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DIFFERENTIAL HYDROGEN EXCHANGE DURING THE BIOSYNTHESIS OF FATTY ACIDS IN ANACYSTIS NIDULANS: THE INCORPORATION OF [2,2,2-$^{2}H_3$, 2-$^{13}C_{o;1}$] ACETATE[1]

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^{13}C NMR studies (with simultaneous ^{1}H, ^{2}H-decoupling) of ^{13}C, ^{2}H-labelled methyl palmitate showed a gradation of ^{2}H-retention along the acyl chain. The results are rationalized within the known steps of fatty acid biosynthesis.

The individual steps and overall pathway of fatty acid synthesis have been understood for some time [1, 2, 3]. Measurements of ^{3}H and ^{14}C incorporation during recent in vitro studies using chirally-labelled acetate [4] and malonate [5] demonstrated an overall stereospecificity in the process, and showed that a partial, non-specific exchange of hydrogen occurs after the dehydration step. Using ^{13}C nmr, we have measured incorporation of ^{13}C, and amounts of ^{2}H and ^{1}H bonded to ^{13}C, in the methyl ester 1 of palmitic acid produced by cultures of the prokaryotic alga Anacystis nidulans, which had been supplemented with sodium [2,2,2-$^{2}H_3$, 2-$^{13}C_{o;1}$] acetate (90% ^{13}C at C-2, >99% ^{2}H).

Cultures (30ℓ) were grown in the medium described by Kraty and Myers [6] under constant illumination. The precursor (30 m moles) was supplied 24 hrs after inoculation and the cells allowed to multiply for a further 60 hrs. A. nidulans produces only saturated and monoenoic acids [7] which were obtained in the usual manner following saponification of an organic extract of freeze-dried cells. Esterification with BF_3-methanol and Ag^{+} chromatography gave methyl palmitate (94% pure by GC analysis: mixture never contained more than 5% stearic acid).

The ^{13}C NMR spectrum (fig) of 1 (85 mg in 0.45 ml $C^{2}HCl_3/C_6F_6$: 6/1, Varian XL-100/15 F.T. spectrometer, 25.16 MHz, acquisition time 3.2s, flip angle 54°, ^{19}F internal lock, simultaneous broadband decoupling of ^{1}H and ^{2}H) showed supplementary peaks due to ^{2}H-induced isotope chemical shifts [8, 9]. Resonance assignments (table) agreed with those previously obtained for corresponding carbons of methyl stearate [9] after correction for slight downfield shifts due to C_6F_6. Nuclear Overhauser enhancements and saturation effects were suppressed by the relaxation reagent [10] Cr $(acac)_3$ (10 mg/ml solvent) and by removal of the ^{1}H decoupling field for 6.8s and 26.8s between data acquisition periods [11]. Fractions of ^{13}C bonded to ^{2}H and ^{1}H, and ^{13}C enrichments (table) were measured from integrals, enhanced resonances being compared with well-separated peaks known to correspond to ^{13}C at natural abundance (C-1, C-3, C-15, OCH_3). In spectra of 1 at natural abundance, under identical conditions, all resonances were of equal intensity (relative error ±0.07).

The ^{13}C enrichments of the labelled material followed the expected pattern: evenly-numbered carbons only were enriched, and the resonances of C-2 and C-14, which were uniformly enriched with ^{13}C, contained a single isotopically shifted signal indicating that each of them bore not more than one deuterium atom. The resonance of C-16 had three isotopically-shifted components corresponding to the species $^{13}C^{1}H_2{}^{2}H$, $^{13}C^{1}H^{2}H_2$ and $^{13}C^{2}H_3$, in addition to the $^{13}C^{1}H_3$ resonance (fig).

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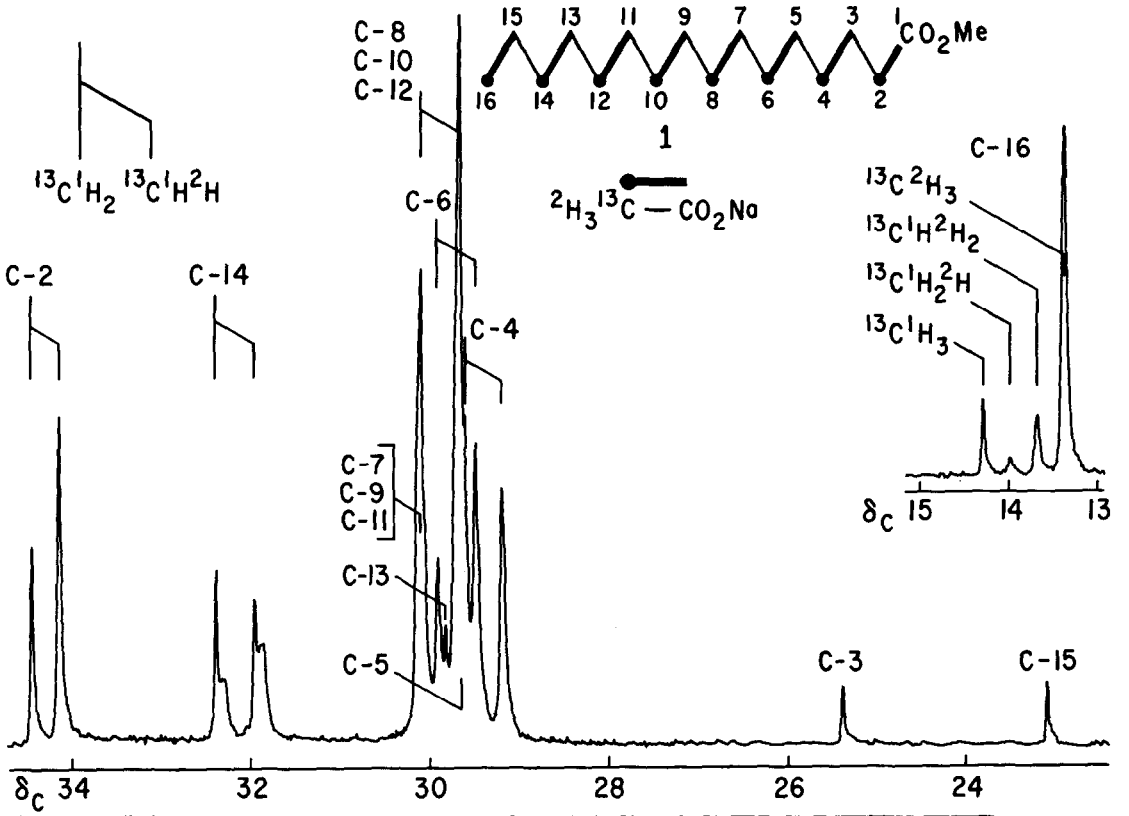

Carbon	δc* ppm	Δδc ppm	Total % ^{13}C§	Probability of ^{13}C‡'' from precursor	Proportions of labelled species†¶ $^{13}C^1H^2H$	$^{13}C^1H_2$
C-2	34.35	0.30	12.2	0.112	0.73	0.27
C-4	29.53	0.41	12.7	0.117		
C-6	29.83	0.42	12.8	0.118	0.69	0.31
C-8 to C-12	29.98, 30.02	0.39	12.7**	0.117	0.66	0.34
C-14	32.30	0.43	12.3	0.113	0.63	0.37
Average C-2 to C-14**			12.6	0.116		

					$^{13}C^2H_3$	$^{13}C^1H^2H_2$	$^{13}C^1H_2{}^2H$	$^{13}C^1H_3$
C-16	14.23	0.31, 0.60, 0.91	14.7	0.138	0.79	0.14	0.04	≤ 0.03

* ^{13}C chemical shift referenced to internal $(CH_3)_4$ Si, no $Cr(acac)_3$ added to solution. Unenriched carbons are not included; their chemical shifts δc are OCH_3 51.47, C-1 174.48, C-3 25.30, C-5 29.61, C-7 to C-11 29.98 to 30.02, C-13 29.73, C-15 23.02 ppm. Solvent C^2HCl_3/C_6F_6 6/1. Error ±0.01 ppm.

† Magnitude of upfield ^2H-induced isotope shift. Error ±0.02 ppm.

§ Including natural-abundance material. $Cr(acac)_3$ added to solution. Average of 2 spectra, absolute error ±2.0% due mainly to errors in measuring integrals of weak natural-abundance peaks (C-3, C-15, C=O, OMe). Comparative error of two ^{13}C enrichments < ca. ± 0.6%, except C-4, C-6 ca. ± 1.3%.

‡ After subtraction of contribution from natural-abundance material; average of 2 spectra.

'' Comparative error ca. ± 0.006, except C-4, C-6 ca. ± 0.013.

¶ Error ±0.02. Proportion for C-4 not measurable due to overlap; assumed 0.71/0.29 for estimating $\%^{13}C$.

** Allowing for signals due to 4.9% methyl stearate, assumed to have some ^{13}C enrichment from C-8 to C-14 as methyl palmitate from C-8 to C-12.

All components of these ^{13}C resonances had "shoulders" to high field, due to isotope shifts produced by ^{2}H atoms three bonds removed (ca. 0.02 ppm if a $^{13}C^{1}H^{2}H$ group is two bonds removed; 0.09 ppm if $^{13}C^{2}H_3$). From the fractional intensity of the shoulders (estimated for the C-2, C-4 and C-14 resonances where they were most clearly resolved, and allowing for splitting due to ^{13}C-^{13}C coupling $^{2}J_{cc}$ 0.9 ± 0.2 Hz) the probability of adjacent incorporation of (^{13}C, ^{2}H)-labelled units was calculated to be 58 ± 10%. This is considerably larger than the average ^{13}C probability for the sample (ca. 13%, table) and indicates that highly-enriched fatty acids had been diluted with material at natural abundance.

The probability that ^{13}C is bonded to ^{2}H at labelled positions in the fatty acid chain can be calculated from the fraction of the total signal intensity arising from ^{13}C directly bonded to ^{2}H, and therefore giving an isotopically-shifted resonance. At C-16, the predominant species is $^{13}C^{2}H_3$ (table), and the loss of ^{2}H is 10 ± 3% (100 x [1-(0.79 + $\frac{2}{3}$ x 0.14 + $\frac{1}{3}$ x 0.04)], table) demonstrating that little of the original labelled acetate had undergone $^{1}H \rightleftharpoons {}^{2}H$ exchange before incorporation. Hydrogen exchange during fatty acid biosynthesis has been observed in previous studies [4, 5]. In our experiments, acetyl coenzyme A $\rightleftharpoons$ malonyl coenzyme A interconversion [12] could be responsible for some or all of the 10 ± 3% loss of ^{2}H at C-16, where acetate is incorporated, in which case at least this degree of exchange would also have occurred at other labelled positions, which arise from the incorporation of malonate. The successively smaller amounts of $^{13}C^{1}H^{2}H_2$, $^{13}C^{1}H_2{}^{2}H$ and $^{13}C^{1}H_3$ (excluding $^{13}C^{1}H_3$ from natural abundance material) at C-16, together with the similarity of ^{13}C enrichment at C-16 compared to other labelled positions, is evidence that the malonate pool is small and rapidly incorporated into fatty acids after formation from acetate, with little chance of reconversion to acetate and uptake of ^{1}H from the medium. The slightly higher incorporation of ^{13}C at C-16 compared to all other carbons (table) is to be expected, since the concentration of ^{13}C in the acetate pool should always exceed that in the malonate pool.

The variation in ^{2}H-loss with length of the acyl chain (table) is more difficult to explain. It could not be accounted for by exchange of methylene protons in malonyl thioesters which, under physiological conditions, is slow on the time-scale of fatty acid synthesis [5, 13]. The possibility of hydrogen exchange *via* an intermediate in the condensation reaction having a methine hydrogen activated by three adjacent carbonyl groups, has been rejected by Sedgwick and Cornforth [4] because of evidence for a concerted condensation mechanism [13]. Arnstadt et. al., [13] have also excluded the possibility of hydrogen exchange with the solvent (water) at the β-ketoacyl thioester stage. Cornforth's group [4, 5] concluded that "post-malonate" exchange, which has been noted in cell-free preparations from a variety of sources [14], occurs after the dehydration step, and *our results show that in A. nidulans this exchange is dependent on chain length*. The only known chain-length dependent step in fatty acid synthesis is the reversible transfer of the growing acyl chain from the acyl carrier protein (ACP) to a cysteine residue of β-keto acyl ACP synthetase [1]. While on the cysteine residue hydrogen exchange may occur, perhaps by activation of the carbonyl group and enolization of the adjacent methylene group. Such a mechanism would show a kinetic isotope effect as observed with "post malonate" exchange in yeast [4]. Our results would be explained by a steady decrease in residence time at the cysteine residue with increasing chain length, and a consequent reduction in ^{2}H-loss. This is comparable to the variation in activity found for β-keto acyl ACP synthetase from *E. Coli* [15]. The markedly lower degree of exchange at

C-16 implies a shorter residence time, and/or a slower rate of ^{2}H-loss, for an acetyl group.

It is noteworthy that the chain-length distribution of saturated fatty acids in A. nidulans is consistent with a model [16] describing such distributions for saturated acids produced by yeast. In this model, the likelihood of removal of the acyl chain from the ACP to coenzyme A by a transferase mechanism increases greatly after a chain length of 14 carbon atoms. Our explanation of ^{2}H-loss requires that, in A. nidulans, such a competing reaction has little effect on the residence time of the acyl chain at the cysteine residue, since there is relatively little difference in ^{2}H-loss between C-2 and other labelled carbons. Hence the rate of removal by the appropriate acyl-transferase is always lower than the rate of transfer to the cysteine residue.

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