Biochemical Pharmacology, Vol. 23, pp. 2595–2602. Pergamon Press, 1974. Printed in Great Britain.

ACCUMULATION OF CHOLINE BY THE SEGMENTAL GANGLIA OF THE LEECH

Š. ADAMIČ

Institute of Pathophysiology, Medical Faculty, University of Ljubljana, 61105 Ljubljana, Yugoslavia

(*Received* 3 *December* 1973; *accepted* 13 *February* 1974)

Abstract—The entry of choline into cells of the segmental ganglia of the leech (*Hirudo medicinalis*) was studied *in vitro* using ^{3}H-choline. The weight of a fresh ganglion was found to be about 53 μg and its water content about 78 per cent. After 30 min of incubation the mannitol space was about 47 per cent and the inulin space about 35% of the total ganglion volume. The accumulation of ^{3}H-choline was considerably faster than that of mannitol or inulin. After 30 min of incubation the intracellular choline concentration calculated on the basis of the mannitol extracellular space was about 10 times higher than the labelled choline concentration in the medium. Paper chromatography showed that about 65 per cent of the total radioactivity inside the ganglion was still in the form of choline and about 25 per cent in the form of acetylcholine. At external choline concentrations ranging from 0·06 to 1 mM the choline entry obeyed the Michaelis–Menten kinetics. The maximum entry rate and the Michaelis constant calculated from these data were about 8 m-moles/30 min/l. of intracellular water and 1 mM, respectively. The choline entry decreased when tetramethylammonium or hemicholinium-3 was added to the incubating solution, tetramethylammonium had a stronger inhibitory effect than hemicholinium-3. Our results suggest that at the choline concentrations studied, the choline entry is mediated by a carrier and that a considerable portion of choline is transformed into acetylcholine.

IN RECENT years a carrier-mediated movement of choline across the plasma membrane was found in different types of cells. This mechanism seems to be a common feature of most cells and plays an important role in phospholipid synthesis. In the nervous tissue the choline entry mechanism probably has an additional role in that it renews the storage of acetylcholine in the synapses. For this reason the choline movement in the central nervous system has been studied rather extensively and a carrier-mediated choline entry system has been found in mammalian brain slices[1] as well as in synaptosomes prepared from the mammalian brain.[2,3] However, the function and structure of the mammalian central nervous system is very complex and therefore a relatively simple nervous system would seem to be useful as a model for choline entry studies. Leech ganglia seem to be convenient for this purpose for many reasons. They lie entirely within a blood sinus without being penetrated by any vessel. Incubation of these ganglia in an artificial medium *in vitro*, therefore, is less unphysiological than is the incubation of mammalian brain slices. In addition, they can easily be isolated and they have been rather extensively studied both morphologically and physiologically. The aim of the present work was to find out whether the leech ganglia accumulate labelled choline and, if so, to investigate the mechanism of its entry.

METHODS

The experiments were carried out on ganglia from the leech *Hirudo medicinalis*. The leeches weighing between 1·5 and 2 g were anesthetized in 10% ethanol, pinned on a cork plate and submerged in leech Ringer solution modified by Nicholls and Kuffler.[4] The ventral sinus with the central nervous system was exposed by cutting longitudinally through the skin along the midventral line. The ventral sinus was opened under a dissecting microscope and the bilateral nerve roots dissected as close to the ganglion as possible avoiding any damage to the latter. Subsequently parts of the ganglionic chain consisting of four segmental ganglia linked by their connectives were carefully removed for experimentation. Four samples of such ganglionic chains were taken from each animal.

The ganglia were weighed on a microbalance (Mettler M-5) to within 1 μg in the following manner. In order to prevent the drying of ganglia during the weighing procedure perspex slides (about 10 × 10 × 2 mm) with a well (about 0·5 mm deep and 2 mm in diameter) were prepared together with glass cover slides (about 6 × 6 mm) and a thin layer of grease put around each well. Each perspex slide together with one glass cover slide was weighed three times. The weights of such sets ranged from 80 to 100 mg. Three to four ganglia dissected from the chain were transferred to the well of one perspex slide using a Holter pipette. The ganglia were carefully blotted with filter paper and the well covered with the glass slide. The perspex slides with ganglia were also weighed three times. Since the weight of ganglia was less than 1 per cent of the total weight, even a small change in the weighing conditions could appreciably affect the results. In order to minimize such errors each blank perspex slide with grease and glass cover was weighed simultaneously in the same way as those loaded with ganglia. The average difference in the weight of the blank perspex slides was used for correcting the results of the ganglia weighing procedure.

The water content was determined by drying the ganglia at 105° to constant weight and weighing them in the same way as fresh ganglia.

For the study of the choline entry into the ganglia, labelled choline was used. Four ganglia linked by their connectives were dissected, slightly blotted an immediately incubated in 20 μl of leech Ringer solution containing radioactive choline. At higher choline concentrations non-radioactive choline was added in order to obtain the desired initial concentration. The incubating solution was mechanically stirred in 5 min intervals. After incubation the ganglionic chain was quickly rinsed with leech Ringer solution, blotted with filter paper and the connectives removed. Ganglia were solubilized overnight in 10 μl of 1 N NaOH. The resulting solution was quantitatively transferred to the counting vials using 90 μl of distilled water. During the incubation samples of the incubating medium were taken for radioactivity measurements.

The mannitol and the inulin extracellular space was determined simultaneously with the choline entry or in separate experiments. For this purpose labelled mannitol or inulin were used. Samples for radioactivity measurement were prepared in the same way as in the choline entry experiments.

Paper chromatography was used to identify the radioactive material in the ganglia. After incubation in labelled choline four ganglia were homogenized in small glass homogenizers containing 10 μl of a cold 10 μM solution of DFP in distilled water. Using HCl the solution was adjusted to pH 4. The homogenate was quantitatively transferred to a small tube and the volume increased to 100 μl. Using a paper indicator,

the pH of the homogenate was checked and readjusted with HCl to the value of 4–5. Subsequently the homogenate was heated to 100° for 10 min and the precipitated protein removed by centrifugation. For the identification of the spots of high radioactivity a mixture of non-radioactive acetylcholine and choline was added to the aqueous extract which was subsequently applied to chromatographic paper. The chromatogram was developed for 14–18 hr with *n*-butanol–ethanol–acetic acid-water (8 : 2 : 1 : 3, by vol.). After development the spots of choline and ACh were visualized by spraying the chromatogram with Dragendorff solution.

In our experiments the following labelled substances were used: choline(*N*-methyl-^{3}H)chloride with a specific radioactivity of 250 mCi/m-mole; mannitol-^{14}C with a specific radioactivity of 10–30 mCi/m-mole, both purchased from the Radiochemical Centre Amersham, and inulin-^{3}H with a specific radioactivity of 130 mCi/g, purchased from the New England Nuclear. The ^{14}C labelled compound was stored at −30°, and the tritiated substances were stored at 4°. To make sure that the radioactive material was still in the form of choline, the stock solution of radioactive choline was periodically checked by paper chromatography using the same solvent system as mentioned above.

Radioactivity was measured by liquid scintillation spectrometry (Unilux II, Nuclear Chicago) using a modified Bray's liquid scintillation mixture containing 5 g of diphenyloxasole (PPO), 0·5 g of *p*-bis-[2-(5-phenyloxazolyl)]benzene (POPOP) and 80 g of naphthalene per litre of solvent consisting of equal volumes of toluene, *p*-dioxane and ethyleneglycol monomethyl ether.

RESULTS

The results of the experiments in which the weight of fresh and dry ganglia was determined are shown in Table 1. From these data it can be seen that the water content represents about 78 per cent of the total ganglion weight.

In order to express the results in terms of intracellular choline concentration it was necessary to determine the ganglion extracellular space. The mannitol space of the ganglia incubated for 30 min in leech Ringer solution containing ^{14}C-mannitol was found to be 46·7 ± 2·8 per cent (means of 20 expts ± S.E.) of the total ganglion volume. During the same period of incubation the ^{3}H-inulin space occupied only 35·3 ± 1·8 per cent of the ganglion volume (mean of 20 expts ± S.E.). The kinetics of mannitol uptake by leech ganglia is shown in Fig. 1. There was a rapid phase of mannitol uptake which was largely completed within 10 min. Thereafter a further slow uptake of mannitol was observed.

When ganglia were incubated in leech Ringer solution containing ^{3}H-choline, they

TABLE 1. THE WEIGHT OF FRESH AND DRY LEECH GANGLIA

Ganglia	No. of samples	Weight (μg)*
Fresh	12	53 ± 1·7
Dry	12	11·2 ± 0·5

Three to four fresh or nine dry ganglia were weighed together as a sample and the average weight of ganglion in the sample was calculated.

* Values are mean ± S.E.

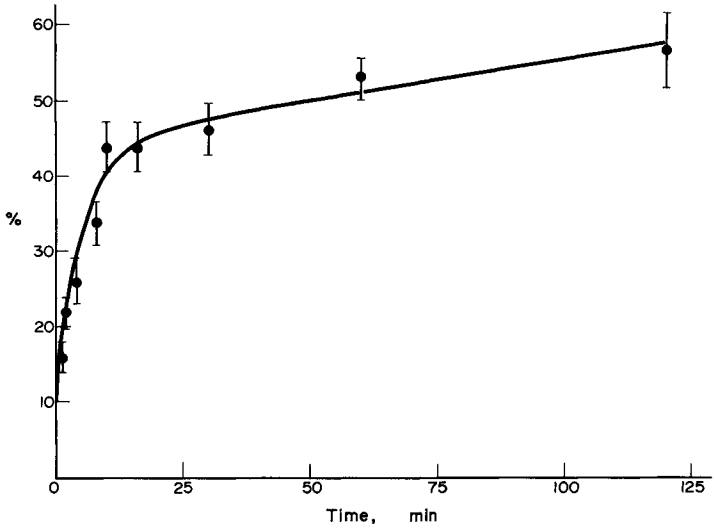

FIG. 1. The dependence of mannitol extracellular space on the incubation time. Chains consisting of four ganglia linked by their connectives were incubated for different periods of time in leech Ringer solution containing labelled mannitol. After incubation the ganglionic chain was rinsed and connectives removed. The mean values and their standard deviation of eight experiments are presented.

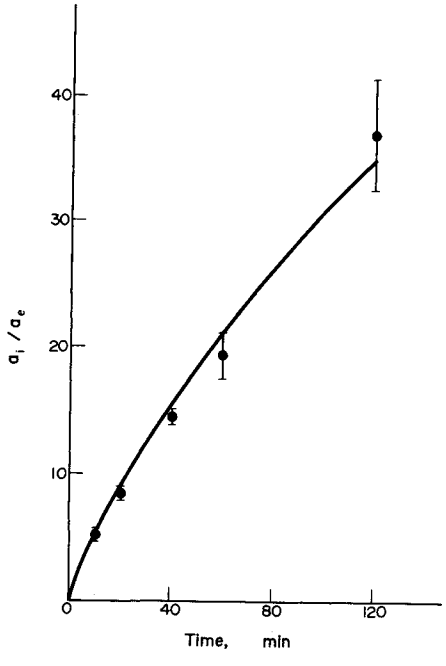

FIG. 2. Time course of choline uptake into leech ganglia. Chains consisting of four ganglia linked by their connectives were incubated for different periods of time in leech Ringer solution containing 60 μM labelled choline. After incubation the ganglionic chain was rinsed and connectives removed. a_i, radioactivity per ml of intracellular water; a_e, radioactivity per ml of medium. The mean values and their standard deviations of six experiments are presented.

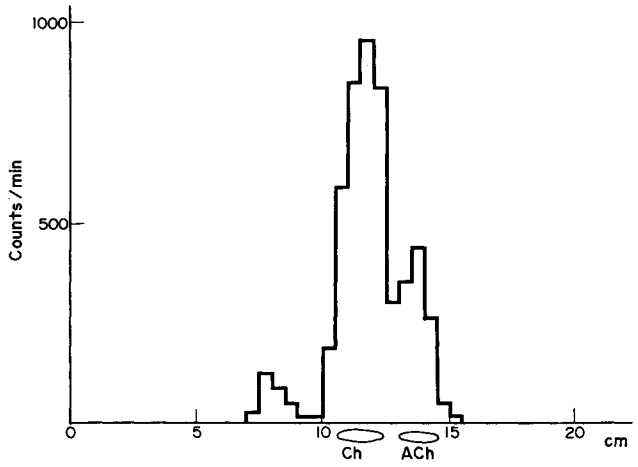

FIG. 3. Paper chromatography of ^3H recovered from leech ganglia after 30 min of incubation in labelled choline. Ch and ACh are spots of unlabelled choline and acetylcholine.

accumulated the radioactive material. The time course for the uptake of ^3H-choline by leech ganglia incubated in 60 μM choline solution is shown in Fig. 2. The ratio between the intracellular concentration of the radioactive material calculated on the basis of mannitol extracellular space and the concentration of the labelled choline in the incubating solution increased with the time of incubation. Apart from showing a faster rate the choline uptake differed from that of mannitol also in that it was not

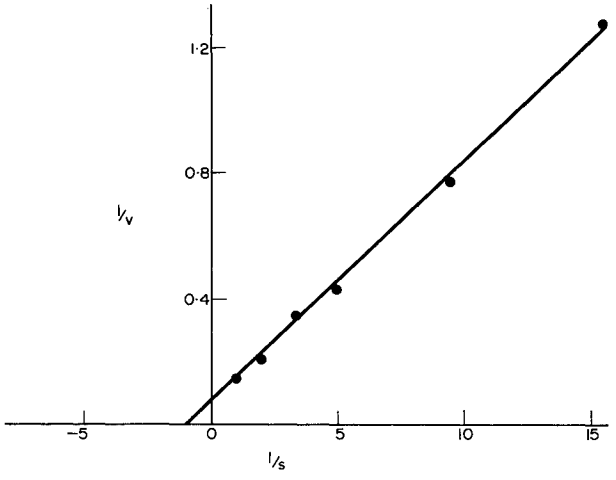

FIG. 4. Lineweaver–Burk plot of choline uptake in leech ganglia. Chains consisting of four ganglia linked by their connectives were incubated for 30 min at room temperature in leech Ringer solution containing choline at different concentrations. After incubation the ganglionic chain was rinsed and the connectives removed. [S]. in m-moles/l.; v, in m-moles/l. of intracellular water/30 min.

TABLE 2. INHIBITION OF CHOLINE ENTRY IN LEECH GANGLIA

	a_i/a_e	Inhibition (%)
Control	8·17	
Tetramethylammonium 6 mM	0·98	88
Tetramethylammonium 1·25 mM	2·34	71
Control	7·80	
Hemicholinium-3 6 mM	3·14	52
Hemicholinium-3 1·25 mM	7·00	20

Chains of ganglia were incubated for 30 min at room temperature in leech Ringer solution containing 0·025 mM labelled choline with or without inhibitor. $a_i a_e$-ratio between radioactivity inside the cells obtained after 30 min of incubation and radioactivity in the incubating medium.

complete even after 2 hr of incubation when an intracellular/extracellular concentration ratio of about 35 was obtained.

In similar experiments the chemical nature of the radioactive material extracted from the ganglia incubated in labelled choline was investigated by paper chromatography and the chromatogram of such an experiment is presented in Fig. 3. The results show that after 30 min of incubation in 60 μM ^{3}H-choline about 65–70 per cent of the radioactive material was still in the form of choline, about 25 per cent in the form of acetylcholine and about 5 per cent in a so far unidentified substance with a R_f of about 0·28. After 2 hr of incubation the acetylcholine portion of the radioactive material in ganglia was unchanged whereas the portion of the substance with R_f 0·28 increased to about 10 per cent and the choline portion decreased to about 60 per cent. In both cases the radioactivity in the sediment obtained after centrifugation of the heated homogenate amounted to about 6 per cent of the total radioactivity.

In the next series of experiments the dependence of the choline entry rate on the external choline concentration was studied. The results of the experiments when the choline entry at external choline concentrations between 0·06 mM and 1 mM was studied, were plotted according to Lineweaver and Burk, and are shown in Fig. 4. At these concentrations the process obeys the Michaelis–Menten kinetics. The maximum velocity and the Michaelis constant obtained from this plot are 8 m-moles/30 min/l. of intracellular water and 1 mM, respectively.

In order to see whether the choline entry can be inhibited by substances which are known as inhibitors of the choline carrier in other tissues, tetramethylammonium and hemicholinium-3 were added to the incubating solution in another series of experiments. The results are presented in Table 2. Both substances inhibited choline entry, but the inhibition with hemicholinium was weaker than that caused by the same concentration of tetramethylammonium.

DISCUSSION

The weight of leech segmental ganglia was found to be rather constant. No difference between ganglia from different parts of the ganglionic chain was noticed. The water content calculated from our results is close to that known for the mammalian brain which differs with the species from 76 to 78 per cent.[5]

The mannitol space obtained in our experiments was considerably larger than the volume of the extracellular clefts estimated from electron micrographs.[4,6,7] On the other hand, our results are rather close to those obtained in experiments in which sodium[8] or sucrose and inulin[9] were used as extracellular space markers. The inulin space obtained in our experiments was smaller than the mannitol space as it is in isolated rat ganglia,[10] and can be accounted for by the difference in the molecular size of the two substances. The mannitol space reached practically its maximum after 10 min of incubation, even though a further slight increase was observed when incubation was continued, a fact suggesting the possibility of slow intracellular penetration.[9] Since the data on the relative extracellular fluid volume were required for the calculation of the intracellular choline concentration, mannitol was chosen as an extracellular space marker because its molecular weight is very close to that of choline. Calculations of the intracellular choline concentrations were, therefore, based on the mannitol space.

Our results indicate that the level of choline concentration in leech ganglia is considerably higher than that of the extracellular fluid. The calculated intracellular concentration of the radioactive material in the ganglia after 30 min of incubation in labelled choline is about 10 times higher than the concentration of the radioactive material in the incubating medium. By prolonging the incubation time to two hours the intracellular concentration of the radioactive material increased at roughly the same rate and an intracellular/extracellular concentration ratio of about 35 was obtained. The concentration ratio obtained in leech ganglia was therefore higher than that in mouse brain slices.[1]

For further studies of the choline entry mechanism it seemed important to find out whether unchanged choline or its metabolites accumulated in the ganglia. Paper chromatography revealed that most of the radioactive material in the ganglia was still in the form of choline (Fig. 3). It seems, therefore, that a permanent low intracellular concentration of choline due to its intracellular metabolism is not the driving force in the choline uptake. Only a small fraction of the radioactive material was built into or trapped by the components of the sediment after centrifugation. The fraction with R_f 0·28 was not further studied. Its R_f value, however, was rather close to that for betaine[11] a product of choline metabolism in some cells. A rather substantial part of the radioactivity in the ganglia was found to be in the form of acetylcholine. A similar transformation of choline into acetylcholine has been observed to occur in slices[1,12] and synaptosomes[13,14] of mammalian brain, in whole brain in experiments *in vivo*,[15,16] and in sympathetic ganglia.[17] On the other hand in non-nervous tissues such as skeletal muscle,[18,19] cardiac muscle[20] or intestine[21] no significant amount of acetylcholine was found after incubation in labelled choline.

Our results (Fig. 4) show that at choline concentrations between 60 μM and 1 mM the choline entry obeys the Michaelis–Menten kinetics. This fact suggests that its entry is mediated by a carrier. The considerable difference between the intracellular and the extracellular choline concentration obtained in our experiments might be, at least partly, due to the fact that owing to its electrical charge choline is distributed across the cell membrane in accordance with its electrochemical potential. In addition, part of the choline might be bound inside the cell and, if so, the real concentration of choline dissolved in the intracellular water would be lower than that calculated from the total intracellular choline. For these reasons, our data cannot

show whether an energy-driven transport against the electrochemical potential or a facilitated diffusion is involved in the carrier-mediated choline entry.

It is known for other tissues that the carrier-mediated choline entry can be inhibited by quaternary ammonium compounds. In our experiments the inhibitory effect of two quaternary ammonium compounds, hemicholinium-3 and tetramethylammonium was tested and it was found that both of them inhibited choline entry. This fact also points to the existence of a carrier-mediated choline entry system. There is, however, a quantitative difference between the effect of the two above mentioned compounds on leech ganglia and their effect on other tissues studied so far. With the leech ganglia a stronger inhibition was obtained with 1·25 mM tetramethylammonium than with 6 mM hemicholinium-3 (Table 2). It can be roughly estimated that tetramethylammonium is, by about one order of magnitude, a stronger inhibitor of choline entry in leech ganglia than hemicholinium-3. In other tissues hemicholinium-3 was found to be a stronger inhibitor of the choline entry than tetramethylammonium.[22,23]

Acknowledgements—This work was supported by the Boris Kidrič Foundation, Ljubljana. The author wishes to express his thanks to Professor A. O. Župančič for his suggestions and encouragement during this work. He is also indebted to Mr. Vasja Loboda and Mrs. Marjeta Dobovišek for valuable technical assistance.

REFERENCES

1. J. SCHUBERTH, A. SUNDWALL, B. SÖRBO and J.-O. LINDELL, *J. Neurochem.* **13,** 347 (1966).
2. R. M. MARCHBANKS, *Biochem. J.* **110,** 533 (1968).
3. I. DIAMOND and E. P. KENNEDY, *J. biol. Chem.* **244,** 3258 (1969).
4. J. G. NICHOLLS and S. W. KUFFLER, *J. Neurophysiol.* **27,** 645 (1964).
5. J. W. T. DICKERSON, in *Applied Neurochemistry* (Eds. A. N. DAVISON and J. DOBBING), pp. 48–115. Blackwell, Oxford (1968).
6. R. E. COGGESHALL and D. W. FAWCETT, *J. Neurophysiol.* **27,** 229 (1964).
7. A. VAN HARREVELD, F. I. KHATTAB and J. STEINER, *J. Neurobiol.* **1,** 23 (1969).
8. J. G. NICHOLLS and S. W. KUFFLER, *J. Neurophysiol.* **28,** 519 (1965).
9. J. G. NICHOLLS and D. E. WOLFE, *J. Neurophysiol.* **30,** 1574 (1967).
10. D. A. BROWN, J. V. HALLIWELL and C. N. SCHOLFIELD, *Br. J. Pharmac.* **42,** 100 (1971).
11. M. ACARA and B. RENNICK, *J. Pharmac.* **182,** 1 (1972).
12. J. A. RICHTER and R. M. MARCHBANKS, *J. Neurochem.* **18,** 691 (1971).
13. R. M. MARCHBANKS, *Biochem. Pharmac.* **18,** 1763 (1969).
14. A. A. ABDEL-LATIF and J. P. SMITH, *Biochem. Pharmac.* **21,** 3005 (1972).
15. J. SCHUBERTH, B. SPARF and A. SUNDWALL, *J. Neurochem.* **16,** 695 (1969).
16. M. V. GOMEZ, E. F. DOMINO and O. Z. SELLINGER, *Biochem. Pharmac.* **19,** 1753 (1970).
17. B. COLLIER and F. C. MACINTOSH, *Can. J. Physiol. Pharmac.* **47,** 127 (1969).
18. C. C. CHANG and C. LEE, *Neuropharmac.* **9,** 223 (1970).
19. L. P. MCCARTY, A. S. KNIGHT and M. B. CHENOWETH, *J. Neurochem.* **20,** 487 (1973).
20. S. BOSTEELS, A. VLEUGELS and E. CARMELIET, *J. gen. Physiol.* **55,** 602 (1970).
21. P. A. SANFORD and D. H. SMYTH, *J. Physiol.* **215,** 769 (1971).
22. K. MARTIN, *Br. J. Pharmac.* **36,** 458 (1969).
23. Š. ADAMIČ, *Biochem. Pharmac.* **21,** 2925 (1972).