hartkamp voor zijn hulp en morele steun bij het persklaar maken van dit proefschrift.

Voorts dank ik de redacties van Biochemical Pharmacology en van het British Journal of Pharmacology voor de toestemming de in deze tijdschriften gepubliceerde artikelen in dit proefschrift op te nemen.

Het Bestuur van de Rijksverdedigingsorganisatie TNO dank ik voor de mij geboden gelegenheid op het in het Medisch Biologisch Laboratorium verrichte onderzoek te promoveren.