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THE METABOLISM OF CHOLINE IN REGIONS OF RAT BRAIN AND THE EFFECT OF HEMICHOLINIUM-3

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Abstract—When [*Me*-^{14}C]choline was injected intracerebrally, the radioactivity rapidly spread throughout the brain but the distribution in the six regions examined was not uniform. HC-3 caused a higher retention of the labelled choline particularly in the side of the brain which was the site of the injection. In the cerebellum and mid-brain the synthesis of phosphorylcholine was inhibited by HC-3 but the synthesis of phosphatidylcholine was stimulated. This may be due to an increase in base exchange in the presence of HC-3. In the cortex, phosphorylcholine synthesis was inhibited by HC-3 but no stimulation of phosphatidylcholine synthesis was found. This regional difference may demonstrate that the pathways for the formation of phosphatidylcholine are not the same throughout the brain.

Hemicholinium-3 (HC-3; α,α′-dimethylethanolamino-4,4′-bisacetophenone) inhibits the formation of acetylcholine [1, 2]. It does not inhibit the activity of the cytoplasmic enzyme, choline acetyltransferase (EC 2.3.1.6) [3] but probably blocks the uptake of choline into membrane-enclosed systems [4]. There have been reports that HC-3 affects the metabolism of choline by pathways other than that involving its acetylation. Thus, Gomez *et al.* [5, 6] produced evidence to show that the uptake of choline into phosphorylcholine, CDP-choline and phosphatidylcholine in the caudate nucleus of dog brain was stimulated by HC-3 *in vivo*. However, the phosphorylation of choline *in vitro* by choline kinase (EC 2.7.1.32) is inhibited by HC-3 [7] which supports our earlier observations *in vivo* reported briefly elsewhere [8]. Nevertheless, Ansell and Spanner [9] also confirmed the apparent stimulation of choline incorporation into phosphatidylcholine and, in the present paper we report on the metabolism of choline in six regions of rat brain and the effect of HC-3 on the movement and metabolism of intracerebrally injected [*Me*-^{14}C]choline in greater detail.

MATERIALS AND METHODS

Materials. The HC-3 dibromide was a gift from Dr. Domino. The [*Me*-^{14}C]choline (61 μCi/μmole) was supplied by the Radiochemical Centre, Amersham, U.K. The chemicals were, where possible, of AR quality. The animals were female rats, 12–14 weeks old and 200–230 g in body weight.

Injection. The rats were injected intracerebrally by the method described by Ansell and Spanner [10] but at a depth of 2 mm not 4 mm. Rats were anaesthetised with an intraperitoneal injection of tribromoethanol (Avertin®) and the head held in a simplified stereotaxic instrument. An incision was made down the midline of the head and the skin reflected over the parietal bones of the skull. A tiny hole was then drilled through the left parietal bone 1·5 mm laterally from the mid-line and 2·0 mm anterior to the *sutura lambdoidea*. A micrometer syringe fitted with a No. 30 gauge needle was lowered until the needle tip touched the brain meninges. It was then lowered exactly 2 mm into the brain and the [*Me*-^{14}C]choline or [*Me*-^{14}C]choline with HC-3 (0·008 μmoles choline with 0·105 μmoles HC-3) in a vol of 10–15 μl injected at a rate of 10 μl/30 sec. The needle was left in position for a further 30 sec; it was then slowly withdrawn and the skin sutured. The animals regained consciousness within 20 min. The position of this injection was in the hippocampal region well above the lateral ventricle. Control animals were always paired with HC-3 treated animals and tissue samples processed in parallel.

Extraction of brain tissue. Rats were killed by decapitation after lightly anaesthetising them in an ether chamber. The brains were removed rapidly and the tissue homogenised in ice-cold distilled water. Brains were either used whole or divided longitudinally into left and right halves or dissected into six regions by the method of Glowinski and Iversen [11]. The aqueous homogenate was rapidly made up to a measured volume; one sample was taken for the assay of the water-soluble choline compounds and the other for lipid analysis.

Determination of water-soluble choline compounds. To the aqueous homogenate in a centrifuge tube was added 50% (w/v) trichloroacetic acid to give a final concentration of 10–12% (w/v). The sample was left in ice for 20 min, then centrifuged. The supernatant was filtered into a stoppered graduated test tube and the precipitate was washed with 1 ml water and, after centrifuging, the supernatant was added to the original supernatant. This washing procedure was repeated once more. The combined supernatants were made up to a measured volume. A sample was taken for scintillation counting and the remainder was washed four times with an equal volume of diethyl ether. After each wash, the ether was discarded.

After adjusting its pH to almost 8 with an ammonia wick, the aqueous extract was applied to a column of Dowex 50 (H^+) resin (0·8 × 3·0 cm) followed by

10 ml of water and the effluent was discarded. The fraction containing phosphorylcholine was eluted with 40 ml 0·1 N HCl and that containing choline, with 10 ml 3 N HCl. These two fractions were taken to dryness under reduced pressure on a rotary evaporator and left in a vacuum desiccator over potassium hydroxide pellets to remove the acid. Each choline-containing fraction was assayed for total radioactivity.

The phosphorylcholine fraction was dissolved in 75 μl of water and 50 μl were applied to Whatman No. 1 chromatography paper. The chromatogram was run for approximately 8 hr in a descending direction in ethanol: 18 N NH_4OH: water (60:30:10 by vol). After drying, the paper was exposed to Blue Band X-ray film and the phosphorylcholine-containing spot located. This was extracted with 2 ml water and 0·8 ml taken for counting and for the determination of phosphate.

Determination of phosphatidylcholine. To a second sample of the aqueous homogenate was added four volumes of $CHCl_3$: MeOH (2:1, v/v) and the mixture well shaken intermittently over a period of an hour at room temperature. After centrifuging, the upper phase was removed, the lower $CHCl_3$ phase filtered and the solvent evaporated under reduced pressure in a rotary evaporator. This was repeated twice after the addition of $CHCl_3$, then the residue suspended in 7·5 ml $CHCl_3$ and washed by the method of Folch *et al.* [12] The phosphatidylcholine was isolated and determined as described by Ansell and Spanner [13].

Scintillation counting. The ^{14}C-labelled samples were counted in a Philips liquid-scintillation counter and corrected to dis/min by the channels' ratio method.

Subcellular fractionation. The subcellular fractions of the cortex were prepared by the method of Gray and Whittaker [14], and those of the cerebellum by the method of Rabié and Legrand [15].

RESULTS

Distribution and recovery of injected [Me-^{14}C]*choline.* Hemicholinium does not pass the blood–brain barrier, a fact contradicted by Csillik *et al.* [16] but recently confirmed by Domino *et al.* [17] using [^{14}C]HC-3. The effect of HC-3 in brain on choline metabolism was therefore investigated by injecting it intracerebrally with [*Me*-^{14}C]choline. When HC-3 was injected into the lateral ventricle of the rat brain the animal died so the previous method [10] was modified in that the HC-3 was injected in the same position as in our earlier experiments [13] but at a depth of 2 mm instead of 4 mm. This modification did not affect the amount of labelled choline found in the brain nor did it affect the formation of labelled choline lipid. A graph of percentage recovery of labelled acid-soluble and lipid-bound components in the brain was coincident with the original curve obtained by Ansell and Spanner [13]. The regional distribution of [*Me*-^{14}C]choline was also unaffected by the depth of injection from 1·5 to 4·0 mm (Fig. 1).

Throughout our experiments it was found that, after the very rapid initial loss of labelled choline from brain following intracerebral injection, the percentage recovered remained remarkably constant at

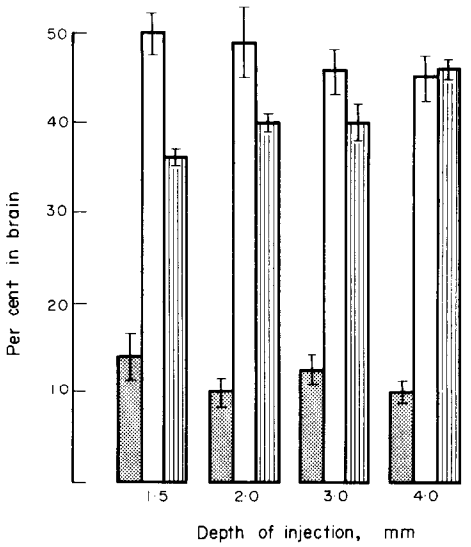

Fig. 1. A comparison of the radioactivity in three regions of the brain after intracerebral injection of [*Me*-^{14}C]choline at different depths. The values are expressed as a percentage of radioactivity in the whole brain after an exchange period of 0·5 hr. Cerebellum (▨), mid-brain (□), cortex (▥). The S.D. is indicated by the vertical bar in each case (minimum of 5 animals).

36–40% of that injected for over 12 hr [13]. When HC-3 was injected with the labelled choline, the retention was consistently and significantly higher from 0·5 to 5·0 hr. This increase was in both the acid-soluble and the lipid fractions. There was a retention of 53%, 13–17% higher than in the control brains. Of this 11·1% could be accounted for in the acid soluble fraction and 4·5% in the lipid. The increase was confined to the left side of the brain, i.e. the side of the injection (Fig. 2).

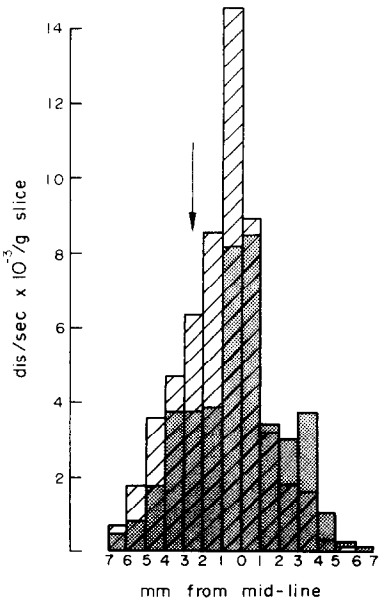

Fig. 2. The distribution of [*Me*-^{14}C]choline (dis/sec per g tissue) in sagittal sections 1 mm thick in the presence (▨) and absence (▩) of HC-3. The arrow indicates the distance of the point of injection from the mid line.

Table 1. The effect of HC-3 on the uptake of [*Me*-^{14}C] choline into three regions of the brain (dis/sec per g fresh wt) 0·5 hr after injection

	Control	HC-3	
Cerebellum	2250 ± 266 (6)	4400 ± 164 (6)	$P < 0.001$
Mid-brain	5131 ± 873 (6)	9449 ± 434 (6)	$P < 0.001$
Cortex	4796 ± 315 (6)	4894 ± 178 (6)	$P > 0.05$

Values are given ± S.D., with the number of determinations in parentheses. The difference between the controls and the HC-3 treated animals was evaluated by the paired *t*-test. The difference is regarded as significant when the P-value was smaller than 0·05.

Regional distribution of [Me-^{14}C]*choline in the presence and absence of HC-3.* Distribution of radioactive choline in different regions of the brain was measured 0·5 hr after an injection of the choline at a depth of 2 mm from the cortical surface. In some experiments the cortex, cerebellum and mid-brain were examined while in others the hypothalamus was separated from the mid-brain and the striatum was also studied. The level of radioactivity in the cortex was little affected by the HC-3 but the uptake of labelled choline into the cerebellum was doubled and there was a smaller but highly significant rise in the midbrain (Table 1). There was very little uptake into the striatum which contained only 1–2% of the radioactivity in the brain as a whole (Fig. 3).

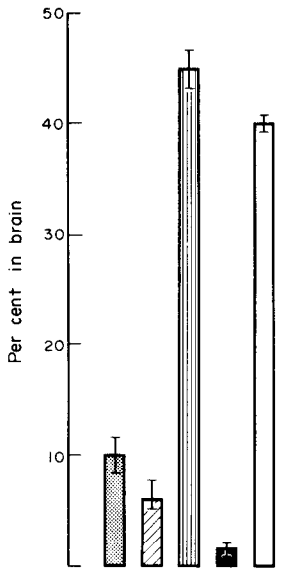

Fig. 3. The distribution of radioactivity in cerebellum (▨), hypothalamus (▨), cortex (▨), striatum (■), mid-brain (□) 0·5 hr after the intracerebral injection of [*Me*-^{14}C]choline at a depth of 2 mm from the cortical surface. Values are expressed as a percentage of the radioactivity in whole brain. The S.D. is indicated by a vertical bar in each case (minimum of five animals).

When the cerebellum was fractionated into subcellular components the uptake of labelled choline into the nerve endings, the microsomes and the cell sap was shown to be greatly increased by the presence of HC-3. This was not found for the subcellular fractions of the cortex (Fig. 4).

Metabolism of choline and the effect of HC-3

(a) *Phosphatidylcholine.* It has been shown recently that HC-3 caused an increased labelling of the lipid fraction of brain *in vivo* [9]. When the lipid fraction was studied in detail, it became clear that the specific radioactivity (dis/sec per μmole) of the phosphatidylcholine was increased at all the times studied for whole brain. However, this increase was not general throughout the brain. The phosphatidylcholine of the cortex was unaffected by HC-3, but there was an 80% increase of specific radioactivity in the cerebellum and a 30–40% increase in the midbrain (Table 2).

(b) *Phosphorylcholine.* Studies *in vitro* have shown that choline kinase activity was inhibited by HC-3 [7]. *In vivo*, the total radioactive free choline in the acid-soluble fraction of the brain was considerably increased in the presence of HC-3. However, in spite

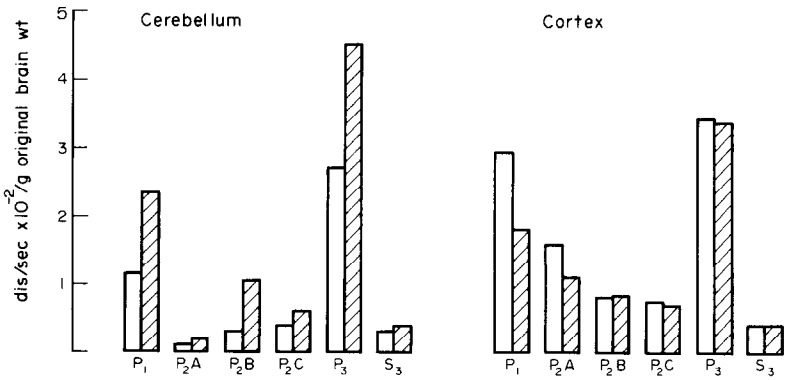

Fig. 4. The radioactivity in the phosphatidylcholine (dis/sec per g original tissue) of the subcellular fractions of cerebellum and cortex 0·5 hr after the intracerebral injection of [*Me*-^{14}C]choline (□) or [*Me*-^{14}C]choline + HC-3 (▨).

Table 2. The effect of HC-3 on the specific radioactivity (dis/sec per μmole) of phosphatidylcholine in three brain regions at different times after the intracerebral injection of [*Me*-^{14}C]choline

Time after injection (hr)		Cerebellum	Mid-brain	Cortex
0·5	control	37·4 ± 1·7 (5)	79·0 ± 4·2 (5)	56·2 ± 3·2 (5)
	HC-3	66·0 ± 2·8 (5)	114·0 ± 2·4 (5)	56·2 ± 2·1 (5)
2·0	control	79·5 ± 8·3 (4)	169·0 ± 18·0 (4)	93·0 ± 10·2 (4)
	HC-3	108·0 ± 12·4 (4)	238·0 ± 25·6 (4)	102·0 ± 12·1 (4)
3·5	control	56·0 (2)	163·0 (2)	172·0 (2)
	HC-3	114·0 (2)	281·0 (2)	135·0 (2)
5·0	control	109·0 (2)	261·0 (2)	214·0 (2)
	HC-3	206·0 (2)	330·0 (2)	206·0 (2)

Values are given ±S.D., where applicable and the number of determinations in parentheses.

of the apparent increased availability of free choline, the phosphorylation was inhibited throughout the brain regions. For example, at 3·5 hr the specific radioactivity in the whole brain showed a decrease from the control value of 1062 to a value of 363 dis/sec per μmole. Cerebellum, cortex and mid-brain were all affected (Table 3).

(c) *Subcellular fractions of cortex and cerebellum.* As can be seen in Fig. 4, the levels of incorporation of labelled choline into phosphorylcholine in the subcellular fractions of cortex were unaffected by HC-3 apart from an apparent reduction in the P_1 fraction. In contrast, in the subcellular fractions of the cerebellum, there was a marked increase of incorporation of ^{14}C-labelled choline in the presence of HC-3. This increase was particularly marked in the synaptosomal fraction (P_2B) and in the microsomes (P_3). The increased incorporation is an increase in the amount of radioactivity incorporated since the levels of phosphorylcholine and phosphatidylcholine remained the same after HC-3 injection. In the soluble fraction (S_3) the specific radioactivity of the phosphorylcholine in the HC-3 treated animals was only 70% of that of the control animals. This reflected the values for the cerebellum as a whole. In both the cortex and the cerebellum, over 96% of the phosphorylcholine of the whole region was recovered in the soluble fraction.

DISCUSSION

When [*Me*-^{14}C]choline was injected into the left cerebral hemisphere of rat brain it was distributed rapidly throughout both sides of the brain. The distribution was not uniform and, as can be seen in Fig. 3, very little choline was taken up by the striatum, which agrees with the recent observation of Loh and Hitzemann [18] who used an intracisternal injection. When expressed per g wt of each region, the cerebellum is seen to have as high a concentration of radioactivity as the cortex after HC-3 (Table 1). HC-3 increases the concentration of radioactivity in the brain [9] particularly on the side of injection, presumably by inhibiting the efflux of choline but this effect was not found throughout the regions (Fig. 3). The cerebellum and midbrain showed a much higher uptake of radioactive choline after HC-3 than did the controls. The cortex, which was the site of injection, appeared to be unaffected. There is a slight parallel here with the distribution of intraventricularly injected HC-3 into the brain of the rabbit which was measured by Slater and Stonier [19]. They showed that after 1 hr the mesencephalon, cerebellum and pons had the highest concentration of HC-3 while the cerebral hemispheres had the lowest.

It was found that HC-3 had at least two effects upon choline metabolism in that it inhibited the phosphorylation of choline and it stimulated the turnover of choline in phosphatidylcholine. Superficially this would appear to be a contradiction since the chief pathway for the formation of phosphatidylcholine in brain is via the Kennedy pathway; choline → phosphorylcholine → CDP-choline → phosphatidylcholine. However, choline can be incorporated directly into phosphatidylcholine by another route, namely an exchange of the base moiety of a phospholipid with the free base [20, 21] and this could explain why Gomez *et al.* [6] using labelled choline found a stimulation of phosphatidylcholine formation in dog caudate

Table 3. The effect of HC-3 on the specific radioactivity (dis/sec per μmole) of phosphorylcholine in three brain regions at different times after the intracerebral injection of [*Me*-^{14}C]choline

Time (hr)		Cerebellum	Mid-brain	Cortex
0·50	control	4540 ± 520 (5)	8672 (3)	5180 ± 540 (5)
	HC-3	3157 ± 386 (5)	6685 (3)	2260 ± 289 (5)
0·75	control	2302 (3)	—	7370 (3)
	HC-3	1250 (3)	—	3640 (3)
3·50	control	1226 (2)	403 (2)	1557 (2)
	HC-3	201 (2)	293 (2)	596 (2)

±S.D. with the number of determinations in parentheses.

nucleus, as is described in this paper for rat brain while Rodriguez de Lores Arnaiz *et al.* [22] demonstrated no such stimulation using $^{32}PO_4^{3-}$ as a precursor. It was found that, while in cerebellum and mid-brain there was a stimulation of phosphatidylcholine synthesis by HC-3 there appeared to be no effect in the cortex (Table 2). However, the inhibition of choline kinase and the formation of phosphorylcholine seemed to occur throughout the brain (Table 3). It is possible, that in cortex the only pathway for the formation of phosphatidylcholine is via the Kennedy pathway while in mid-brain and cerebellum the exchange mechanism may play a significant part and be stimulated by HC-3. This apparent stimulation of phosphatidylcholine synthesis may therefore be a consequence of the inhibition of choline kinase. The pool of radioactive free choline was raised after HC-3 and, if the cytidine pathway is inhibited by the drug, more labelled choline would be available for the exchange pathway, giving rise to phosphatidylcholine of higher specific radioactivity than in the absence of the drug.

When the subcellular fractions of cortex and cerebellum were compared after the injection of [^{14}C]choline with HC-3 a marked difference was demonstrated. As had been found when examining the regions, HC-3 had very little effect upon the formation of labelled phosphatidylcholine in the subcellular fractions of the cortex. In the cerebellum, however, there was a considerable stimulation of phosphatidylcholine labelling in the microsomes (P_3) and the nerve endings (P_2B) (Fig. 4). This was also demonstrated in the caudate nucleus of dog by Gomez *et al.* [6]. Bjerve [23] demonstrated a marked base exchange of free choline with phosphatidylcholine in the microsomes of liver *in vitro* which was Ca^{2+} dependent, but the effect of HC-3 on the base exchange in brain microsomes *in vitro* has still to be investigated.

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