Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 251, No. 19, Issue of October 10, pp. 6147-6149, 1976
Printed in U.S.A.

Deuterium Magnetic Resonance Spectroscopy of Isotopically Labeled Mammalian Cells*

(Received for publication, July 12, 1976)

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SUMMARY

Choline completely deuterated in the methyl groups has been incorporated into two mammalian cell systems. The first was the chemically transformed mouse fibroblast LM cell line, grown in suspension culture. The second system consisted of mitochondria from Sprague-Dawley rats which were fed upon a choline-deficient diet enriched with deuterated choline chloride. Two classes of deuterium nuclear magnetic resonance (NMR) signal were obtained from each system. The first class of signal exhibited no residual quadrupole coupling constant and was assigned to the natural abundance of ^2H in water, together with free labeled choline. The second class of signal had a residual quadrupole coupling of about 1 KHz and was assigned to ^2H-labeled choline headgroups of phospholipids. These results are in conflict with a previous study of deuterated rat liver mitochondrial membranes. Deuterium magnetic resonance spectra can be obtained on mammalian membranes that have incorporated specific deuterium-labeled compounds and, therefore, provides a very powerful method for studying the dynamic structure of membranes.

The deuterium nucleus has a spin $I = 1$ and thus possesses an electric quadrupole moment. The interaction of this moment with the electric field gradient at the deuteron nucleus causes a weak perturbation of the nuclear Zeeman levels so that separate transitions corresponding $+1 \leftrightarrow 0$ and $0 \leftrightarrow -1$ are observed. From the magnitude of this "quadrupole splitting" it is often possible to extract information about the rate and type of motion of the deuterium nuclei involved (1-3). Recently, it has been shown that deuterium magnetic resonance (DMR) spectroscopy is an extremely powerful technique with which to study the structural organization of both model (4-6) and natural cell membranes (7, 8). In this communication, experiments are reported on the possible uses of deuterium magnetic resonance spectroscopy in studying the structural organization of mammalian cell surfaces. This work has been facilitated by the recent development of techniques to specifically manipulate the polar headgroup composition of the membrane phospholipids of LM cells, which have a requirement for choline or choline analogs (*e.g.* dimethylethanolamine), in tissue culture (9). For example, when growth medium was supplemented with choline analogs for 3 days, the total membrane phospholipids contained up to 50% of the analog that was supplied (9). The results given here show that it is relatively easy to incorporate deuterium-labeled species into specific membrane lipid components and observe their deuteron nuclear magnetic resonance spectra.

* This work was supported by National Science Foundation Grant PCM 76-01491 and by National Institutes of Health Grants HL-19481 and GM-21953.

‡ Supported by National Institutes of Health Research Career Development Award GM-00193 from the Institute of General Medical Sciences.

MATERIALS AND METHODS

$[^2H_9]$Choline chloride, $(C^2H_3)_3N^+CH_2CH_2OH\ Cl^-$, was prepared as described previously (10). Male Sprague-Dawley rats (80 to 100 g) were obtained from the Holtzman Co., Madison, Wisc. and were fed on a choline-deficient diet (Nutritional Biochemicals, Cleveland, Ohio) supplemented with either $[^2H_9]$choline chloride or choline chloride (Aldrich Chemical Co., Milwaukee, Wisc.) for 7 days. The rats were "stunned" using a blunt instrument and liver mitochondria were prepared as described by Arvidson and co-workers (11). Mitochondrial lipids were extracted by freeze-drying the membranes and then extracting with chloroform/methanol (2/1, v/v ratio). Mouse LM cells in suspension culture were grown in Higuchi's medium (12) containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (Sigma Chemical Co., St. Louis, Mo.), 1 g/liter of methyl cellulose (Fisher Scientific Co., Chicago, Ill.), and 0.02 g/liter of sodium dextran sulfate (Sigma Chemical Co., St. Louis, Mo.), basically as described previously (9). Choline chloride was added as a supplement at 40 μg/ml of medium. Lipids were extracted from the LM cells using the method of Bligh and Dyer (13) as modified by Ames (14). Deuterium spectra were obtained using the Fourier transform method at 33.8 MHz on a "home-built" instrument.

RESULTS AND DISCUSSION

Mouse LM cells grown in suspension culture contain approximately 45 to 50% phosphatidylcholine and 5 to 10% sphingomyelin. When choline deuterated in the methyl groups was used to supplement the media instead of normal choline for approximately six generations, the choline-containing phospholipids should have contained practically all deuterated headgroups. The first deuterium magnetic resonance experiments with these cells indicated two types of signal, as shown in Fig. 1. The first type of signal had a splitting of about 1 KHz at 18°, and a shape characteristic of the resonance of a spin $I = 1$ nucleus with a low asymmetry parameter, Fig. 1*A*. The second type of signal shown in Fig. 1*A* again showed a splitting, but the magnitude of this splitting was only about 56 Hz. This splitting was almost identical with that reported by Arvidson *et al.* (11) for deuterium-labeled rat liver mitochondria, when compared on a parts per million basis.

Spectra of extracted membrane lipids dispersed in water, Fig. 1*B*, again revealed two types of signals, although in this case one of the central lines was absent. Because of the similarity of the splittings of the 1 KHz component of the spectra of intact cells and isolated lipids, and because the magnitude of this splitting was approximately that obtained by Seelig and co-workers (6) and Smith and co-workers (15) for synthetic lecithins deuterated in the choline methyl groups, it can be attributed to the methyl groups of the choline headgroup of lecithin and sphingomyelin in the LM cell membrane lipids.

The chemical shift difference between the two narrow central lines was 56 ± 8 Hz, or about 1.6 ppm. The chemical shift difference between water and the methyl groups of choline was about 1.7 ppm. Consequently, the two narrow signals of Fig. 1*A* must be attributed to natural abundance of deuterium in water (low field component) and free deuterium-labeled choline inside the LM cells (high field component). This interpretation is supported by the observation that the chemical shift of the low field component was identical with that of an external reference sample of deuterium oxide and the fact that the high field component was not observed in the lipid extract of these cells dispersed in water, Fig. 1*B*.

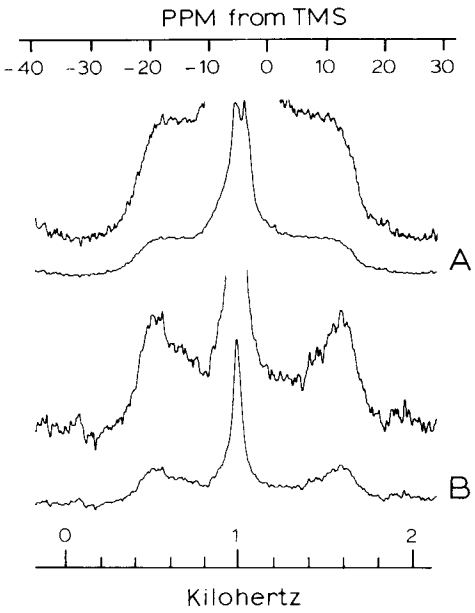

FIG. 1. *A*, deuterium NMR spectrum of intact mouse LM cells containing phospholipids biosynthetically enriched with [$^2H_9$]choline headgroups, in fresh growth medium without deuterated choline, at 18° ± 1°. The spectrum was recorded at 33.8 MHz using 2,048 data points, a spectral width of 5,000 Hz, and a recycle time of 0.11 s. Also, 2,048 "zeros" were added to the time-domain data before Fourier transformation, to improve peak definition. *B*, lipids extracted from sample of *A* by the methods described in Refs. 13 and 14 and dispersed in H_2O. Spectra conditions as in *A* except that recycle time was 0.21 s. The traces above the main spectral are 3× vertical expansions. The narrow central components arise from free [$^2H_9$]choline or natural abundance of ^2H in water, or both. The broad doublet feature arises from [$^2H_9$]choline biosynthetically incorporated into membrane phospholipid. *TMS*, tetramethyl silane.

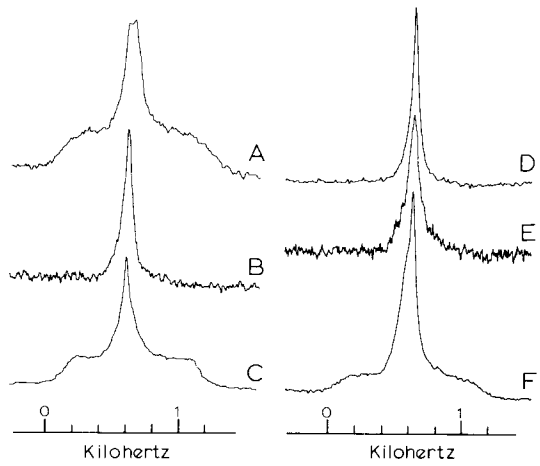

FIG. 2. *A*, deuterium NMR spectrum of rat liver mitochondria obtained from rats fed a diet supplemented with [$^2H_9$]choline chloride, in buffer (0.25 M sucrose, 0.01 M Tris, 1 mM EDTA, pH 8.0) at 18° ± 1°. The spectrum was recorded at 33.8 MHz using 2,048 data points, a spectral width of 5,000 Hz, and a recycle time of 0.21 s. Also, 2,048 "zeros" were added to the time-domain data before Fourier transformation. *B*, as *A* except mitochondria obtained from rats grown on a [^1H]choline-supplemented diet. *C*, lipids of mitochondria of *A*, obtained by chloroform/methanol extraction, dispersed in buffer: other spectrometer conditions as in *A*. *D*, [$^2H_9$]choline chloride (20 mg ml^{-1}) in H_2O, at ~24°, other conditions as in *A*. *E*, lipids of *C* dissolved in chloroform, other conditions as in *A*. *F*, whole liver slice from rats fed on a diet enriched with [$^2H_9$]choline chloride, spectral conditions as in *A*.

In Fig. 2, results are presented for the rat liver system. Fig. 2*A* shows the deuterium NMR spectrum of a small sample of liver mitochondria, obtained from Sprague-Dawley rats grown on a deuterated choline-supplemented medium. Again, two classes of signal were apparent: a narrow central component together with a broader one. To test the assumption that the broad component represented predominantly deuterated lecithin of the mitochondrial membranes, identical procedures were used to prepare membranes from rats grown on [^1H]choline chloride-supplemented media. As shown in Fig. 2*B* only the natural abundance water peak was visible. The broad component and the high field component of the (partially split) narrow central signal must have originated from the deuterated choline supplement. Confirming this, extracted mitochondrial lipids dispersed in a buffer solution, Fig. 2*C*, exhibited only a single narrow central line (which must have arisen from HO^{2}H) and a broad doublet (which must have arisen from deuterium-labeled lecithin and sphingomyelin molecules). Finally, it should be noted that deuterium-labeled choline chloride dissolved in water exhibited one intense peak, Fig. 2*D*, having the same chemical shift as the high field component of the LM cell system (Fig. 1*A*) and the rat liver mitochondrial system (Fig. 2*A*), and also that membrane lipids dissolved in chloroform exhibit an identical chemical shift, Fig. 2*E*.

Interpretation of the mitochondrial results conflicts with that proposed by Arvidson and co-workers (11) who attributed the narrow central components to a quadrupole-split doublet arising from specifically labeled membrane phospholipids undergoing either almost isotropic motion or some strong interaction with proteins. It is possible that these workers were observing only HO^{2}H and free labeled choline in their spectra of "mitochondrial membranes," and that the real membrane signals were obscured because of low signal-to-noise ratios. Unfortunately, because of the low homogeneity of the solenoid used in experiments reported here, the narrow central components have not been resolved very well. This poor field homogeneity also prevented accurate comparisons of reduced quadrupole couplings between intact membranes and extracted lipids. Nevertheless, it seems clear that the degree of order of the mouse LM cell lecithin headgroup was appreciably higher than that of the rat liver mitochondrial lecithin headgroup. It also appears that in both cases the extracted lipids had slightly higher order parameters than the intact membrane systems. Further studies are in progress to clarify this point.

Since spectra from the enriched mouse cells and rat liver mitochondria could be obtained with only a few minutes of data acquisition, experiments were attempted on intact liver. The results are shown in Fig. 2*F*. Clearly, a doublet splitting of about 900 Hz was present in the enriched cells and this signal must represent deuterated choline headgroups of lecithin and sphingomyelin in the rat liver, since it was absent from ^1H-supplemented cells. The magnitude of the quadrupole coupling was, within experimental error, the same as that obtained from isolated lipids, eliminating any possibilities of "bound choline" not covalently incorporated into lipid contributing to the spectrum.

This ability to selectively enrich and manipulate the lipid composition of cells with deuterated compounds, coupled with the use of high field deuterium magnetic resonance techniques provides a powerful method for studying the dynamic structure of mammalian cell membranes.

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