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Accumulation of choline by the rat diaphragm

Choline is a precursor for both acetylcholine and phospholipids. The problem of its entry, therefore, concerns not only the nervous tissue but the cells in general. On account of the electrical charge of choline and the structure and function of the cell membrane, the entry of choline by simple diffusion is thought to be rather slow. It is not surprising that recently choline transport into squid axon[1], brain slices[2], synaptosomes[3] and erythrocytes[4] has been established. In the present work the uptake of choline by the rat diaphragm was studied in order to find out whether this substance possibly enters the cell by a carrier-mediated process.

The experiments were carried out on albino rats (80–120 g). [*N*-*Me*-^{14}C]Choline was supplied by the Radiochemical Centre (Amersham), eluted from the paper with water and stored at −20°. Radioactivity was determined by liquid scintillation spectrometry using a modified Bray's liquid scintillation mixture[5]. The water content was determined by the loss in weight after keeping the muscle overnight at 110°. The inulin extracellular space was determined using the method of ROE *et al.*[6].

The diaphragm was excised under ether anesthesia and incubated at 38° in 10 ml of the incubating solution (Krebs bicarbonate buffer, pH 7.4, with 200 mg/100 ml of glucose) containing radioactive choline (0.05 μC/ml). After incubation the preparation was rinsed with saline and blotted, the muscle weighed, dissolved in 0.5 ml of 1 M NaOH and its radioactivity measured. Fig. 1 shows that diaphragm incubated with labeled choline accumulates radioactive material. The concentration of this material obtained after 1 h of incubation was about 6–7 times higher in the fibre water than in the medium. No equilibrium was obtained even after 2 h of incubation.

In these experiments, however, the total radioactivity in the muscle was measured and that trapped in the interspace was only estimated. The rest of the labeled substance was assumed to be mainly located within the cell itself and an increase in this fraction was taken as a measure of the substance entry into the muscle

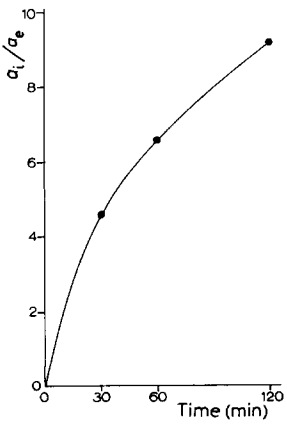
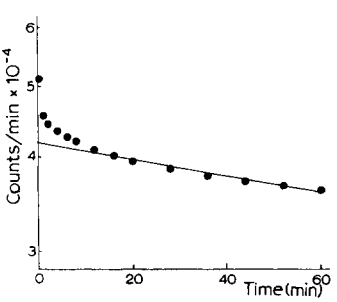


Fig. 1. Time-course of choline uptake in the rat diaphragm. The rat diaphragm was incubated at 38° in Krebs bicarbonate buffer containing labeled choline at 5 μM concentration. a_e = radioactivity per ml fibre water; a_i = initial radioactivity per ml medium. The mean values obtained in 4–6 experiments are presented.

fibre. This assumption is supported by our experimental findings. Since the latter fraction kept increasing for at least 2 h, the possibility of the radioactive material having been adsorbed outside the cell must be excluded. Further, in parallel experiments, the radioactive material in the muscle tissue extracts was identified by paper chromatography. The tissue was homogenized in cold distilled water, adjusted to pH 4–5 with HCl. Subsequently, the extract was heated at 100° for 10 min, and the precipitated protein was removed by centrifugation. The aqueous extract was spotted on chromatographic paper. The chromatogram was allowed to develop for 16–18 h with the following solvent system: *n*-butanol–ethanol–acetic acid–water (8:2:1:3, by vol.). For measuring radioactivity the chromatogram was cut into small pieces and placed in counting vials together with the liquid scintillation mixture. For the identification of the spots of high radioactivity, non-radioactive choline had been spotted on the same chromatogram. After the development, choline was visualized by exposing the chromatogram to iodine vapor. The results revealed that about 80–85 % of all the radioactive material had the same R_F as choline. In some other experiments the muscle preincubated in the radioactive medium was cut into small pieces and kept for 2 h in distilled water. After centrifugation the amount of the radioactive material diffused into the water was compared with that precipitated with the tissue. It was found that about 75–80 % of the radioactive material diffused out of the muscle fibre segments. It seems, thus, that a greater part of labeled choline was not built into the cell membranes though a lesser part of choline might have been built into the cell phospholipids in a similar way as it was reported with other tissue[7–10].

The choline efflux was measured on a diaphragm muscle strip. The strip mounted on a perspex frame was first incubated in the medium with labeled choline (0.5 μC/ml) and after 1 h was transferred into another vessel in which the non-radioactive solution was changed at different periods of time. The solution was collected and prepared for measuring radioactivity. At the end of the washing procedure, the muscle strip was blotted, weighed, dissolved in the NaOH solution, and its radioactivity was measured. One of the efflux experiments is shown in Fig. 2. After a fast initial drainage



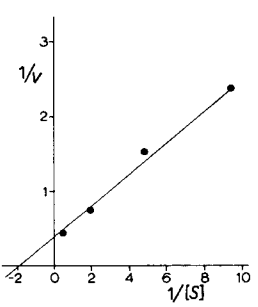


Fig. 2. The efflux curve of the radioactive material from the rat diaphragm strip preparation. The strip was preincubated for 1 h at 38° in Krebs bicarbonate buffer containing 10 μM labeled choline. The volume of the washing solution was 5 ml.

Fig. 3. Lineweaver–Burk plot of choline uptake in the rat diaphragm. The diaphragm was incubated for 1 h at 38° in Krebs bicarbonate buffer with labeled choline at different concentrations. [S] in mM, v in mmoles/l of fibre water per h. The mean values obtained in 4–6 experiments are presented.

of the radioactive material, probably corresponding to the material's extracellular fraction, its intracellular fraction was washed out rather slowly with a half time of about 5–6 h. Similar results were found by RENKIN[11], working on the frog sartorius muscle. The rather large half time for the efflux of choline from the muscle fibre indicates either that the passive diffusion of choline across the membrane is slow or that choline is bound inside the cell. The efflux of choline is probably also influenced by the electrical potential across the cell membrane.

In order to study the mechanism of the accumulation of labeled choline in the muscle fibre and to test whether it enters the cell by a carrier-mediated mechanism, the entry of choline at different concentrations was studied. For this purpose the non-radioactive choline was added to the incubating medium in order to obtain the desired initial concentration. The results graphically evaluated in a LINEWEAVER–BURK[12] plot, are shown in Fig. 3. The entry of choline at concentrations from 0.1 to 2 mmoles/l obeyed Michaelis–Menten kinetics. The maximal velocity of entry into the fibre water was about 2.8 mmoles/l per h and the Michaelis constant about 0.5 mmole/l. The Michaelis constant obtained in our experiments is considerably higher than that for choline in erythrocytes[4] but rather close to that for decamethonium in the guinea pig diaphragm[13].

The present results show that choline is accumulated inside the muscle fibre and that its entry obeys Michaelis–Menten kinetics. This fact suggests that its entry is mediated by a carrier. The difference between the choline concentration in the medium and inside the cell can be partly due to the fact that choline is electrically charged and, therefore, distributed according to its electrochemical potential across the membrane. Further, a similar concentration difference would be obtained if choline were bound inside the cell; it is known, for example, that acetylcholine is practically bound in the nerve endings[14, 15] and decamethonium in the diaphragm muscle fibre[16]. There is not enough data on the possible energy-driven transport mechanism which could also cause the concentration difference.

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The effect of copper on membrane enzymes

Copper has a variety of effects on cells, modifying the facilitated diffusion of glycerol across erythrocytes[1,2], Schwann cell[3] and squid axon membranes[3] and altering the Cl^- permeability of frog skin[4] and molluscan neurones[5]. $CuCl_2$ is the copper salt frequently used in permeability studies and so was used in the present investigation, although it is little dissociated in aqueous solution. However, $CuCl_2$ is still able to attack lysine, histidine and cysteine residues[6]. Cu(II) also induces mitochondrial swelling[7] and inhibits the oxygen consumption of brain homogenates[8], and subarachnoid injections in pigeons cause the rapid onset of convulsions and death[9]. This convulsive action is believed to be due to a direct effect on the plasma membrane of brain cells. These workers conclude that it is primarily the microsomal Mg^{2+}-ATPase and not the (Na^+-K^+-Mg^{2+})-ATPase which is inhibited by copper[10] although EPSTEIN AND MCILWAIN[11] found both enzymes to be sensitive to 150 μM Cu(II).

We have suggested elsewhere[12,13] that both these enzyme systems could be implicated in the passage of ions across the cell membrane and have therefore studied their differential sensitivity to $CuCl_2$ in greater detail, using a microsomal preparation from rat brain, and rat erythrocyte ghosts. We have not used a Tris buffer system when the preparation is exposed to Cu(II), since these two agents interact strongly in dilute solution[14].

Microsomal preparations were made from rat brain as previously described[15] except that EDTA was not used at any stage, and the microsomal pellet was gently homogenised in 10 mM Mg^{2+} and recentrifuged to reduce Ca^{2+} concentration to a level which did not inhibit ATPase activity. The final microsomal pellet was suspended in neutralised, deionised water, and the resulting specific activity and the (Na^+-K^+-Mg^{2+})-ATPase: Mg^{2+}-ATPase activity ratio both proved to be the same as that found previously[15]. 1-ml aliquots of the preparation were mixed with 1 ml of $CuCl_2$ of appropriate concentration and left for 10 min. 0.5-ml samples were then taken and assayed for ATPase activity over 10 min at 37°. The reaction medium was made up in 50 mM histidine–HCl buffer.

Ghosts were prepared from rat erythrocytes by haemolysis of washed cells in 3 mM $MgCl_2$ *plus* 0.5 mM EDTA, followed by three washings with 10 mM $MgCl_2$ and one wash with deionised water. The ghosts were suspended in deionised water, and 0.5-ml samples were mixed with 0.5 ml $CuCl_2$ of appropriate concentration and left for 10 min at 37°. The reaction was started by addition of 1 ml of ions *plus* ATP in 1 mM imidazole–HCl buffer and was stopped after 30 min.