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The Metabolic Fate of [^{3}H-Methyl]Choline in Cultured Human Neuroblastoma Cell Lines, LA-N-1 and LA-N-2

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ABSTRACT

The conversion of choline in cultures of the human neuroblastoma cell lines, LA-N-1 and LA-N-2 cells, was investigated in order to identify potential precursors in acetylcholine (AcCho) synthesis. LA-N-1, a catecholaminergic and LA-N-2, a cholinergic, cell line were incubated with [^{3}H-methyl]choline (Cho) for varying periods of time up to 72 h. The radioactivity present in lipids and water-soluble metabolites increased linearly up to 24 h in both cell lines. Approximately 20% of the radioactivity associated with the water-soluble metabolites in both control (untreated) and retinoic acid-induced differentiated (RA-treated cells) LA-N-2 cells was present as Cho and AcCho. There was no detectable AcCho in the catecholaminergic cell line, LA-N-1.

The untreated and RA-treated LA-N-1 and LA-N-2 cells were labeled for 24 h with [^{3}H-methyl]Cho, followed by a chase in growth medium containing 100 μM unlabeled choline. The distribution of radioactivity in the LA-N-2 cells was 6–10% of AcCho, 84–89% as phosphocholine (*P*Cho), 1–3% as glycerophosphocholine (Gro*P*Cho), and 2–4% as Cho. The distribution of radioactivity in the LA-N-1 cells was similar except for the absence of AcCho. The distribution of ra-

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dioactivity in the culture medium of LA-N-1 cells was 70–80% as Cho, 20–30% as *P*Cho, and 1–3% as Gro*P*Cho. In contrast, the radioactivity was equally distributed between Cho (50%) and *P*Cho (50%), with only 1–3% as Gro*P*Cho in the medium of LA-N-2 cells.

Index Entries: Human neuroblastoma cells; LA-N-1; LA-N-2; choline; acetylcholine; phosphocholine; glycerophosphocholine; retinoic acid.

ABBREVIATIONS

Cho: Choline
AcCho: Acetylcholine
RA: Retinoic acid
*P*Cho: Phosphocholine
Gro*P*Cho: Glycerophosphocholine
L-15: Leibovitz's L-15 medium

INTRODUCTION

The metabolic fate of labeled choline (Cho) in cholinergic and noncholinergic neuroblastoma cell lines has been examined (Lanks et al., 1974). The majority of label incorporated by these cells was present either as phosphocholine (*P*Cho) or as Cho phospholipids. There is an uptake of choline by both a high- and low-affinity system in cultured brain cells (Massarelli et al., 1974a) and cloned cell lines of neural origin (Richelson and Thompson, 1973; Massarelli et al., 1974b). It is well known that Cho serves as a precursor for the formation of the neurotransmitter acetylcholine (AcCho) in the cholinergic nerve terminals in addition to serving as a phosphatidylcholine precursor. The bulk of the choline is presumably derived from the blood circulation. AcCho is released presynaptically into the cleft and hydrolyzed postsynaptically by acetylcholinesterase into free Cho and acetate. The choline moiety is then taken up into nerve terminals, a process that may be directly linked to the process of AcCho synthesis (Barker and Mittag, 1974; O'Regan et al., 1982; Schmidt and Wecker, 1981). Ansell and Spanner (1979) demonstrated that phosphatidylcholine, a major component of biological membranes, may also serve as a reservoir of Cho for AcCho formation. Suggestive evidence was offered that glycerophosphocholine (Gro*P*Cho) may also be a Cho source (Jope and Jenden, 1979; Jope, 1982).

In pathological conditions that lead to selective degeneration of cholinergic neurons (e.g., Alzheimer's disease), the availability of a cholinergic cell line of human origin as a suitable model would be useful. To further investigate these observations on Cho utilization, we chose a cell line, LA-N-2, which was originally established and characterized by Seeger and his colleagues (Seeger et al., 1977). The cholinergic characteristics of LA-N-2 cells include storage of AcCho (504 nmol/g tissue) and a veratridine-stimulated, tetradotoxin-sensitive sodium ionophore (West

et al., 1977). It also possesses choline acetyltransferase activity (ChAT) responsible for the synthesis of acetylcholine (Singh et al., 1990). In this communication, we investigate the choline utilization by control and retinoic acid (RA) differentiated LA-N-1 and LA-N-2 cells.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H-methyl]Choline (Cho) chloride (81.8 Ci/mmol) was purchased from Amersham Canada Limited, Oakville, Ontario, Canada. Ethanol solutions of RA were prepared in subdued light at a concentration of $10^{-3}M$ and stored at −20°C in the dark. Falcon T-25 flasks, Leibovitz's L-15 medium, and heat-inactivated fetal bovine serum were obtained from Flow labs. Physostigmine, choline chloride, all-trans RA, and gentamicin were purchased from Sigma Chemical (St. Louis, MO).

Cell Culture

The human neuroblastoma cell lines, LA-N-1 (Passage 49) and LA-N-2 (Passage 81), were obtained from Robert Seeger, University of California, Los Angeles. Cells were routinely maintained in Leibovitz's L-15 medium, which yields a final Cho concentration of 8 μ*M* supplemented with 15% heat-inactivated serum, 100 m*M* glutamine, and 50 μg/mL of gentamicin, were grown as monolayers on the surface of 25 cm^2 (T-25) plastic tissue-culture flasks tightly capped, and the medium was changed every 4th d. Cultures were maintained at 37°C in a humidified atmosphere.

Cells, 10^5, in 5 mL of culture medium were transferred to a flask and the next day, the medium was replaced with medium containing $10^{-5}M$ RA or with medium containing an equivalent quantity of ethanol at a final concentration of 0.1%. The culture medium was replaced every 4th d with either the RA or the solvent containing medium and cultures were examined daily with a phase-contrast microscope to observe morphological changes.

Protein Determination

Protein was determined according to the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Cell Labeling Conditions

LA-N-1 and LA-N-2 cell monolayers containing about 3–4 × 10^6 cells that had been grown either in the absence or in the presence of $10^{-5}M$ RA for 8 d in vitro (DIV) were labeled with medium containing 5–10 μCi of Cho and incubated for different times, up to 72 h. The cells were harvested with a rubber policeman, washed twice with phosphate-

buffered saline (pH 7.4), and processed for lipid extraction by the method of Folch et al. (1957).

For pulse-chase studies, LA-N-1 and LA-N-2 cell monolayers were incubated with 5–10 μCi [^{3}H-methyl]Cho for 24 h at which time the labeled medium was removed and replaced with fresh L-15 medium containing 15% heat-inactivated fetal calf serum and 100 μ*M* unlabeled Cho. Cells were harvested at 0 time, 15 min, 30 min, 45 min, 1 h, 3 h, 6 h, and 24 h after the addition of nonradioactive medium. Gross changes in the growth patterns of LA-N-1 and LA-N-2 cells in the presence or absence of 100 μ*M* Cho were not observed under microscopic observation.

Procedures for Extraction of Labeled Products

The procedure of Jenden et al. (1973) was employed for obtaining the labeled water soluble products from the cell pellets harvested as described above. In brief, 1 mL of ice-cold formic acid:acetone (15:85, v/v) was added to the pellet of labeled cells, homogenized, and centrifuged for 10 min at 0°C in an Eppendorff centrifuge. The supernatant was removed, brought to dryness with a nitrogen stream, and suspended in 200 μL of cold 0.4 *N* HCl.

In order to obtain the labeled products from the media, each sample was brought to 6% ice-cold trichloroacetic acid (TCA) and the denatured protein from the fetal calf serum removed by centrifugation at 2500*g* for 10 min. The supernate was extracted three times with diethyl ether to remove excess TCA, and the aqueous phase retained for TLC.

The total cellular lipids were obtained by the classical procedure of Folch-Pi (Folch-Pi et al., 1957).

Thin Layer Chromatography (TLC) and Determination of Radioactivity Associated with Various Products

The water-soluble components, *P*Cho, Gro*P*Cho, Cho + AcCho, were separated on Silica Gel G60 plates with a solvent composed of 1.2% NaCl/CH_3OH/conc. NH_4OH (50:50:5 v/v/v) (Yavin, 1976). This system does not resolve AcCho from Cho, which were separated on cellulose TLC plates (Polygram CEL 300 PEI) with a solvent composed of butanol/ethanol/acetic acid/water (40:10:1:15, v/v/v/v) (Blusztajn and Wurtman, 1981). The lipids were separated by TLC with chloroform/methanol/water (65:25:4, v/v/v/) as solvent. Appropriate radioactive standards were applied to each plate in separate lanes to facilitate product identification.

The plates were air-dried and processed for autoradiography with Kodak X-OMAT film. The individual areas of the TLC corresponding to a radioactive spot on the film was removed and the amount of radioactivity quantitated by liquid scintillation counting.

RESULTS

Time-Course of Cho Labeling in Undifferentiated and Differentiated LA-N-1 and LA-N-2 and AcCho Formation in LA-N-2 Cells

When control and RA-treated LA-N-1 and LA-N-2 cells were incubated with [^{3}H-methyl]Cho for varying times, the incorporation of radiolabel in water-soluble metabolites progressively increased up to 24 h. There was greater incorporation of radioactivity into the water-soluble metabolites of the RA-treated LA-N-2 cells as compared with untreated LA-N-2 cells. There were no apparent differences in the labelings of the water-solubles of untreated and RA-treated LA-N-1 cells (data not shown).

Turnover of Water-Soluble Metabolites in Untreated and RA-treated LA-N-1 and LA-N-2 Cells

There was only a slight decrease of radioactivity from phosphatidylcholine in pulse-chase experiments. It thus seemed more reasonable to focus attention on the distribution of radioactivity in the water-soluble components in the LA-N-1 and the LA-N-2 cells. Radioautograms of the TLC of the formic acid:acetone (15:85, v/v) cell extracts showed that both Cho and AcCho were present in the LA-N-2 cells, but that the LA-N-1 cell extracts contained exclusively Cho without any detectable AcCho (Fig. 1). The radioactive spot close to the origin in Lanes 2 and 4 is a mixture of *P*Cho and Gro*P*Cho.

The distribution of cell-associated radioactivity in the LA-N-2 cells was 10–6% in AcCho; 84–89% in *P*Cho; 1–3% in Gro*P*Cho; and 2–4% in Cho, as shown in Fig. 2. An appreciable change was noticed in the turnover of labeled AcCho, as shown in Fig. 2, in the presence of 100 μM unlabeled Cho in L-15 medium during the chase period. Approximately 20% of the cellular radioactivity of the water-soluble metabolites of control and RA-treated LA-N-2 cells was present as Cho plus AcCho, as measured by the extraction procedure developed by Fonnum (1975). The labeling of Cho and AcCho was higher in the RA-treated LA-N-2 cells as compared to control cells (data not shown). There were no differences in the percentage distribution of radioactivity between control and RA-treated LA-N-2 cells. There was little change in these distributions over a 24-h period.

The distribution of radioactivity in the water-soluble cellular components of the untreated and RA-treated LA-N-1 cells was 95–85% as *P*Cho; 4–20% as Gro*P*Cho; and 1–3% as Cho (Fig. 3). There was a very small gradual decrease in the percent label present in *P*Cho, accompanied by a small gradual increase in the label present in Gro*P*Cho over a 24-h period.

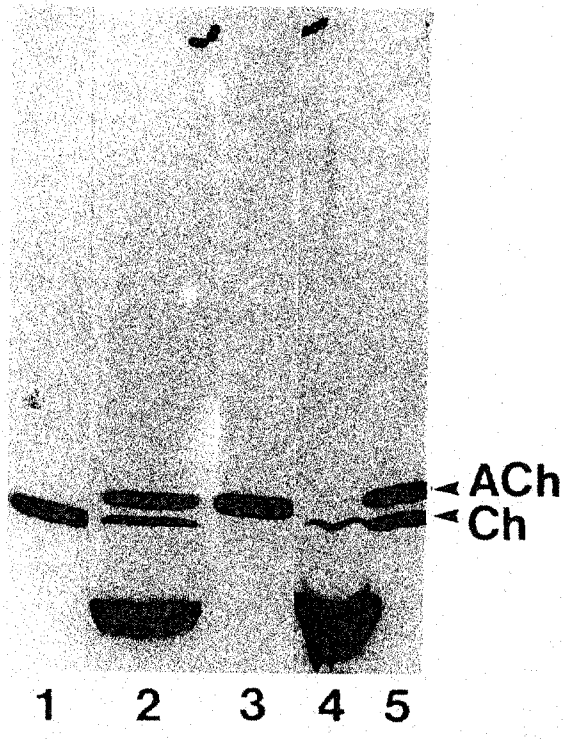

Fig. 1. Autoradiogram from thin-layer chromatography (TLC) plates of extracts of LA-N-2 and LA-N-1 cells. Procedures for Cho and AcCho extraction from the cells and for TLC were as described under "Experimental Procedures." Lane 1, standard of [^{3}H-methyl]Cho; lane 2, 0.4 MHCl extract from LA-N-2 cell line (control); lane 3, standard [^{3}H]AcCho; lane 4, 0.4 MHCl extract from LA-N-1 cell line (control); lane 5, standard mixture of [^{3}H-methyl]Cho and [^{3}H]AcCho.

Turnover of Cho-Containing Compounds in the Medium of Untreated and RA-treated LA-N-1 and LA-N-2 Cells

The turnover of Cho-containing compounds in the growth medium of LA-N-1 and LA-N-2 cells was measured to determine if there was any similarity to that of the cellular components. As shown in Fig. 4, there was no apparent difference in labeling pattern of *P*Cho and Gro*P*Cho between the control and RA-treated LA-N-2 cells. There was no AcCho detectable in the medium. The distribution of radioactivity in the medium of LA-N-2 cells was equally divided between Cho (50%) and *P*Cho (50%), with only 1–3% as Gro*P*Cho (Fig. 4). This is in contrast to the cells

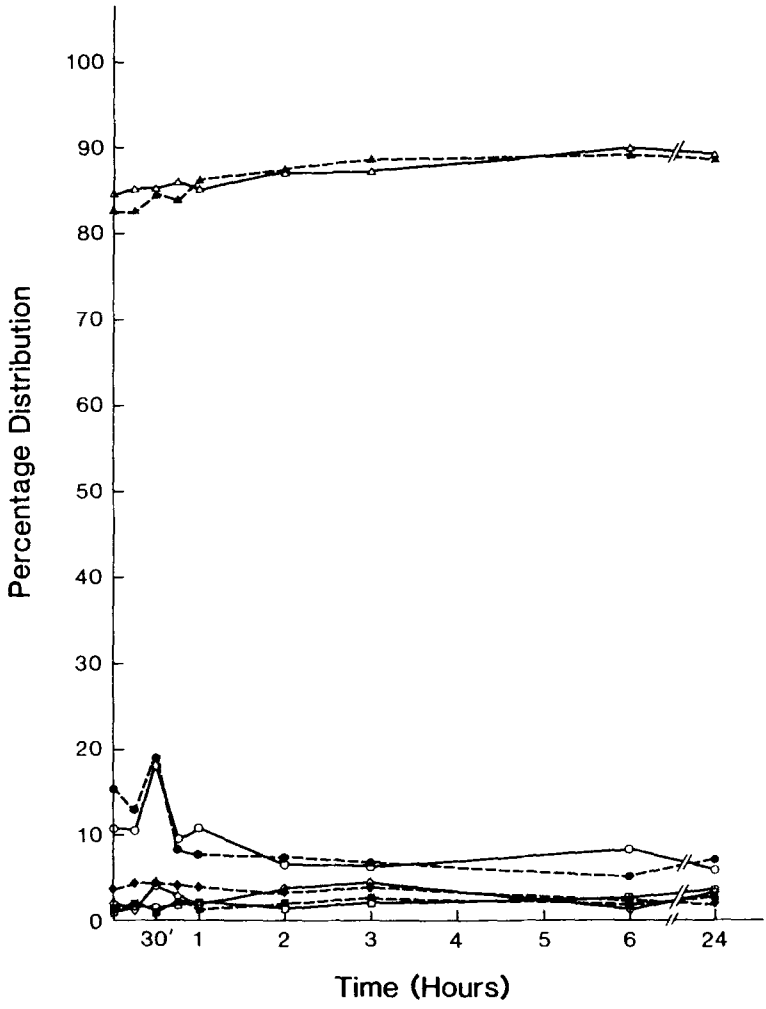

Fig. 2. Pulse-chase experiments with control and RA-treated LA-N-2 cells. LA-N-2 cells were incubated with 5–10 μCi of [³H-methyl]choline for 24 h and then with a medium containing 100 μ*M* unlabeled choline. Cells were harvested at the indicated times and incorporation into water-soluble material was determined as described under "Experimental Procedures." Each point represents the average of two different experiments.

- ◇—◇ Cho (control; the radioactivity at zero time was 1889 ± 1318).
- ◆···◆ Cho (RA-treated; the radioactivity at zero time was 2432 ± 1564).
- △—△ *P*Cho (control; the radioactivity at zero time was 65,070 ± 23, 541).
- ▲···▲ *P*Cho (Ra-treated; the radioactivity at zero time was 70,878 ± 23, 913).
- ○—○ AcCho (control; the radioactivity at zero time was 9248 ± 4460).
- ●···● AcCho (RA-treated; the radioactivity at zero time was 11,962 ± 4236).
- □—□ Gro*P*Cho (control; the radioactivity at zero time was 777 ± 414).
- ■···■ Gro*P*Cho (RA-treated; the radioactivity at zero time was 824 ± 60).

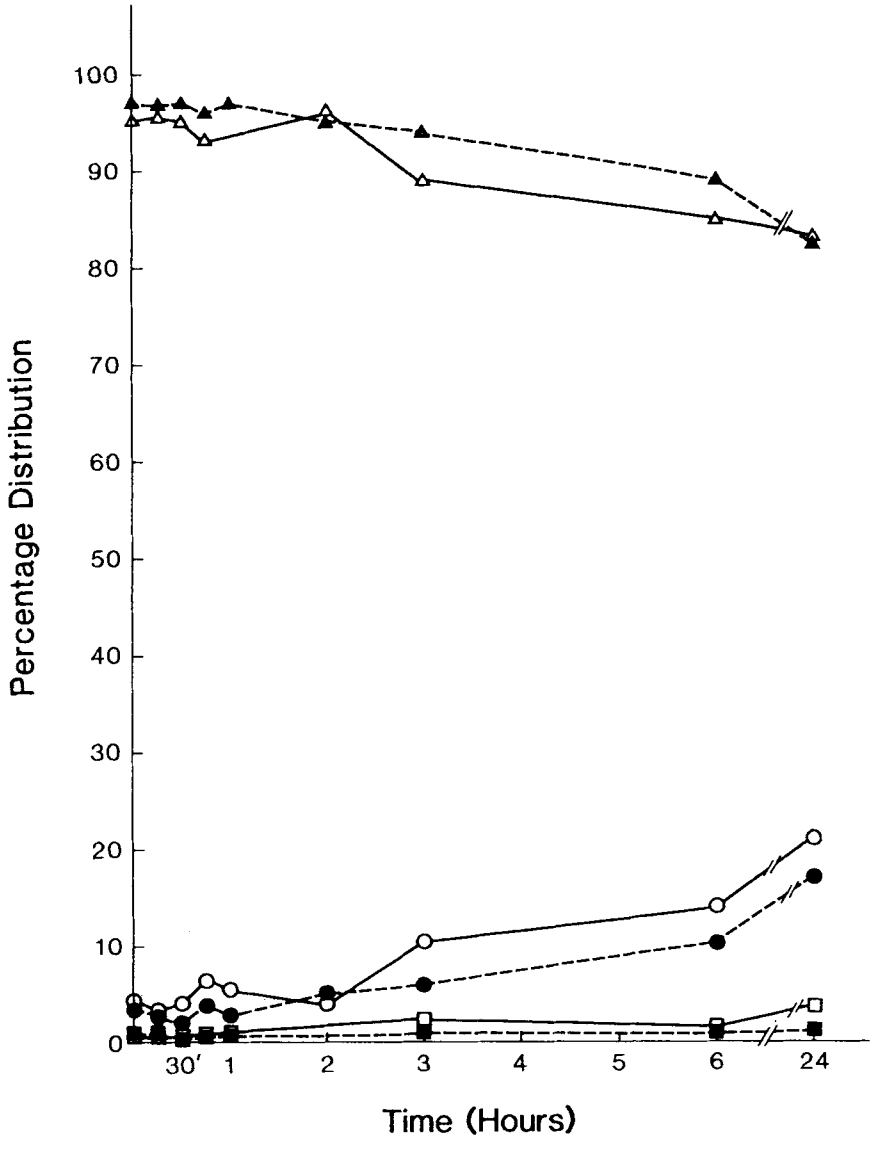

Fig. 3. Pulse-chase experiments in control and RA-treated LA-N-1 cells. The experiment is as described in the legend to Fig. 2. Each point is the average of the data from two different experiments.
- ○—○ Cho (control; the radioactivity at zero time was 664 ± 229).
- ■····■ Cho (RA-treated; the radioactivity at zero time was 581 ± 313).
- △—△ PCho (control; the radioactivity at zero time was 96,099 ± 25,176).
- ▲····▲ *P*Cho (RA-treated; the radioactivity at zero time was 98,445 ± 43,755).
- ○—○ Gro*P*Cho (control; the radiactivity at zero time was 4658 ± 2952).
- ●····● Gro*P*Cho (RA-treated; the radioactivity at zero time was 2829 ± 912).

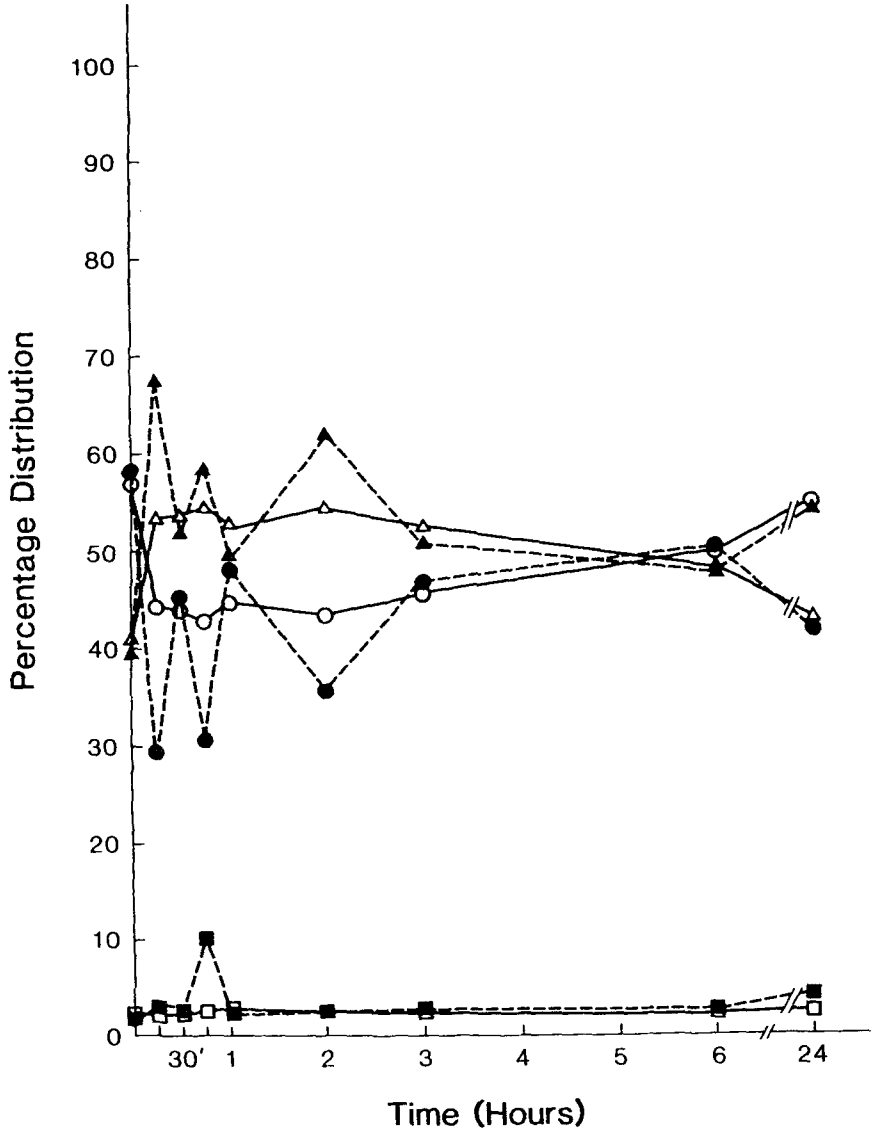

Fig. 4. Distribution of radioactivity in the growth medium of control and RA treated LA-N-2 cells in pulse-chase experiments. The isolation and analysis of water-soluble products from the growth medium are as described under "Experimental Procedures." Each point represents the average of two different experiments.

- ○—○ Cho (control; the radioactivity at zero time was 2153 ± 635).
- •···• Cho (RA-treated; the radioactivity at zero time was 4780 ± 145).
- △—△ *P*Cho (control; the radioactivity at zero time was 1524 ± 301).
- ▲···▲ *P*Cho (RA-treated; the radioactivity at zero time was 3269 ± 254).
- □—□ Gro*P*Cho (control; the radioactivity at zero time was 82 ± 43).
- ■···■ Gro*P*Cho (RA-treated; the radioactivity at zero time was 160 ± 11).

that have only a few percent choline (Fig. 2). There was little change in distribution over 24 h. In contrast, the distribution of radioactivity in the culture medium of LA-N-1 cells was about 50–90% present as Cho; 10–45% as *P*Cho; and 1–3% only as Gro*P*Cho (Fig. 5) for the first 6 h. During the following 18 h, there was a redistribution of radioactivity between choline and phosphorylcholine.

DISCUSSION

The characteristic of the human neuroblastoma cell lines, LA-N-2, as being cholinergic may provide a potentially useful model for determining the factors contributing to AcCho synthesis. These cells grown in the presence of $10^{-5}M$ RA undergo a morphological differentiation, principally evidenced by the extending of neurites. The characteristic neurotransmitter biosynthetic enzyme activity ChAT was increased twofold in LA-N-2 cells exposed to RA as compared to control cultures (Singh et al., 1990). The AcCho content of LA-N-2 cells was reported to be 504 nmol/g (West et al., 1976) compared to 50 nmol/g in rat caudate (Cohen and Wurtman, 1976), 2 nmol/mg protein in NS 20 neuroblastoma cells (Kato et al., 1977), and 130 nmol/mg protein in cholinergic synaptosomes of the Torpedo electric organ (Morel et al., 1977). The amount of AcCho present after a 5-min exposure varies with the availability of extracellular Cho supplied to LA-N-2 cells (Richardson et al., 1989).

In the presence of [^{3}H-methyl]Cho, LA-N-2 cells grown in the absence and presence of RA have approx 20% of the cell-associated radioactivity as AcCho and Cho, but with LA-N-1 cells there is only Cho (data not shown). LA-N-1 cells being catecholaminergic, therefore, provides a suitable control for observations on a cholinergic cell line, such as LA-N-2, in experiments focusing on AcCho formation and secretion.

As enumerated by Richardson et al. (1989), the principle water-soluble compound into which labeled Cho is incorporated is *P*Cho, as shown with NS-20 neuroblastoma cells (Lanks et al., 1974); neuroblastoma X glioma hybrid cells, NG108-15 (Blusztajn et al., 1986); dissociated neurons of chick brain and rat brain synaptosomes (Massarelli and Wong, 1981; Wong et al., 1983); and cultures of septal slices of neonatal rats (Keller et al., 1987) in vivo, which may be a possible precursor for AcCho synthesis.

The pulse-chase experiments with control and RA-treated LA-N-1 and LA-N-2 cells showed that the majority of the cellular radioactivity in the water-soluble metabolites was *P*Cho, which comprised of 84–89% of the label followed by Cho (2–4%) and Gro*P*Cho (1–3%). The radioactivity in AcCho was about 6–10% in both untreated and RA-treated LA-N-2 cells. Yavin (1976), using rat cerebral hemisphere cultures, showed that the relative incorporation of [^{3}H-methyl]Cho in AcCho increased from 1.1 to 27.4% when the Cho concentration was elevated from 10^{-6} *M* to 2 $\times$ $10^{-3}M$. The levels of free intracellular Cho also increased with higher

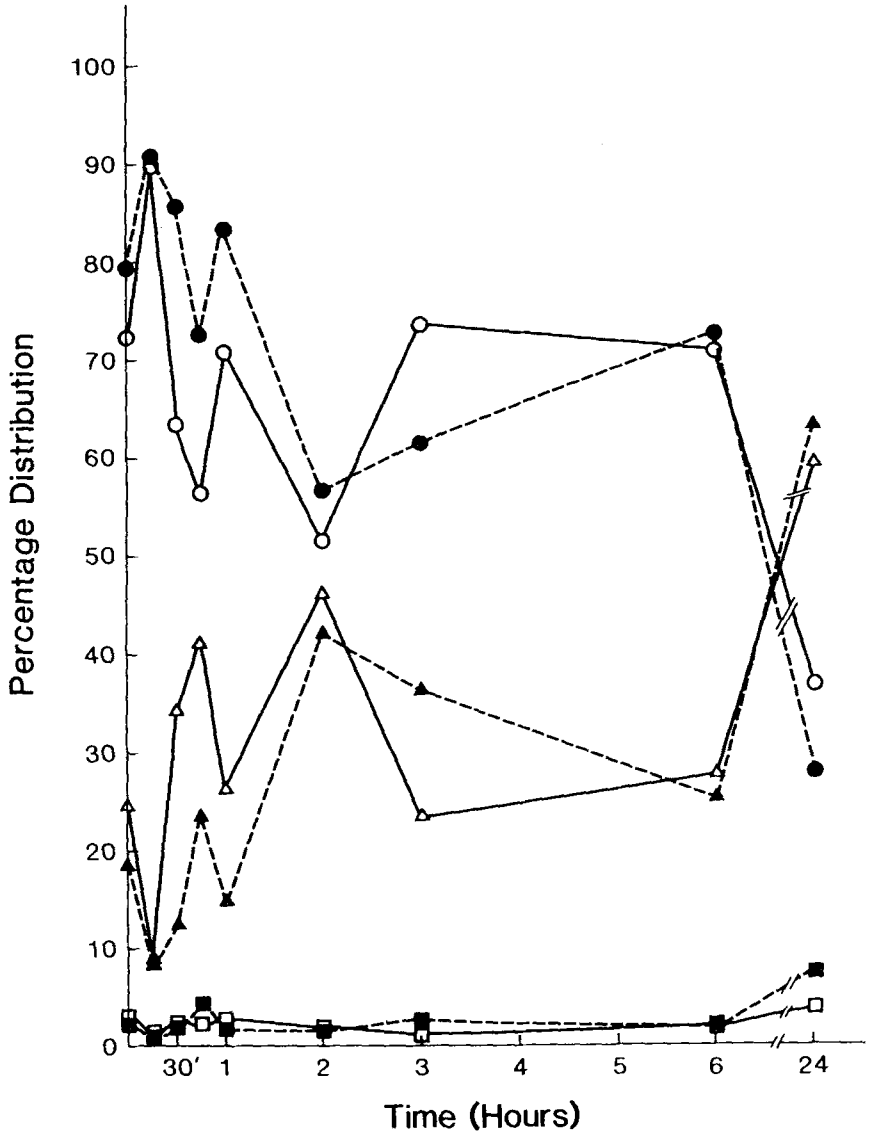

Fig. 5. Distribution of radioactivity in the growth medium of control and RA treated LA-N-1 cells from pulse-chase experiments. The isolation and analysis of water-soluble products from the growth medium are as described under "Experimental Procedures." Each point represents the average of two different experiments.

- o—o Cho (control; the radioactivity at zero time was 1398 ± 586).
- •····• Cho (RA-treated; the radioactivity at zero time was 4758 ± 188).
- Δ—Δ *P*Cho (control; the radioactivity at zero time was 462 ± 129).
- ▲····▲ *P*Cho (RA-treated; the radioactivity at zero time was 1154 ± 937).
- o—o Gro*P*Cho (control; the radioactivity at zero time was 62 ± 36).
- •····• Gro*P*Cho (RA-treated; the radioactivity at zero time was 55 ± 32).

concentrations of extracellular Cho. Pulse-chase experiments in HeLa cell extracts with [^{3}H-methyl]Cho showed that less than 3% of the label present in the water-solubles was associated with Cho, CDP-choline, and Gro*P*Cho, whereas more than 97% was associated with *P*Cho (Vance et al., 1980). Using rat brain cell cultures, Yavin (1976) demonstrated that at 1–5 $\times$ $10^{-6}M$ [^{3}H-methyl]Cho, approx 79% of total radioactivity was incorporated into *P*Cho, and that this proportion decreased progressively as the concentration of extracellular Cho was increased. At 2 $\times$ $10^{-3}M$, the low-affinity uptake region, only 22% of the radioactivity was present as *P*Cho.

Pulse-chase experiments with NIE-115, C_6 and primary glia cells revealed that Gro*P*Cho was the major intracellular water-soluble metabolite. In contrast, Morash et al. (1988) have shown that in fibroblasts, Gro*P*Cho accounts for only 30% of the water-soluble label compared with *P*Cho being 50% of the radioactive. *P*Cho was also present as major water-soluble component (30%) in primary glia.

The distribution of radioactivity in the growth medium of LA-N-2 cells was equivalent between choline and *P*Cho, with only 1–3% as Gro*P*Cho. In contrast, the LA-N-1 cells had 70–80% of the radioactivity in Cho, followed by 20–30% as *P*Cho and 1–3% of Gro*P*Cho. In pulse-chase studies, Yavin (1976) demonstrated that Gro*P*Cho was the major Cho-containing compound released into the medium. It is evident that *P*Cho is the major intracellular and extracellular water-soluble metabolite with these two human neuroblastoma cells.

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