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The influence of drugs on the uptake of acetylcholine by slices of rat cerebral cortex

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

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

3. The uptake of acetylcholine 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 acetylcholine in the cortical slices, but soman and ethyl N,N-dimethyl phosphonoamidocyanate (tabun) had no effect on the uptake of acetylcholine.

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

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

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

8. Autoradiography of sections from slices in which ^{3}H-acetylcholine 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.

In the course of an investigation of the release and synthesis of acetylcholine from cortical slices of rat brain, treated with the irreversible cholinesterase inhibitor 3,3-dimethyl-*n*-butyl 2-methylphosphonofluoridate (soman), acetylcholine added to the medium was found to accumulate in the tissue. The amounts of acetylcholine 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 acetylcholine, 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 acetylcholine 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 acetylcholine 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 (5×10^{-6}M) had been added to inactivate the cholinesterase were set up simultaneously for pre-incubation. During the incubation period the vessels contained 5 ml. of medium to some of which acetylcholine (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 acetylcholine 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) in 2 min and then left at room temperature for 60 min. Thereafter it was centrifuged, the precipitate was re-suspended 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 acetylcholine.

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 5×10^{-3}. 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 acetylcholine 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 acetylcholine content of the extracts and incubating media was determined by bio-assay on the eserinized dorsal muscle of the leech 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 acetylcholine, which might influence the sensitivity of the assay preparation.

Presentation of the results and statistical evaluation

Results are expressed as means ± 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 5×10^{-6}M soman. Thereafter incubation was carried out for 30 min in the same medium to which (^{3}H-acetyl) choline chloride (4 μg/ml.) had been added. In a few experiments a different procedure was followed. With the intention of displacing the labelled acetylcholine chloride from aspecific sites in the tissue, a 15 min exposure to (^{3}H-acetyl) choline chloride was followed by exposure to non-radioactive acetylcholine (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 method 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-*n*-butyl 2-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.

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

Results

When slices of cerebral cortex were incubated in a medium to which acetylcholine had been added, some of the acetylcholine accumulated in the tissue. After 30 min incubation in a medium containing 25 mM KCl and acetylcholine (4 μg/ml.) the acetylcholine concentration in the slices amounted to 24·01 ± 0·37 μg/g (49 observations), whereas it was only 7·17 ± 0·28 μg/g (14 observations) after 30 min incubation in the same medium without added acetylcholine. Practically the same value, 6·7 ± 0·3 μg/g (seven observations), was obtained in previous experiments without acetylcholine (Bertels-Meeuws & Polak, 1968).

Recovery of added acetylcholine

The ratio between the amount of tissue and the volume of incubation medium was chosen in such a way that the amounts of acetylcholine taken up by the slices were too small to influence the acetylcholine concentration in the medium perceptibly during the 30 min of incubation. In sixty-one experiments using an acetylcholine concentration in the medium of 4 μg/ml. an acetylcholine concentration of 3·88 ± 0·04 μg/ml. was found in the medium at the end of the incubation period. When the recovery of added acetylcholine (20 μg/5 ml. of medium) was calculated it amounted to 19·92 + 0·20 μg or 99·6%. The calculation was made by determining the difference between the total acetylcholine 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 acetylcholine.

Influence of KCl concentration on the uptake of acetylcholine

The uptake of acetylcholine 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 acetylcholine concentration in the tissue after 30 min incubation in the presence of acetylcholine (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 acetylcholine in slices of cerebral cortex in a medium containing acetylcholine (4 μg/ml.) and 25 mM KCl. This is shown in Table 1. From these data linear and parallel regression lines representing the acetylcholine 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 acetylcholine uptake.

The effect of physostigmine on the uptake of acetylcholine 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 acetylcholine in cortical slices high concentrations of soman or tabun, which also have potent anticholinesterase actions, did not influence the uptake of acetylcholine (Table 1).

Metabolic poisons

The accumulation of acetylcholine 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 acetylcholine 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 acetylcholine in the slices, as shown in Table 2. Its log concentration-effect curve was not

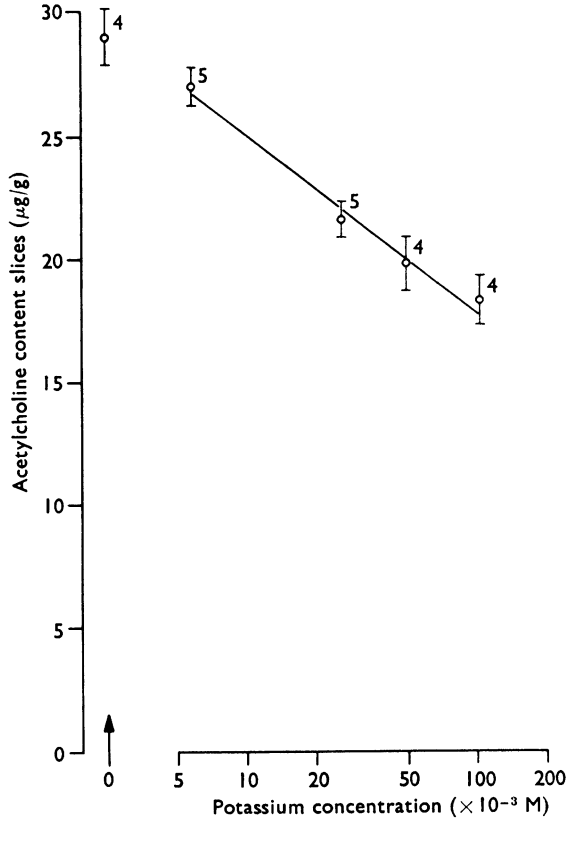

FIG. 1. Influence of potassium on the accumulation of acetylcholine in cortical slices. Mean values of four or five observations (indicated by the numbers) ± S.E.M. and the calculated least square regression line.

TABLE 1. *Influence of drugs on the uptake of acetylcholine by cortical slices*

Drug	Conc. (M)	Acetylcholine conc. slices (μg/g) $\pm$ S.E.
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)
	$2·9\times10^{-6}$	24·0±0·7 (4)
	$2·9\times10^{-5}$	18·5±0·9 (4)
	$8·6\times10^{-5}$	14·8±0·6 (4)
	$2·9\times10^{-4}$	9·8±0·3 (4)
Physostigmine	0	25·4±0·6 (5)
	3×10^{-6}	22
	6×10^{-6}	16, 16
	$1·2\times10^{-5}$	17, 18
	$2·4\times10^{-5}$	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 acetylcholine 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.

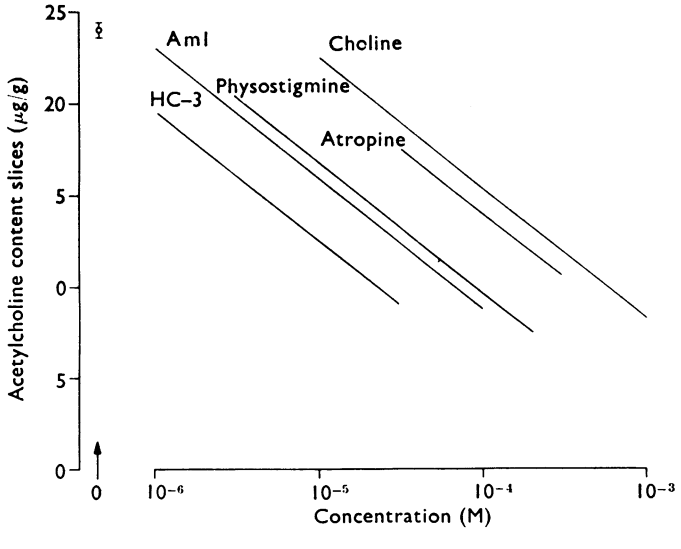

FIG. 2. Influence of drugs on the accumulation of acetylcholine in cortical slices. Mean slope of the regression lines calculated from the data in Table 1.

linear. It was very steep between 1×10^{-4}M and 2×10^{-4}M. Oligomycin, sodium azide, amylobarbitone sodium and *p*-chloromercuribenzoate also inhibited acetylcholine 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 acetylcholine uptake only slightly.

Anaerobic incubation

The uptake of acetylcholine 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 acetylcholine 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

TABLE 2. *Influence of some metabolic poisons on the uptake of acetylcholine by cortical slices.*

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

Details as in Table 1.

TABLE 3. *Influence of anaerobic incubation on the endogenous acetylcholine content of slices of cerebral cortex and on the uptake of added acetylcholine*

	Acetylcholine content of slices and amounts of acetylcholine released into medium (μg/g of tissue) after incubation for 30 min in a medium:			
	Without added acetylcholine		Containing acetylcholine 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.

in a atmosphere of 95% nitrogen and 5% carbon dioxide the accumulation of acetylcholine was strongly inhibited. The results summarized in Table 3 further demonstrate that during anaerobic incubation part of the endogenous acetylcholine 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 acetylcholine uptake was temperature-dependent. The acetylcholine concentration in the tissue after incubation for 30 min in a 25 mM KCl medium containing acetylcholine 4 μg/ml. was $21{\cdot}7 \pm 0{\cdot}6$ μg/g (11 observations) at 37° C, $16{\cdot}3 \pm 0{\cdot}5$ μg/g (eight observations) at 27° C and $10{\cdot}8 \pm 0{\cdot}4$ μg/g (six observations) at 17° C. Since the acetylcholine concentration in the slices immediately after preincubation (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) choline showed a diffuse and uniform labelling 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) choline was followed by incubation with non radioactive acetylcholine or not.

Discussion

When slices of rat cerebral cortex were incubated in a medium containing acetylcholine after their cholinesterase activity had been inhibited by soman acetylcholine 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 acetylcholine is an energy requiring process. The suggestion is supported by the observation that the uptake of acetylcholine 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, Aml, physostigmine, atropine and choline on the uptake of acetylcholine, 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 acetylcholine 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 acetylcholine uptake, whereas the cholinesterase inhibitors soman and tabun did not. In older unpublished experiments DFP was also found to have no effect on acetylcholine 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 acetylcholine by cortical slices, the inhibitory effect on acetylcholine 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 acetylcholine 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 acetylcholine uptake by cortical slices of mouse brain.

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

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