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UPTAKE OF ACETYLCHOLINE IN RAT BRAIN CORTEX SLICES

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Abstract—Acetylcholine is taken up against a concentration gradient by rat brain cortex slices, incubated aerobically in a physiological saline medium, in the presence of paraoxon. Optimal rates of acetylcholine uptake are obtained with slices 0·2–0·4 mm thick, in the presence of glucose (> 3 mM), in oxygen at 37° and in the presence of paraoxon (> 5 μM) which completely inactivates the choline esterase. Little or no uptake apart from that due to passive diffusion occurs in the presence of eserine. It is evident that the uptake is carrier-mediated. The concentration ratio (tissue:medium) of acetylcholine varies from approximately unity with relatively high external concentrations of acetylcholine (e.g. 5 mM) to 21 with low concentrations (0·01 μM) of acetylcholine. The optimal pH for acetylcholine uptake is 8·3 and the amount of uptake in 1 hr, corrected for passive diffusion, is doubled on increasing the temperature of incubation from 17° to 27°. The rate of acetylcholine uptake, corrected for the passive diffusion rate, is approximately proportional to the respiratory rate (in the presence of oxygen or air or in the absence of glucose). It is inhibited by 2:4 dinitrophenol (10 μM) and by ouabain (10 μM), their inhibitions being independent of the acetylcholine concentration. The rate of uptake declines (from its optimal value) in the absence of potassium and calcium ions, or in the presence of relatively high concentrations of potassium chloride (> 50 mM) or calcium chloride (8 mM) or magnesium sulfate (6 mM). It is also diminished, but not abolished, by the omission of sodium ions from the medium. It is concluded that the rate of uptake of acetylcholine (apart from passive diffusion) in the brain slices is partly dependent on the operation of the sodium pump. The possible involvement of exchange diffusion in the process of uptake is discussed.

WHEREAS a variety of early results[1] showed that uptake of acetylcholine against a concentration gradient into brain slices either does not occur, or does so only to a small extent, it is now known that concentrative uptake of acetylcholine takes place in rat or mouse brain cortex slices[2, 3] so long as a suitable choline esterase inhibitor is present. Such an inhibitor is the organophosphate, pinacoloxymethylphosphoryl-fluoride (Soman), used at a concentration of 5 μM,[2] or isopropoxymethylphosphoryl-fluoride (Sarin), used at a concentration of 1 μM.[3] Eserine blocks the concentrative uptake of acetylcholine into brain slices,[2, 3] accounting for the negative (or almost negative) results obtained in previous investigations, where eserine was used to inactivate the choline esterase. Atropine inhibits the uptake of acetylcholine into brain slices[2, 3] and it is known that hemicholinium (HC-3), eserine, atropine, oxo-tremorine and morphine inhibit the uptake competitively.[3] Moreover, the main part of the uptake process requires oxygen, and is inhibited by 2:4 dinitrophenol.[3]

In view of the possibility that the sites on the brain cell membrane concerned

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with the uptake of acetylcholine against a concentration gradient may be related to the receptor sites concerned with the pharmacological behavior of acetylcholine, or with acetylcholine metabolism,[4] we have carried out experiments to throw further light on the nature of the uptake process for acetylcholine and on the manner in which drugs affect this process. The following paper is concerned with a description of the results of these experiments.

MATERIALS AND METHODS

We have used, as choline esterase inhibitor, the organophosphate, diethyl *p*-nitrophenylphosphate (paraoxon). This substance has been extensively used in studies of choline esterase inactivation[5–8] and for suppression of electrical activity in nerve fibers.[9, 10] We have used it at a concentration of 20 μM, which experiment showed was ample for complete inhibition of the choline esterase in the brain tissue. Radioactive acetylcholine chloride ($N\text{-}^{14}CH_3$) was purchased from the Radiochemical Centre, Amersham, Bucks., U.K. (specific activity = 57·3 μc/mg).

Preparation of brain slices and incubation procedure

Wistar rats, of either sex, weighing 200–300 g, were killed by dislocation of the neck and the brains were quickly removed and placed in a moist chamber surrounded by crushed ice. Brain cortex slices (40–60 mg wet wt.) were cut from the cerebral hemispheres with a chilled Stadie–Riggs slicer (about 0·3–0·4 mm thick). The slices were weighed at once on a torsion balance and suspended in the incubation medium in chilled Warburg manometric vessels. Each vessel contained 2·8 ml Krebs–Ringer phosphate medium containing 128 mM NaCl, 5 mM KCl, 2·7 mM $CaCl_2$, 1·3 mM $MgSO_4$, 10 mM Na_2HPO_4 (brought to pH 7·4 with 1N HCl), together with 10 mM glucose and 20 μM paraoxon. The center well contained small rolls of filter paper moistened with 0·2 ml 20% KOH. The side arm contained 0·2 ml of a solution of acetylcholine. The solution consisted of 0·1 ml of a solution of radioactive acetylcholine hydrochloride (0·1 μc) dissolved in Krebs–Ringer phosphate medium together with 0·1 ml of a solution of (unlabeled) acetylcholine hydrochloride dissolved in the same medium. Different concentrations of acetylcholine were prepared by having differing quantities of unlabeled acetylcholine dissolved in 0·1 ml of the carrier solution.

After gassing the manometer vessels with oxygen for 5 min and equilibration in the thermostat at 37° for 10 min, the acetylcholine was tipped from the side arm into the main vessel and incubation was continued for 1 hr except where otherwise stated. Measurements were made of the rates of oxygen consumption, CO_2 being absorbed by the KOH in the center wells. Such measurements were useful for assessing the metabolic activities of the tissue, under the conditions prevailing when estimations of acetylcholine transport were made.

In experiments in which the cation concentrations were varied, a potassium-free medium was prepared by omitting KCl from the incubation medium. When the potassium concentration was increased, the sodium chloride concentration was correspondingly reduced to preserve isotonicity. For preparation of calcium- or magnesium-free media, the corresponding salts were omitted from the incubation medium. When a sodium-free medium was required, sodium chloride was replaced by 280 mM sucrose, and Tris-HCl buffer, 0·1 M pH 7·4, replaced phosphate buffer.

Estimation of radioactivities in tissue and medium

At the end of the incubation period, the slices were removed from the vessels, quickly rinsed with 20 ml fresh acetylcholine containing incubation medium (in which, however, the radioactive acetylcholine was replaced by an equal concentration of unlabeled acetylcholine), lightly blotted and weighed to estimate the tissue swelling. The slices were then transferred to a weighed-graduated tube and dried at 105° overnight and the dry weight estimated. One ml 10% KOH was added to the dry tissue and the mixture was heated at 75° for 15 min in a water bath. After cooling, 1 ml methanol was added and the tube was stoppered and stirred with a magnetic stirrer. Two-tenths ml was added to 10 ml of a scintillation medium. This consisted of a mixture of equal volumes of toluene, dioxane and 95% (v/v) ethanol containing 2,5-diphenyloxazole (5 g/l.), 1,4-*bis*-(4 methyl-5 phenyloxazol-2 yl) benzene (0·5 g/l.) and naphthalene (80 g/l.). The radioassay was performed with a Nuclear Chicago model 720 liquid scintillation counter.

Calculations of the concentration ratio (tissue:medium)

After assay of the radioactivity of the acetylcholine present in the tissue slice, the concentration ratio (i.e. the ratio of concentration of labeled acetylcholine in the wet tissue to the concentration of labeled acetylcholine in the medium) was calculated by dividing the amount of radioactivity in 100 mg wet wt. tissue by the amount of radioactivity in 0·1 ml of the suspending medium at the end of the experiment. Corrections were routinely made for the amount of swelling, which, except where otherwise stated, amounted to approximately 17 per cent. These consisted in subtracting from the amount of radioactivity accumulated in the slice that amount present in a volume of the medium equivalent to 17 per cent of the volume of the slice.

Reproducibility of results

Each experiment was carried out at least four times. The figures below record the averages of all experiments, together with the standard deviations from the mean.

RESULTS

Stability of acetylcholine in the presence of rat brain slices and paraoxon

The fact that acetylcholine is not broken down by rat brain cortex slices in the presence of 20 μM paraoxon is demonstrated by the following observations: (1) Manometric estimations of choline esterase activity of the brain, using acetylcholine as substrate, show at least 99 per cent inhibition of the enzyme activity by the paraoxon. (2) Incubation of radioactive acetylcholine, in which the acetyl group is labeled, with the brain tissue in the presence of 20 μM paraoxon shows no evolution of labeled carbon dioxide. Hydrolysis of acetylcholine, with release of labeled acetate, results in the formation of labeled carbon dioxide in the presence of respiring rat brain cortex slices.[11] Under the conditions of our experiments, 4 per cent of the radioactivity of the acetyl-labeled acetylcholine (40 μM) is found as labeled CO_2 at the termination of 1 hr aerobic incubation at 37° of rat brain cortex slices in the absence of the paraoxon. In the presence of the paraoxon, no radioactivity is found in the evolved CO_2.

The labeled acetylcholine taken up by the brain tissue in the presence of 20 μM paraoxon is not incorporated into tissue constituents to a measurable extent. This is shown by the fact that the tissue, after incubation for 1 hr in the presence of 40 μM

radioactive acetylcholine, on homogenisation in Krebs–Ringer phosphate–paraoxon medium and subsequent washing three times with this medium loses 94 per cent of its radioactivity.

Effects of the thickness of the brain slices on the acetylcholine uptake

When the concentration of acetylcholine in the medium bathing the rat brain tissue is 40 μM, a concentration ratio (tissue:medium) of 6·3 $\pm$ 0·4 is obtained after an incubation period of 1 hr. This value is obtained at slice thicknesses between 0·25 and 0·4 mm. The concentration ratio diminishes at a thickness exceeding 0·4 mm. It is 4·0 $\pm$ 0·4 at a thickness of 0·5 mm and 2·5 $\pm$ 0·3 at a thickness of 0·6 mm. Doubtless, the low values of the concentration ratio at thicknesses exceeding 0·4 mm are partly due to the diminished rates of cerebral respiration under such conditions. When the slices are thinner than 0·2 mm, erratic results are obtained due to the partial disintegration of the tissue which may occur during the incubation shaking procedure.

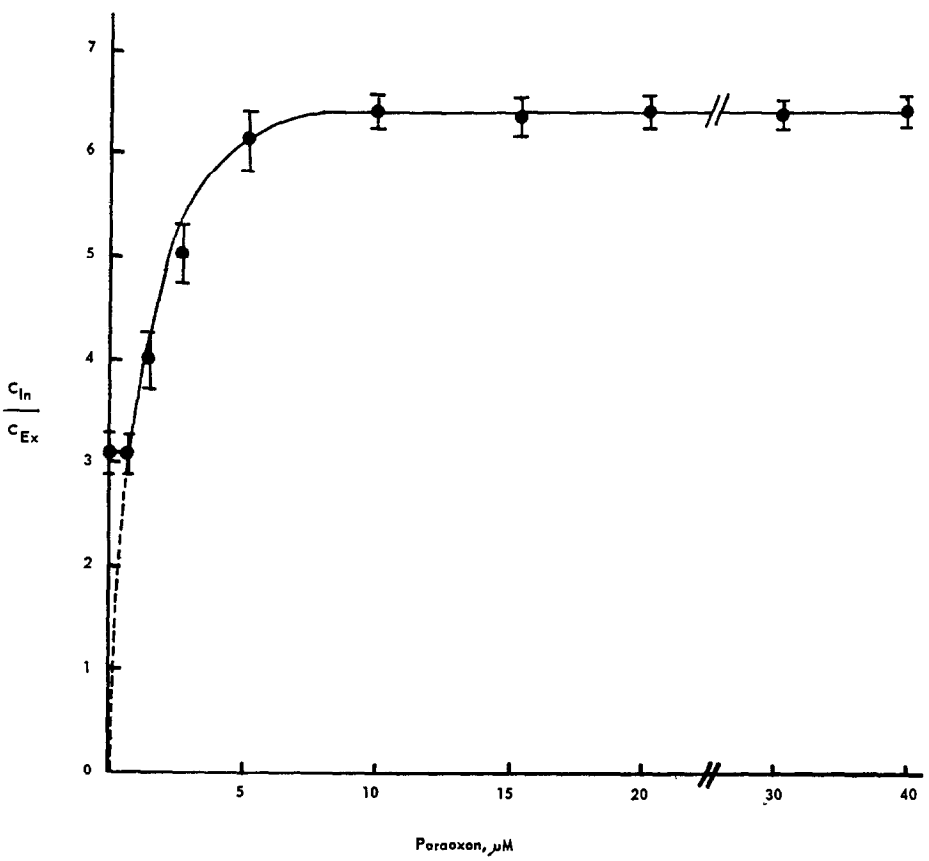

FIG. 1. Effects of incubation of rat brain cortex slices in physiological media containing varying concentrations of paraoxon on the uptake of acetylcholine. Concentration of acetylcholine in the medium = 40 μM. *Ordinate*: C_{In}/C_{Ex} = Concentration ratio. This is the ratio of the quantity of labeled acetylcholine in 100 mg wet tissue to that in 0·1 ml medium. *Abscissa*: Concentration of paraoxon (μM).

Effects of varying concentrations of paraoxon on the acetylcholine concentration ratio

Results given in Fig. 1 show that the concentration ratio of acetylcholine (tissue: medium) under the given experimental conditions is almost at a maximum when the paraoxon concentration is 5 μM. It does not fall when the paraoxon concentration is raised to 20 μM. The latter concentration of paraoxon has been used systematically by us to ensure complete inhibition of the choline esterase in the brain tissue. It should be noted that in the absence of a choline esterase inhibitor, the labeled acetylcholine is hydrolyzed to form labeled choline (assuming the label is on the *N*-methyl group) which is, itself, taken up by an active transport process in the brain slice.[12, 13] However, the transport processes of acetylcholine and choline in the isolated brain tissue may easily be distinguished by the fact that eserine inhibits the former process[2, 3] but not the latter.[13] The value of the concentration ratio equal to 3·2, found in the absence of paraoxon (Fig. 1), is presumably that due to the influx of labeled choline as the added acetylcholine under these conditions is completely hydrolyzed.[14]

Effects of variation of pH on acetylcholine uptake

The incubation media, in these experiments on the effects of variation of pH, were the standard medium containing phosphate buffer or Tris buffer, at different hydrogen ion concentrations, and a Krebs–Ringer bicarbonate medium of the following composition: 133 mM NaCl, 5 mM KCl, 2·8 mM $CaCl_2$, 1·3 mM $MgSO_4$, 15·2 mM $NaHCO_3$, and gassed with a mixture of 95 per cent O_2 plus 5 per cent CO_2.

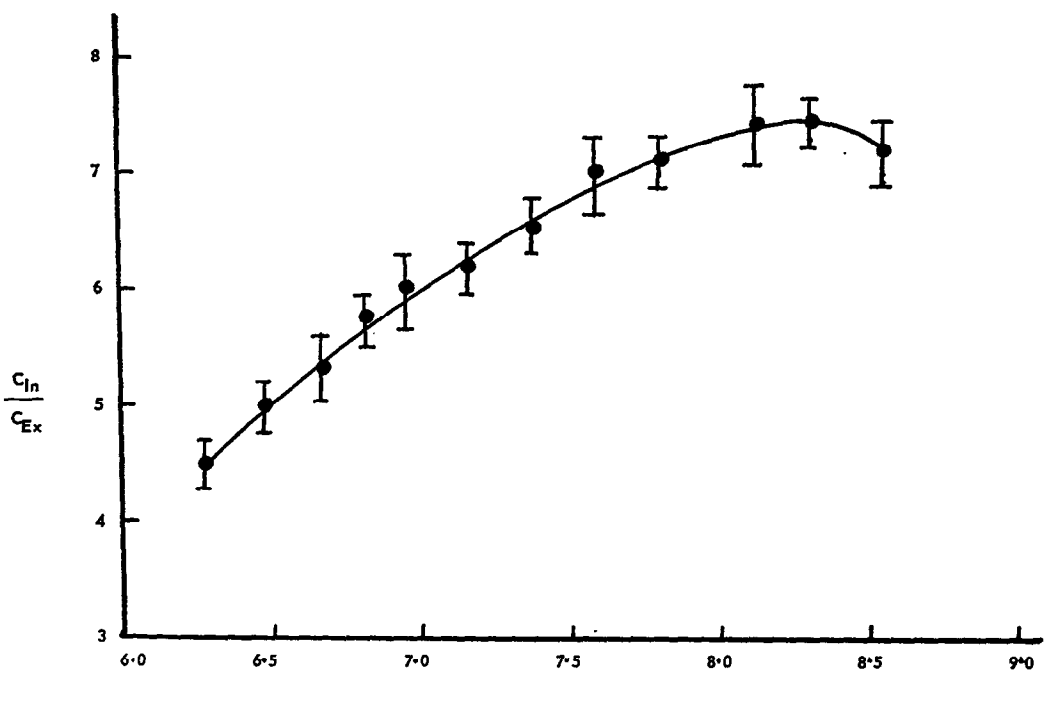

FIG. 2. Effects of variation of pH on the uptake of acetylcholine in rat brain cortex slices in presence of 20 μM paraoxon. Concentration of acetylcholine in the medium = 40 μM. *Ordinate*: Concentration ratio (tissue:medium) of acetylcholine. *Abscissa*: pH.

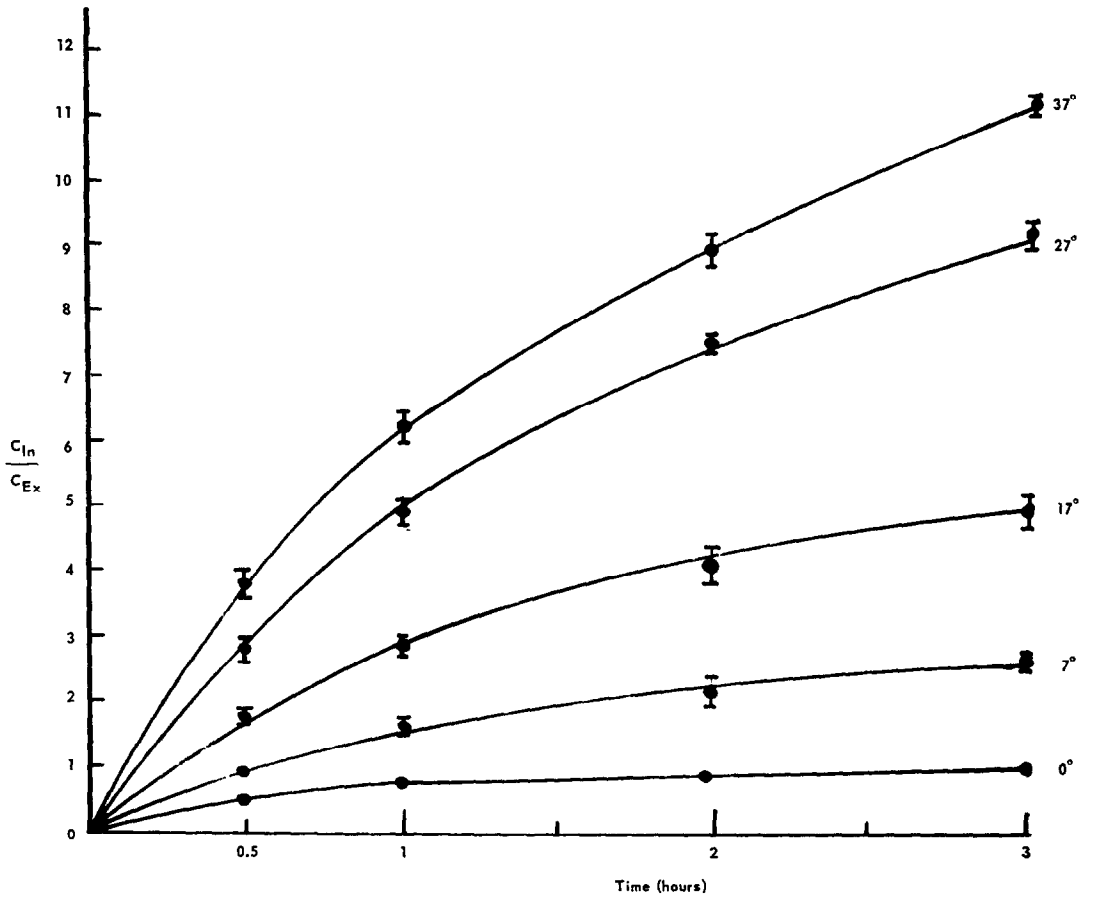

FIG. 3. Effects of variation of temperature and period of incubation on the uptake of acetylcholine in rat brain cortex slices in presence of 20 μM paraoxon. Concentration of acetylcholine in the medium = 40 μM. *Ordinate*: Concentration ratio (tissue:medium) of acetylcholine. *Abscissa*: Time (hr).

Different pH values in the medium were obtained by changing the concentration of $NaHCO_3$. The pH of the incubation medium was always determined at the end of each incubation experiment.

It was found that the same results of the effects of variation of pH in the concentration ratio are obtained with either the phosphate or the bicarbonate media.

The results are given in Fig. 2 and show that an optimum value for the concentration ratio occurs at pH 8·3.

Variation of uptake of acetylcholine with temperature

Results given in Fig. 3 show that the concentration ratio (tissue:medium) of acetylcholine is temperature dependent. The velocity of the transport process between 17° and 27° is increased by a factor of 2·05, after correction for passive diffusion.

Incubation at 0° results in a cessation of the active uptake of acetylcholine. The concentration ratio after 1 hr is 0·89, a slightly higher value being obtained after 3 hr

of incubation. This is to be expected if passive diffusion of acetylcholine into the brain slice alone takes place at 0°. The fact that the concentration ratio does not significantly exceed unity after 3 hr of incubation at 0° indicates that there must be, with an external concentration of acetylcholine of 40 μM and under the given experimental conditions, only little binding of acetylcholine to tissue constituents. We have taken the value of the concentration ratio equal to 0·89 as representing the accumulation of acetylcholine by passive diffusion, in 1 hr, under our experimental conditions. This compares favorably with the ratio equal to 0·85 found with mouse brain slices.[3]

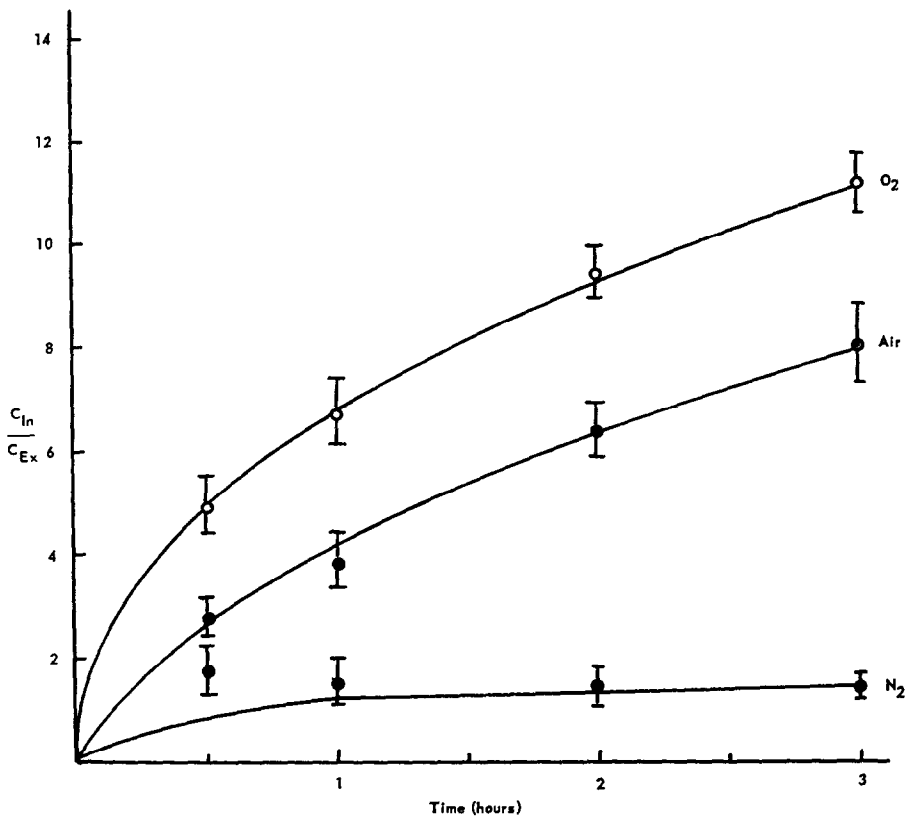

FIG. 4. Effects of oxygen tension and absence of oxygen on the uptake of acetylcholine in rat brain cortex slices in presence of 20 μM paraoxon. Concentration of acetylcholine in the medium = 40 μM. *Ordinate*: Concentration ratio (tissue:medium) of acetylcholine. *Abscissa*: Time (hr).

Effects of concentration of acetylcholine on the concentration ratio

Typical results given in Table 1 demonstrate the manner in which the concentration ratio of acetylcholine (tissue:medium) varies with the concentration of acetylcholine and with the time of incubation. They show that, with very dilute concentrations of acetylcholine, a concentration ratio of 20:1 may be rapidly reached. With a relatively high concentration of acetylcholine (5 mM), the concentration ratio (tissue:medium)

is approximately 0·9, after incubation for 1 hr. This is the passive diffusion value and indicates, as mentioned above, that if there is any incorporation of acetylcholine into the tissue constituents, the amount must be small and not detectable by our technique.

TABLE 1. EFFECTS OF VARIATION OF ACETYLCHOLINE CONCENTRATION IN THE MEDIUM, AND OF THE TIME OF INCUBATION, ON THE UPTAKE OF ACETYLCHOLINE IN RAT BRAIN CORTEX SLICES IN THE PRESENCE OF 20μM PARAOXON

Acetylcholine concn in the medium (μM)	Acetylcholine concn ratios (corrected for passive diffusion) (tissue:medium)	
	After 30 min incubation	After 1 hr incubation
0·01	19·7	21·0
0·08	14·8	18·5
0·2	9·3	14·2
4	5·2	8·4
40	4·0	6·0
100	2·8	4·2
1000	1·2	2·0
5000	0·8	0·9

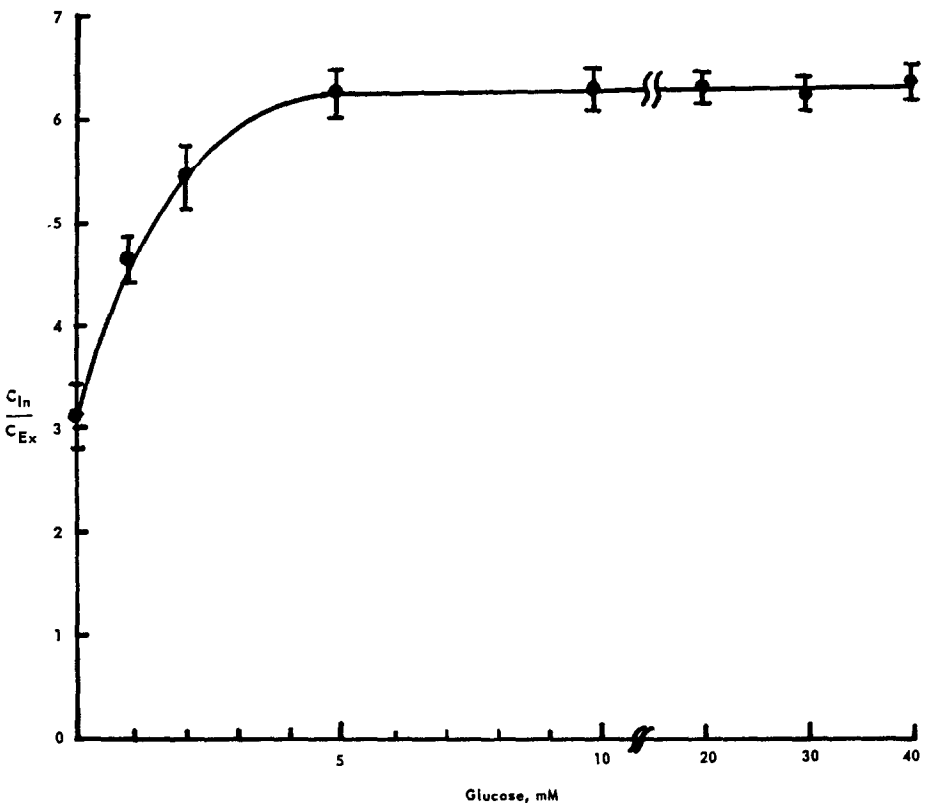

FIG. 5. Effects of variation of glucose concentration on the uptake of acetylcholine in rat brain cortex slices in presence of 20 μM paraoxon. Concentration of acetylcholine in the medium = 40 μM. *Ordinate*: Concentration ratio (tissue:medium) of acetylcholine. *Abscissa*: Glucose concentration (mM).

Effects of oxygen tension on acetylcholine uptake

Results given in Fig. 4 show that optimal rates of acetylcholine uptake against a concentration gradient are obtained in the presence of oxygen. In the absence of oxygen, and in the presence of nitrogen, the ratio of the concentration of acetylcholine in the tissue to that in the medium, at the end of 1 hr incubation, is a little higher than unity.

Variation of uptake of acetylcholine with concentration of glucose

In the absence of glucose in the incubation medium the concentration ratio of acetylcholine (tissue:medium) exceeds unity and has a value under the given experimental conditions of 3·4. The results are shown in Fig. 5. The presence of glucose brings about a marked increase in the concentration ratio which reaches the maximum value of 6·4 with 4 mM glucose. This value remains constant with increase of glucose concentration to 40 mM. A partial dependence of acetylcholine uptake on the presence of glucose with mouse brain slices has already been demonstrated.[3]

Variation of uptake of acetylcholine with respiratory rate

Results given in Table 2 record values of the rates of acetylcholine uptake in rat brain tissue in the presence of 20 μM paraoxon, corrected for passive diffusion, and

TABLE 2. EFFECTS OF OXYGEN TENSION, AND ABSENCE OF GLUCOSE, ON THE UPTAKE OF ACETYLCHOLINE AND ON THE RESPIRATORY RATE OF RAT BRAIN CORTEX SLICES, IN PRESENCE OF 20 μM PARAOXON, INCUBATED AT 37° FOR 1 hr*

Conditions	Uptake of acetylcholine (mμmoles/g) corrected for passive diffusion	O_2 consumption (μl/mg dry wt/hr)	Ratio: Uptake of ACh / O_2 consumption
100% O_2, glucose (10 mM)	224	12·5	17·9
Air, glucose (10 mM)	128	7·0	18·3
100% O_2, glucose absent	120	6·4	18·7

* Acetylcholine concentration in the medium equal to 40 μM.

the corresponding respiratory rates when the slices are incubated in oxygen or in air in the presence of glucose or in oxygen in the absence of glucose. It will be seen that the amount of acetylcholine uptake in 1 hr is approximately proportional to the respiratory rate under the given experimental conditions.

Control experiments have shown that 20 μM paraxon does not affect the respiratory rate of rat brain cortex slices incubated in a Ringer–glucose medium.

Effects of 2:4 dinitrophenol on acetylcholine uptake

The addition of 2:4 dinitrophenol (10 μM) brings about a marked diminution of the rate of uptake of acetylcholine. The percentage diminution of the concentration ratio is independent of the concentration of acetylcholine. Typical results are shown in Table 3 where it is evident that the ratio of the uptake of acetylcholine in the presence of 2:4 dinitrophenol (10 μM) to that in the absence of the inhibitor is approximately constant at concentrations of acetylcholine varying from 0·02 mM to 1 mM. This result is to be expected if the rate of uptake of acetylcholine (corrected

TABLE 3. EFFECTS OF 2:4 DINITROPHENOL (10μM) AND OF OUABAIN (10 μM) ON THE UPTAKE OF ACETYLCHOLINE IN RAT BRAIN CORTEX SLICES IN THE PRESENCE OF PARAOXON (20 μM) WHEN INCUBATED IN OXYGEN AT 37° FOR 1 hr

Acetylcholine concentration ($\times 10^{-3}$M) in the medium	Acetylcholine concn ratios (corrected for passive diffusion) (tissue:medium)				
	A	B		C	
	No inhibitor present	In presence of DNP	Ratio A/B	In presence of ouabain	Ratio A/C
0·02	6·4	4·0	1·6	4·2	1·5
0·04	5·5	3·3	1·7	3·7	1·5
0·05	1·4	0·8	1·7	0·9	1·5
1·0	0·8	0·5	1·6		

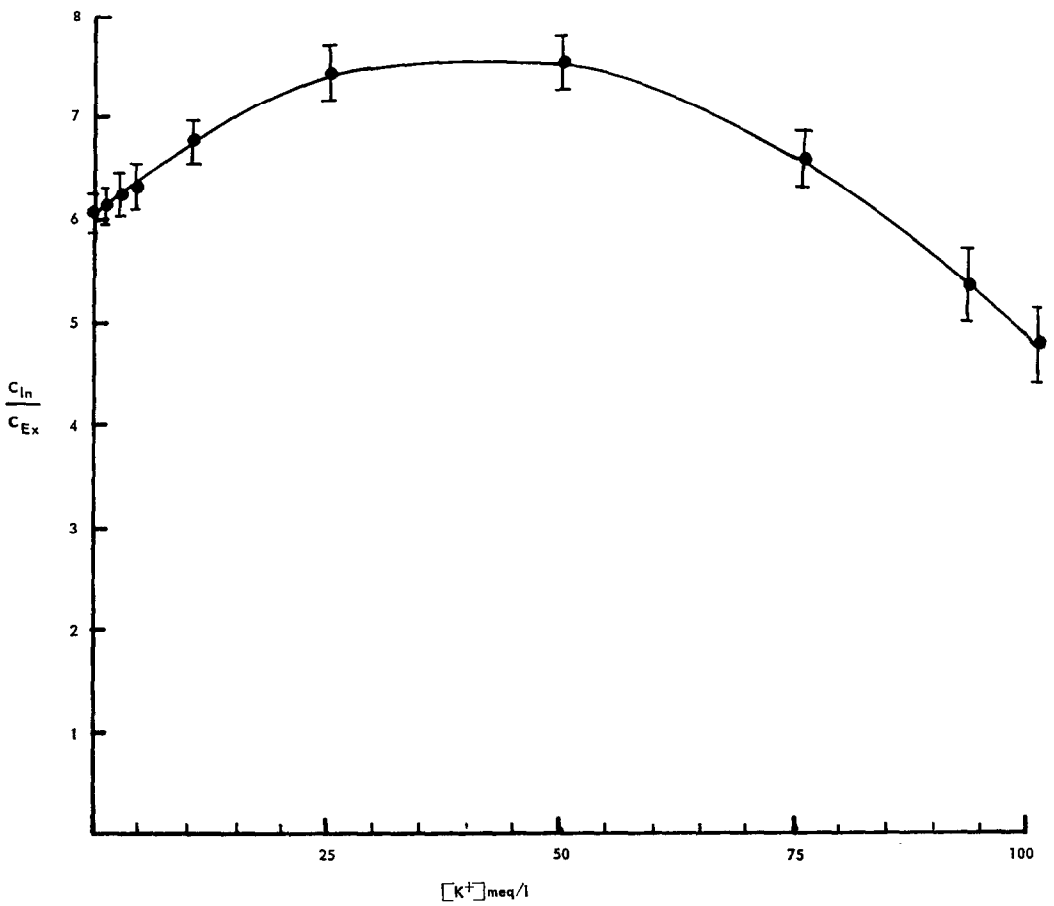

FIG. 6. Effects of variation of potassium ion concentration on the uptake of acetylcholine in rat brain cortex slices in presence of 20 μM paraoxon. Concentration of acetylcholine = 40 μM. *Ordinate*: Concentration ratio (tissue:medium) of acetylcholine. *Abscissa*: Potassium ion concentration in the medium (m-equiv./liter)

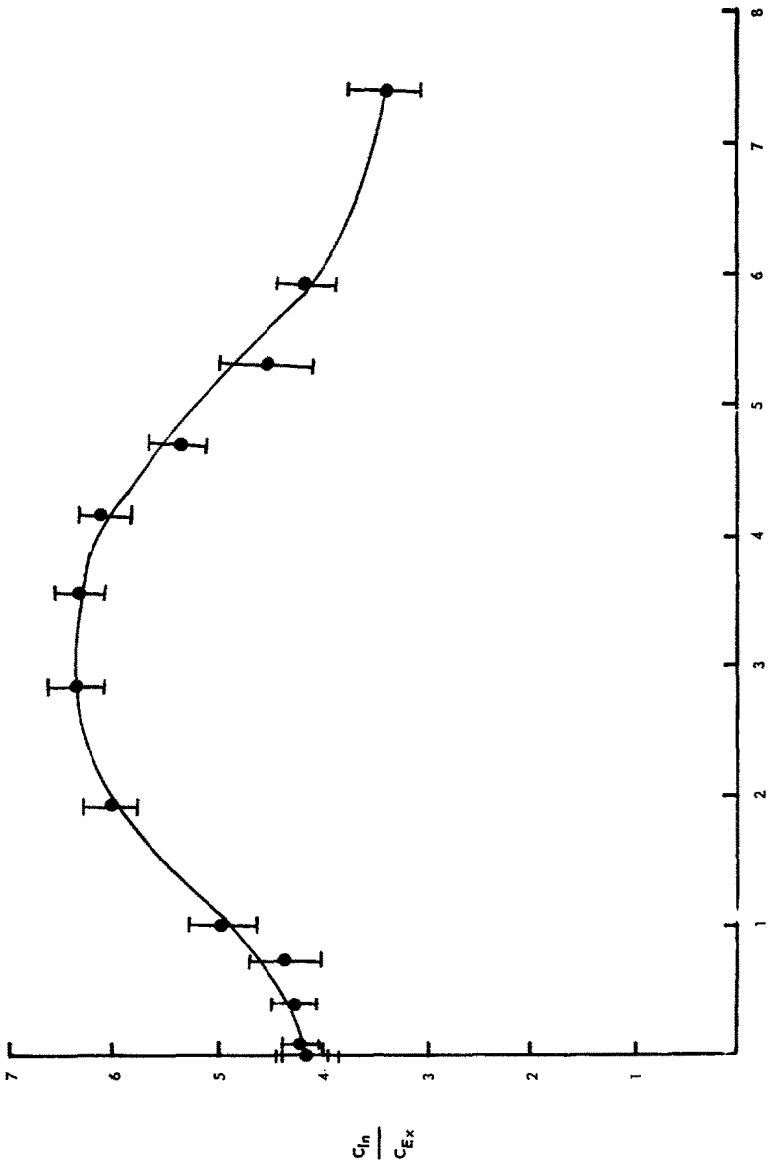

FIG. 7. Effects of variation of calcium ion concentration on the uptake of acetylcholine in rat brain cortex slices in presence of 20 μM paraoxon. Concentration of acetylcholine = 40 μM. *Ordinate*: Concentration ratio (tissue:medium) of acetylcholine. *Abscissa*: Calcium chloride concentration in the medium (mM).

for the passive diffusion rate) is a function of the level of brain cell ATP which is known to be diminished in the presence of 2:4 dinitrophenol.

Effects of ouabain on acetylcholine uptake

Typical results, recorded in Table 3, show that the presence of ouabain (10 mM) brings about an inhibition of acetylcholine uptake in the brain tissue. The ratio of the concentrative uptake of acetylcholine in the presence of ouabain to that in the absence of the inhibitor is constant at concentrations of acetylcholine varying from 0·02 mM to 0·5 mM. This result indicates the dependence of the uptake of acetylcholine on the activity of the membrane-bound brain cell sodium-potassium-dependent ATPase whose activity is well known to be highly sensitive to ouabain and this inhibitor is believed to be specific for this ATPase.

Effects of variation of the concentrations of inorganic ions on the uptake of acetylcholine

Potassium ions. Optimal accumulation of acetylcholine takes place in rat brain cortex slices at potassium ion concentrations between 20 and 50 m-equiv./l. (Fig. 6). Absence of potassium ions leads to a fall in the concentrative uptake of acetylcholine,

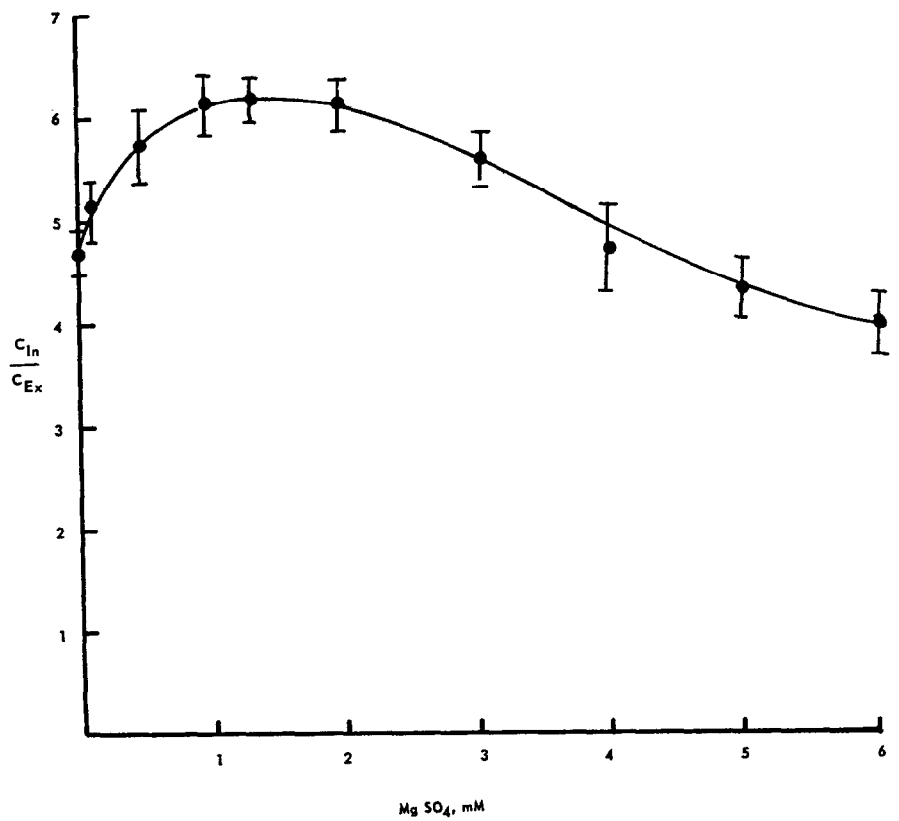

FIG. 8. Effects of variation of magnesium ion concentration on the uptake of acetylcholine in rat brain cortex slices in presence of 20 μM paraoxon. Concentration of acetylcholine = 40 μM. *Ordinate*: Concentration ratio (tissue:medium) of acetylcholine. *Abscissa*: Magnesium sulfate concentration in the medium (mM).

and the presence of potassium chloride concentrations in excess of 50 mM also leads to a fall. The results in Fig. 6 have been corrected for the changes of tissue swelling, which occur in the presence of different potassium ion concentrations.

Calcium ions. Absence of calcium ions, or the presence of relatively high concentrations of calcium chloride (e.g. 8 mM), leads to a decline in concentrative accumulation of acetylcholine during the period of incubation. Results are shown in Fig. 7.

Magnesium ions. Results given in Fig. 8 show that the presence of relatively high concentrations of magnesium sulfate (e.g. 6 mM) brings about a depression of the concentrative uptake of acetylcholine.

Sodium ions. The absence of sodium ions leads to a fall in the rate of acetylcholine uptake (Fig. 9). However, under the given experimental conditions, the fall in the absence of sodium ions is not as large as that known to take place with the active transport, in rat brain slices, of glycine,[15] glutamate[16] or thiamine.[17] The reason for this is not yet known.

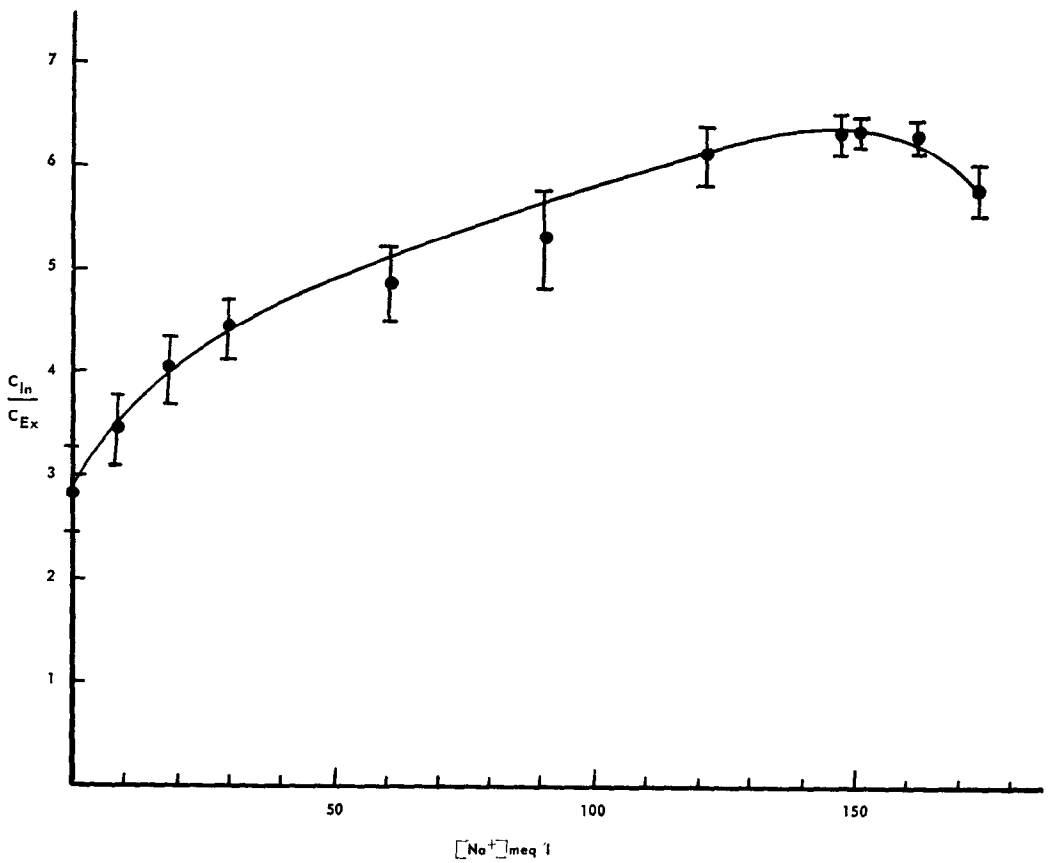

FIG. 9. Effects of variation of sodium ion concentration on the uptake of acetylcholine in rat brain cortex slices in presence of 20 μM paraoxon. Concentration of acetylcholine = 40 μM. *Ordinate*: Concentration ratio (tissue:medium) of acetylcholine. *Abscissa*: Sodium ion concentration in the medium.

Release of labeled acetylcholine from rat brain cortex slices

Preliminary experiments were carried out to observe the rates of release of labeled acetylcholine from rat brain slices after their incubation for 1 hr at 37° in Krebs–Ringer phosphate medium in the presence of glucose (10 mM), paraoxon (20 μM) and labeled acetylcholine (40 μM). These brain slices were transferred, after rinsing with 20 ml fresh incubation medium, free from labeled acetylcholine but containing 40 μM unlabeled acetylcholine, and after lightly blotting, into 3 ml Krebs–Ringer phosphate–glucose medium containing 20 μM paraoxon but no acetylcholine. Incubation was carried out in oxygen for 1 hr at 37° and the radioactivity in the incubation medium was then estimated. It was found that the radioactivity released into the medium amounted to 43 per cent of that present in control tissue slices. The addition of 2:4 dinitrophenol (10 μM) or of eserine (10 μM) to the acetylcholine-free medium apparently increased the rates of release of radioactivity from the brain slices into this medium, these amounting to 72·5 per cent and 60·6 per cent of the control values respectively.

The results show that the rate of release of acetylcholine from the brain tissue is enhanced under conditions that suppress concentrative uptake of the acetylcholine. Possibly the effect is due to the suppression of uptake of acetylcholine normally released in the absence of the inhibitor.

DISCUSSION

The demonstration of the uptake against a concentration gradient of acetylcholine, in rat brain cortex slices, in the presence of paraoxon (20 μM), which inactivates the brain choline esterase, confirms the work of earlier investigators[2, 3] who used different organophosphates as inhibitors of choline esterase and is in accord with the fact that concentrative uptake of the acetylcholine analogue, carbamylcholine, takes place in rat brain slices.[18]

In considering the nature of the uptake process of acetylcholine into brain slices (apart from that of passive diffusion) it is of interest to assess the possible role of exchange diffusion. This process will bring about an accumulation of labeled acetylcholine against a concentration gradient, by exchange of labeled with unlabeled acetylcholine, if the concentration of the acetylcholine in the brain slice exceeds that in the medium. Such an exchange, however, will not add to the total acetylcholine concentration in the brain slice. Recorded values of the quantities of acetylcholine present in rat brain slices previous to, and after, incubation in the presence of oxygen and in a physiological-glucose medium may help to determine the limits of the exchange process. According to Richter and Crossland[19] the average cerebral content of acetylcholine of rats, killed by dropping into liquid air, is 7 mμmoles/g, i.e. approximately 9 μM, taking the cerebral tissue water to be 80 per cent of the wet wt. Obviously this concentration is too small to account for the concentrative uptakes of acetylcholine recorded, for example, in Table 2, where it is seen that the concentration of labeled acetylcholine in the tissue, even in the absence of glucose in the incubation medium, exceeds 120 μM with an external concentration of acetylcholine of 40 μM. However, during the process of incubation of rat brain slices for 1 hr in the presence of oxygen in a physiological-glucose medium, containing eserine, acetylcholine accumulates. The usual quantity of acetylcholine formed was 12·7 μg/g* corresponding

* Estimated as the chloride.

to 69 mμmoles/g and, of this quantity, 30 per cent (i.e. 21 mμmoles/g) was in the free condition, the rest being in the bound form.[20] Incubation of the brain slices under the same conditions, but in the absence of glucose, led to the formation of only 3·1 μg/g of acetylcholine (free plus bound) corresponding to 17 mμmoles/g. Such yields of acetylcholine, formed during the incubation process in the presence of a choline esterase inhibitor, cannot account for the concentrative accumulation, by exchange diffusion, of acetylcholine in the slices amounting to at least 224 mμmoles/g in the presence of glucose, or 120 mμmoles/g in the absence of glucose, with an external concentration of acetylcholine of 40 μM (Table 2). However, with very small external concentrations of labeled acetylcholine, e.g. 0·2 μM (Table 1), where the concentration of labeled acetylcholine in the tissue after 1 hr incubation reaches 2·8 mμmoles/g, the exchange diffusion process may certainly account for the concentrative accumulation of labeled acetylcholine in the brain slice. It seems, therefore, that exchange diffusion may play a significant role in the concentrative uptake process in brain with very small external concentrations of acetylcholine but that it becomes less important as the concentration of acetylcholine in the incubation medium rises.

The facts described in this paper lead to the conclusion that part of the uptake of acetylcholine against a concentration gradient is probably a function of the cell level of ATP. This conclusion is supported by the following facts concerning the rate of uptake:

(a) It is diminished by the presence of 10 μM 2:4 dinitrophenol, whose inhibitory effect is independent of the acetylcholine concentration. (b) It is optimal in the presence of 4 mM glucose at which concentration the rate of rat brain slice respiration and the cell level of ATP are known also to be optimal. (c) It falls from its optimal value in the absence of either potassium or calcium ions or in the presence of a high concentration of potassium ions, under which conditions the cell level of ATP also falls.[15] The conclusion is also supported by the observations that the concentrative uptake of acetylcholine is proportional to the rate of respiration of the rat brain slices both in the presence and absence of glucose, and that the uptake is diminished almost to the passive diffusion value in the absence of oxygen though glucose is present.

It is worthy of note that the active transport of glycine into rat brain slices is affected in a similar manner by the absence of potassium or calcium ions or by their presence in relatively high concentrations.[15] These effects were attributed to falls in cell levels of ATP or to the inhibition of membrane-bound ATPase activity.[15, 21–24]

The uptake of acetylcholine in rat brain slices in the presence of paraoxon is dependent on the activity of the membrane-bound ATPase as shown by the inhibitory effect of 10 μM ouabain, whose action is independent of the acetylcholine concentration, and also by the inhibitory effects of relatively high concentrations of magnesium and calcium ions which are known to depress membrane-bound ATPase activity.[15, 21–25]

Results of preliminary experiments show that the rate of release of acetylcholine from rat brain cortex slices in the presence of 20 μM paraoxon is enhanced under conditions that suppress concentrative uptake of acetylcholine.

These observations support the conclusion that the transport of acetylcholine in rat brain slices, in the presence of paraoxon, like that of glycine in rat brain slices in the absence of any inhibitor, is a process that is partly dependent on both the ATP level and the activity of the membrane ATPase and, presumably, partly there-

fore on the operation of the sodium pump. Such a conclusion would also account for the suppression of carbamylcholine transport in rat brain slices by high potassium ion concentrations.[18]

The influx of acetylcholine into the brain slice to a concentration ratio of the order of 20:1, with a small external concentration of acetylcholine, in the rat brain slices may also be considered as due to its entry as a charged ion accumulating because of the resting potential of the tissue, an explanation already put forward for carbamylcholine.[26] A concentration ratio for acetylcholine equal to that for potassium ions may then be expected and the conditions (e.g. operation of the sodium pump) necessary for the concentration of potassium ions in the brain cell would then apply for the concentration of acetylcholine. Nevertheless, as already pointed out, the process of exchange diffusion may account for the concentrative uptake of acetylcholine with small external concentration of acetylcholine.

The process of concentrative uptake of acetylcholine into the brain cell is controlled by a membrane constituent, or transport carrier, so that it is greatly affected by substances having affinities for the carrier. It is blocked, for example, by eserine or atropine. A discussion of the nature of these substances, and of their possible modes of action, will be considered in a succeeding publication.

Further experiment is needed to determine whether compartmentation of the labeled acetylcholine taken up by the brain cell occurs but the evidence would seem to indicate that it may be, for the most part, in free solution in the cytoplasm.[27]

The physiological significance of the carrier mediated uptake process for acetylcholine at the brain cell membrane is obviously, at present, a matter for conjecture. It seems possible that the energy-dependent uptake mechanism may represent a process alternative (or additional) to the well known action of choline esterase for the removal of acetylcholine. There seems to be increasing evidence that active transport systems, that will remove a released compound from its site of action, may prove to be a general feature of transmitter inactivation.[28]

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