In vivo detection of cryoprotectants and lipids in overwintering larvae using carbon-13 nuclear magnetic resonance spectroscopy

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Natural abundance carbon-13 nuclear magnetic resonance (NMR) spectra of intact, acclimated *Eurosta solidaginis* larvae have been obtained. The spectra show peaks assignable to lipid components and the presence of one major type of monounsaturated fatty acid. Chloroform-soluble extracts of whole larvae support this. The major nonlipid carbon components were the two cryoprotectant polyols glycerol and sorbitol. Carbon-13 NMR is a useful tool for analysis of cryoprotectant molecules in whole, living cold acclimated freezing tolerant insects.

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Nous avons obtenu plusieurs spectres de résonance magnétique nucléaire (NMR) du carbone-13 avec des larves d'*Eurosta solidaginis* intactes, acclimatées au froid. Les spectres montrent des pics que l'on peut assigner à des constituants lipidiques et la présence d'un type important d'acide gras monoinsaturé. Les extraits de larves entières solubles dans le chloroforme confirment ces résultats. Les principaux composés carbonés non lipidiques sont deux polyols cryoprotecteurs, le glycérol et le sorbitol. La NMR du carbone-13 est un instrument utile pour l'analyse des molécules de cryoprotecteurs dans des insectes vivants, entiers, acclimatés au froid et capables de tolérer le gel.

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Introduction

The third instar larvae of the goldenrold gall fly *Eurosta solidaginis* overwinter in galls on goldenrod plants. The larvae are freeze tolerant and survive temperatures as low as −40°C (1).

The larvae accumulate two polyhydric alcohols, glycerol and sorbitol, which act as cryoprotectants. Both laboratory acclimations and studies on outdoor populations have shown that polyols are accumulated in a temperature-dependent manner (2, 3).

Glycerol is produced first, starting at temperatures near 10°C and accumulating during cooling to concentrations of 250–660 μmol/g wet weight. Exposure to temperatures below 5°C induces sorbitol synthesis with up to 250 μmol/g wet weight being accumulated.

The present study reports the first application of natural abundance ^{13}C NMR to an investigation of the metabolism of a living insect larva. This technique permits the detection of lipids and cryoprotectant compounds *in vivo* in a noninvasive, nondestructive manner. Component identification is based on spectral comparison with authentic polyol samples as well as spectra of chloroform-soluble extracts of the larvae.

ABBREVIATIONS: NMR, nuclear magnetic resonance; o.d., outside diameter; APT, attached proton test; TMS, tetramethylsilane.

Materials and methods

Third instar larvae of *E. solidaginis* were collected in the fall and acclimated to −5°C for at least 8 weeks in a cold room. Before use in the ^{13}C-NMR experiments the whole larvae were removed from their plant gall.

For the NMR experiments, about 50 larvae were gently placed in a 10-mm o.d. NMR tube, to a depth of about 3 cm. Fourier transform ^{13}C spectra were recorded at 50.3 MHz on a Varian XL-200 NMR spectrometer under conditions of complete ^{1}H decoupling at 20°C. The sample was not spun. Spectral width was 10 000 Hz and satisfactory signal to noise ratios were obtained after about 200 scans (5 min). For processing the free induction decay, a line broadening of 10 Hz was introduced. The acquisition time was 1.6 s and 32 K data points were employed. The observed pulse width was 8 μs, corresponding to a 60° pulse for ^{13}C.

For the J-modulated spin echo (APT) (4) experiments standard conditions were used to produce CH_3 and CH signals having opposite phase to CH_2 and nonprotonated carbons. A delay (D2) of 7.0×10^{-3} s was employed.

The ^{13}C spectrum of the $CHCl_3$-soluble larval extract was obtained under the same conditions as that of the live larvae, with the exception that the sample was spun at a rate of 20 Hz and no line broadening was introduced during the processing

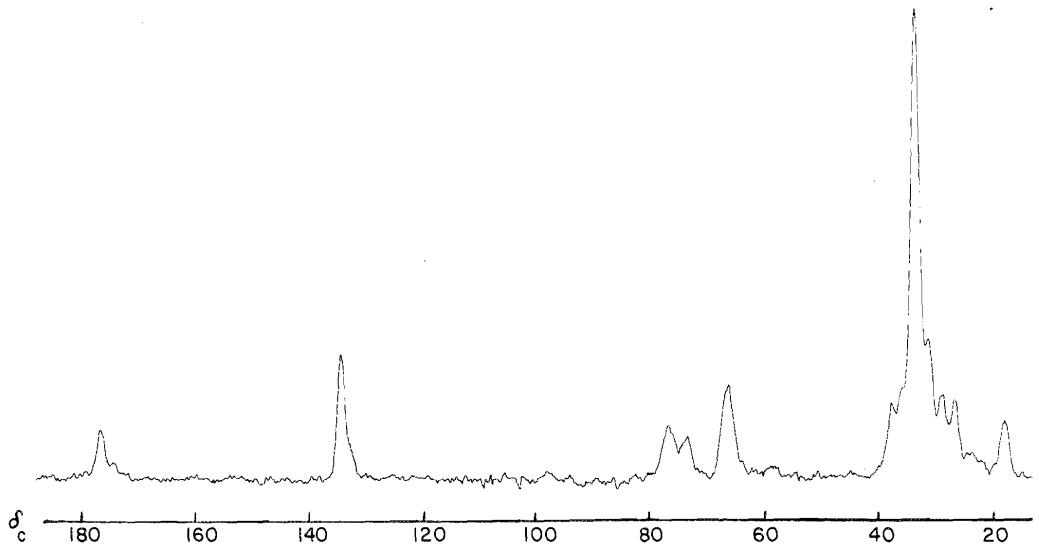

FIG. 1. ^{13}C-NMR spectrum of living *E. solidaginis* larvae.

of the free induction decay. All spectra were recorded without the use of a field-frequency lock.

Results and discussion

The ^{13}C-NMR spectrum of the living larvae, recorded under normal conditions of complete ^{1}H-noise decoupling, is shown in Fig. 1. This spectrum is the result of 554 accumulations of data, with a total time for the experiment of about 15 min. Due to the severe inhomogeneities in the ensemble of larvae in the sample tube, the carbon resonances are an order of magnitude broader than those obtained for organic molecules in solution. Despite this limitation, however, valuable information can be extracted based on the observed chemical shifts (δ_c), measured in parts per million from an external TMS reference.

It is known (5) that resonances in the range of 0–40 ppm arise from saturated aliphatic carbon types. The range of chemical shifts from 60 to 80 ppm is normally characteristic of saturated carbons bearing oxygen (5), while resonances in the 100–140 ppm range are due to carbons involved in C=C bonds. From 160 to 180 ppm, resonances for carboxylic acids and derivatives, such as esters, normally appear.

Additional information regarding the carbon environments can be obtained from the APT experiment (4). In this experiment carbons bearing one directly bonded hydrogen have the same phase as carbons with three directly bonded hydrogens, while resonances for CH_2 and nonprotonated carbons will appear ca. 180° out of phase. Figure 2 shows the *in vivo* APT spectrum of the third instar larvae, in which the resolution is enhanced over the spectrum in Fig. 1.

Near $\delta_c = 18$, there is a small positive peak which is likely a CH_3 group, since CH carbons do not appear at such low δ_c values (5). In the range of 22–38 ppm a large number of negative peaks appear, which can be assigned to CH_2 carbons and are probably due to the hydrocarbon chain in the lipid components of the larvae. Fully substituted (nonprotonated) carbons do not normally appear at such low δ_c values (5). From $\delta_c = 62$ to $\delta_c = 68$, there are at least three apparent types of CH_2—O carbons, two of which have about equal intensity. Several positive peaks appear in the range of 70–78 ppm, probably arising from CH—O type carbons, since CH_3 groups do not normally appear at such high δ_c values (5). Near $\delta_c = 133$, a positive peak appears, denoting a proton-bearing carbon in a C=C moiety. Since there are no negative resonances in the 100–140 ppm range, we suggest that all olefinic carbon types present to any significant extent have one attached proton. Finally, several small negative peaks appear in the 172–178 ppm range indicating nonprotonated carbons of carboxylic acids or their derivatives.

As noted in the introduction, *Eurosta* larvae which are cold acclimated build up high concentrations of glycerol and sorbitol which are deemed to act as cryoprotectants for their cells (1, 2). In the larvae studied here, extraction and analysis as previously described (2) gave measured levels of glycerol and sorbitol as 215.0 ± 10.0 and 110.0 ± 10.0 m*M*, respectively.

Such high concentrations will undoubtedly produce ^{13}C-NMR signals for these components. In an attempt to specifically identify resonances, ^{13}C-NMR spectra of authentic polyol samples were recorded. For glycerol

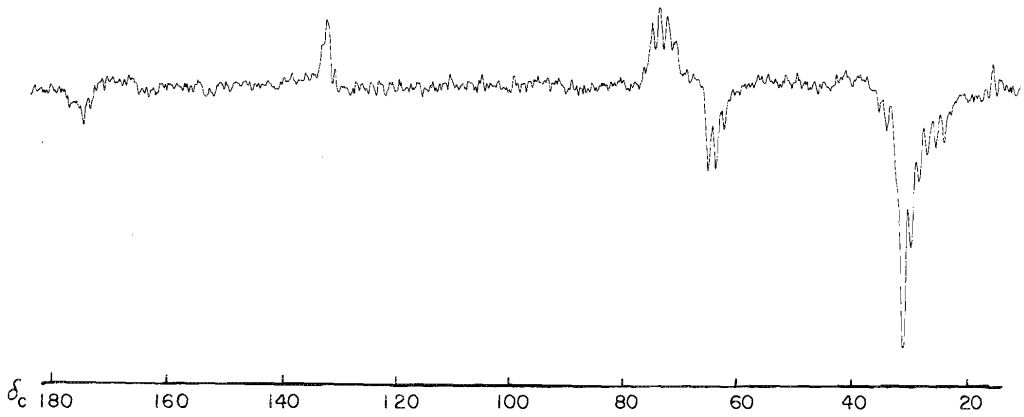

FIG. 2. ^{13}C APT NMR spectrum of living *E. solidaginis* larvae.

(250 m*M* in H_2O), two resonances were found at δ_c = 64.8 and 74.4, assigned to C-1,3 and C-2, respectively, in good agreement with literature ^{13}C data (6). (See Fig. 3 for a ^{13}C spectrum of glycerol and Fig. 4 for the APT spectrum.) For D-sorbitol, also known as hexitol or D-glucitol, in which the configuration is asymmetric, one ^{13}C-NMR signal for each of the six carbon atoms has been reported (7), although completely unambiguous distinction between resonances was not possible. Below is shown the structure of sorbitol with the ^{13}C chemical shifts determined previously (7).

	δ_c
$^1CH_2OH$	(65.7, 66.1)
H—^{2}C—OH	(74.3, 74.5)
HO—^{3}C—H	72.9
H—^{4}C—OH	(74.5, 74.3)
H—^{5}C—OH	76.1
$^6CH_2OH$	(66.1, 65.7)

Values in parentheses denote uncertain assignments. Our ^{13}C-NMR chemical shifts for D-sorbitol (120 m*M* in H_2O) are as follows: δ_c = 65.2, 65.4 (assignable to C-1/C-6 as before (6)); δ_c = 73.4 (two overlapping resonances from C-2/C-4 as before (6)); δ_c = 75.2 (assigned to C-5); and δ_c = 72.0 (C-3). (See Fig. 3 for the sorbitol ^{13}C spectrum and Fig. 4 for its APT spectrum.)

The other major components of the ^{13}C spectra shown in Figs. 1 and 2 are a saturated aliphatic carbon region (δ_c = 17–38 ppm), a C=C region ($\delta_c \approx 133$), and the carbonyl (ester) resonances around δ_c = 178 ppm. It is known (8) that animal lipids contain unsaturated fatty acid esters of glycerol, the two most abundant of these being derived from the unsaturated fatty acids oleic and palmitoleic acids.

$CH_3—(CH_2)_7—CH=CH—(CH_2)_7—COOH$
Oleic acid

$CH_3—(CH_2)_5—CH=CH—(CH_2)_7—COOH$
Palmitoleic acid

The remaining resonances in the ^{13}C spectra as previously described, as well as some of the additional complexity in the CH_2—O and CH—O region of the spectra, can be explained via the presence of glycerol esters of the illustrated fatty acids. The results of the APT experiment (*vide supra*) are consistent with terminal CH_3 groups, long $(CH_2)_n$ chains, one or more HC=CH, and several ester carbons as well as CH_2—O and CH—O resonances present in the lipids. Unfortunately, the spectral resolution does not permit unambiguous identification of which of the two triglycerol esters are present.

Support for the suggestion that the lipid components contribute substantially to the *in vivo* ^{13}C spectrum of the larvae comes from examination of the ^{13}C NMR of the chloroform–methanol extracted larvae. The chloroform layer should contain mainly lipid-type molecules, and the spectrum is shown in Fig. 5 (with chloroform and residual methanol responances subtracted out).

Comparison of this spectrum with the published ^{13}C NMR of the triglyceride of oleic acid (9) reveals many similarities. In neither case are all the CH_2 resonances resolved, but in Fig. 5 there is clearly a CH_3 group near δ_c = 18, the group of CH_2's in the range of δ_c = 20–38, a CH_2—O resonance near δ_c = 61, a CH—O resonance near δ_c = 68, two olefinic CH resonances near δ_c = 133, and ester resonances in the δ_c = 178 region.

In principle, one expects to observe a distinct ^{13}C resonance for each chemically unique carbon environment, but completely resolved ^{13}C spectra of such triglycerides await the use of NMR spectrometers operating at extremely high magnetic field.

The presence of triglycerides in the ^{13}C spectra of these larvae is consistent with the relatively high content

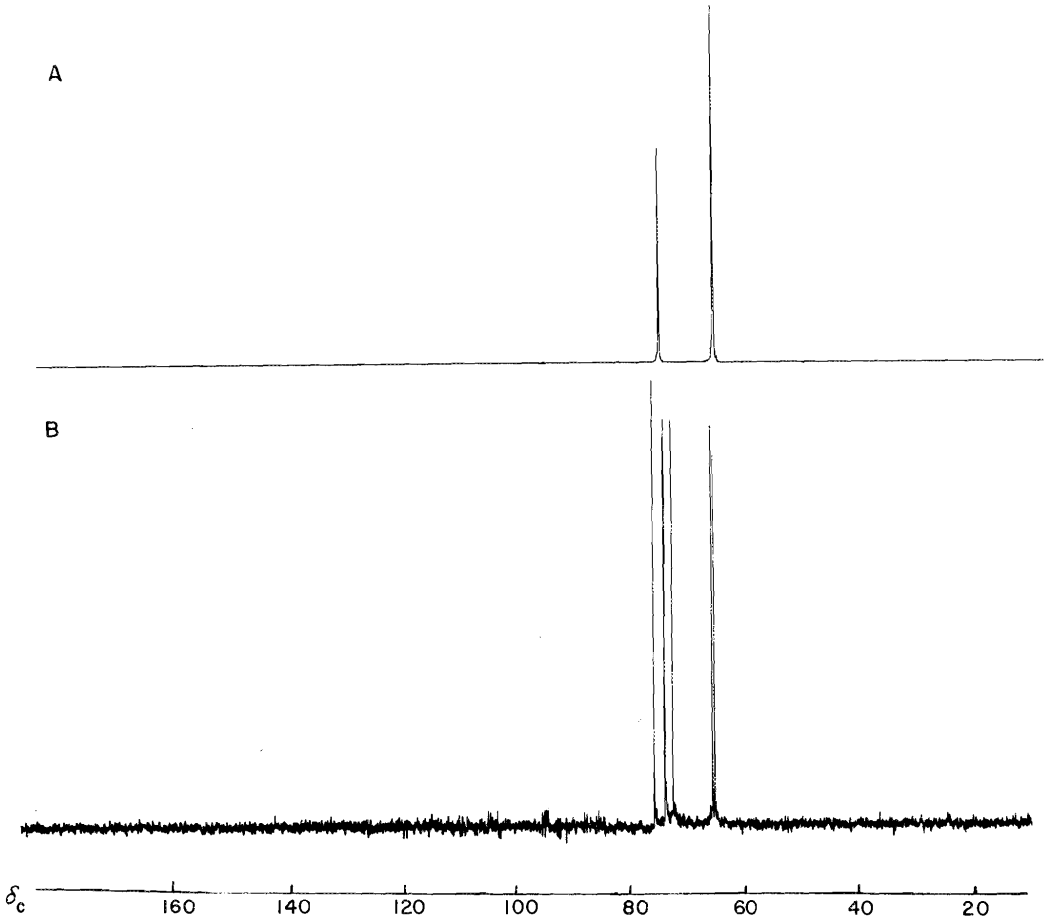

FIG. 3. ^{13}C-NMR spectra of glycerol (A) and sorbitol (B), both in H_2O solution.

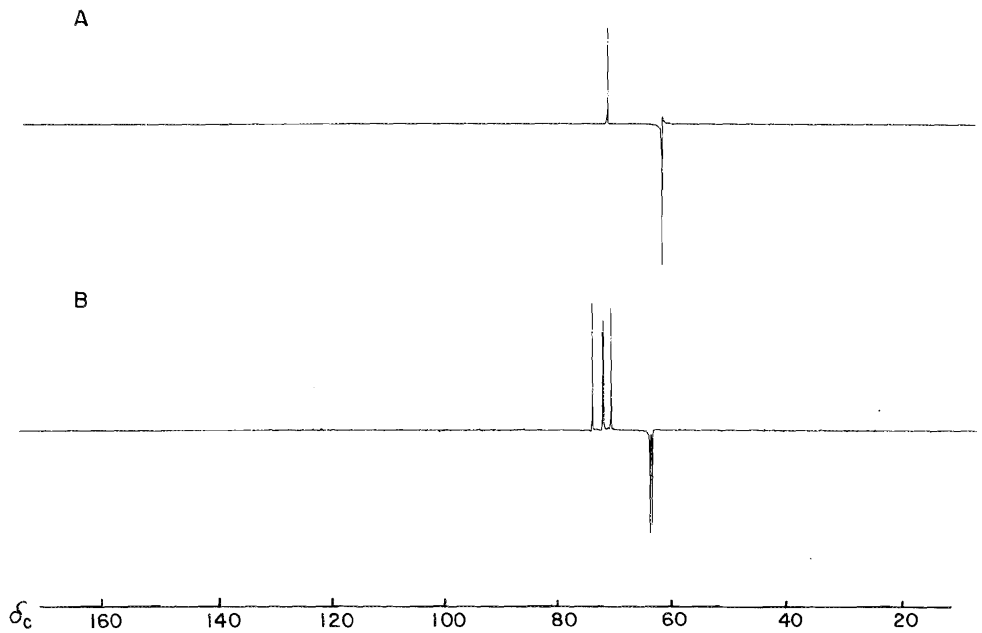

FIG. 4. ^{13}C APT NMR spectra of glycerol (A) and sorbitol (B).

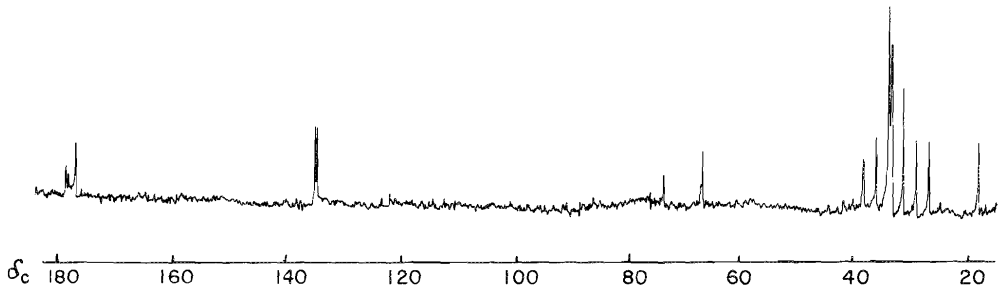

FIG. 5. ^{13}C-NMR spectrum of chloroform-soluble extract from *E. solidaginis* larvae.

(ca. 500 μmol fatty acid/g wet weight) previously reported (3). Very recently (10) ^{13}C NMR has been used to examine live cestodes. Many ^{13}C spectral features similar to the lipid fractions noted herein were found, as well as additional resonances near δ_c = 100 ppm attributed to the C-1 or C-1′ of glycogen.

Although analysis (3) of the present larvae indicates ca. 160 μmol glycogen (determined as glucose equivalent) per gram wet weight of larvae, there are no clear indications of resonances near δ_c = 100 in Figs. 1 or 2.

To summarize, it is clear that ^{13}C NMR represents an exciting method for detection of carbonaceous components in living organisms. The technique is nondestructive, as evidenced by a larval pupation rate of over 80% after completion of the NMR experiments. Routine use of the technique is envisaged to monitor qualitatively the presence of low molecular weight carbon containing cryoprotectants in other small overwintering organisms.

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