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REGIOCHEMICAL DISTRIBUTION OF DEUTERIUM DURING FATTY ACID BIOSYNTHESIS FOLLOWING INCORPORATION OF [2-^{13}C, 2-$^{2}H_3$] ACETATE

A ^{13}C NMR STUDY OF EXCHANGE, DESATURASE AND ENOYL REDUCTASE STEREOSPECIFICITIES IN THREE ALGAE AND A YEAST†

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Abstract—^{13}C NMR analysis of [^{13}C, ^{2}H]-enriched fatty acids from cultures of *Saccharomyces cerevisiae* (yeast), *Anacystis nidulans* (blue-green alga), *Chlorella pyrenoidosa* (green alga) and *Phaeodactylum tricornutum* (diatom) has established the regiochemical distribution of ^{13}C–^{2}H bonds, the efficiency of ^{2}H incorporation at labeled sites, the chiral purity of $^{13}C^{1}H^{2}H$ groups, enoyl reductase and desaturase stereospecificities, and that hydrogen exchange during fatty acid biosynthesis is stereospecific. Possible mechanisms for this exchange are discussed.

Synthesis of long-chain fatty acids *de novo* from acetate[1] requires prior activation of the latter through formation of acetyl-CoA[2] and subsequent participation of two well-characterized enzyme systems, acetyl-CoA carboxylase and fatty acid synthetase. The synthetase, primed with a molecule of acetyl-CoA, utilizes successive molecules of malonyl-CoA, generated by acetyl-CoA carboxylase, in a repeating series of condensation-reduction-dehydration-reduction reactions to yield the fatty acids.[3] Stereochemical studies of these reactions in yeast[4–7] have shown that the acetyl-CoA carboxylase step proceeds with retention of configuration, and the formation of β-ketothiolester with inversion. Furthermore, as dehydration of (3*R*)-hydroxyacyl thiolester, formed on reduction of β-ketothiolester,[8,9] involves *syn* elimination of water, it follows that it is the *pro-S* hydrogen of malonyl-enzyme which is retained at C-2 of enoyl thiolester.

There is a growing consensus that the preceding reactions are characteristic of fatty acid biosynthesis in all cells. In contrast, recent evidence suggests that *trans*-2-enoyl thiolester reductases can utilize different prochiral hydrogens of NADPH and differ in the stereochemistry of reduction. Reductases from yeast, *E. coli* and *B. ammoniagenes* use the *pro*-4*S* hydrogen of NADPH[10–12] or NADH[11] and add hydrogen by 2*si*-3*si*,[7] 2*re*–3*si*[13,14] and 3*si*[15] attack, respectively. On the other hand, the corresponding enzyme from rat liver utilizes the *pro-R* hydrogen of NADPH[16] for insertion at C-3 by *re* attack,[15] the stereochemistry of hydrogen addition at C-2 of enoyl thiolester, as in the case of *B. ammoniagenes*,[15] remaining as yet undetermined. Three (*si–si*, *re–si* and *si–re* or *re–re*) of the four possible modes of addition of hydrogen to enoyl thiolester have therefore been observed. Most of the above information was obtained from *in vitro* studies with purified enzymes or cell-free extracts. In the only *in vivo* study[14] a desaturase of known stereospecificity from another organism was used to derive configurations of isotopic labels.

Although these studies established the positions and stereochemistries of H-isotopic labels incorporated into fatty acids from acetate, malonate, pyridine nucleotides and water at a limited number of sites, they provided no information on the concentrations of incorporated isotope throughout the molecule. Our recent study[17] showing ^{2}H retention at labeled sites in palmitic acid enriched from [2-^{13}C, 2-$^{2}H_3$] acetate by *A. nidulans*, was the first attempt to fill this gap in the literature.

An intriguing and unexplained feature of the work of Sedgwick *et al.*[4,5] was the unexpected loss of 19–36% of H-isotope at carbons derived from chirally labeled malonate, by an exchange process exhibiting a kinetic isotope effect similar to that observed for enolizations. The process was named "post-malonate" exchange because the loss of isotope was in addition to that observed for malonyl-CoA. Because chiral substrates were used, Sedgwick *et al.* were able to restrict possible mechanisms to (i) a stereospecific exchange process occurring prior to the dehydration step and involving the hydrogen which is not lost during dehydration of (3*R*)-hydroxyacyl thiolester, or (ii) exchange at C-2 of acyl-enzyme, which if not stereospecific (as they preferred) would entail epimerization of hydrogen label at these sites. This loss of isotope was also an important aspect of our work on palmitic acid enriched from [2-^{13}C, 2-$^{2}H_3$] acetate by *A. nidulans*[17] and was also noted by White[14] in his study on fatty acids labeled with [2-$^{2}H_3$] acetate by *E. coli*.

In this study we report the regiochemical distribu-

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Table 1. Fatty acid methyl esters from yeast and algae enriched from [2-^{13}C, 2-^{2}H$_3$] acetate (90% ^{13}C; ≥ 98% ^{2}H): percentage of $^{13}C^{2}H$ or $^{13}CH^{2}H$ species and % ^{13}C from labelled precursor (underlined) after subtraction of ^{13}C from natural abundance materials, percentage of ^{2}H retained at methyl groups and distributions of labelled species at methyl groups, average % ^{13}C from labelled precursor[a]

Organism:	*Saccharomyces cerevisiae* Yeast				*Anacystis nidulans* Alga				*Chlorella pyrenoidosa* Alga			*Phaeodactylum tricornutum* Alga		
[k] Fatty acid n:m	[e] 16:1	16:1	[f] 16:0	[f] 18:0	[e] 16:0	[f] 16:0	[h] 16:1	[f] 18:1	[e] 16:0	18:2	[e] 18:3	14:0	16:0	16:1
Labelling expt:	1st	2nd	3rd	3rd	1st	2nd	2nd	2nd	1st	1st	1st	1st	1st	1st
[i] Amount of sample:	14 mg	2 mg	8 mg	3 mg	85 mg	150 mg	70 mg	30 mg	16 mg	<5 mg	<5 mg	<5 mg	2 mg	2 mg
Carbon position	Percentage of $^{13}C^{2}H$ or $^{13}CH^{2}H$ species and %^{13}C from labelled precursor (underlined). Vertical bars indicate averages													
n-16				[l] 62 1.02				62 16		33 0.9	48 2.0			
n-14	64 38	59 [l] 0.93	62 29		73 12.2	69.4 20.5	72 33	[q] 33	43 1.4	[c] 57 0.6	41 2.1		60 0.8	48 1.3
n-12	66 42	67 [l] 0.95		70			67 36					49 1.3		36 1.5
n-10			72	[l] 75 1.25	69 12.8	67.5 ±2.0		70 30		58 0.4 av. with C-14	49 1.9			
n-8	75 41	76 [l] 1.02	77 31			65.2 22.0	64 33	61 33	48 1.4	[d] 45 0.6	[b] 46 2.0	[g] 1.1	[g] 1.1	46 1.5
n-6	[j]$_2$ 42	[j]$_2$ [l] 0.95			66 12.7		64 33	[q] 33		av. with C-8				42 1.2
n-4	79 41	71 [l] 0.99					69 33			58 0.4	51 2.0			38 1.6
n-2	40 40	44 [l] 0.98	40 29	39 [l] 1.06	63 12.3	59 21.2	60 32	59 32	60 1.5	51 0.4	51 1.9	48 0.6	43 0.7	50 1.0
n (methyl)	90 42	90 [l] 1.00	88 29	88 [l] 1.00	90 14.7	91.1 22.0	91 35	90 34	91 1.6	90 1.2	95 2.0	77 0.9	81 0.6	89 0.9
[p] Error	±2 ±2	±2.5 ±0.05	±2 ±1	±3 ±0.15	±2 ±0.6	±0.6 ±1.0	±1.5 ±3	±2 ±2	±5	±0.4 ±20	±0.4 ±8	±0.4 ±15	±0.5 ±10	±0.4 ±10 ±0.4

CH_3	0.01	0.0	0.02	0.02	0.03	0.013	0.02	0.02_0	0.0	0.0	0.0	0.03	0.03	0.0
$CH_2{}^2H$	0.05	0.05	0.06	0.07	0.04	0.044	0.04	0.05_2	0.03	0.0	0.04	0.13	0.12	0.08
CH^2H_2	0.18	0.21	0.20	0.19	0.14	0.141	0.13	0.12_3	0.18	0.3	0.09	0.33	0.22	0.17
C^2H_3	0.76	0.75	0.73	0.73	0.79	0.802	0.81±0.04	0.80_4	0.79	0.7	0.87	0.51	0.63	0.75
Error	±0.02	±0.03	±0.02	±0.03	±0.03	±0.010	±0.02	±0.02	±0.06	±0.2	±0.08	±0.15	±0.10	±0.10
Av. % ^{13}C from precursor	41	>20	30	>25	12	21.8	33	C-2:16 Others:32	1.5	0.6	2.0	1.0	0.9	1.3
Error	±8		±7		±2	±1.0	±4	±2	±0.3	±0.4	±0.5	±0.3	±0.3	±0.4

a/ Large absolute error in measurement of ^{13}C enrichment is due to difficulty in obtaining integrals accurately for signals from carbons at natural abundance (e.g. C-3, OMe). Comparitive enrichments of evenly numbered carbons may be more accurately measured (see footnotes ℓ and p).

b/ Isotopically-shifted resonance of C-12 $^{13}C^2H$ species overlaps C-10 ^{13}CH resonance. Figures in table obtained by assuming that C-13 resonance (which overlaps C-12 ^{13}CH resonance) has the average intensity for unlabelled sites, and that F_D is the same for C-10 and C-12.

c/ Resonances overlap.

d/ Isotopically-shifted resonances definitely present but not resolved. Average intensity used.

e/ Average measured from 2 spectra.

f/ Averages measured from 3 spectra.

g/ Not measurable due to low enrichment and small amount of compound.

h/ Averages measured from 4 spectra.

i/ Samples (amounts shown above) were dissolved in 0·2 to 0·5 ml of C^2HCl_3/C_6F_6 under conditions similar to those given in refs 17 and 30. (^1H,^2H-broadband decoupling, relaxation reagent added, nuclear Overhauser effect suppressed). Where enrichment was poor and amount of sample small (e.g. C. pyrenoidosa and P. tricornutum acids) the delay between acquisitions was reduced to 1·6 s to improve sensitivity.

j/ Barely detectable signal coinciding with expected position of C-10 $^{13}C^2H$ resonance.

k/ n = number of carbons, m = number of double bonds.

ℓ/ Absolute % ^{13}C could not be measured; ^{13}C enrichments relative to the terminal methyl carbon are shown.

p/ Error for comparison of enrichments at different positions within one sample.

q/ Not measureable due to overlap of resonances.

tion and concentration of deuterium in saturated and unsaturated fatty acids enriched from [2-^{13}C, 2-$^{2}H_3$] acetate by *Saccharomyces cerevisiae*, *Anacystis nidulans*, *Chlorella pyrenoidosa* and *Phaeodactylum tricornutum*. In addition the study provides information on the stereospecificities of the desaturases and enoyl reductases in these organisms as well as the origin of "post-malonate" exchange.[4,5] This information was obtained by locating and measuring the concentration of ^{13}C–^{2}H bonds at even-numbered carbons displaying isotopically shifted resonances in the ^{13}C NMR spectra recorded with simultaneous ^{1}H- and ^{2}H-broadband decoupling, and by using CD measurements[18] to identify the chirality of monodeuteromethylene groups at C-2 of labeled saturated fatty acids.

RESULTS AND DISCUSSION

^{2}H *Distribution*. Unequivocal evidence for survival of intact chemical bonds throughout a biochemical process cannot be obtained by radiotracer experiments or by studies using precursors singly-labeled with a stable isotope. Correlated labels in a metabolite derived from a multiply-labeled precursor are an absolute requirement. They can be monitored by spin–spin coupling and isotopic chemical shift effects in NMR, or with greater difficulty, by mass spectrometry. We have identified the locations of ^{13}C–^{2}H bonds, and the number at each site, from the presence of isotopically-shifted resonances in the [^{1}H, ^{2}H]-broadband-decoupled ^{13}C spectra of fatty acids enriched from [2-^{13}C, 2-$^{2}H_3$] acetate. Furthermore, after subtracting natural ^{13}C-abundance contributions at enriched positions, the percentage of the theoretical maximum number of ^{13}C–^{2}H bonds which have survived in the fatty acids is given by the intensity ratio 100 $^{13}C^{1}H^{2}H/(^{13}C^{1}H^{2}H + {}^{13}C^{1}H_2)$ for methylene groups and 100 $(x + 2/3y + 1/3z)$ for methyl groups, where x, y and z are the fractional intensities of the isotopically-substituted species $^{13}C^{2}H_3$, $^{13}C^{1}H^{2}H_2$ and $^{13}C^{1}H_2{}^{2}H$, respectively Table 1).

The presence of $^{13}C^{2}H_3$, $^{13}C^{1}H^{2}H_2$ and $^{13}C^{1}H_2{}^{2}H$ species in addition to $^{13}C^{1}H_3$ at the Me groups of the fatty acids, and the retention of single ^{13}C–^{2}H bonds as well as uniform ^{13}C-labeling at the even numbered methylene groups, are characteristic features of the data in Table 1. Moreover, the uniform ^{2}H loss at the Me groups (*ca* 10%), combined with the similarity of ^{13}C-enrichment at Me groups compared with other positions and the successively smaller amounts of $^{13}C^{1}H^{2}H_2$, $^{13}C^{1}H_2{}^{2}H$ and $^{13}C^{1}H_3$, is evidence that the malonyl–CoA pool in these organisms is small and rapidly incorporated into the fatty acids. This evidence shows there is little chance of reconversion to acetyl–CoA and uptake of ^{1}H from the medium *via* the acetyl–CoA carboxylase reaction.[19] It is also clear that ^{2}H loss varies in amount, and along the chain length, in a way characteristic for each organism. The reproductibility of the quantitative data obtained for palmitoleic acid from yeast and palmitic acid from *A. nidulans* is particularly noteworthy as these data were obtained from experiments carried out months apart.

^{2}H *Chirality and enoyl reductase stereospecificity*. Recent CD studies on 2*S*- and 2*R*-deuteromonocarboxylic acids have shown that they exhibit, respectively, positive and negative Cotton effects with the maximum at 209 nm, due to the larger contribution of C–H as compared to C–^{2}H bonds.[18] In our study, this approach was used to provide direct evidence for the chirality of the centres retaining ^{2}H in the saturated fatty acids biosynthesized from [2-^{13}C, 2-$^{2}H_3$] acetate by yeast and three algae. This in turn was related to the stereochemistry of the enoyl reductase reaction at C-2, and hence the other even-numbered chiral deuteromethylene groups. As previous work on other chiral acids and their esters had established that derivatization had little effect on the sign of Cotton effects[20] we carried out the CD measurements on the methyl esters of the fatty acids isolated by GLPC, in order to avoid the possibility of racemization of the label during hydrolysis to the free acid.

CD curves with reasonably high peak amplitudes were obtained (Table 2) from the highly [^{13}C, ^{2}H]-enriched samples of palmitic acid from yeast and *A. nidulans*, whereas the corresponding samples from *C. pyrenoidosa* and *P. tricornutum*, with lower isotopic enrichments, gave only weak reproducible bands relative to baselines obtained from standard solutions of unlabeled acid recorded under the same conditions. The sign of Cotton effects in Table 2 establishes that the ^{2}H of the monodeuteromethylene groups at C-2 in the palmitic acids from the three algae has a *pro-S* configuration and, in the acid from yeast, a *pro-R* configuration. The absolute magnitudes of the molar ellipticities for the samples of palmitic acid from yeast and *A. nidulans*, corrected on the basis of [^{13}C, ^{2}H]-enrichments, are in good agreement with the value $[\theta]_{209} = -116$ reported for (2*R*)-deuteropalmitic acid.[18] This suggests that extensive racemization of chiral centres in the labeled acids of these organisms had not occurred and would seem to confirm the proposition that esterification of the acids would not significantly affect the magnitude of molar ellipticities.[20] Consequently we can conclude that in each algal sample of palmitic acid the *pro*-2*R* hydrogen, originating from the medium, was added by *re*-attack during the enoyl reductase step. By contrast in yeast, the corresponding hydrogen in palmitic acid is inserted in the *pro*-2*S* configuration following *si*-attack. The latter result confirms the conclusions on the specificity of yeast enoyl reductase reached by Sedgwick *et al.*,[7] whereas the former is reminiscent of the behaviour of the enzyme from *E. coli*.[13,14]

Desaturase stereospecificity. As we have shown that the ^{2}H at the chiral deuteromethylene groups have a *pro-R* configuration in saturated fatty acids from yeast and a *pro-S* configuration in those from the three algae, it is possible to deduce the stereospecificities of the desaturases in each organism from the presence or absence of ^{2}H at olefinic carbons in the labeled unsaturated fatty acids. Thus the almost complete absence of ^{2}H at C-10 of palmitoleic acid from yeast and the retention of this label at all similar sites in the unsaturated acids from the three algae establishes that the desaturases in all of these organisms stereospecifically remove the *pro-R* hydrogen from even-numbered positions of the saturated fatty acids (Table 1). Interestingly, desaturases from *Corynebacterium diphtheriae*,[21] the green alga *Chlorella vulgaris*[22,23] hen[23] and pig[24,25] liver also remove *pro-R* hydrogens from adjacent carbons, and indeed

Table 2. Molar ellipticities $[\theta]_{209}$ of ^{13}C, ^{2}H-enriched palmitic acid from algae and yeast in isooctane

Origin	Concentration (ML^{-1})	$[\theta]_{209}$ (deg-cm²/decimole)	Corr. $[\theta]_{209}$‡	Chirality at C-2
S. cerevisiae	9.61×10^{-2}	-27±2	-150±11	R
A. nidulans	12.55×10^{-2}	+19±2	+134±14	S
C. pyrenoidosa	11.27×10^{-2}	+*	---	S
P. tricornutum	6.50×10^{-2}	+*	---	S

‡ Corrected for ^{13}C, ^{2}H-enrichments from $[\theta]_{209}/xy$ where $[\theta]_{209}$ is experimental value and x is the % ^{13}C and y the $^{13}C^{1}H^{2}H/(^{13}C^{1}H^{2}H+^{13}C^{1}H_{2})$ intensity ratio due to isotopic enrichment at C-2 of palmitic acid (see Table 1)

* Gave weak positive bands relative to baselines obtained from solutions of unlabeled acid recorded under the same conditions.

the collective evidence at this stage would suggest that all desaturases, irrespective of their origin, will exhibit the same stereospecificity. If this is true, the stereospecificity of an enoyl reductase could be deduced from the presence or absence of resonances for olefinic deuteromethine groups in the ^{13}C spectra of unsaturated acids enriched from [2-^{13}C, 2-$^{2}H_3$] acetate. The presence, or absence, of an olefinic ^{13}C–^{2}H bond would correspond to insertion of hydrogen by *si* or *re* attack at C-2 of enoyl thiolester, respectively. Indeed this approach could have been used to determine enoyl reductase stereospecificity in *C. pyrenoidosa*, without recourse to configurational studies with circular dichroism, because the desaturase stereospecificity in this organism would be expected to be identical to that observed for *C. vulgaris*.[22,23]

Isotopic exchange. A comparison of the fraction of the theoretical maximum number of ^{13}C–^{2}H bonds retained at olefinic and methylene groups provides information on the chiral purity of monodeuteromethylene groups in the fatty acids (Table 1). A high degree of chiral purity is indicated for the fatty acids of yeast because of the high retention of ^{13}C–^{2}H bonds at labeled methylene groups and the lack of such bonds at C-10 of palmitoleic acid. Racemization of chiral monodeuteromethylene groups would result in retention of ^{13}C–^{2}H bonds at unsaturated positions. Comparable ^{2}H-retention at unsaturated and saturated sites in the acids from the three algae leads to the same conclusion; racemization in these cases would lead to a lower proportion of ^{13}C–^{2}H bonds at the olefinic centers.

In our study, the fraction of ^{13}C–^{2}H bonds lost by "post malonate" exchange at sites derived from [^{13}C, ^{2}H]-enriched malonate in palmitoleic acid from yeast can be calculated as follows from the duplicate results given in Table 1. It is obvious that the average proportions of isotopic species at C-16 [$^{13}C^{2}H_3$: $^{13}C^{1}H^{2}H_2$:$^{13}C^{1}H_2{}^{2}H$:$^{13}C^{1}H_3$ = 0.75:0.19:0.05:0.01] reflects the species of acetyl–CoA from which malonyl–CoA is formed by carboxylation. This reaction is known to exhibit a kinetic isotope effect $k_{1H}/k_{2H} = 1.15$.[4] Thus the proportions of $HO_2C^{13}C^{2}H_2COSCoA$, $HO_2C^{13}C^{1}H^{2}HCOSCoA$ and $HO_2C^{13}C^{1}H_2COSCoA$ derived from the labeled acetyl-CoA is calculated to be $[0.75 + 0.19/(1 + 2k_{2H}/k_{1H})] = 0.819$, $[(0.19 \times 2k_{2H}/k_{1H})/(1 + 2k_{2H}/k_{1H}) + (0.05 \times 2)/(2 + k_{2H}/k_{1H})] = 0.156$ and $[(0.05 \times k_{2H}/k_{1H})/(2 + k_{2H}/k_{1H}) + 0.01] = 0.025$ respectively. The fraction of the theoretical maximum number of ^{13}C–^{2}H bonds retained in [^{13}C, ^{2}H]-enriched malonyl–CoA is therefore 89.7% [100 (0.819 + 0.156/2)], which is higher than the retention of 89.3% [100 (0.75 + 2 × 0.19/3 + 0.05/3] in acetyl–CoA because of the kinetic isotope effect operating in the carboxylation reaction.

The degree of nonstereospecific exchange of ^{2}H at C-2 of the [^{13}C, ^{2}H]-labeled malonyl–CoA can now be calculated assuming $k_{1H}/k_{2H} = 5$ for both inter- and intramolecular kinetic isotope effects, and the conditions used by Sedgwick *et al.*, *viz*: 25% exchange of ^{1}H in 5 min and a mean lifetime for malonyl–CoA of 2.5 min (p. 476 Ref. 4). For $HO_2C^{13}C^{2}H_2COSCoA \rightarrow HO_2C^{13}C^{1}H^{2}HCOSCoA$ this would be $(0.819 \times 0.25 \times 2.5/5 \times 1/5) = 0.02$ and for $HO_2C^{13}C^{1}H^{2}HCOSCoA \rightarrow HO_2C^{13}C^{1}H_2COSCoA$ $(0.156 \times 0.25 \times 2.5/5 \times 1/5) = 0.004$. The fraction of the theoretical maximum number of ^{13}C–^{2}H bonds retained in [^{13}C, ^{2}H]-labeled malonyl–CoA after $^{1}H/^{2}H$-exchange at C-2 would therefore be $100[0.819 - 0.02 + (0.156 - 0.004 + 0.02)/2] = 88.5\%$ and half of these bonds would be lost subsequently during the dehydration step in fatty acid biosynthesis. Thus, in the absence of further exchange 44.25% of the ^{13}C–^{2}H bonds would be retained at sites of palmitoleic acid derived from malonyl–CoA, so we may assume that this would also be true for the corresponding sites in palmitic acid from which palmitoleic acid is formed. The ^{13}C NMR intensity ratio $^{13}C^{1}H^{2}H/(^{13}C^{1}H^{2}H + ^{13}C^{1}H_2)$ corresponding to this retention would be 88.5% as $^{13}C^{1}H^{2}H/2(^{13}C^{1}H^{2}H + ^{13}C^{1}H_2) = 44.25\%$. The experimental intensity ratio for C-10 of palmitic acid might be expected to be close to the average value of 75.3% [(75 + 76 + 79 + 71)/4] observed for C-8 and C-12 of palmitoleic acid, which agrees well with the average result of 77% actually observed for C-4, C-8, C-10 and C-12 of palmitic acid (Table 1). Loss of ^{13}C–^{2}H bonds due to a "post-malonate" exchange process at C-10 of palmitic acid was therefore 88.5 − 75.3% = 13.3%. It should be noted that the close agreement between intensity ratios for corresponding sites in palmitoleic, palmitic and stearic acid from yeast clearly establishes that they had a

common biosynthetic history (a property also shared by the fatty acids of the three algae).

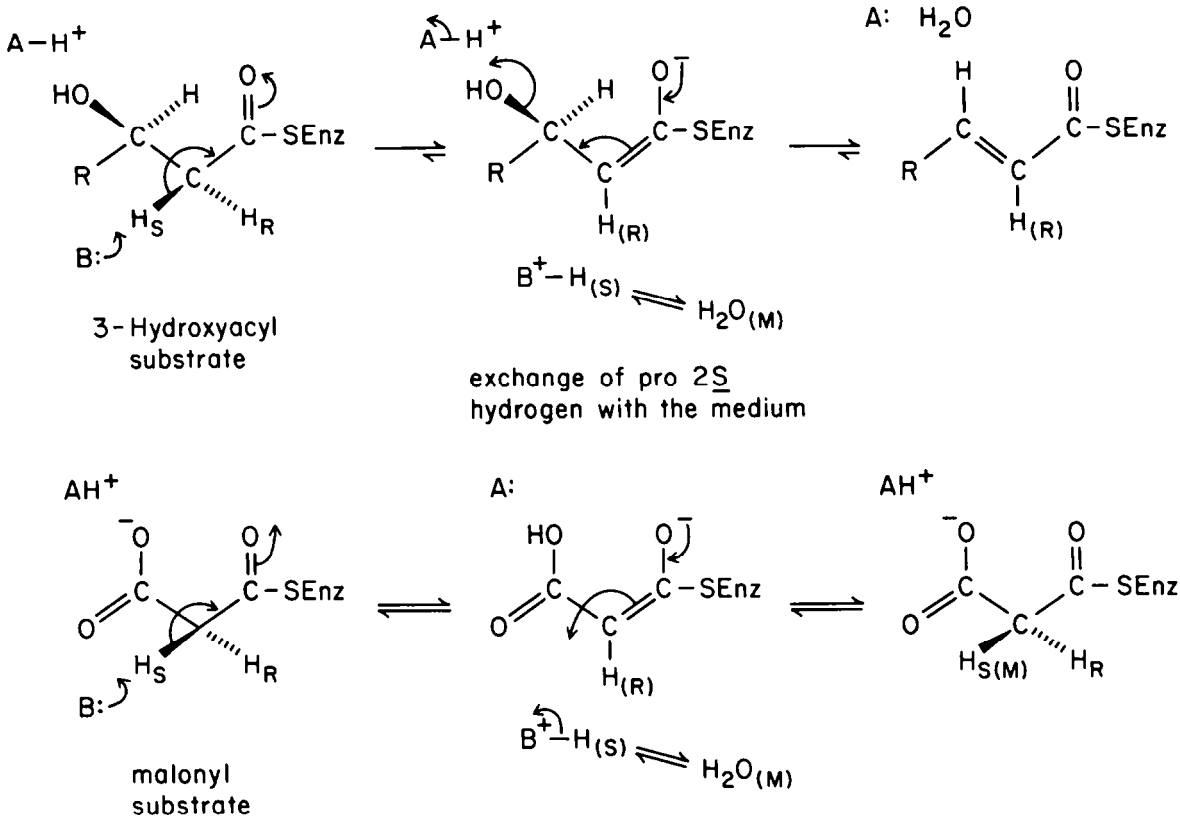
If the fraction of ^{13}C–^{2}H bonds at C-10 of palmitic acid was reduced from 88.5 to 75.3% by a nonstereospecific exchange process exhibiting a kinetic isotope effect $k_{1H}/k_{2H} = 5$ (Ref. 4, p. 477), as suggested by Sedgwick *et al.* (Ref. 4, p. 478), it can be shown by equation 6 in Ref. 4 that $1/2[1 + (0.753/0.885)^5] \times 75.3 = 54.4\%$ of the chiral groups remaining at C-10 of palmitic acid would have *R*- and 20.9% *S*-configurations. As the desaturase of yeast removes *pro*-*R* hydrogens it follows that the NMR intensity ratio $^{13}C^1H^2H/(^{13}C^1H^2H + ^{13}C^1H_2)$ at C-10 of palmitoleic acid under these circumstances would have been 20.9%. With the signal to noise ratio obtained in ^{13}C NMR spectra of the acid this value could have been measured to an accuracy better than ± 2%. However, the experimental value for the intensity ratio for C-10 of palmitoleic acid from yeast was ⩽ 2% (Table 1) thereby indicating a chiral purity of at least $100(75.3 - 2)/75.3 = 97\%$ at C-10 of palmitic acid, and this presumably holds for other $^{13}C^1H^2H$ groups in the fatty acids of yeast. This exchange is therefore a *stereospecific* process, the small retention of ^{13}C–^{2}H bonds at C-10 of palmitoleic acid representing the proportion of chiral $^{13}C^1H^2H$ groups which had been racemized by an as yet unidentified nonenzymic process.

The degree of exchange varied along the chain length of each fatty acid (Table 1). Thus by using the calculation procedure outlined above it was determined that loss of ^{2}H from the three fatty acids of yeast by this process varied between 12 ± 1 and 48 ± 1% with a mean value for all positions of 21 ± 3%. This is in excellent agreement with the result of 26% found for palmitic acid enriched from dideuteromalonate by Sedgwick *et al.* (Ref. 4, p. 473). The corresponding low, high and average ^{2}H losses for the fatty acids of *A. nidulans* were 19 ± 0.4, 30 ± 0.6 and 25 ± 1% and for *C. pyrenoidosa* 35 ± 6, 52 ± 4 and 43 ± 4%. In the case of *P. tricornutum*, the values were 38, 52 and 46% for palmitoleic acid, the only fatty acid from this organism for which a complete set of intensity ratios was obtained. These results indicate that the degree of ^{2}H loss is not related in a simple way to the type of fatty acid synthetase.

Origin of exchange. From the discussion above it is clear that the exchange of hydrogen during fatty acid biosynthesis is stereospecific and occurs after the transfer of malonate to the acyl carrier protein. The exchange involves either the *pro*-2*S* hydrogen of the bound malonyl unit or, because of inversion during the condensation step, the *pro*-2*R* hydrogen of β-ketoacyl or 3-hydroxyacyl thiolester, the olefinic hydrogen at C-2 of enoyl thiolester or the *pro*-2*R* (or *pro*-2*S*) hydrogen of the acyl-enzyme. The seemingly ubiquitous nature of the stereospecific exchange process and the observation that the final configuration of ^{2}H depends on the origin of the labelled fatty acids, clearly indicates that in all organisms examined to date exchange must occur at a common stage in the fatty acid biosynthetic cycle. A further constraint is that the exchange process necessarily involves cleavage of a C–H bond and so must be promoted by a component enzyme of the synthetase capable of performing this. The only known cleavage of a C–H bond during fatty acid synthesis is the stereospecific removal of the *pro*-2*S* hydrogen from 3-hydroxyacyl thiolester during the dehydration step. Significantly, this occurs at the same site previously occupied by a malonyl unit prior to the condensation step. Furthermore, the malonyl enzyme is the only common intermediate which has the hydrogen, which must eventually undergo exchange, in a *pro*-2*S* configuration. It seems highly probable therefore that the *pro*-2*S* hydrogen of malonyl enzyme is stereospecifically exchanged by the 3-hydroxyacyl thiolester dehydratase leading to the observed loss of isotope in labeled fatty acids.

Exchange probably occurs by the following mechanism. The dehydration reaction would be expected to proceed *via* the biochemical equivalent of an E_1, Cb elimination reaction in which ionization of the *pro*-2*S* hydrogen would precede, but certainly not follow, elimination of the OH group. On the dehydratase, there is presumably a basic group oriented to assist removal of the *pro*-2*S* hydrogen and an acidic group to facilitate removal of the hydroxyl substituent. The malonyl moiety bears a marked resemblance to the C-1 through C-3 fragment of the 3-hydroxyacyl residue, and both attach to the same sulphydryl group (Scheme 1). Interaction between the carboxylate group of malonyl–enzyme and the acidic group of the dehydratase could therefore orient the malonyl residue in such a way that the activated *pro*-2*S* hydrogen is appropriately aligned with respect to the basic group of this enzyme to promote ionization and exchange with the medium.

The following observations also support exchange at the malonyl–enzyme stage. Exchange *via* an acyl malonic thiolester intermediate with an activated methine hydrogen in the condensation reaction can be rejected,[4] because recent investigations with yeast fatty acid synthetase and purified β-ketoacyl thiolester synthetase from *E. coli* produced evidence for a concerted mechanism.[26] Additional studies with purified β-ketoacyl thiolester synthetase and reductase from *E. coli* in the presence of tritiated water also excluded the possibility that these enzymes could facilitate exchange of hydrogen in β-ketoacyl and (3*R*)-hydroxyacyl thiolesters.[26] The dehydratase probably exchanges the *pro*-2*S* hydrogen of β-ketoacyl thiolester by the same mechanism suggested for malonyl–enzyme but this hydrogen is the one subsequently lost in the dehydration reaction. Indeed, there appears to be no plausible mechanism for exchange of the *pro*-2*R* hydrogens of β-ketoacyl and (3*R*)-hydroxyacyl thiolester. Furthermore, it is known that acyl–CoA dehydrogenase in the absence of electron transfer and acceptor agents, stereospecifically exchanges the *pro*-2*R*, but not the *pro*-3*R*, hydrogen of butyryl–CoA[27] and that succinate dehydrogenase likewise exchanges deuteriums of tetradeuterosuccinic acid to give the (*RS*)-dideutero derivative.[28] As bonds broken and reformed during exchange are the same as those cleaved during the dehydrogenation reaction itself, it follows that the pattern of these enzymatic reactions involving abstraction of β-methylene hydrogens is reversible. However, our results on fatty acids cannot be explained by similar exchange at C-2 of acyl–enzyme. The hydrogen to be exchanged at this carbon is derived from acetate and can have a *pro*-2*S*



Scheme 1.

or *pro*-2*R* configuration depending on the stereospecificity of individual enoyl reductases. Even if, by analogy with the acyl dehydrogenase study,[27] the addition of hydrogen at C-2 of enoyl thiolester during the enoyl reductase reaction was reversible, it would be the hydrogen that was introduced from the medium, and not the one derived from acetate, that would be expected to undergo exchange. Furthermore, there is no obvious mechanism whereby the other component enzymes of fatty acid synthetases which have identical stereospecificities, or indeed the enoyl reductases themselves, could be responsible for exchange of the olefinic hydrogen at C-2 of enoyl thiolester or the stereospecific exchange of hydrogens of opposite configuration in acyl–enzymes of different origin. Evidence for exchange at the malonyl–enzyme stage would therefore seem to be overwhelming.

EXPERIMENTAL

The yeast *S. cerevisiae* (strain AG 17) was grown at 23° on an enriched YM-1 medium[29] and *Anacystis nidulans* and *Chlorella pyrenoidosa* were maintained and grown as described elsewhere.[30] *Phaeodactylum tricornutum* was maintained in ASW medium[31] and an aliquot (100 ml) was used to inoculate 30 l of the same medium in a special sterilized glass apparatus.[32]

Conditions for feeding [2-^{13}C, 2-$^{2}H_3$] acetate, for isolating, esterifying and separating the labeled fatty acids, for recording the ^{13}C NMR spectra, as well as the methods for calculating the data on Table 1, are reported in the previous and similar study on palmitic acid from *A. nidulans*.[17]. The ^{13}C spectrum of a sample of unlabeled methyl palmitate recorded under the same conditions used for labeled samples gave resonances with equal intensity (relative error ± 0.07).

Circular dichroism measurements were carried out on isooctane (2,2,4-trimethylpentane; Fisher spectral grade) solns of labeled methyl palmitates in quartz cells, 0.1 cm pathlength, at 27 ± 2°, using a Cary–Varian Spectrometer model 61, which was calibrated by the method of Gillen and Williams.[33] Isooctane was the solvent used to measure the molar ellipticity of 2*R*-deuteropalmitic acid.[18]

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