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INFLUENCE OF CHOLINE, HEMICHOLINIUM-3 AND NAPHTHYLVINYLPYRIDINE ON UPTAKE AND ACETYLATION OF ^{3}H-LABELED CHOLINE INTO HIPPOCAMPUS SLICES

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Abstract—In slices of rat hippocampus the influence of choline (Ch), hemicholinium-3 (HC-3), and naphthylvinylpyridine (NVP) on the uptake of [^{3}H]-Ch and its incorporation into acetylcholine (ACh) was studied. Increasing concentrations of Ch, added to the incubation medium, led to an elevation of the uptake of labeled precursor and its acetylation. The release of radioactive labeled ACh from slices of hippocampal tissue was also enhanced. A kinetic analysis of the transport of Ch revealed two distinct uptake systems for the precursor with K_m-values of 0.87 μM and 30.3 μM, respectively. The uptake of tritiated Ch into hippocampal slices could be inhibited by HC-3. NVP, an inhibitor of choline acetyltransferase, reduced the uptake of Ch to a similar degree as observed by HC-3, whereas the subsequent acetylation to ACh was not affected. Using an intraventricular application of 400 nmoles unlabeled Ch, the influence of the precursor on uptake and incorporation of [^{3}H]-Ch was also tested *in vivo*. Both the uptake of Ch and its subsequent acetylation were enhanced, whereas no changes in the ACh content of the hippocampus were observed. The data suggest that the increase of the precursor supply yields an elevation of Ch transport, its acetylation and the transmitter release.

It was reported by several workers [6, 15] that after oral and parenteral application of choline (Ch) an increase in Ch concentration in plasma and different organs including the brain might be evident: this increase was accompanied by a rise in the concentration of the cholinergic transmitter in the brain. Furthermore, most of these studies revealed that the elevation of Ch concentration in the brain was of greater magnitude than that of acetylcholine (ACh) [6, 8, 11, 14].

The kinetic analysis of the transmembranal Ch transport in brain structures exhibited the existence of two different uptake systems for Ch [12, 35]. The sodium-dependent high affinity uptake system seems to be regulated by or reflects neuronal activity [17, 27]. It was demonstrated by MOREL [21] that increased concentrations of Ch led to a stimulation of ACh release.

On the other hand the intraneuronal precursor supply can be reduced by inhibitors of the high affinity sodium-dependent Ch uptake, like hemicholinium-3 (HC-3). The uptake inhibition led to a decrease of the releasable ACh pool. An additional inhibitory action of HC-3 on the ACh synthesizing enzyme could not be detected *in vitro* [7, 18].

There are some contradictions concerning the *in vivo* efficacy and specifity of the action of naphthylvinylpyridine (NVP), a potent inhibitor of choline acetyltransferase *in vitro* [32]. It is suggested that, apart from the inhibitory action on the actylcholine synthesizing enzyme, an additional effect may exist which can also influence the synaptosomal uptake of Ch [2].

Thus, the present work was started with the aim to study the importance of the available precursor pool for the uptake and acetylation of Ch and the release of ACh. Therefore *in vitro* experiments were performed using hippocampus slices. *In vivo* experiments were carried out to study the uptake and acetylation in the hippocampus of rats after intraventricular application of high Ch doses.

MATERIALS AND METHODS

Labeled and unlabeled compounds. Choline chloride methyl-^{3}H (sp. act. 16 Ci/mmole), choline chloride methyl-^{14}C (sp. act. 60 mCi/mmole), acetyl-1-^{14}C choline chloride (sp. act. 10 mCi/mmole) were obtained from the Radiochemical Centre Amersham (Great Britain); hemicholinium-3 from Aldrich Chemical Co. Ltd.; naphthylvinylpyridine from Calbiochem, San Diego; butyl-ethyl-ketone (3-heptanone) from Fluka AG, Switzerland; sodium tetraphenylboronate from VEB Jenapharm; Dowex (2 × 8; 200–400 mesh; Cl^-form) from Serva Entwicklungslabor Heidelberg; TLC aluminium sheets cellulose without fluorescent indicator from Merck Darmstadt; 2,5-diphenyloxazole (PPO) from Koch-Light Laboratories, Ltd.: 1,4-bis 2-(5-phenyloxazolyl)-benzene (POPOP) from New England Nuclear; naphthalene from Germed (VK Labor- und Feinchemikalien).

Animals. All experiments were carried out using male Wistar rats from our own breeding stock weighing 210–240 g.

Preparation and preincubation of hippocampus slices. Rats were decapitated after a blow on the

neck. The hippocampi were carefully removed and cut into 0.4 mm thick slices. The latter were preincubated for 15 minutes in Krebs–Henseleit solution containing 10^{-5} M physostigmine at 37° and pH 7.3 according to the method described by Molenaar [20].

Incubation. (i) To estimate the influence of Ch on the uptake and acetylation of the labeled precursor the slices were incubated in presence of different concentrations of labeled Ch for 5 min. The specific radioactivity of Ch in the medium was identical for all concentrations used.

(ii) In a second series the slices were incubated with 5 μCi[^{3}H]-Ch per 1 ml incubation medium for 30 min. Unlabeled Ch was added to reach final concentrations from 1 μM to 1 mM.

(iii) The inhibitors HC-3 (final concentration 10 μM) and NVP (final concentration 30 μM) were added to the medium in a second preincubation period (10 min), followed by the incubation with 10 μCi[^{3}H]-Ch (10 μM) for 2 or 30 min, respectively. Generally, after incubation the slices were rinsed with cold Ca^+-free Krebs–Henseleit solution containing 10 μM Ch and frozen on dry ice. Ch and ACh were extracted using the TCA-procedure.

Extraction procedures. (i) *TCA extraction.* The hippocampus slices were homogenized in 5% TCA containing 180 μM physostigmine, for 90 sec. Then the homogenate was centrifuged at 11,000 r.p.m. for 40 min. From the supernatant the TCA was removed by agitating twice using a threefold volume of water-saturated ether.

(ii) *Fractionated extraction.* The fractionated extraction of Ch and ACh was performed according to the method described by Beani *et al.* in a modification by Matthies *et al.* [19] to differentiate between the 'free', 'labile bound' and 'stable bound' fractions.

(iii) *TLC separation of Ch and ACh.* The separation of Ch and ACh was performed using the method described by Barker *et al.* [1]. From the supernatants yielded by the extraction procedures, as well as from the incubation medium, Ch and ACh were transferred into an organic solvent by shaking the supernatants with 500 μl butyl-ethyl-ketone containing 10 mg of sodium tetraphenylboronate. After centrifugation the organic supernatants were added to a Dowex column and eluted with 2 ml methanol. The eluates were dried and then dissolved 50 μl methanol containing 14 mM Ch and 25 mM ACh as carriers. 2 × 10 μl of this methanol solution were then applied to a cellulose thin-layer plate. The chromatograms were developed by use of a butanol–ethanol–acetic acid–water mixture (8:2:1:3), dried in air and stained with iodine vapour. The marked spots were scraped out from the cellulose plates and shaken with 2 ml methanol–1N HCl (19:1). The radioactivity was measured in a toluene solvent system using a liquid scintillation counter (MULTI MAT Intertechnique, France).

The values of [^{3}H]-Ch and [^{3}H]-ACh were calculated on the basis of the recovery of internal ^{14}C-labeled standards added to each sample and expressed as disintegrations per minute (dpm) per mg protein for *in vitro* experiments or as dpm per 100 mg fresh tissue for *in vivo* studies. An exception was represented by the series (ii). Because of different specific activities of Ch in the incubation medium, the values have been multiplied by the reciprocals of these specific activities. The protein determination was performed using an amido black procedure [23].

In vivo *experiments.* (i) Rats received chronically implanted cannulas under hexobarbital–urethane-anaesthesia (100 and 600 mg/kg respectively). One week after implantation of cannulas 50 μCi [^{3}H]-Ch plus 400 nmoles Ch dissolved in 10 μl artificial cerebrospinal fluid were applied into the right lateral ventricle. Control animals received 50 μCi [^{3}H]-Ch dissolved in artificial cerebrospinal fluid. After an incorporation time of 40 min the animals were sacrificed immediately after a blow on the neck. The hippocampi of each animal were rapidly dissected out and frozen on dry ice. Ch and ACh were extracted using the fractionated extraction procedure. All other procedures were identical with those of *in vitro* experiments.

(ii) 400 nmoles of unlabeled Ch were administered via chronically implanted cannulas as described above. Forty min later the rats were decapitated. After dissection the hippocampi were rapidly frozen on dry ice up to the estimation of ACh content. The ACh content of TCA extracts (see TCA extraction) was determined by bioassay using guinea-pig ileum.

Statistics. The statistical calculations were done by use of Mann-Whitney's U-test.

RESULTS

Uptake and acetylation of [^{3}H]-*Ch in hippocampus slices.* In the first series of *in vitro* experiments, slices of hippocampus tissue were incubated with concentrations of Ch ranging from 0.1 μM to 10.0 μM. It could be observed that uptake and acetylation of tritiated Ch were enhanced in the dependence on elevation of the labeled precursor (Fig. 1). The release of [^{3}H]-ACh into the incubation medium exhibited a similar dependence on the concentrations of Ch (Fig. 1). Plotting the uptake of the [^{3}H]-Ch into hippocampus slices according to Lineweaver and Burk, K_m-values of 0.87 μM for the high affinity

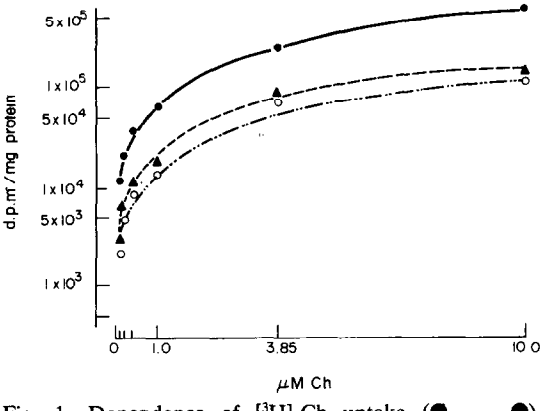

Fig. 1. Dependence of [^{3}H]-Ch uptake (●——●), [^{3}H]-Ch acetylation (▲---▲) and [^{3}H]-ACh release (○···—···○) on Ch concentration of the incubation medium using the same specific activity. Each point represents the mean of seven separate determinations.

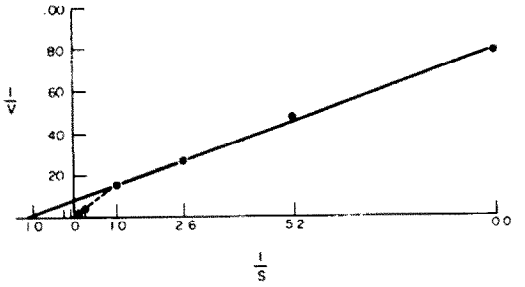

Fig. 2. Lineweaver–Burk plot of Ch uptake by the hippocampus slices as a function of Ch concentration. The rate of Ch uptake (V) is expressed as dpm [^{3}H]-Ch/mg protein/5 min. Each point represents the mean of seven determinations.

uptake system and of 30.3 μM for the low affinity uptake system could be estimated (Fig. 2). The quotient (Q) between acetylation and uptake of [^{3}H]-Ch, was enhanced by Ch concentrations up to 3.85 μM. It was going back using a concentration of 10.0 μM in the incubation medium (Fig. 3).

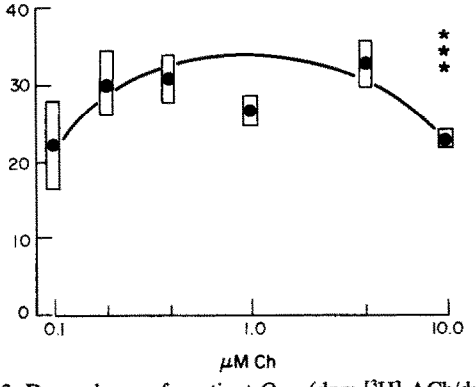

Fig. 3. Dependence of quotient Q = (dpm [^{3}H]-ACh/dpm [^{3}H]-Ch × 100) on Ch concentration of the incubation medium using the same specific activity. The means ± S.E.M. of seven determinations are represented. *** Differs significantly from result with 0.386 or 3.85 μM Ch at $P \leq 0.002$.

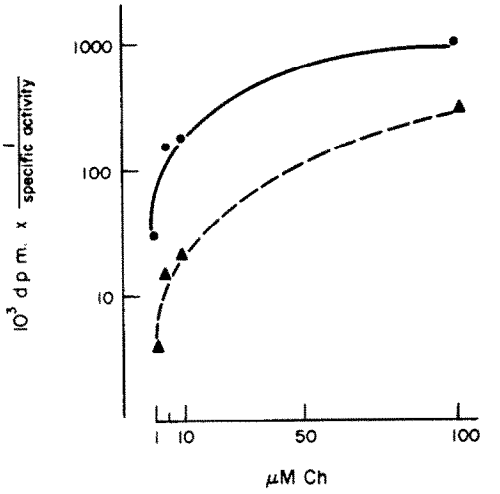

Fig. 4. Dependence of [^{3}H]-Ch uptake (●——●) and [^{3}H]-Ch acetylation (▲——▲) on Ch concentration of the incubation medium using different specific activities. The values are corrected by multiplication with the reciprocals of the specific radioactivities in the medium. Each point represents five separate determinations.

To study the influence of Ch concentrations above the K_m-value obtained for the low affinity uptake system, it was necessary to add unlabeled precursor to the incubation medium containing a constant amount of 5 μCi [^{3}H]-Ch per ml. The measured values for [^{3}H]-Ch and [^{3}H]-ACh in the incubated hippocampus slices were lowered following the range of Ch concentrations from 1 to 1,000 μM, because the specific activity of [^{3}H]-Ch in the incubation medium was decreased by adding of unlabeled compound. If the measured radioactivity was corrected by the reciprocals of the specific radioactivities, a similar dependence of uptake and acetylation on the Ch concentration was elucidated as described in the first series (Fig. 4).

The influence of HC-3 and NVP on uptake and acetylation of [^{3}H]-Ch *in hippocampus slices.* The

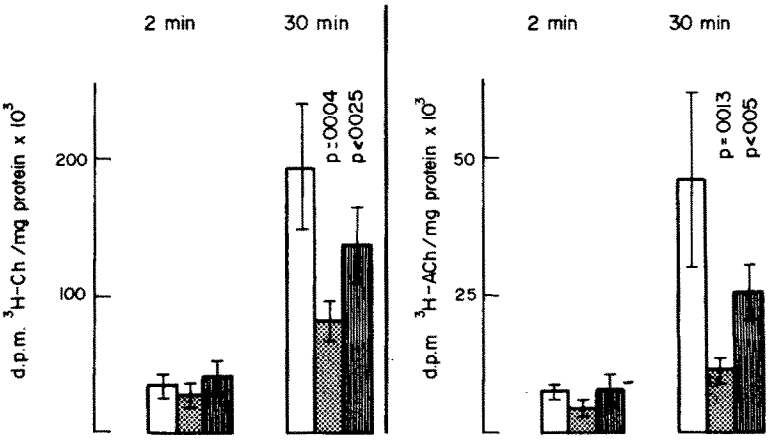

Fig. 5. Effects of HC-3 (pointed columns) and NVP (striped columns) on [^{3}H]-Ch uptake and [^{3}H]-Ch acetylation are expressed as dpm [^{3}H]-Ch and dpm [^{3}H]-ACh after 2 and 30 min incubation. Empty columns indicate control slices incubated in Krebs–Henseleit solution. Each value is the mean ± S.E.M. of five to six separate determinations.

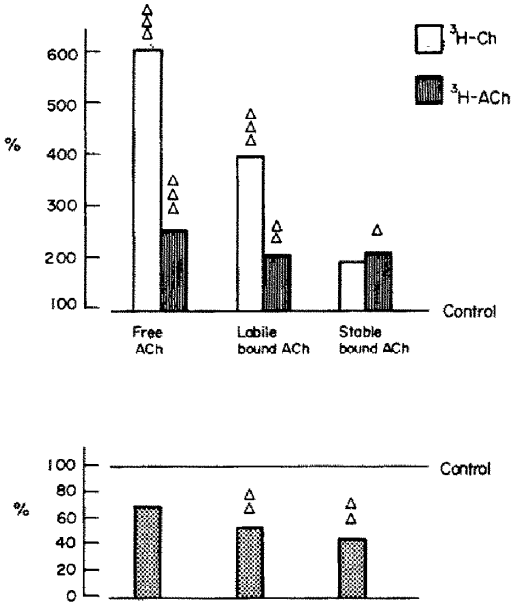

Fig. 6. Upper panel: *in vivo* changes of [^{3}H]-Ch uptake (empty columns) and [^{3}H]-Ch acetylation (striped columns) into three ACh fractions after intraventricular application of 400 nmoles Ch; percentage values compared with controls being 100 per cent. The mean of seven separate determinations are represented. △ △ △ Significantly different from untreated ($P \leq 0.002$). △ △ Significantly different from untreated ($P \leq 0.004$). △ Significantly different from untreated ($P \leq 0.047$). Lower panel: the quotient Q = (dpm [^{3}H]-ACh/dpm [^{3}H]-Ch of labile bound fraction) × 100 obtained for three ACh fractions after intraventricular application of 400 nmoles Ch; percentage values compared with controls being 100 per cent. Significantly different from untreated ($P = 0.004$). The means of seven separate determinations are represented.

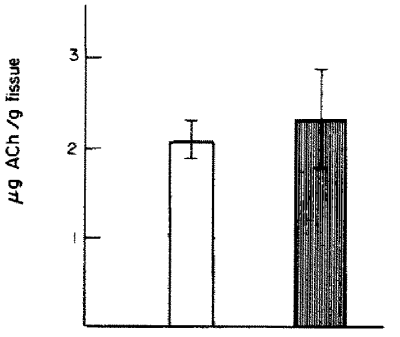

Fig. 7. Effect of 400 nmoles intraventriculary applied Ch on the ACh content of the hippocampus. Empty columns represent control values, striped columns values obtained for experimental animals. Values are the means ± S.E.M. for five animals.

effects of HC-3 and NVP were proved after incubation periods of 2 or 30 min. Using the short-lasting incubation, neither HC-3 nor NVP were able to effect the uptake and acetylation of Ch.

After a 30 min incubation in the presence of HC-3 or NVP the uptake as well as the acetylation of tritiated precursor were reduced (Fig. 5). Considering the radioactivity taken up during the incubation with HC-3, the [^{3}H]-Ch was lowered by 56 per cent, whereas the [^{3}H]-ACh was reduced by 77 per cent. In contrast to these findings, in the experiments with NVP no differences between the diminution of [^{3}H]-Ch (51%) and the reduction of [^{3}H]-ACh (55%) could be detected.

The influence of Ch on the uptake and acetylation of ^{3}H-labeled precursor in vivo. Forty minutes after the application of 50 μCi [^{3}H]-Ch, combined with 400 nmoles of unlabeled Ch, the estimated [^{3}H]-Ch and [^{3}H]-ACh in hippocampus tissue were enhanced in comparison with control animals. The greatest increase of [^{3}H]-Ch was observed in the 'free' fraction, following by those in the 'labile bound' and 'stable bound' fractions. The [^{3}H]-ACh of all of these fractions was also enhanced, but to a smaller extent compared to the Ch radioactivity (Fig. 6; upper part). Despite of higher amounts for [^{3}H]-Ch and [^{3}H]-ACh, respectively, the quotient between labeled ACh and its precursor was reduced under the influence of high doses of unlabeled Ch (Fig. 6; lower part).

In vivo *influence of high extracellular Ch concentrations on ACh content.* The content of total ACh in the hippocampus was not changed by the application of 400 nmoles unlabeled Ch. Thus, for control animals the ACh content was 2.13 ± 0.22 μg ACh/g hippocampal tissue, while after application of 400 nmoles Ch the ACh content rose only unessentially to 2.32 ± 0.59 μg/g tissue (Fig. 7).

DISCUSSION

The results presented here demonstrate the influence of Ch availability on the uptake and acetylation as well as the release of ACh in hippocampus tissue of rats. The *in vitro* studies performed on hippocampus slices exhibit an active uptake process for Ch, which is inhibited by HC-3 and NVP. Despite of preincubation of slices with HC-3 or NVP we were unable to find an inhibitory action of these drugs on the uptake of Ch using a two minutes lasting incubation with tritiated precursor.

The well documented decrease of Ch uptake [2, 4, 16, 26, 30] produced by HC-3 was also found by us prolonging the incubation with [^{3}H]-Ch up to 30 minutes.

Surprisingly, the acetylation of Ch was more strongly reduced by HC-3 than its uptake. In our opinion, a direct inhibitory action on the choline acetyltransferase can be excluded, since *in vitro* the ACh synthesizing enzyme was not influenced by 10 μM HC-3 [7, 18].

The assumption of Guyenet *et al.* [11] concerning effects of HC-3 on the compartmentation of Ch taken up seems to be of the same speculative degree as the HC-3 induced release of ACh, postulated by Rodriguez *et al.* [24].

It must be mentioned that unlabeled Ch was added to a concentration of 10 μM in the experiments with HC-3. Considering the K_m-values of the high and low affinity uptake systems one can conclude that under these conditions a considerable amount of Ch was taken up by the low affinity carrier system. The

latter would be influenced by concentrations of HC-3 higher than that used in our studies and seems to be of minor importance for the synthesis of the cholinergic transmitter [3, 33, 35].

This assumption is supported by the observed decrease of relationship between [^{3}H]-ACh and [^{3}H]-Ch radioactivity in hippocampus slices using tritiated Ch in a concentration of 10 μM (Fig. 3).

After a 30 min incubation in the presence of NVP a marked decrease in the uptake and acetylation of [^{3}H]-Ch was observed. Both processes were reduced to an approximately equal extent. These findings are in a good agreement with those of Barker *et al.* [2] and Simon *et al.* [28] demonstrating an inhibitory effect of NVP on the Ch uptake into synaptosomes. The concentration of NVP used in our experiments was reported by Smith *et al.* [32] as the IC_{50} of the substance on the choline acetyltransferase *in vitro*. Taking into consideration the nonexistence of substrate saturation of the choline acetyltransferase under physiological conditions [10], it is not surprising that in our studies no additional effects of NVP on the acetylation of intracellular precursor supply could be demonstrated. Therefore, the doubts concerning the advantages of NVP as a useful inhibitor of choline acetyltransferase *in vivo* should be emphasized.

The kinetic analysis of Ch uptake into hippocampal slices revealed the existence of two different uptake systems with K_m-values of 0.87 μM and 30.0 μM, respectively (see Fig. 2). These results are comparable with other findings on synaptosomes and slices of cerebral cortex [12, 29, 35]. It is depicted in Fig. 3 that not only the uptake of [^{3}H]-Ch, but also its acetylation and the release of newly synthesized labeled transmitter are enhanced following the increase of Ch concentration in the incubation medium. Furthermore, a saturation of these processes was reached between 3.85 and 10 μM Ch.

As mentioned above, the relationship of [^{3}H]-ACh radioactivity to the [^{3}H]-Ch radioactivity in hippocampus slices was reduced by concentrations of Ch exceeding the K_m-value of the high affinity uptake system. Since the portion of Ch, taken up by the high affinity uptake mechanism is thought to be used primarily in ACh synthesis, the observed reduction would be explainable. The Ch dependent increase of ACh release reported also by Morel [21] in the electric organ of Torpedo and by Richter *et al.* [25] on superfused slices of rat brain requires further elucidation to clarify its physiological importance.

To investigate a possible saturation of the low affinity uptake system a second series of experiments was performed using concentration of Ch from 1 μM to 1000 μM. Naturally, such high concentration could not be realized by the labeled precursor itself.

Therefore, the slices were incubated with 5 μCi of [^{3}H]-Ch and unlabeled Ch was added to reach the final concentration. The measured values of [^{3}H]-Ch and [^{3}H]-ACh of the slices have been multiplied by the reciprocals of resulting specific activities in the incubation medium. Using this correction, a concentration dependent enhancement of uptake and acetylation could be observed.

Because preferentially that part of Ch should be utilized for the ACh synthesis, which is taken up by the high affinity system [33, 35], it is surprising that the acetylation increased also in a concentration range saturating the low affinity transport mechanism.

In this connection the findings of Eisenstadt and Schwartz [9] should be remembered. They injected [^{3}H]-Ch directly into the cell bodies of cholinergic neurons of Aplysia and observed an efficient conversion to ACh. There is some other evidence in the literature [5, 33], that the Ch taken up by the low affinity system can also be acetylated. We intend to conclude that generally the intraneuronal Ch, independent of its source, may be acetylated. The question arises, if under physiological conditions *in vivo* such concentrations of Ch are present to be transported by other carriers than the high affinity uptake system. In our opinion, the regulation of precursor availability *in vivo* is realized preferentially, by the high affinity uptake system which seems to be connected with the neuronal impulse flow [17, 22, 27, 29, 31].

The intraventricular application of 400 nmoles Ch plus 50 μCi [^{3}H]-Ch led to a rise in [^{3}H]-Ch uptake into the hippocampal terminals as well as in its acetylation. As shown in Fig. 6 the uptake was more enhanced than the acetylation. These findings are in good agreement with those of Fernstrom [10], Cohen and Wurtman [6] and Eckernäs [8], which reported a higher increase of Ch content than of ACh content in the brain after systemic precursor administration. To obtain more detailed information on the Ch influence on ACh synthesis we performed an extraction from tissue in three fractions, 'free', 'labile bound' and 'stable bound' fractions.

Considering the limited knowledge of compartmentation of Ch methodical reasons made us choose the data for labeled Ch of one of the three fractions as reference value. The Ch in the 'free' fraction was not suitable as reference since it may be contaminated by extracellular Ch to a great extent. Because the 'labile bound' fraction was contaminated by extracellular Ch in very low extent, this part of Ch can be regarded as a representative Ch pool useful to study changes in Ch metabolism. When relating the values of [^{3}H]-ACh of the three fractions to those obtained for [^{3}H]-Ch in the 'labile bound' fraction, it can be found that under increased supply of Ch this ratio showed a statistically significant reduction for the 'labile bound' and 'stable bound' fractions. This can be explained in a similar manner as discussed for our *in vitro* experiments. The intraventricular applied Ch leads to an increase of the extra- and intraneuronal Ch in the hippocampus. We assumed that only a portion of the intraneuronal Ch can be utilized for the synthesis of ACh. More detailed information about the compartmentation of Ch is necessary to elucidate the functional significance of different intraneuronal pools for the availability of releasable transmitter.

The ACh content of the hippocampus remained unchanged after intraventricular application of 400 nmoles Ch. Considering the Ch induced enhancement of acetylation on the one hand and the unchanged transmitter content on the other hand the conclusion can be drawn that the intraventricular application of Ch leads to a raising of ACh release.

These results confirm findings of Ulus and Wurtman [34] on the Ch stimulated transmitter release. As pointed out in connection with the *in vitro* studies, characteristics and physiological role of this enhanced release require further investigation in the near future.

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