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IN VITRO METABOLISM OF [2-^{13}C]-ETHANOL BY ^{1}H NMR SPECTROSCOPY USING ^{13}C DECOUPLING WITH THE REVERSE DEPT POLARIZATION-TRANSFER PULSE SEQUENCE

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The metabolism of [2-^{13}C]-ethanol by alcohol dehydrogenase purified from *Drosophila melanogaster* has been observed by proton nuclear magnetic resonance spectroscopy (NMR). The reverse-DEPT pulse sequence, with composite pulse ^{13}C decoupling to simplify and increase the signal-to-noise of spectra, has been used to eliminate the strong water signal while still observing the proton signals of metabolites of interest. Using these techniques the rates of synthesis of acetaldehyde, its diol and acetate from [2-^{13}C] ethanol by alcohol dehydrogenase were measured simultaneously.

One of the major problems of ^{13}C-NMR is the relatively weak signal strength obtained compared to ^{1}H-NMR. However, in aqueous solutions, ^{1}H-NMR is compromised by the powerful signal emitted by water, which overloads the preamplifier and thus reduces the efficiency of detection of any weaker signal of interest. We have recently developed techniques using reverse ^{13}C → ^{1}H DEPT and POMMIE polarization transfer pulse sequences of ^{13}C-labelled compounds, in which water suppression factors of 2 x 10^{4} - 1 x 10^{5} facilitate the elimination of the water interference of the ^{1}H spectra of [^{13}C]-ethanol in concentrations less than 10 mM (1,2,3). However, the spectra acquired using these procedures have been complicated by ^{13}C - ^{1}H scalar couplings. We show in this paper that it is possible by the use of composite pulse decoupling (4) to efficiently decouple ^{13}C during the data acquisition cycle thereby simplifying the proton spectra. Using these procedures the rates of synthesis of acetaldehyde, the hydrated form of acetaldehyde and acetate from [2-^{13}C]-ethanol by alcohol dehydrogenase from *Drosophila melanogaster* have been recorded.

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MATERIALS AND METHODS

The pulse sequence used in this study was the reverse-DEPT sequence: randomize ^1H, $\frac{\pi}{2}[C,\pm x] - \frac{1}{2J} - \theta[H,x]\pi[C] - \frac{1}{2J} - \frac{\pi}{2}[C,y]\pi[H,y] - \frac{1}{2J}$ - acquire ^1H, decouple ^{13}C (5). The initial carbon $\frac{\pi}{2}$ pulse was phase inverted on alternate scans together with the receiver phase, allowing polarization transfer signals to add. Signals due to protons not coupled to ^{13}C nuclei, H_2O and natural ^1H magnetization, cancel. To enable observation of methyl signals and signals of any other CH_n multiplicity at close to maximum strength, the θ-pulse was set at $\frac{\pi}{4}$. The ^{13}C-composite pulse scheme MLEV-16 (6) was used in these studies because it had been shown to give a significant improvement in the decoupling bandwidth compared with noise decoupling methods at constant power. It is particularly suited to this system because of the wide spectral range exhibited by ^{13}C resonances as well as the fact that because of the high dielectric constant of aqueous biological systems they are susceptible to radiofrequency loss. ^1H-NMR spectra were determined at 200 MHz using an extensively modified BRUKER spectrometer. A wide-bore 130 mm OXFORD magnet was interfaced to a CXP-type spectrometer computing and pulse programming facilities. A field frequency lock built at Griffith University was used in these studies.

Alcohol dehydrogenase was purified from *D. melanogaster* by the method of Moxon *et al* (7). Kinetic studies were performed at 27 ± 2°C in a 10 mm diameter NMR tube. The reaction mixture consisted of 20 mM-glycine-sodium hydroxide buffer (pH 9.2); 10 mM NAD^+; 50 mM [2-^{13}C]-ethanol (C.E.A., France); 27 mM sodium pyruvate; 1.4 IU of lactate dehydrogenase; 1 mm dithiothreitol in a final volume of 2.7 mls. Lactate dehydrogenase and pyruvate were added to regenerate NAD^+. The reaction was initiated by the addition of 3.5 IU of alcohol dehydrogenase.

RESULTS AND DISCUSSION

Using the reverse DEPT sequence, the ^1H spectra of [1-^{13}C] and [2-^{13}C] ethanol (50 mM) in 90% water and 10% D_2O as lock signal is shown in Figure 1. Figure 1A shows water suppression without ^{13}C-decoupling while Figure 1B shows the spectrum obtained using phase modulated ^{13}C-decoupling with the carbon spectrometer frequency set midway between the two carbon resonances. The inefficient decoupling which results is obvious. Figure 1C shows the excellent results obtained with the use of MLEV-16 composite pulse decoupling at the same carbon frequency. In both cases the same decoupling power (3 W) was used. The effective improvement in the signal-to-noise with ^{13}C decoupling was by a factor of two.

The data in Figure 2 shows the improvement in signal-to-noise and simplification of spectra using ^{13}C-decoupling in the metabolism

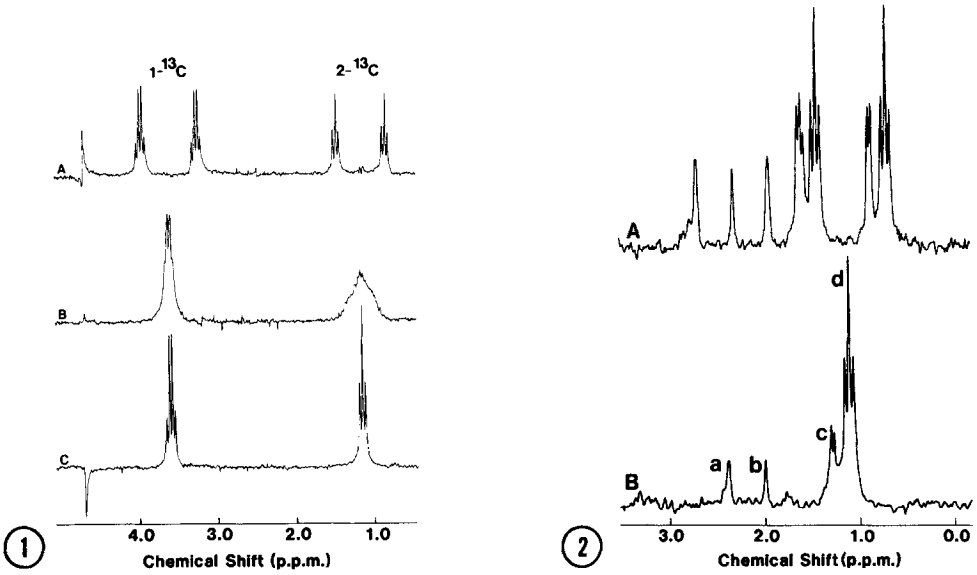

Figure 1. ^{1}H NMR spectra of [1-^{13}C] and [2-^{13}C] labelled ethanol (50 mM) in H_2O obtained using the Reverse-DEPT pulse sequence with 4 seconds of proton magnetization randomization and averaging 64 scans. (A) with no ^{13}C decoupling. (B) with phase modulated ^{13}C decoupling and (C) with MLEV-16 ^{13}C decoupling.

Figure 2. Reverse-DEPT ^{1}H NMR spectra of [2-^{13}C] ethanol metabolized by *D. melanogaster* ADH. (A) with no decoupling (128 scans) (B) with MLEV-16 ^{13}C decoupling (32 scans). The assignments are (a) CH_3CHO, (b) CH_3COO^-, (c) $CH_3CH(OH)_2$, and (d) CH_3CH_2OH with only the methyl proton signals being displayed. The weak, residual H_2O signal is seen downfield of these signals. Chemical shifts are referred to tetramethylsilane at 0.00 p.p.m.

of [2-^{13}C]-ethanol by alcohol dehydrogenase from *D. melanogaster*. Peaks have been assigned to acetaldehyde, the hydrated form of acetaldehyde and acetate. Assignments were made from similar studies using [1-^{13}C] ethanol, when the polarization transfer signals due to acetaldehyde and its hydrated form only were detected. The presence of [^{13}C]-acetate confirms that alcohol dehydrogenase from *D. melanogaster* also possesses aldehyde dehydrogenase activity (7).

Figure 3 shows the changes in concentration of [^{13}C]-ethanol, [^{13}C]-acetaldehyde, its diol and [^{13}C]-acetate when the oxidation of [2-^{13}C]-ethanol by alcohol dehydrogenase is monitored by ^{1}H-NMR using

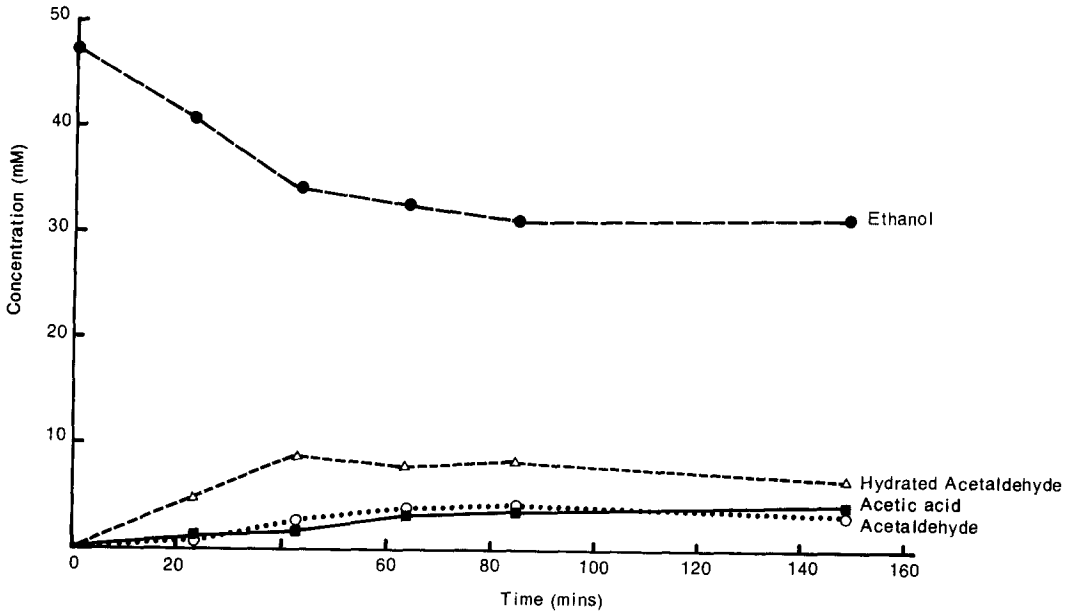

Figure 3. Kinetic studies of the oxidation of 50 mM-[2-^{13}C] ethanol by alcohol dehydrogenase purified from *D. melanogaster*. Concentrations of ethanol and its metabolites were determined by ^{1}H-NMR using the Reverse-DEPT pulse sequence as described in material and methods.

the reverse-DEPT pulse sequence. Examination of this data shows that during the first forty minutes of the reaction sequence, acetaldehyde formed from [2-^{13}C]-ethanol is rapidly hydrated, with 74% of the acetaldehyde produced being in the hydrated form. However with the further production of H^+ from acetic acid, the ratio of aldehyde to its diol increases. After 80 minutes the system came to equilibrium, even in the presence of an NAD^+ regenerating system. However, in the presence of hydrazine sulphate (which forms an irreversible Schiffs base with acetaldehyde), no hydrated acetaldehyde was detected, and the [2-^{13}C]-ethanol was rapidly and completely oxidised. These results indicate that an equilibrium exists between free and hydrated forms of acetaldehyde *in vitro*, and that under conditions where free acetaldehyde is trapped, this equilibrium is perturbed and optimal enzyme activity is observed.

The experiments reported here demonstrate the use of reverse-DEPT to generate proton polarization transfer signals to study *in vitro* metabolism of aqueous samples. This technique eliminates the strong water resonance by use of a spherical randomizing field applied for 4 seconds prior to the initial pulse, and the use of composite pulse ^{13}C-decoupling using the MLEV-16 pulse train to improve the signal-to-noise ratio as well as to simplify spectral analysis. Because the signals of interest are generated via polarization transfer from ^{13}C-nuclei only, the metabolism of a specified carbon atom can be observed. With respect to the oxidation of ethanol by alcohol dehydrogenase this procedure yields far more information than conventional analytical procedures. The enzymatic oxidoreduction of acetaldehyde is usually followed spectrophotometrically by the change in absorption of NADH at 340 nm. Using this technique very little, if any, aldehyde dehydrogenase activity can be detected in alcohol dehydrogenase purified from *D. melanogaster* or yeast at pH 7.4. However, using NMR spectroscopy, not only can the synthesis of acetic acid be confirmed but the production of acetaldehyde and its diol can be simultaneously quantified. The degree of hydration of acetaldehyde is largely ignored in kinetic studies of alcohol and aldehyde dehydrogenases, even though it may offer an explanation for the biphasic kinetics reported for cytosolic aldehyde dehydrogenases in sheep and human liver, human erythrocytes and *D. melanogaster* alcohol dehydrogenase (7,8). Further studies using reverse polarization-transfer pulse sequences may determine whether hydrated acetaldehyde can act as a substrate and or competitor of aldehyde and alcohol dehydrogenases as proposed by Sheridan *et al* (9).

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