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Preferential release of newly synthesized ^{3}H-acetylcholine from rat cerebral cortex slices *in vitro*

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Summary

1. Slices of rat cerebral cortex after treatment with the irreversible cholinesterase inhibitor soman, were incubated for 5 min in a Krebs-Henseleit solution containing 25 mM KCl and ^{3}H-choline. Subsequently incubation was continued in a medium containing non-radioactive choline and this medium was replaced at 5 min intervals. The amounts of labelled and total acetylcholine (ACh) released into the medium and extracted from the slices were determined at intervals.

2. After the initial 5 min contact with ^{3}H-choline, 44% of the newly synthesized ACh contained a choline moiety originating from the choline in the medium. During the initial 5 min and the subsequent incubation part of the labelled ACh was released. While the rate of total ACh release remained constant, that of the release of labelled ACh was highest in the 5 min period following the initial incubation with ^{3}H-choline and then declined exponentially.

3. The ratio of labelled ACh/total ACh in the ACh released during the initial 5 min incubation with ^{3}H-choline and during the subsequent 5 min was about three times as high as that in the ACh extracted from the slices at the end of these incubation periods.

4. The ratio of labelled ACh/total ACh in superficial layers of the slices was not higher than that in the total slices.

5. The rates of release of labelled and unlabelled ACh decreased when calcium was omitted from the incubation medium and were restored when the calcium was added. This suggests that both labelled and unlabelled ACh were released from nerve endings. The efflux of ^{3}H-choline was not calcium dependent.

6. It is concluded that labelled ACh newly synthesized from externally applied ^{3}H-choline does not exchange immediately with all other ACh in the tissue and has a greater chance of being released than unlabelled ACh.

Introduction

Recently, evidence has been obtained in the perfused and electrically stimulated superior cervical ganglion of the cat (Collier, 1969), as well as in the isolated phrenic nerve diaphragm preparation of the rat (Potter, 1970) that newly synthesized acetylcholine (ACh) has a greater chance of being released from the nerve endings than preformed ACh stored in the tissue. The present paper deals with similar findings in slices of rat cerebral cortex in which the release of ACh was stimulated

by a high concentration of potassium in the medium. A preliminary account of part of this work has appeared previously (Molenaar, Nickolson & Polak, 1971).

Methods

Incubation of cortex slices

Adult female small-Wistar (WAG) rats weighing 160–180 g were lightly anaesthetized with ether and decapitated. The brains were immediately removed from the skull. At room temperature one slice was cut from each hemisphere, with the aid of a recessed perspex guide. The slices, which were thinner than 0·4 mm, were weighed after removal of the adherent fluid and stored for 20–60 min at room temperature in an oxygenated phosphate buffered medium (pH 7·4) of the following composition (mM): NaCl 118·5, $CaCl_2$ 0·8, $MgSO_4$ 1·2, Na_2HPO_4 9·2, glucose 10. The procedure was similar to that described by McIlwain & Rodnight (1962).

The slices were then incubated in slightly modified 25 ml Erlenmeyer vessels, 6 slices (200–250 mg) per vessel. The vessels had an outlet in the bottom which was closed by a silicone stopper, pierced by two stainless steel injection needles. Through one of the needles medium could be withdrawn whereas the other needle permitted replenishment of medium. In order to prevent plugging of the needles by the slices a sintered glass sieve was mounted over the outlet. Whenever incubation medium was withdrawn only 75% of the fluid was removed from the Erlenmeyer vessels as the remaining 25% adhered to the slices and the glass sieve. The values for ACh release have been corrected for this 25% ' dead space '.

Incubation was in 2·5 ml Krebs–Henseleit medium at 37° C with continuous shaking in an atmosphere of 95% O_2 and 5% CO_2. The pH of the medium was 7·3 and its composition 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, choline chloride 0·01. In addition, the medium contained soman (0·005 mM) in order to inhibit irreversibly cholinesterases. After 45 min incubation in this medium (at $t=-15$ min, see Fig. 1) incubation was continued without soman in Krebs–Henseleit medium which contained 25 mM KCl in order to stimulate ACh release, and 0·05 mM physostigmine sulphate in order to prevent reuptake of released ACh (Polak, 1969). No osmotic compensations were made for the extra KCl. After 15 min incubation (at $t=0$ in Fig. 1) the medium was withdrawn through the injection needle at the bottom of the Erlenmeyer vessel and replaced by medium of the same composition, and this was repeated every 5 minutes. In most experiments 6 μCi [NMe-^{3}H]-choline chloride (Amersham) per ml was added to the medium used during the first of the 5 min periods of incubation. The specific activity of the added labelled choline was so high (20

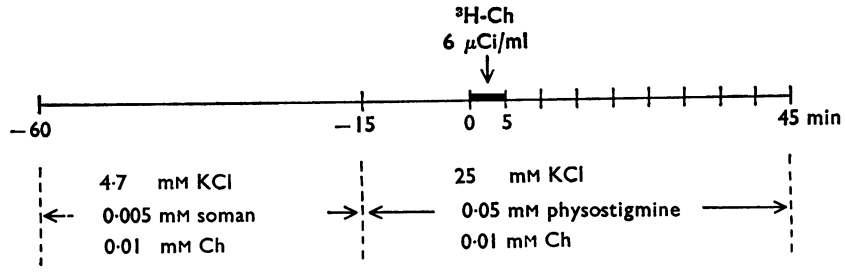

FIG. 1. Time scheme of incubation of rat cortex slices. Ch=choline.

Ci/mmol) that its amount was negligible compared to the choline already present in the medium. Consequently, the concentration of the choline in the medium remained 0·01 mM and the final specific activity was 600 pCi/pmol (164 cpm/pmol). In some experiments $CaCl_2$ was omitted during part of the incubation. The media collected at the end of each 5 min incubation were stored at −12° C after lowering the pH to 4 with HCl. When the incubation was terminated, the ACh of the slices was extracted with Krebs–Henseleit medium which was acidified with HCl to pH 2, essentially according to the method of Elliott, Swank & Henderson (1950). Extracts were stored at −12° C after adjustment to pH 4.

The slices did not disintegrate significantly during the incubation. In other experiments under similar conditions in which cortical slices were incubated for 60 min in Krebs–Henseleit medium they lost 1·5% of their protein content. When incubation was continued for another period of 60 min in a 25 mM KCl containing medium, 3·5% of the protein was lost into the medium.

Subdivision of cortex slices into different layers parallel with the pial surface

Six cortex slices were incubated as described in the preceding section with the following differences in procedure. After the 5 min incubation with labelled choline the slices were rinsed twice with fresh medium containing non-radioactive choline and then incubated for 2·5 min in this medium. Thereafter the incubation medium was collected and the slices were rinsed once more. The rinsing fluid was added to the medium and in the combined fluids the total ACh and labelled ACh released from the six slices during the 2·5 min incubation were determined. In order to prevent diffusion of total and labelled ACh between different layers of the cortical slices they had to be frozen very rapidly. For this two slices only were used. With the pia upwards, each was spread on a 2·5 mm thick layer of 3% agar and 10·8% sucrose in aqueous solution fixed on a metal block. The two blocks carrying the slices on the agar were rapidly frozen by immersion in liquid nitrogen, and each slice was subdivided with a freezing microtome into 6 fractions containing tissue from successive layers parallel with the pial surface. The ACh of the fractions was extracted as described in the preceding section.

Determination of labelled acetylcholine and labelled choline

^{3}H-ACh and ^{3}H-choline were separated from other labelled compounds by a selective co-precipitation with acetylcarnitine and KI_3 followed by paper electrophoresis. The details of the purification procedure were as follows.

To 2·5 ml of each sample [NMe-^{14}C]-choline chloride (Amersham) was added as an internal standard. The added amount of ^{14}C-choline was chosen in such a way that about 1 cpm ^{14}C-choline was added to 3 cpm ^{3}H in the sample. Subsequently protein was removed by the addition of 60% trichloroacetic acid (TCA) to a final concentration of 10% followed by centrifugation. The protein pellet was resuspended in 0·5 ml 5% TCA and after centrifugation the supernatants were combined and the volume was made up to 4 ml with water. To each 4 ml sample thus obtained was added 0·05 ml of a solution containing 50 μg acetylcarnitine-HCl and 0·4 ml of a solution containing 40 mg KI and 36 mg I_2. The sample was kept for 1 h at 0° C and then centrifuged at 0° C. The precipitate was washed with 2 ml water containing 10 mg KI and 9 mg I_2. After centrifugation the residual KI_3 solution adhering to the precipitate was removed with filter paper. Subsequently

the precipitate was heated to 100° C in 0·1 ml water in order to remove iodine from the complex by sublimation. The resulting clear solution of quaternary ammonium iodides was transferred as a droplet to a paraffin sheet (' parafilm ') and dried *in vacuo*. The dried material was dissolved in 10–20 μl water and pipetted on Whatman 1 paper which had been soaked in a solution of 0·75 M formic acid and 1·5 M acetic acid. Near both edges of the paper 210 nmol of choline chloride and 110 nmol of ACh iodide were applied as references. The material was submitted to electrophoresis at 45 V/cm for 1 hour. The electrophoresis procedure was essentially that of Potter & Murphy (1967). The reference spots were stained with iodine vapour. Paper strips were cut at the position indicated by the references. The strips were rinsed with ether to remove acetic and formic acid. Acetylcholine and choline were eluted with Locke solution, diluted by adding 40 ml H_2O to 100 ml, and acidified with HCl to pH 4. These eluates were tested for ACh by bioassay and for ^{3}H-ACh and ^{3}H-choline by radioactivity measurement.

Radioactivity was determined in a liquid scintillation medium that was composed of 1·5 ml of the diluted sample and 7·5 ml of a scintillator mixture containing per litre: toluene 500 ml, triton X-100 500 ml, PPO (2,5-diphenyloxazole) 4 g, dimethyl POPOP (2,2′*p*-phenylene-bis-methyl-5-phenyloxazole) 0·1 g. The isotopes ^{3}H and ^{14}C were counted in two channels of a liquid scintillation spectrometer (Packard Tricarb) and a correction for isotope overlap in the two channels was made. In the sample containing choline purified by electrophoresis both ^{3}H and ^{14}C due to ^{3}H-choline and ^{14}C-choline were measured.

During the precipitation of ACh and choline with KI_3 and acetylcarnitine and the subsequent electrophoresis, losses of ACh and choline occurred. The ^{14}C-choline added to each sample before purification served as internal standard. From its recovery after purification and from the ^{3}H/^{14}C ratio in the purified choline the amount of ^{3}H-choline originally present in each individual sample was calculated. The recovery of choline varied in different experiments between 10 and 30%. The endogenous total ACh present in the crude sample served in the same way as internal standard for the recovery of ^{3}H-ACh. By bioassay of the amounts of total ACh before and after purification its recovery (and consequently also that of ^{3}H-ACh) was determined and used for the calculation of the ^{3}H-ACh originally present in each individual sample before purification. The recovery of ACh varied between 40 and 60%. The difference between the recoveries of ACh and choline arose because ACh was precipitated more efficiently by KI_3 and acetylcarnitine than choline. The sample containing purified ACh was contaminated by very small amounts of ^{14}C originating from the added ^{14}C-choline. Since the ratio ^{3}H/^{14}C in the purified choline had been determined the amount of ^{14}C contaminating the purified ACh sample was used as a measure of the contamination of the ACh with ^{3}H-choline. When necessary a correction for this contamination which never exceeded 10% was made.

In control experiments in which ^{3}H-choline had been incubated without cortex slices electrophoresis showed that some radioactivity migrated with the same velocity as ACh. Only a part of this radioactive material was due to ^{3}H-choline as indicated by its ^{14}C-choline content. The remainder could not be distinguished from ^{3}H-ACh and its identity was not clarified. The ^{14}C-choline did not contain this impurity. The values obtained for the release of ^{3}H-ACh from the cortex slices during the 5 min of contact with ^{3}H-choline were corrected for the impurity which amounted to about 35% of the ^{3}H-ACh apparently released during this interval. Since, as

mentioned before, the 'dead space' of the incubation system was 25%, not all of the impurity was removed from the vessels when at the end of the first 5 min incubation the medium was replaced by medium containing non-radioactive choline. The values obtained for apparent ^{3}H-ACh release during the second 5 min of incubation, therefore, also had to be corrected but the correction was now less than 9%. For the ^{3}H-ACh released in the subsequent 5 min periods no correction was made.

Labelled ACh values were expressed as pmol per mg tissue (wet weight). They were obtained by dividing cpm ^{3}H-ACh/mg by the specific activity of the ^{3}H-choline in the medium (164 cpm/pmol).

Bioassay of acetylcholine

The ACh contents of the tissue extracts and the media collected at the end of 5 min intervals were determined by bioassay on the physostigmine-treated dorsal muscle of the leech and expressed as pmol per mg tissue. For reference ACh perchlorate standard solutions were used in suitable dilutions either of the incubation medium or of extracts of slices in which ACh had been destroyed by alkali (Feldberg, 1945). The ACh purified by precipitation and electrophoresis was bioassayed against standard solutions of ACh perchlorate to which appropriate dilutions of eluates from parts of the electropherogram on which no ACh was present, were added in order to compensate for possible interactions between paper eluate and assay preparation.

Identification of ^{3}H-acetylcholine

The ^{3}H-ACh synthesized by cortex slices was not only identified as ACh by precipitation with KI_3 and acetylcarnitine followed by electrophoresis, but in two experiments also by the subsequent determination of the rate of its hydrolysis by electric eel acetylcholinesterase (Sigma Cy).

One sample of ^{3}H-ACh purified by electrophoresis containing 2,595 cpm (900 pmol) and another sample containing 3,660 cpm (260 pmol) were made up to end volumes of 3 ml containing the following substances in the concentrations indicated: ACh iodide 4 mM, KH_2PO_4 50 mM, $MgCl_2$ 10 mM, gelatine 0·1% and acetylcholinesterase 0·05 units/ml. (One unit is the amount of acetylcholinesterase which hydrolyzes 1 μmol of ACh (concentration 4 mM) per min at 37° C at pH 8·0.) Before the acetylcholinesterase was added the pH of the mixtures was adjusted to 7·5 by KOH and their temperature to 37° C. At different times in the course of the incubation of the mixtures, test samples of 0·350 ml and 0·125 ml were taken simultaneously. The enzyme reaction in these test samples was stopped by mixing the 0·350 ml sample with 4·5 ml containing 5% TCA and ^{14}C-choline in varying known amounts and the 0·125 ml sample with 0·25 ml containing NH_2OH 2 M, NaOH 1·5 M, NaCl 2 M. The time course of hydrolysis of labelled ACh was followed by the disappearance of labelled ACh in the 0·35 ml samples and that of the added ACh by the disappearance of non-radioactive ACh in the 0·125 ml samples. Labelled ACh was measured after precipitation with acetylcarnitine and KI_3 and purification by electrophoresis as described above. The unlabelled ACh and the ^{14}C-choline were used as internal standards. The non-radioactive ACh was measured by the colorimetric method of Hestrin (1949).

Results

Release and synthesis of labelled acetylcholine by cortex slices

The results are summarized in Table 1. In a high potassium medium containing physostigmine the cortex slices released a practically constant amount of ACh into the medium during seven 5 min periods of incubation (about 5 (pmol/mg tissue)/5 min). This is shown in column 3. Part of the released ACh consisted of labelled ACh. As shown in column 4 the amount of labelled ACh appearing in the medium was 0·32 pmol/mg during the first 5 min incubation, i.e. during contact with the labelled choline and 0·46 pmol/mg during the next 5 min of incubation. Thereafter the release of labelled ACh fell by approximately 50% for every 10 minutes.

Since no hydrolysis or metabolic degradation of ACh occurs under the experimental conditions used (Polak, 1970), the rate of synthesis of ACh could be calculated from the values given in Table 1 for the ACh content of the slices at different times and for the amounts of ACh released. During the 35 min of incubation the total ACh content of the cortical slices decreased by 18 pmol/mg (i.e. from 42 to 24), whereas 37 pmol ACh/mg were released during this period into the incubation medium so that 19 pmol ACh/mg was synthesized. This corresponds to an average rate of synthesis of 2·7 (pmol/mg)/5 minutes. During the first 5 min incubation in the presence of labelled choline 0·88+0·32=1·20 pmol labelled ACh/mg was formed, which corresponds to 44% of the 2·7 pmol. During the second 5 min 1·10–0·88+0·46=0·68 pmol/mg was synthesized per mg tissue. During the subsequent 25 min only 0·21 pmol labelled ACh/mg was synthesized. Thus the synthesis of labelled ACh decreased much more rapidly after removal of the labelled choline from the medium than the release of labelled ACh.

The ratio of labelled ACh/total ACh in the ACh released during the first two 5 min periods of incubation was about 3 times as high as that in the ACh extracted from the tissue at the end of each of these periods (column 5 in Table 1). In the subsequent periods of incubation the difference became progressively less.

Ratio of labelled acetylcholine/total acetylcholine in different layers of the cortex slices

If the ratio of labelled ACh/total ACh were much higher in the outer than in the inner regions of the slices, this could explain why this ratio was higher in the

TABLE 1. *Total acetylcholine (ACh) and labelled ACh released from cortex slices during seven consecutive 5 min periods of incubation and the ACh contents of the tissue at the beginning of the first 5 min period (t=0) and 5, 10 and 35 min afterwards*

	Time (min)	*n*	Total ACh (pmol/mg)	Labelled ACh (pmol/mg)	$\frac{\text{Labelled ACh}}{\text{Total ACh}} \times 100$
ACh release	0– 5	12	5·0±0·26	0·32±0·020	6·4±0·6
	5–10	8	5·3±0·27	0·46±0·065	8·7±1·2
	10–15	2	5·4±0·22	0·33±0·002	6·1±0·3
	15–20	2	5·3±0·24	0·20±0·010	3·8±0·3
	20–25	2	5·3±0·12	0·16±0·007	3·0±0·2
	25–30	2	5·3±0·12	0·13±0·018	2·5±0·3
	30–35	2	5·5±0·08	0·08±0·004	1·5±0·2
ACh content	0	6	42·0±2·0	0	0
	5	6	39·0±0·8	0·88±0·079	2·3±0·3
	10	6	36·0±1·2	1·10±0·041	3·0±0·2
	35	2	24·0±4·7	0·41±0·092	1·7±0·2

The slices were pre-incubated as described in **Methods**. The incubation medium contained 25 mM KCl, 0·05 mM physostigmine and 0·01 mM choline. During the first 5 min period of incubation the choline was labelled by ^{3}H (164 cpm/pmol). Mean values±S.E.M. *n*=Number of observations.

released ACh than in the ACh extracted from the slices. However, when this ratio was determined in six separate fractions representing successive tissue layers parallel with the pial surface it was found that there was no great difference between the outer and the inner fractions. In fact, the ratio of labelled ACh/total ACh in the outer layers of the slices was equal to or somewhat lower than that in the inner layers, and was much lower than that of the ACh released from the slices.

Influence of calcium on acetylcholine release

The finding that the ratio of labelled ACh/total ACh in the medium was much higher than that in the tissue might be explained if the labelled ACh had been synthesized extracellularly by choline acetylase which had leaked from partially disrupted neurones. This possibility was tested by investigating the effect of calcium on the amounts of labelled ACh released into the medium. If the labelled ACh originated from nerve endings, one might expect a reduction of its release with a calcium deficient medium (Randič & Padjen, 1967 ; Hemsworth & Mitchell, 1969 ; Molenaar & Polak, 1970).

In experiments in which the calcium was omitted from the medium during part of the incubation both total ACh release (Fig. 2) and labelled ACh release (Fig. 3) were reduced. Re-addition of calcium to the medium stimulated the release of labelled ACh at least as much as that of total ACh when it is taken into account that the release of labelled ACh decreased with time.

By adding together the amounts of labelled ACh released during incubation and extracted from the tissue at the end of the incubation a value of 2·24 pmol/mg was obtained for the synthesis in those experiments in which $CaCl_2$ was present during the whole incubation, and of 2·21 pmol/mg in those in which the calcium was omitted during parts of the incubation. The synthesis of labelled ACh was therefore not influenced significantly by the temporary withdrawal of the $CaCl_2$.

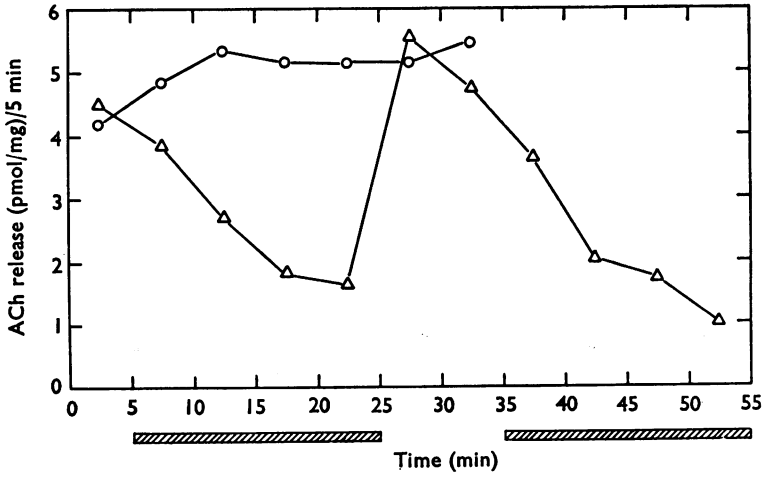

FIG. 2. Acetylcholine (ACh) release ((pmol/mg)/5 min) from rat cortex slices incubated as described in the legend of Table 1 but without radioactive choline. ○——○, ACh release into medium containing 2·5 mM $CaCl_2$; △——△, ACh release into medium from which the $CaCl_2$ was omitted during two periods of 20 min indicated by the hatched bars. Each result is the mean of 2 experiments.

The efflux of labelled choline from the slices was found to decrease exponentially with time. Unlike the release of labelled ACh that of labelled choline was not influenced by the calcium concentration of the medium.

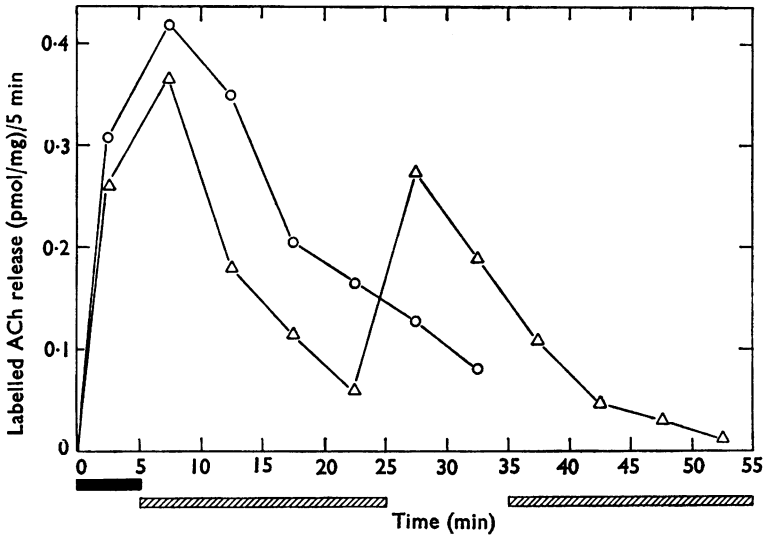

FIG. 3. Labelled acetylcholine (ACh) release from rat cortex slices incubated as described in the legend of Table 1. During the first 5 min of incubation, indicated by the black bar, the medium contained labelled choline (164 cpm/pmol, 0·01 mM). ○——○, Labelled ACh release into 2·5 mM $CaCl_2$ containing medium; △——△, labelled ACh release into medium from which the $CaCl_2$ had been omitted during two periods of 20 min indicated by the hatched bars. Each result is the mean of 2 experiments.

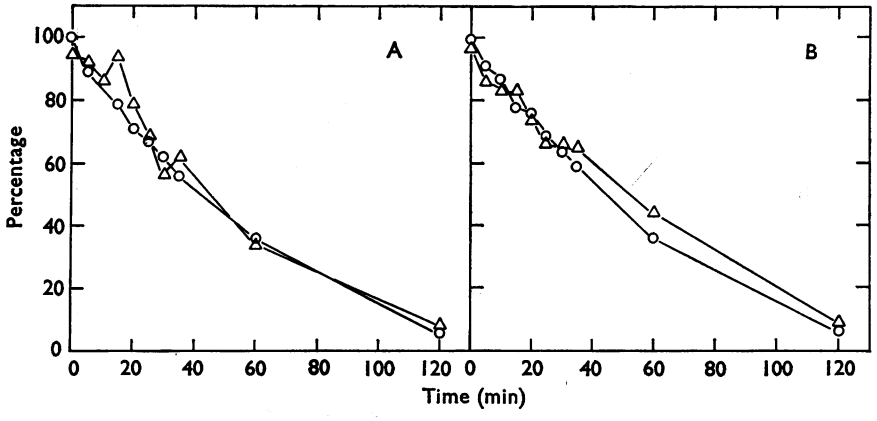

FIG. 4. Hydrolysis of labelled ester and of acetylcholine (ACh) iodide by acetylcholinesterase. The labelled ester was isolated by precipitation and electrophoresis from an extract of cortex slices after incubation with ^{3}H-choline (A) and from medium used for incubation (B). Each sample was mixed with 12 μmol ACh iodide and, at zero time, with 0·15 units acetylcholinesterase in 3 ml buffered medium. For the triangles (△——△) the ordinates refer to the non-hydrolyzed percentages of 2,595 cpm in (A) and of 3,660 cpm in (B). For the open circles (○——○) the ordinates refer in both (A) and (B) to the non-hydrolyzed percentages of the added ACh-iodide.

Identification of the labelled acetylcholine

Although the separation of labelled ACh and choline by electrophoresis was good, it was desirable to use a different method to test whether the labelled choline ester was identical with ACh. For this purpose two samples obtained by precipitation and electrophoresis were incubated with electric eel acetylcholinesterase and the rate of hydrolysis compared with that of authentic ACh. One sample was a purified extract of slices which had first been incubated for 5 min with labelled choline and then kept during 30 min in a medium containing non-radioactive choline. The other sample was derived from medium in which the cortex slices had been incubated during the 5 min interval that followed the 5 min period of contact with labelled choline.

As shown in Fig. 4 the labelled ester isolated from the tissue and from the medium was hydrolyzed at the same rate and practically to the same extent as the authentic ACh iodide. In a control experiment in which no acetylcholinesterase had been added no hydrolysis of ACh occurred. Furthermore, the rate of formation of labelled choline from the labelled ester isolated from the medium and the rate of hydrolysis of the ester were found to proceed at the same rate.

Discussion

In the present experiments with incubated slices from the rats' cerebral cortex a procedure was used which made it possible to study the fate of newly formed ACh. The cortex slices were incubated for the short time of 5 min in a medium containing labelled choline, so that labelled ACh would be formed during this period. The labelled choline in the medium was then replaced by unlabelled choline and incubation was continued. The amounts of labelled and unlabelled ACh released into the medium during the initial 5 min period and during seven subsequent 5 min periods were determined and also the amounts of labelled and unlabelled ACh in the cortex slices at different stages of the incubation. Release and synthesis of ACh were stimulated in these experiments by an increased KCl concentration in the medium.

The labelled ACh had to be separated from the labelled choline before its radioactivity could be determined. The yield of the purification procedure was determined accurately for each sample by use of its endogenous total ACh as internal standard.

As far as the yield of the extraction of ACh from the cortex slices is concerned only indirect evidence is available. Important losses probably did not occur because the cholinesterases were completely inhibited. The efficiency of the extraction was probably good since extraction with perchloric acid according to the method of Morris, Bull & Hebb (1965) which has been reported to be highly efficient for the extraction of ACh from fresh brain tissue (Lederis & Livingston, 1969) does not give higher yields of ACh from cortex slices treated with soman than the method used in the present experiments (Polak, unpublished experiments).

In order to exclude the possibility that the labelled choline ester isolated by electrophoresis was not identical with ACh its identity was confirmed in two additional experiments by the demonstration that it was hydrolyzed at the same rate and to the same extent as authentic ACh.

Some of the present results were complicated by the fact that the ^{3}H-choline contained traces of a labelled impurity which could not be distinguished from ACh by electrophoresis. It was therefore necessary to correct for this impurity the values obtained for the release of labelled ACh during the first 5 min of incubation when the medium contained labelled choline. As the labelled choline was removed after this, the labelled ACh released during the second 5 min period was contaminated by a much lower amount of this impurity, and the correction for it was consequently small (less than 9%). For ACh release in the subsequent 5 min periods no correction was necessary.

It was found that about 44% of the new ACh synthesized by the slices during the initial 5 min was formed from the labelled choline. This agrees with the recent finding by Collier, Poon & Salehmoghaddam (1972) that choline added to the medium in which slices from rat cerebral cortex are incubated, competes with endogenous choline for ACh synthesis. After the labelled choline was replaced by unlabelled choline the synthesis of labelled ACh rapidly decreased. Apparently soon after the replacement of the medium little of the labelled choline taken up by the slices was still available for ACh synthesis. Consequently the 5 min incubation of the slices in a medium containing labelled choline resulted in a rapidly beginning and rapidly ending synthesis of labelled ACh, as was intended with this technique of ' pulse labelling '.

Of the ACh released during the first and second 5 min period of incubation a greater proportion was labelled than was found for the ACh present in the cortex slices at the end of these periods. This means that the part of ACh which was newly synthesized from the choline in the medium was preferentially released, a result which is in accordance with the view expressed by Collier (1969) and Potter (1970) that newly formed ACh has a greater chance of being released than preformed ACh stored in the tissue. Recently Richter & Marchbanks (1971) who investigated synthesis and release of labelled ACh by minced cerebral cortex of the guinea-pig during incubation with ^{3}H-choline also came to this conclusion. The situation appears to be the same with regard to the release and synthesis of transmitter from adrenergic and dopaminergic neurones. For instance, Kopin, Breese, Krauss & Weise (1968) observed preferential release of newly synthesized noradrenaline from the perfused cat spleen during sympathetic stimulation and Besson, Cheramy, Feltz & Glowinski (1969) formulated a similar view concerning the release of dopamine from nerve terminals in the rat striatum.

As the ratio of labelled ACh/total ACh in the ACh released during the first and second 5 min period of incubation was about three times greater than that in the ACh extracted from the cortex slices at the end of these periods, the released ACh would appear to have originated from a separate pool of readily releasable ACh and the size of this pool must have been less than one-third of the total ACh present in the tissue.

During the initial 5 min incubation of the slices in contact with labelled choline the ratio of labelled ACh/total ACh in the released ACh was somewhat lower than that in the subsequent 5 min period. This is probably due to the fact that the amount of released labelled ACh which was collected after the initial 5 min incubation with labelled choline was the mean of a value which began at zero and then rapidly rose to a value well above that found for the ACh collected after the second 5 min period of incubation.

The release of labelled and unlabelled ACh was calcium dependent. This was not a non-specific effect since the efflux of labelled choline from the cortex slices was not affected by the calcium concentration of the medium. The dependence of the release of ACh on the calcium concentration resembles the dependence on calcium of the ACh release from motor nerve endings (Hubbard, 1961 ; Gage & Quastel, 1966) and therefore suggests that in the cortex slices too, both labelled and unlabelled ACh were released from nerve endings.

One possibility of explaining why the ratio of labelled ACh/total ACh in the ACh released from the cortex slices was so much higher than that in the ACh that was extracted from the slices, was that the released ACh originated from the outer layers of the cortex slice which might be in better contact with the medium than more internally situated tissue and, therefore, participate more actively in the processes of uptake of labelled choline from the medium and of synthesis and release of labelled ACh. However, this explanation was refuted by the finding that the ratio of labelled ACh/total ACh in the superficial layers of the slice was equal to or even slightly less than the specific activity of the ACh in the internal layers, and much less than in the released ACh. Consequently the compartment containing releasable ACh with a high proportion of labelled ACh must have been represented either by a separate population of nerve endings or by a subcellular compartment within the nerve endings.

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