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THE EFFECT OF HEMICHOLINIUM-3 ON THE *IN VIVO* FORMATION OF CEREBRAL PHOSPHATIDYLCHOLINE*

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SUMMARY

The effect of hemicholinium-3 on the incorporation of [*Me*-^{14}C]choline into the phospholipids of the subcellular fractions of the canine caudate nucleus was studied. A correlation between the amount of hemicholinium-3 administered and increased choline incorporation into phospholipids was obtained. The analysis of the labeled phospholipids revealed that 93% of the radioactivity was in phosphatidylcholine and the remainder in lysophosphatidyl choline and sphingomyelin + choline plasmalogens. The specific radioactivity of phosphatidylcholine (counts/min per μg phosphorus) determined after its isolation by thin-layer chromatography, increased markedly as a result of the administration of hemicholinium. An unequal time-dependence in the labeling of the phosphatidylcholine in the subcellular fraction containing nerve endings, mitochondria and lysosomes, as opposed to the phosphatidylcholine of the microsomal fraction was also noted, particularly after the administration of hemicholinium and choline in a weight ratio of 500.

Discontinuous density gradient centrifugation of the fraction containing the nerve endings, the mitochondria and the lysosomes showed that phosphatidylcholine formation was most affected by hemicholinium in the fraction containing the "heavy" nerve endings, followed by the fraction containing the mitochondria + lysosomes.

It is suggested that hemicholinium acts by accelerating the intracellular rate of movement of choline from the water-soluble into the lipid pool of the caudate nucleus where, by virtue of its presence[19], it subsequently also stimulates the incorporation of choline into membrane phosphatidylcholine.

INTRODUCTION

In spite of the essential nature of choline as an obligatory precursor of acetylcholine, a neurotransmitter substance in brain[1,2], and of phosphatidylcholine, a ubiquitous component of cerebral membranes[3,4] the metabolism of this substance in brain

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tissue has not been studied extensively. Knowledge of the details governing its metabolic disposition in the various neuroanatomical regions of the brain is most desirable, especially since it may turn out that its metabolic fate is geared to the functional competence of a given brain region. The synthesis of choline by successive methylations of ethanolamine, a biosynthetic pathway in extracerebral tissues[5] appears not to be quantitatively significant in brain[6]. Choline must be supplied preformed from the liver[7]. The question of its uptake by brain tissue is therefore particularly relevant. A previous report dealing with the intracellular disposition of choline in the canine caudate nucleus addressed itself to this issue[8]. With regard to its further metabolism, it is known that phosphorylcholine[9,10] and phosphatidylcholine[7,10] are its major metabolites, while acetylcholine is a minor one [11–13].

Hemicholinium-3 (ref. 14) the bishemiacetal form of α,α-dimethylethanolamino-4,4′-bisacetophenone, is a pharmacologic agent frequently used in studies of the uptake of choline and of its further conversion to acetylcholine in membrane-enclosed systems. It has been known for some time[11,15,16] that this drug may decrease the uptake of choline by such systems, and hence influence the formation of acetylcholine necessary for maintenance of normal tissue levels[10,17,18].

Recently, we have examined the dynamics of the cellular uptake, the intracellular movement and the intracellular localization of [^{14}C]hemicholinium in the caudate nucleus of the dog[19], a region in which acetylcholine levels are greatly reduced following its intraventricular administration. We noted that, 4 h after its administration, [^{14}C]hemicholinium was tightly bound to the intracellular membranes of the caudate. Also in a previous paper[8], we reported that the proportion of the injected choline appearing as radioactive phosphorylcholine and cytidine diphosphocholine was appreciably increased if hemicholinium was administered with choline. The present paper describes the subsequent steps of cytidine diphosphocholine utilization in the caudate nucleus of the dog; namely its conversion to phosphatidylcholine. The particularly novel aspect of the present study, documented in detail below, is the observed acceleration of the conversion of choline to phosphatidylcholine by hemicholinium.

MATERIALS

Animals. Mongrel, beagle-like dogs were used[8].

Chemicals. The following were used: silica G-25 (Merck and Co.) silicic acid (UNISIL, Clarkson Chem. Co., Williamsport, Pa.), Biosolv-3 (Beckman Co., Fullerton, Calif.) and [1,2-^{14}C]phosphatidylcholine chloride (Tracerlab, Lot No. 867–165–8), specific activity: 75.4 mC/mmole. [*Me*-^{14}C]choline chloride 7.6 mC/mmole (New England Nuclear) and 54 mC/mmole (Amersham-Searle) were used.

METHODS

Animals. The surgical procedure as well as the intraventricular injection and sacrifice techniques were described previously[8,19].

Centrifugation. Caudate homogenates were subjected to differential and density gradient centrifugation as described in a previous communication[19]. The particulate fraction sedimenting at 10000 × *g* contained myelin, nerve endings, mitochondria and lysosomes. It is termed the heavy particulate fraction. The microsomal fraction

sedimented at 100000 × *g* after a 1-h centrifugation. The procedure was modeled after that of DE ROBERTIS *et al.*[20].

Separation of the water-soluble metabolites from tissue lipids. The water-soluble components, choline, acetylcholine, phosphorylcholine and cytidine diphosphocholine were extracted by the procedure of CROSSLAND AND MERRICK[21]. Most of the lipids were first extracted into ether[21] and two chloroform–methanol (2:1, v/v) washes of the aqueous phase were combined with the ether extract. The total lipids thus obtained were subjected to silicic acid chromatography (Unisil) for separation into neutral and phospholipids. The latter fraction was counted directly or was separated into several components by thin-layer chromatography.

Thin-layer chromatography of phospholipids. This was done on silica G-25 using a mixture of chloroform–methanol–water (65:25:4, by vol.) as solvent[22]. While choline plasmalogens and phosphatidylcholine are not separated, a good separation of sphingomyelin, lysophosphatidyl- and phosphatidylcholine is achieved. Authentic [^{14}C]-phosphatidylcholine was used as marker. The dry plates were exposed to iodine vapors for visualization of the spots.

Chemical hydrolysis of phospholipids. The hydrolytic method of DAWSON *et al.*[23] was used for the identification of and the assignment of the radioactivity among choline plasmalogens, sphingomyelin and phosphatidylcholine.

Analytical. The method of BARTLETT[24] was used for the determination of total phosphorus. When phosphorus was determined on scrapings of silica G-25 removed from thin-layer chromatography plates, a standard sample was applied to a blank region of the plate after chromatographic development and was then processed as the experimental phospholipid spots. The values obtained for that standard were identical to those given by a sample analyzed directly.

Radioactivity. The radioactivity was determined by liquid scintillation techniques. Samples were prepared for counting in scintillation vials by evaporation of all solvents at which time 15 ml of a counting fluid of the following composition were added: 1 l of dioxane, 8 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis-(5-phenyloxazolyl-2) benzene and 110 g of naphthalene. Appropriate quenching corrections were applied and the counts/min were converted to disint./min using a suitable program on the PDP-8 computer. The counting efficiency of the spectrometers used ranged between 80 and 90%. Radioactivity derived from scrapings of thin-layer chromatography plates was determined as described previously[8], except that bleaching of the iodine color was done in an oven (100° for 10 min).

RESULTS

The effect of the hemicholinium/choline ratio on the conversion of [Me-^{14}C]choline to phospholipids

The partition of radioactivity 4 h after the administration of choline between the water-soluble choline metabolites and the choline phospholipids in the heavy particulate and the microsomal fractions is shown in the first set of bars of Fig. 1 (hemicholinium/choline = 0). In the heavy particulate fraction about 80% of the radioactivity was in the phospholipids as against only 40% in the microsomal fraction. The corresponding 1-h values (not shown graphically) were 62% for the heavy particulate and 55% for the microsomal fraction. When hemicholinium and [*Me*-^{14}C]choline were ad-

ministered together in varying ratios and the phospholipid radioactivity again examined 4 h later, the percentage in the heavy particulate fraction increased to about 90% (hemicholinium/choline = 500), with intermediate values for intermediate hemicholinium/choline ratios (10 and 100). The increase in the microsomal fraction was

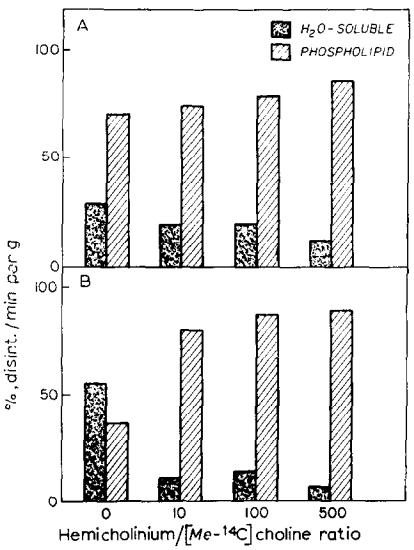

Fig. 1. The effect of hemicholinium on the distribution of [*Me*-^{14}C]choline radioactivity between the water-soluble and the phospholipid extracts of the heavy particulate (A) and the microsomal (B) fractions of the dog caudate nucleus. The dose of [*Me*-^{14}C]choline was 10 μg (0.73 mC). Unlabeled hemicholinium was administered jointly at doses of 0 (three animals), 100 (two animals), 1000 (two animals) and 5000 μg (two animals). After isolation, they were lyophilized and the residues extracted[21]. The lipid extract was further separated into its neutral and phosphorylated components (see METHODS) before determination of the radioactivity. The height of the bars indicates mean percent values.

from 37 to 90% for a hemicholinium/choline ratio of 500. At 1 h post-administration no significant changes in phospholipid radioactivity as a function of the hemicholinium/choline ratio could be detected. Also, at no time were the neutral lipids labeled.

The effect of hemicholinium on the time-course of [Me-^{14}C]choline incorporation into phospholipids

Although an obvious effect of hemicholinium on phospholipid labeling in the heavy particulate and the microsomal fractions could not be detected 1 h after the administration of [*Me*-^{14}C]choline, it was possible to see increases in the specific activity (disint./min per μg phosphorus) of the labeled phospholipids as early as 15 min after the simultaneous administration of hemicholinium and choline. The results (Figs. 2 and 3) show that the specific radioactivity of phospholipids in both fractions was markedly elevated over that of the controls receiving no hemicholinium and that, irrespective of the hemicholinium/choline ratio (Fig. 2) or of the time of administration (Fig. 3), it was higher in the microsomal than in the heavy particulate fraction (by about 300% in the former fraction at 4 h and with a hemicholinium/choline ratio of 500 (Fig. 3)). Yet, as shown in Fig. 4, when the specific radioactivity of the phospho-

lipids in the hemicholinium-treated fractions was divided by that of the controls, the response to the drug of the two fractions revealed time-dependent individual differences. Thus, while the magnitude of the hemicholinium effect on the phospholipids of the heavy particulate fraction reached a maximum at 1 h and then abated, the effect of hemicholinium on the specific activity of the phospholipids of the microsomal fraction maintained a steady increase throughout the entire experimental period.

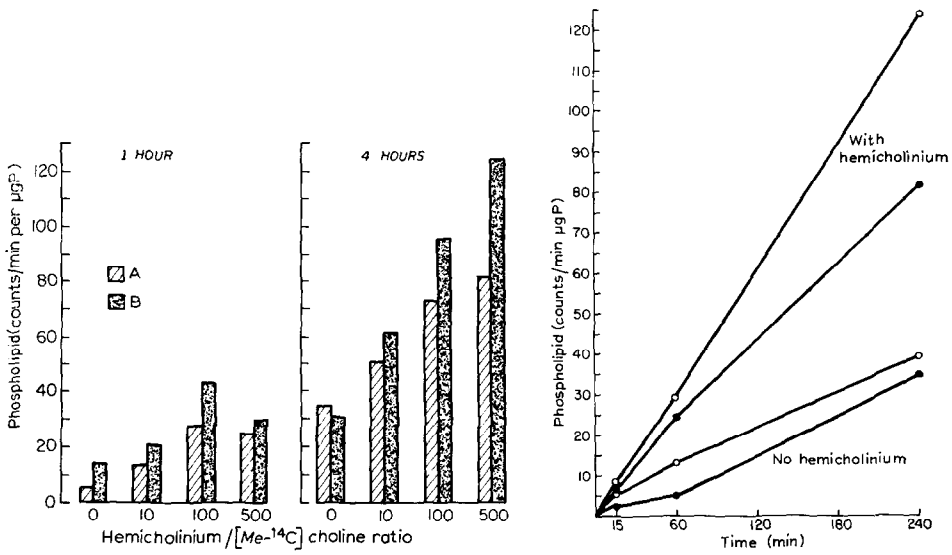

Fig. 2. The effect of hemicholinium on the specific radioactivity of the phospholipid extract of the heavy particulate and the microsomal fractions at 1 and 4 h after the administration of [*Me*-^{14}C]-choline. For the isolation of the heavy particulate fraction (A) and the microsomal fraction (B), see METHODS. The phospholipid extract was obtained and its radioactivity and phosphorus content determined as outlined in METHODS.

Fig. 3. The time-course of the hemicholinium effect on the specific radioactivity of the phospholipids of the heavy particulate (●) and the microsomal (○) fraction. The hemicholinium/[*Me*-^{14}C]-choline ratio was 500 (see legend Fig. 1). The high-specific-activity [*Me*-^{14}C]choline (54 mC/mmole) was used for the 15-min time point while, for the 1- and 4-h time points, the low-specific-activity [*Me*-^{14}C]choline (7.6 mC/mmole) was injected.

The isolation of phosphatidylcholine

Thin-layer chromatography of the phospholipid extracts of both particulate fractions (see METHODS) followed by the determination of the radioactivity in the resolved phosphatidylcholine (+ choline plasmalogen), lysophosphatidyl choline and sphingomyelin revealed that 93% of the radioactivity was in the phosphatidylcholine spot with the remaining 7% divided between sphingomyelin (4%) and lysophosphatidyl choline (3%). These results were verified independently by subjecting a phospholipid extract to alkaline hydrolysis[23] as a result of which it became possible to assign 93.9% of the radioactivity to the combination phosphatidyl- + lysophosphatidyl choline, 2% to choline plasmalogens and 4% to sphingomyelin.

The effect of the hemicholinium/choline ratio on the specific activity of phosphatidylcholine

The specific activity of phosphatidylcholine isolated from thin-layer chromatographic plates was determined at 1 and 4 h after the administration of hemicholinium and

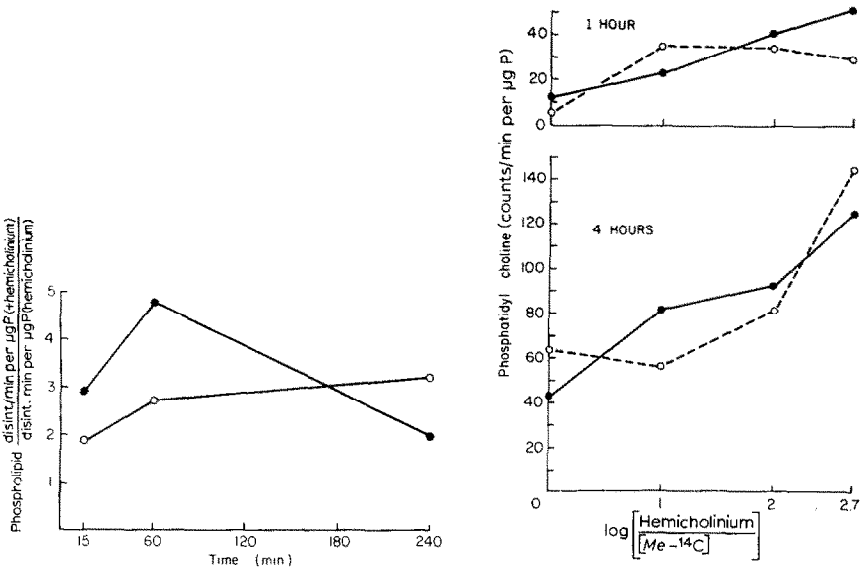

Fig. 4. The ratio of the specific activities of phospholipids extracted from the heavy particulate (●) and the microsomal (○) fractions 15, 60 and 240 min after hemicholinium + [*Me*-^{14}C]choline *vs.* [*Me*-^{14}C]choline alone. For experimental details and the definition of the fractions see legends to Figs. 1 and 3 and text (METHODS).

Fig. 5. Effect of hemicholinium on the specific radioactivity of phosphatidylcholine in the heavy particulate (○) and the microsomal (●) fractions. Phosphatidylcholine was isolated from the thin-layer chromatographic plates and its phosphorus content and radioactivity were determined (see METHODS). The former analysis was performed in duplicate on two samples which were chromatographed side by side. The upper panel refers to 1 h and the lower panel to 4 h after the administration of [*Me*-^{14}C]choline and hemicholinium. For the definition of the abscissa (note log values), see the legend to Fig. 1. See METHODS for the isolation of the heavy particulate and the microsomal fraction.

[*Me*-^{14}C]choline in ratios of 10, 100 and 500. As illustrated in Fig. 5, hemicholinium caused its increase both at 1 and 4 h and which was more pronounced in the microsomal fraction than in the heavy particulate fraction.

The effect of hemicholinium on membrane phosphatidylcholine

Since the results of the experiments depicted in Figs. 2 and 4 clearly indicated that phosphatidylcholine was the main phospholipid affected by hemicholinium and since, on the other hand, phosphatidylcholine is an essential constituent of myelin, and of the membranes of mitochondria and nerve endings[3,4] these intracellular components were isolated by density gradient centrifugation of the heavy particulate fraction[19,25] and the specific radioactivity of the extracted phospholipid (phosphatidylcholine) was determined. The effect of hemicholinium (Table I) was highest on the phosphatidylcholine of the nerve endings sedimenting through 1.2 M sucrose (Fraction D)[25] and second highest on mitochondrial (or lysosomal)[26] phosphatidylcholine (fraction E). The specific activity of phosphatidylcholine in the remaining three fractions (myelin and "light" nerve endings) also showed a moderate increase as a result of the administration of hemicholinium.

TABLE I

THE SPECIFIC RADIOACTIVITY OF THE PHOSPHOLIPIDS IN SUBFRACTIONS OF THE HEAVY PARTICULATE FRACTION OF THE DOG CAUDATE NUCLEUS

5 mg of hemicholinium and 10 μg of [*Me*-^{14}C]choline (specific activity: 54 mC/mmole) were administered jointly or only [*Me*-^{14}C]choline was administered and the animals were killed 4 h later. The heavy particulate subfractions were isolated by the procedure of DE ROBERTIS *et al.*[25]. They contain: A, myelin; B, synaptic debris and myelin; C, nerve endings; D, nerve endings, mitochondria and lysosomes; and E, free mitochondria. The numbers in parentheses refer to the molarity of sucrose separating a given fraction from the one below. P, pellet. The phospholipids were extracted from the fractions isolated as bands after their sedimentation at $104000 \times g$ for 3 h and suspension of the resulting pellets in water. Fraction P, suspended in 0.25 M sucrose, was also subjected to resedimentation, as above.

Subfraction	*After [Me-^{14}C]choline (disint./min per μg phosphorus)*	*After [Me-^{14}C]choline + hemicholinium (disint./min per μg phosphorus)*	*Effect of hemicholinium (%)*
A (0.8)	160	206	+28
B (0.1)	222	257	+15
C (1.2)	281	341	+21
D (1.4)	451	759	+68
E (P)	705	1063	+50

DISCUSSION

While the intraventricular administration of hemicholinium is known to result in a marked reduction of cerebral acetylcholine levels[9,11,17], its use in experiments with cerebral tissue slices has demonstratated its ability to block the influx of choline[27,28] and hence, by virtue of a secondary effect, the synthesis of acetylcholine. Recently, the incorporation of uniformly labeled [^{14}C]glucose (into the acetyl moiety) and of [*Me*-^{14}C]choline (into the choline moiety) of acetylcholine has been shown to be markedly and equally inhibited by 0.1 mM hemicholinium[28]. The uptake of choline by isolated nerve endings is affected equally severely[12,13].

From the bulk of the metabolic studies it would seem, therefore, that the primary metabolic action of hemicholinium is to block the transport of choline across biological membranes. Yet, perhaps because of an overtly unitary approach to the study of its mode of action, other aspects of its biochemical action spectrum appear to have been neglected.

In a recent paper[8], we have reported an effect of hemicholinium on the distribution of [*Me*-^{14}C]choline radioactivity among the subcellular fractions of the dog caudate nucleus. Previously[19], we also reported the time constants of [^{14}C]hemicholinium entry into, and its subsequent movement through this tissue. The conclusions drawn from both of these studies[8,19] were that hemicholinium most probably acts to accelerate the movement of [*Me*-^{14}C]choline from the soluble into the structured portions of the caudate cells (nerve endings, mitochondria, myelin, *etc.*). Of particular significance in this regard was the finding that such an acceleration of the choline flux through the cell occurred in synchrony with the movement of hemicholinium, for the effect of the drug on the mobilization of choline was maximal at 4 h after the latter's administration which is also the time[19] of maximal accumulation of hemicholinium in the caudate subcellular membranes.

The effect of hemicholinium on the conversion of [*Me*-^{14}C]choline to cytidine diphosphocholine was shown to be highest at a drug/choline ratio of 500 (ref. 8). The subsequent fate of cytidine diphosphocholine, namely its conversion to phosphatidylcholine and the concomitant shift of the radioactivity from the water-soluble to the lipid phase was also highest under these conditions, as depicted by the bar graphs in Fig. 2. Calculation reveals that at 4 h post-administration, 35-45% more radioactivity was present as phosphatidylcholine in the heavy particulate and the microsomal fractions of the hemicholinium-treated animals that in corresponding controls; interestingly at 1 h post-administration, the net effect of hemicholinium on this conversion was a mere 6-8%. Recently, the effect of hemicholinium on phospholipid formation from choline has been investigated by COLLIER AND LANG[10], who perfused the superior cervical ganglion of the cat with [*Me*-^{3}H]choline and showed that, after 1 h of perfusion, the radioactivity which could be extracted from the ganglion in lipid form was about one fourth of that present in water-soluble form; hemicholinium added to the perfusion fluid at a concentration of 10 μg/ml failed to change this proportion. These results are compatible with our 1-h results, inasmuch as they also emphasize the necessity of longer exposures of membrane systems to hemicholinium if the latter's effects on lipid accumulation are to be demonstrated. When, however, the specific activity of the phospholipids was examined, immediate effects of the drug on the rate of [*Me*-^{14}C]choline incorporation into phosphatidylcholine became apparent. Thus, at 1 h, the specific activity of the control heavy particulate and microsomal fractions was 5 and 14 counts/min per μg phosphorus, while after hemicholinium + choline (ratio 500) these values were 25 and 45 counts/min per μg phosphorus, respectively, representing a 5- and a 3-fold increase over controls. At 4 h, the increases were 2.3-fold for the heavy particulate fraction and 4.1-fold for the microsomal fraction (Fig. 2). These results as well as those depicted in Fig. 3 and which include the 15-min time point, reveal that the formation of phospholipid in the two subcellular fractions was unequally affected by hemicholinium. In the microsomal fraction the effect of hemicholinium was sustained through the period 1–4 h, resulting in a net rise of the ratio of specific activities: hemicholinium/control (ordinate, Fig. 4) from 2.7 to 3.3 while in the heavy particulate fraction this ratio decreased from 3.8 to 2. Interestingly, during the first hour after hemicholinium administration, the effect of the drug on [*Me*-^{14}C]-choline incorporation into phosphatidylcholine was more intense in the heavy particulate than in the microsomal fraction. It should be noted, however, that these differences in the incorporation of choline into the phospholipids of these two fractions were probably a reflection of the high hemicholinium/choline ratio (500) used, for, as shown by inspection of Fig. 5 and by suitable calculation of the specific activity ratios, they were not seen with hemicholinium/choline ratios of 10 and 100.

To test whether the drug effect related specifically to choline, the incorporation of [1,2-^{14}C]ethanolamine into the phosphatidylethanolamine of the heavy particulate and microsomal fractions was investigated. A marked increase in specific radioactivity was noted in the microsomal fraction (from a value of 58 in the controls to 135 in the hemicholinium-treated caudates) but not in the heavy particulate fraction (from a value of 50 in the controls to 59 in the hemicholinium-treated caudates). Since only 4-h experiments with a hemicholinium/choline ratio of 500 have been carried out to date, conclusions regarding an effect of the drug on phospholipid formation in general, while attractive, may admittedly be somewhat premature.

The combined results of this and a previous paper[8] confirm the existence in the canine caudate nucleus, of the pathway of choline metabolism found to be dominant in rat brain[7]. This pathway involves the initial phosphorylation of choline followed by the formation of cytidine diphosphocholine and the subsequent transfer of the phosphorylcholine to a diglyceride. McCAMAN AND COOK[29] have recently investigated the intracellular localization in rat brain of the enzymes catalyzing the first and the last step of the above sequence, namely choline phosphokinase and phosphocholine-diglyceride transferase, and have concluded that the activity of the former is mostly in the soluble phase of the cell, while the latter shares a largely microsomal + "mitochondrial" localization. Further analysis[29] revealed that areas of the brain possessing a high neuronal density, such as the caudate nucleus, are particularly rich in both enzymes.

Our results on the presence of choline-derived radioactivity in membranes other than the microsomal contrast with the recent observations of NAGLEY AND HALLINAN[30] who noted that, in liver, choline is an exclusive marker of the microsomal phosphatidylcholine. The different experimental conditions and the different tissues used most probably account for our finding a large proportion of the phosphatidylcholine radioactivity in the phosphatidylcholine of the membranes of nerve endings, mitochondria as well as in myelin (Table I). Our results are in substantial agreement, however, with the findings of PLAGEMANN[31], who observed choline incorporation into the nuclear, mitochondrial, plasma and microsomal membrane phosphatidylcholine of rat hepatoma cells grown in culture, as well as with the findings *in vitro* of KAISER[32], who has recently described a phosphatidylcholine-synthesizing system in liver mitochondria.

The relation of a few drugs to cerebral phospholipid formation has recently been reviewed by ANSELL AND HAWTHORNE[33] and ANSELL[34,35]. Subsequently, acetylcholine has been shown to inhibit the incorporation of ^{32}P into the phospholipids of nerve ending membranes of rat brain[36] and tetraphenylborate has been found to stimulate phospholipid labeling in goldfish brain[37]. With regard to effects of hemicholinium, in addition to the observations of COLLIER AND LANG[10] discussed above, the only other report concerns the "significant" decrease of phosphatidylcholine noted in extracts of Ehrlich ascites tumors isolated from mice receiving hemicholinium[38]. Since those studies did not include measurements of isotope incorporation rates, they cannot be compared to the present results.

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