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# ACTION OF HEMICHOLINIUM-3 ON PHOSPHOLIPID METABOLISM IN KREBS II ASCITES CELLS

JACQUES LLOVERAS, MOHAMED HAMZA, HUGUES CHAP and LOUIS DOUSTE-BLAZY*
Inserm Unité 101, Biochimie des Lipides, Hôpital Purpan, 31059 Toulouse, France

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**Abstract**—Incorporation of [Me-$^{14}$C]choline or/and [2-$^{14}$C]ethanolamine into phospholipids of Krebs II ascites cells *in toto* have been tested in the presence of hemicholinium-3. With [Me-$^{14}$C]choline, labelling of cell pellet, intracellular choline, phosphocholine and total lipid extract is inhibited by hemicholinium-3 in a dose-dependent way between $6.25 \times 10^{-6}$ M and $10^{-3}$ M. These effects are caused by a diminution of the choline or/and ethanolamine transport across the cell membrane and by a choline–kinase inhibition. In Krebs cells, choline is taken up by a low affinity $Na^+$ sensitive uptake system ($K_T = 46 \times 10^{-6}$ M) which is competitively inhibited by hemicholinium-3 ($K_{Ti} = 161 \times 10^{-6}$ M). Krebs cells exert a counter-transport (i.e. an exchange of choline across the membrane) against a concentration gradient of 10 mM choline whereas 10 mM hemicholinium-3 has no effect. Choline–kinase is also inhibited ($I_{50} = 57 \times 10^{-6}$ M) in Krebs cells *in toto* and time-course data suggest that choline transport and phosphorylation might be tightly coupled. Specific radioactivities of phosphocholine and choline–glycerophospholipids decrease owing to the effect of the drug on the uptake and phosphorylation system. With $4 \times 10^{-5}$ M hemicholinium-3 and [Me-$^{14}$C]choline as a marker, labelled choline–glycerophospholipids are decreased by 22%. With [2-$^{14}$C]ethanolamine, labelled ethanolamine–phospholipids are decreased by 26% and choline–glycerophospholipids remain unlabelled. With the two markers, the additional effect produces a 35% decrease. It is concluded that hemicholinium-3 might be able to induce a depression of the intracellular choline and phosphocholine pool which could provoke a serious quantitative deficiency of major phospholipids in Krebs cells.

Numerous organic compounds have been assayed as choline antimetabolic agents either against its transport in synaptosomes [1, 2] and erythrocytes [3, 4] or as inhibitors of choline acetylation [5, 6] and phosphorylation [7, 8] in the central nervous system. In tumoral tissues, choline–phospholipids represent the major fraction of total phospholipids [9, 10]. Therefore, we have initiated the study of choline–phospholipids and ethanolamine–phospholipids synthesis inhibitors in Krebs II ascites cells as a model of experimental tumor. In a previous paper [11], we have established that hemicholinium-3 {[2,2′-(4-4′-biphenylene) - bis - (2 - hydroxy - 4 - 4 - dimethylmorpholinium)]bromide} is an inhibitor of choline–kinase (EC 2.7.1.32) from Krebs cells and rat liver. The phosphatidyl–choline synthesis is diminished in the first case but not in the other one. Whereas in that study results were obtained using cytosol and post-nuclear supernatant, the present investigation deals with the action of hemicholinium-3 on the choline transport which is strongly inhibited in all nervous tissues [12–14]. Our results indicate that inhibition of choline transport in Krebs II cells leads to a decreased labelling of phosphocholine and choline–glycerophospholipids which may be useful for hindering phospholipid biosynthesis and membrane biogenesis in tumor cells.

## MATERIALS AND METHODS

### *Chemical products and enzymes*

They were purchased as follows: hemicholinium-3, fatty acid free albumin bovine, phosphocholine and CDP-choline from Sigma (St Louis, Missouri, U.S.A.); choline–oxydase, peroxydase, alkaline phosphatase from Boehringer (Mannheim, F.R.G.); 4-aminoantipyrine and betaine from Aldrich (Strasbourg, France); antibiotics ($10^6$ units penicillin plus $10^6$ μg streptomycin per 5 ml) from Diamant (Puteaux, France); silica gel analytical plates from Merck (Darmstadt, F.R.G.); other products from Merck or Prolabo (Paris, France).

### *Labelled compounds*

[Me-$^{14}$C]Choline (58 mCi/mmole), [2-$^{14}$C]ethanolamine (60 mCi/mmole), [9–10,$^3H_2$]palmitic acid (500 mCi/mmole), [U-$^{14}$C]sucrose (555 mCi/mmole) were supplied from Amersham International (Amersham, U.K.). Radioactivity determination of samples solubilized in 10 ml of Instagel was performed using a Packard-Tricarb 4530 liquid scintillation counter (Downers Grove, Illinois).

### *Cellular suspensions and incubations*

Krebs II ascites cells were maintained in female N.M.R.I. Swiss mice as previously described [11]. All manipulations, except incubations, were performed at 1–4°. The harvested cells were added with the solution of penicillin plus streptomycin (1%, v/v), separated from the exsudate by centrifugation at 700 *g* (10 min) and washed twice in three volumes of PBS (Dulbecco's phosphate buffered saline) containing antibiotics. The cell pellet (0.25 ml corresponding to about $50 \times 10^6$ cells) was suspended (1.47%, v/v) in medium M (100 mM Tris, 27 mM $NaHCO_3$, 11 mM glucose, 1.8 mM KCl, 32.9 mM

* To whom correspondence should be addressed.

NaCl, 0.1 mM albumin, 1% v/v antibiotics, pH 7.35 ± 0.05, 300 ± 10 mOs/kg) added with the radioactive marker(s) and various concentrations of hemicholinium-3. It was incubated at 37° with gentle shaking. The incubation vessel (50 ml erlen flasks containing 17 ml of the cell suspension) was flushed with $CO_2$:$O_2$:$N_2$ (5.7:22.4:71.9, v/v/v) during the whole incubation (30 min for the experiments on choline phosphorylation and 3 hr for those on choline–glycerophospholipids and ethanolamine–phospholipids synthesis). The Krebs cell adherent water space was determined in preliminary experiments using [U-$^{14}$C]Sucrose (1 $\mu$Ci per assay). At the end of the incubation, cells were separated at 700 *g* (10 min) from medium M, which was used for lactate dehydrogenase determination according to Wrobleski and La Due [15] using a commercial kit (Boehringer). Then cell pellets were washed thrice in 60 volumes PBS and suspended in 20 volumes of the same to measure the fixed [$^{14}$C] and for further analysis.

To determine choline uptake and counter-transport, Krebs cells were incubated under the standard conditions related above except that the marker was [Me-$^{14}$C]choline diluted successively with unlabelled choline. Details of the experiments are reported in the figure legends. At the end of the incubation, cells were sedimented, washed as before and lysed in 11 ml of Instagel:water (10:1, v/v) to determine their radioactivity. To establish the phenomenon of counter-transport, Krebs cells were preloaded under the standard conditions for 1 hr at 37° in the presence of [Me-$^{14}$C]choline diluted to 400 $\mu$M with unlabelled choline. Cells were pelleted and washed and again suspended in fresh medium (1.47%, v/v) containing either 10 mM unlabelled choline or 10 mM hemicholinium-3 and 10 mM sucrose in controls. For each addition, osmolality was adjusted to its physiological value. To measure the counter-transport, incubations were performed at various times always under standard conditions. Cells were pelleted and washed before measuring the remaining uptaken [Me-$^{14}$C]choline. Released products were analysed by thin-layer chromatography as mentioned below.

*Analytical determinations*

Choline and phosphocholine were measured from a perchloric acid extract using an enzymatic assay [16] where phospholipase D was replaced by an alkaline phosphatase. Lipid phosphorus was determined according to Chen *et al.* [17]. The perchloric soluble components were separated on silica plates using methanol:0.6% NaCl:$NH_4OH$ (50:50:5, v/v/v) as solvent [18] and pure standards of choline, phosphocholine, betaine and CDP-choline. Total lipids were extracted and washed according to the method of Folch *et al.* [19] slightly modified after adding internal standards [$^3H_2$]phosphatidylcholines (3985 dpm/nmole) and [$^2H_2$]phosphatidylethanolamines (1257 dpm/nmole) biosynthesized in our laboratory [20] and randomly labelled by [9-10,$^3H_2$] palmitic acid (about 100,000 dpm each one per assay). After measuring the total lipid extract radioactivity, choline–glycerophospholipids and ethanolamine–phospholipids were separated on silica plates in chloroform:methanol:water:acetic acid (60:30:6:1, v/v/v/v). Statistical significances were computed according to the Student's independent *t*-test. The effects of hemicholinium-3 on the [Me-$^{14}$C] choline incorporation were examined in the range 6.25–1000 $\mu$M using 17 drug concentrations. Linear correlations for cell pellet, total lipid extract, choline and phosphocholine were evaluated by means of the Hill plots construction: log [$Y$/(100-$Y$)] as function of log [hemicholinium-3] where $Y$ corresponds to percentages of the controls.

## RESULTS

After a 3-hr incubation, the cell morphology remained normal under phase microscopy control, pH was above 7.3 and the lactate dehydrogenase extruded into the medium was less than 3% of the total cell enzymatic activity. Furthermore, the third washing of the cell pellet contained about 0.1% of the radioactivity added to the cell suspension. With [U-$^{14}$C]sucrose as indicator, the adherent water-space was 0.07% of the total [$^{14}$C]. It was not modified by hemicholinium-3 and could be neglected with respect to the cell pellet labelling by the radioactive bases.

*Effects of hemicholinium-3 on [Me-$^{14}$C]choline and [2-$^{14}$C]ethanolamine incorporation into phospholipids*

Results relating the cell pellet fixation of [Me-$^{14}$C]choline or/and [2-$^{14}$C]ethanolamine followed by their incorporation after a 3 hr incubation into the total lipid extract, choline–glycerophospholipids and ethanolamine–phospholipids are summarized in Table 1. Such a time was chosen to allow a significant labelling of phospholipids, a previous study having shown that [$^{14}$C]choline incorporation into phosphatidylcholine remained linear for at least 3 hr [21]. The fact that the percentage in choline and ethanolamine–glycerophospholipids slightly exceed the total lipid extract might be explained because the latter was solubilized in chloroform–methanol (2:1, v/v) whereas glycerophospholipids were scrapped from silica-plates before liquid scintillation counting. Under our conditions, hemicholinium-3 caused a 28% inhibition of the choline fixation. Because the labelled base entered the cells at a lesser extent, it follows that the metabolized radiodetectable choline was diminished (25% for choline–glycerophospholipids); but compared to the cell pellet labelling, these results suggested a non-intracellular inhibition of choline–glycerophospholipids synthesis by hemicholinium-3. As foreseen, ethanolamine–glycerophospholipids were not labelled. The intracellular penetration of ethanolamine was 26% inhibited by hemicholinium-3 (Table 1) and this effect will be discussed below. Accounting that ethanolamine–kinase (EC 2.7.1.82) is not inhibited *in vitro* by hemicholinium-3 [8], these experiments with [2-$^{14}$C]ethanolamine were carried out to test the ability of phosphatidylethanolamine–methyltransferases (EC 2.1.1.17) to compensate in Krebs cells *in toto* the choline–glycerophospholipids defect. Total lipid extract and ethanolamine–phospholipids were strongly labelled and their synthesis ratios with and without hemicholinium-3

Table 1. Action of hemicholinium-3 on choline or/and ethanolamine incorporation into lipids of Krebs II ascites cells *in toto*

| Labelled precursor | [$^{14}$C]Labelled sample | Radioactivity incorporated (%) Plus hemicholinium-3 (A) | Without hemicholinium-3 (B) | P | Ratio A/B |
|---|---|---|---|---|---|
| [Me-$^{14}$C]Choline | Cell pellet | 29.3 ± 0.98 | 40.8 ± 1.29 | < 0.001 | 0.72 |
| | Total lipid extract | 15.3 ± 1.55 | 19.5 ± 0.75 | < 0.001 | 0.78 |
| | Choline–glycerophospholipids | 16.5 ± 2.21 | 22.0 ± 3.46 | < 0.01 | 0.75 |
| | Ethanolamine–phospholipids | 0.08 ± 0.07 | 0.08 ± 0.06 | N.S. | — |
| [2-$^{14}$C]Ethanolamine | Cell pellet | 25.8 ± 1.11 | 35.1 ± 2.35 | < 0.001 | 0.74 |
| | Total lipid extract | 14.9 ± 0.49 | 19.1 ± 1.73 | < 0.01 | 0.78 |
| | Choline–glycerophospholipids | 0.39 ± 0.03 | 0.56 ± 0.07 | < 0.01 | 0.74 |
| | Ethanolamine–phospholipids | 15.9 ± 0.66 | 21.7 ± 1.72 | < 0.001 | 0.74 |
| [Me-$^{14}$C]Choline plus | Cell pellet | 28.6 ± 0.80 | 38.2 ± 2.67 | < 0.001 | 0.75 |
| | Total lipid extract | 12.5 ± 0.89 | 19.0 ± 1.66 | < 0.001 | 0.65 |
| [2-$^{14}$C]Ethanolamine | Choline–glycerophospholipids | 5.3 ± 0.34 | 7.9 ± 0.61 | < 0.001 | 0.68 |
| | Ethanolamine–phospholipids | 7.2 ± 1.09 | 10.0 ± 1.32 | < 0.01 | 0.72 |

For experimental techniques, see Materials and Methods. Each assay (in the absence or in the presence of 40 $\mu$M hemicholinium-3) contained 2.5 $\mu$Ci (taken as 100% value) of [Me-$^{14}$C]choline (2.5 $\mu$M) or/and [2-$^{14}$C]ethanolamine (2.4 $\mu$M) and $50 \times 10^6$ cells in a total volume of 17 ml. Incubations: 3 hr at 37°. Radioactivity values were corrected by the [$^3H_2$] internal standard and each value is the mean ± S.D. of six separate experiments performed in duplicate; P = statistical risk; N.S. = not significant.

related to the ethanolamine fixation have shown that the base was totally incorporated in ethanolamine–phospholipids. As to the question of a sequential methylation pathway, choline–glycerophospholipids had a poor labelling (less than 3% of the cell pellet radioactivity) despite the high deficiency in radioactive choline–glycerophospholipids caused by hemicholinium-3. This result demonstrated that phosphatidylethanolamine–methyltransferases were inefficient at synthesizing choline–glycerophospholipids *de novo* from ethanolamine–phospholipids. When markers were [Me-$^{14}$C]choline plus [2-$^{14}$C] ethanolamine (Table 1), hemicholinium-3 always inhibited the intracellular entry of the two bases (25%) and induced a defect in radioactive total lipid extract (35%), which was more important than in the case of a single base. Furthermore, the incorporation into phospholipids remained elevated compared to the cell pellet labelling. These results gave evidence that in all cases, these radioactive choline–glycerophospholipid and ethanolamine–phospholipid defects appeared at least to be caused by the inhibition of the two-base entrance.

*Effects of hemicholinium-3 choline uptake*

As shown in Fig. 1, [Me-$^{14}$C]choline incorporation proceeded by a non-specific uptake (diffusion) and a specific uptake. The latter was linear with time up to 30 min between 100 and 400 $\mu$M choline with a plateau after 60 min. Under the standard conditions reported above, hemicholinium-3 was inefficient against the non-specific uptake and in the experiments related below, only the specific uptake was considered. In this one, hemicholinium-3 produced a competitive inhibition as shown in Fig. 2 ($K_T = 46.4 \times 10^{-6}$ M, $V_{Tm} = 7.0$ nmoles/min per $50 \times 10^6$ cells; $K_{Ti} = 160.8 \times 10^{-6}$ M, $V_{Tim} = 6.3$ nmoles/min per $50 \times 10^6$ cells). We have checked the $Na^+$-dependence of the choline transport without hemicholinium-3. Although it was not possible to suppress totally $Na^+$ in the medium M because of the bicarbonate buffer, we replaced 32.9 mM NaCl by 65.8 mM sucrose, causing a 55% decrease of $Na^+$ concentration. This induced a competitive inhibition with an important increase of $K_T$ ($K_T = 53.3 \times 10^{-6}$ M, $V_{Tm}$ 5.2 nmoles/min per $50 \times 10^6$ cells; $K_{Ti} = 206.4 \times 10^{-6}$ M, $V_{Tim} = 5.4$ nmoles per min per $50 \times 10^6$ cells, $n = 2$, average values). Counter-transport of choline is reported in Fig. 3. When released products were separated on silica plates, one radioactive peak co-migrating with standard choline was identified, betaine being less than 1% of

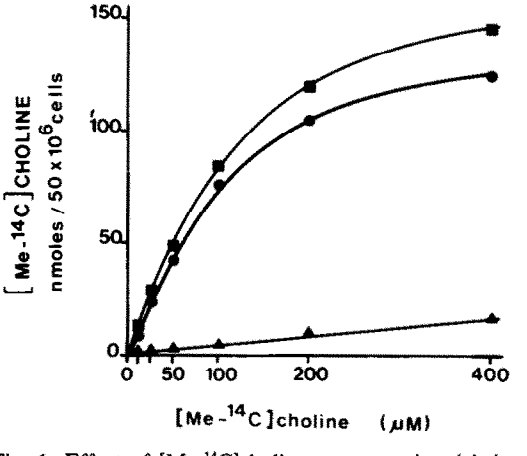


Fig. 1. Effect of [Me-$^{14}$C]choline concentration (abcissa) on choline uptake (ordinates) into Krebs ascites cells *in toto*. Equivalent to $50 \times 10^6$ cells were incubated under standard conditions for 30 min at 37°. Non-specific uptake (▲) was measured adding 50 mM unlabelled choline. Specific uptake (●) was the difference between total uptake (■) and non-specific uptake ($n = 2$, average values).

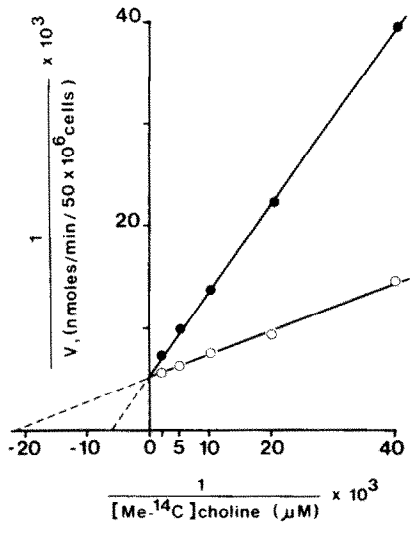


Fig. 2. [Me-$^{14}$C]Choline uptake into Krebs ascites cells *in toto* with (●) 200 μM hemicholinium-3 and without (○). Equivalent to 50 × $10^6$ cells were incubated under standard conditions according Materials and Methods section for 30 min at 37° ($n$ = 2, average values).

the total radioactivity recovered. After the preloading by [Me-$^{14}$C]choline and incubation with hemicholinium-3 or sucrose, cell pellet radioactivity presented a 3–5% decrease up to 1 hr without any significant difference, whatever the presence of the drug. This result indicates that hemicholinium-3 is not acting on the exit of radioactive choline in standard conditions. On the contrary, the addition of a large excess of unlabelled choline induced a 21% decrease of the radioactivity uptaken during the preloading, showing that Krebs cells were able to exert a counter-transport against a concentration gradient.

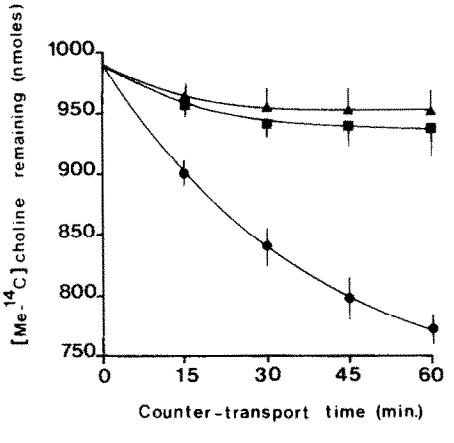


Fig. 3. Time course of [Me-$^{14}$C]choline counter-transport from Krebs ascites cells *in toto*. Equivalent to 50 × $10^6$ cells were preloaded with 400 μM labelled choline for 1 hr at 37° under standard conditions according to Materials and Methods section. After sedimentation and washings, cells were incubated again in fresh medium containing either 10 mM hemicholinium-3 (▲) or unlabelled 10 mM choline (●). Controls received 10 mM sucrose (■); $n$ = 3.

*Effects of hemicholinium-3 on choline phosphorylation*

The inhibition by hemicholinium-3 of the choline carrier mediated transport and its impact on the base incorporation into phosphocholine and phospholipids were closely related to the inhibitor concentration. [Me-$^{14}$C]choline uptake by cell pellet was reduced in proportion of increasing hemicholinium-3 (Fig. 4A) with $I_{50} = 160.3 \times 10^{-6}$ M. Under the same conditions, the total lipid extract labelling was affected in a similar manner [$I_{50} = 111.4 \times 10^{-6}$ M, see Fig. 4B). Concerning the phosphocholine synthesis evaluated by separation of the perchloro-soluble products on silica-plates, betaine and CDP-choline were less than 1.5% of the radioactivity loaded. Cholinephosphorylation was tightly related to hemicholinium-3 ($I_{50} = 57.5 \times 10^{-6}$ M, see Fig. 4C). [Me-$^{14}$C]Choline intracellular level was also proportionally affected by hemicholinium-3 ($I_{50} = 15.4 \times 10^{-6}$ M, see Fig. 4D). As previously established [11], hemicholinium-3 produces a mixed inhibition of choline–kinase in Krebs cell cytosol. In the same cell *in toto* the kinetics of [Me-$^{14}$C]choline and phospho[Me-$^{14}$C]choline labelling was of linear type for 30 min in the presence or in the absence of hemicholinium-3 (Fig. 5). The phosphocholine synthesis was sharply inhibited by hemicholinium-3. It is noteworthy that the proportion of [Me-$^{14}$C]choline was greater without incubation when hemicholinium-3 was present while phosphorylation was already strongly diminished. The slope ratio with and without hemicholinium-3 was 0.43 for [Me-$^{14}$C]choline and 0.24 for phospho[Me-$^{14}$C]choline, showing that the

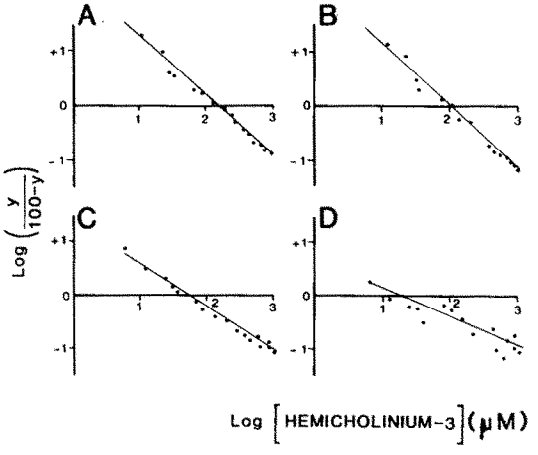


Fig. 4. Action of hemicholinium-3 concentration on choline uptake and metabolism by Krebs ascites cells *in toto*. Experimental data are expressed according to Hill plots where $Y$ is the labelling by [Me-$^{14}$C] choline corresponding to percentages of controls. For experimental techniques, see Materials and Methods section; $n$ = drug concentrations; P = statistical risk. A: Cell pellet, $r = -0.998$ ($n$ = 16), $P < 0.001$, $y = -1.115x + 2.459$, $I_{50} = 160.3$ μM. B: Total lipid extract, $r$, = −0.996 ($n$ = 15), $P < 0.001$, $y = -1.175x + 2.406$, $I_{50} = 111.4$ μM. C: Phosphocholine, $r$ = 0.998 ($n$ = 17), $P < 0.001$, $y$, = $-0.807x + 1.420$, $I_{50}$ = 57.5 μM. D: Choline, $r = -0.899$ ($n$ = 16), $P < 0.001$, $y$ = $0.584x + 1.188$, $I_{50}$ = 15.4 μM.

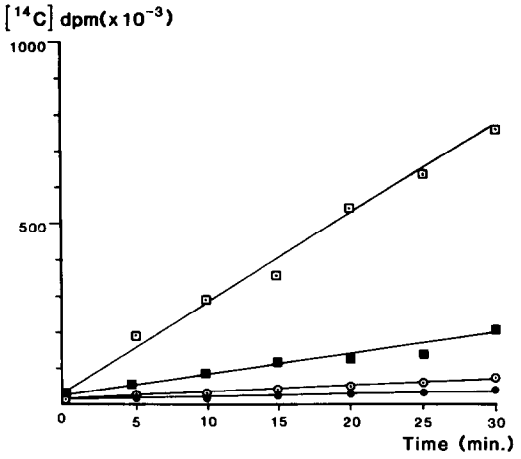


Fig. 5. Action of 100 μM hemicholinium-3 on the time course of choline phosphorylation in Krebs ascites cells *in toto*. Equivalent to $50 \times 10^6$ cells were incubated under standard conditions according to Materials and Methods section [to compute linear correlations, time (min) was × 5000); P = statistical risk. (●) [Me-$^{14}$C]Choline in assays: $r = 0.812$, $P < 0.05$, $y = 0.188x + 8150$. (■) Phospho[Me-$^{14}$C]choline in assays: $r = 0.966$, $P < 0.001$, $y = 1.073x + 19702$. (⊙) [Me-$^{14}$C]Choline in controls: $r = 0.989$, $P < 0.001$, $y = 0.435x + 3698$. (⊡) Phospho[Me-$^{14}$C]choline in controls: $r = 0.994$, $P < 0.001$, $y = 4.620x + 41089$.

hemicholinium-3 effect was more drastic on the [Me-$^{14}$C]choline phosphorylation than on its intracellular concentration. Furthermore the phospho[Me-$^{14}$C]choline/[Me-$^{14}$C]choline slope ratio was 5.71 in the presence of hemicholinium-3 and 10.62 in its absence, that anticipated a 0.54 minimum value for the ratio of assay with and without 100 μM hemicholinium-3 all along choline–glycerophospholipids synthesis from these precursors. This expectation was in agreement with Fig. 4B related above where the same drug concentration gave with hemicholinium-3 a computed 53.4% value of controls without hemicholinium-3 for the total lipid extract labelling. Specific radioactivities of products after a 30-min incubation are reported in Table 2. For [Me-$^{14}$C]choline it was not modified between assays with and without 100 μM hemicholinium-3. On the contrary, under the same conditions, the specific radioactivity of phospho[Me-$^{14}$C]choline was about threefold lower in assays with hemicholinium-3 compared to controls without; however, the same difference was also observed for phosphatidylcholine. Using the ratio of specific radioactivities of phosphatidylcholine to phosphocholine, it could be estimated that about 2.5% of the phosphatidyl molecules were renewed within 30 min in both cases without any effect of hemicholinium-3. One can then conclude that CTP:phosphocholine cytidylyl–transferase (EC 2.7.7.15), the limiting enzyme of phosphatidylcholine biosynthesis also in Krebs cells [21] was not inhibited by hemicholinium-3.

## DISCUSSION

In their broadest sense, the results reported above establish that hemicholinium-3 induces in Krebs cells *in toto* an apparent inhibition of the choline–glycerophospholipid and ethanolamine–phospholipid synthesis. The mechanism involved takes effect at once on choline or/and ethanolamine transport and choline–kinase activity.

Choline uptake was mainly studied in nervous tissues and erythrocytes. Generally it is distinguished between a specific uptake (carrier mediated system) and a non-specific uptake (diffusion). According to earlier kinetics [22], the findings reported here provide evidence for a specific choline transport system in Krebs cells. In nervous tissue, several studies have shown that there are two carrier-mediated systems [23, 24], the first one with a high affinity ($K_T \leq 4 \times 10^{-6}$ M) is $Na^+$ dependent [25, 26] and sensitive to low concentrations of hemicholinium-3 ($10^{-9}$ M) or related drugs [14, 27]. The other one presents a low affinity ($K_T > 30 \times 10^{-6}$ M) and is sensitive only at hemicholinium-3 concentrations greater than $10^{-6}$ M. The meaning of the two transport modes might be explained by the different choline pathways [27, 28]. Synaptosomal acetylcholine synthesis depends on high affinity choline uptake whereas it is likely that choline is equally phosphorylated after being taken up by low or high affinity transport. Besides, it has been established that $K_T$ may be modified by enzymes like neur-

Table 2. Action of hemicholinium-3 on specific radioactivities of choline, phosphocholine and choline–glycerophospholipids into Krebs Ascites cells *in toto*

| | Specific radioactivities (dpm/nmole) | | |
|---|---|---|---|
| | Controls without hemicholinium-3 | 100 μM hemicholinium-3 | P |
| [Me-$^{14}$C]Choline | 202 ± 37 | 152 ± 31 | N.S. |
| Phospho[Me-$^{14}$C]Choline | 2559 ± 176 | 887 ± 46 | < 0.001 |
| [Me-$^{14}$C]Choline–glycerophospholipids | 67 ± 12 (0.026)* | 21 ± 5 (0.024)* | < 0.01 |

For experimental techniques, see Materials and Methods section. Incubation: 30 min at 37°. Results are expressed as mean ± S.D. of four separate experiments; P = statistical risk; N.S. = not significant.

* [Me-$^{14}$C]choline–glycerophospholipids/phospho[Me-$^{14}$C]choline specific radioactivity ratio.

aminidase (EC 3.2.1.18) [25] and phospholipase $A_2$ (EC 3.1.1.4) [29] or by the extracellular choline concentration as reported for erythrocytes [30] or primary nerve cell cultures [31]. In our experimental conditions, Krebs cells exhibit a choline uptake by low affinity carrier-mediated transport which is $Na^+$-sensitive. Our results relative to the choline release from preloaded Krebs cells are partly in agreement with those reported concerning a synaptosomal fraction [32]. Moreover an excess of hemicholinium-3 produces a choline release in synaptosomes but not in Krebs cells. This discrepancy may be due to the fact that hemicholinium-3 enters the synaptic vesicles whereas it probably remains on the outside of the ascites cells. In other respects, ethanolamine is competitive for the choline carrier of rat lens [33] and this effect is in accordance with the inhibition of ethanolamine uptake by hemicholinium-3 in Krebs cells in spite of the fact that choline and ethanolamine appeared to be phosphorylated by separate kinases in lens [33] and brain [8]. As aforesaid, choline counter-transport in Krebs cells releases about 21% of the activity uptaken during the preloading. We have previously established [11] that choline was more than 90% phosphorylated after 30 min. It is under consideration that the released choline might partly originate from phosphocholine which can be accumulated [33] or from choline phospholipids as demonstrated in erythrocytes [34].

Concerning choline phosphorylation, we have reported that hemicholinium-3 is a potent inhibitor of the cytosolic choline–kinase [11]. In the experiments with Krebs cells *in toto* related here, choline phosphorylation is a quick-acting mechanism since it was already observed in experiments without incubations. It has been shown in synaptic vesicles that choline phosphorylated without incubation is uptaken by a non-specific system active at low temperature but insensitive to hemicholinium-3 [35]. The statistical analysis of Fig. 5 shows that some labelling of both choline and phosphocholine occurred during the time of cell handling, indicating that choline entrance and phosphorylation are a very rapid process. Even at this short time, choline phosphorylation is inhibited by hemicholinium-3. One interpretation of these results might be that the specific proteins catalyzing transport and phosphorylation would be coupled in the plasma membrane to constitute a group translocation [36], the choline–kinase being partly solubilized during the subcellular fractionation procedures. This is to be compared with the description in rat striatum of a membrane-bound choline–kinase not exclusively localized in cytosol and displaying no difference in kinetic data between the solubilized and the particulate form [37, 38]. It is very likely that hemicholinium-3 does not enter the cell and inhibits the choline transport and the particulate choline kinase with consequently an only quantitative deficiency of choline–glycerophospholipids. It was reported that, at low concentrations, choline transport and incorporation into phospholipids had the same apparent $K_m$ [39]. The ratios of assays with and without hemicholinium-3 measured for total lipid extracts and choline–glycerophospholipids are slightly higher than for cell pellet one and could indicate a stimulation of the choline-glycerophospholipid biosynthesis in order to compensate the uptake and phosphorylation defects. It is worthwhile noting that enzymes catalyzing the choline–glycerophospholipids synthesis from phosphocholine are not inhibited by hemicholinium-3 as it is demonstrated by the dose-dependent effect on the total lipid extract, compared to the choline–kinase activity slope ratio of assays with or without hemicholinium-3 and the identical [Me-$^{14}$C]choline–glycerophospholipid/phospho[Me-$^{14}$C]choline specific radioactivity ratio. However since choline–glycerophospholipids represent 46% of Krebs cells total phospholipids [40], no mass diminution can be detected after the 30-min incubation used for the specific radioactivity determination of their precursors. The results with [2-$^{14}$C]ethanolamine indicate a sharp uptake inhibition by hemicholinium-3 showing that the same carrier protein is involved in the choline and ethanolamine transport [33, 41] although choline–kinase and ethanolamine–kinase (EC 2.7.1.82) are reported to be separate enzymes [42, 43] as established by the co-purification performed from rat liver [44, 45]. Furthermore, ethanol-amine-kinase is not inhibited *in vitro* by hemicholinium-3 [8] and the whole ethanolamine uptake is incorporated in ethanolamine–phospholipids, because the sequential methylation pathway synthesizing *de novo* choline molecules is weakly active in Krebs cells as in other tumoral tissues [46]. Moreover the base exchange pathway, described for the phosphatidyl–choline synthesis [47] seems unlikely to compensate the choline–glycerophospholipid deficiency caused by hemicholinium-3. These results are in good agreement with those related in our previous paper about Krebs cells post-nuclear supernatant [11].

The general conclusion of this study is that Krebs cells take up choline through a carrier-mediated transport coupled to choline phosphorylation. This system is strongly inhibited by hemicholinium-3 and its efficiency is a rate-limiting step in the choline incorporation into choline–glycerophospholipids. We have determined that choline, phosphocholine and phosphatidylcholine contents are around 220, 900 and 1700 nmoles/$10^8$ cells, respectively. This indicates that the intracellular pool of choline and phosphocholine is insufficient to provide enough substrate for phosphatidylcholine synthesis during a cell division. One could thus predict that drugs, like hemicholinium-3, able to inhibit choline uptake could severely impair the growth rate of rapidly dividing tumoral tissues. In order to obtain more data on the choline transport and phosphorylation, we have undertaken an approach to the isolation of the choline-specific protein.

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