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Biosynthesis of a Specifically Deuteriated Diunsaturated Fatty Acid ($18:2^{\Delta 6,9}$) for ^2H NMR Membrane Studies†

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ABSTRACT: A unique procedure for the biosynthesis and subsequent isolation of a series of specifically deuteriated *cis,cis*-octadeca-6,9-dienoic acids has been developed. An auxotroph of *Tetrahymena*, which lacks Δ9 and Δ12 desaturase activity, is supplemented with specifically deuteriated oleic acid and converts it to the corresponding deuteriated *cis,cis*-octadeca-6,9-dienoic acid, $18:2^{\Delta 6,9}$. The deuteriated fatty acid is subsequently isolated by argentation chromatography and HPLC. To demonstrate the utility of the procedure, we describe here the biosynthesis of *cis,cis*-octadeca-6,9-dienoic acid deuteriated at positions 9 and 10. Gas and thin-layer chromatography of the isolated fatty acid showed that it was greater than 99% pure while ^{13}C NMR and mass spectrometry of the *O*-(trimethylsilyl) derivative confirmed that the 18-carbon fatty acid contains two double bonds located at positions 6 and 9. The yield, from an 11-L culture, was typically 100 mg of which 35% was found to be deuteriated at both the 9- and 10-positions. The deuteriated fatty acid was esterified to 1-hexadecanoyl-*sn*-glycero-3-phosphocholine, and aqueous, multilamellar dispersions of the lipid were studied by ^2H NMR. Each spectrum consists of two overlapping powder patterns and therefore yields two quadrupolar splittings. Over a temperature range from 0 to 40 °C, one splitting decreases from 6.6 to 1.8 kHz while the other increases from 4.5 to 5.3 kHz. The magnitudes of the two splittings are equivalent between 10 and 15 °C. The values of the splittings, and their response to temperature, differ significantly from those of the corresponding deuteriated oleic acid in microbial membranes [Rance, M., Jeffrey, K. R., Tulloch, A. P., Butler, K. W., & Smith, I. C. P. (1980) *Biochim. Biophys. Acta 600*, 245–262] and in bilayers of 1-hexadecanoyl-2-*cis*-octadec-9-enoyl-*sn*-glycero-3-phosphocholine (POPC) [Seelig, J., & Waespe-Šarčevič, N. (1978) *Biochemistry 17*, 3310–3315]. The results suggest that a fatty acyl chain containing two double bonds has physicochemical properties very different from those of the corresponding acyl chain with a single double bond.

In recent years considerable evidence has accumulated suggesting a unique role for polyunsaturated lipids in eukaryotic membranes. These lipids modulate a variety of membrane-associated processes [for a review see Spector and Yorek (1985)] and are thought to play an essential role in neural tissue (Lamptey & Walker, 1976; Crawford et al., 1984) and in the retina (Neuringer et al., 1984).

The effect of polyunsaturated lipids is generally attributed to their ability to "fluidize" membranes. A high degree of unsaturation in a membrane is thought to correlate with a low gel to liquid crystal transition temperature and a high degree of mobility and disorder of the lipids. Although the double bond itself is a relatively ordered, immobile structure (Seelig & Waespe-Šarčevič, 1978; Rance et al., 1980; Dufourc et al., 1984), this correlation appears to hold for membranes containing saturated and monounsaturated acyl chains (Davis & Keough, 1983; Stubbs et al., 1981; Seelig & Seelig, 1977). The extension to highly unsaturated systems, however, has no rigorous physicochemical basis. For example, differential scanning calorimetry has shown that liposomes containing a variety of polyunsaturated lecithins have similar transition temperatures (Coolbear et al., 1983), and fluorescence depolarization of diphenylhexatriene, in similar bilayers, suggests that the order and rates of motion of the lipid acyl chains are very similar (Stubbs et al., 1981). Both techniques suggest

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that there is no simple correlation between the degree of unsaturation of lipid acyl chains and the "fluidity" of a membrane. It is therefore possible that polyunsaturated lipids have unique properties that are not amenable to analysis by these techniques.

Deuterium NMR[1] is one of the few techniques that can yield sufficiently detailed information for investigating the structural and dynamic properties of polyunsaturated acyl chains in membranes. The application of the technique to polyunsaturated lipids has, unfortunately, been limited to bilayers composed of a mixed-acid lecithin containing one vinyl perdeuteriated docosahexaenoyl chain (Dratz & Deese, 1986). The initial results suggest that this highly unsaturated acyl chain may adopt a "helical" conformation in a bilayer and are consistent with the computer modeling studies of Applegate and Glomset (1986). The NMR spectra, however, are difficult to interpret because they consist of twelve superimposed powder patterns; a detailed analysis of the structural and dynamic properties of this fatty acid may therefore be difficult to obtain.

A deuterium NMR analysis of model membranes composed of lipids containing specifically deuteriated polyunsaturated acyl chains would give tremendous insight into the physicochemical properties and thus the biological function of polyunsaturated lipids. Unfortunately, there are many difficulties associated with the chemical synthesis of these deuteriated compounds. We, therefore, considered it worthwhile to develop a biological approach to their synthesis.

A series of specifically deuteriated oleic acids have been synthesized (Tulloch, 1979), and we proposed that a biological organism could introduce additional double bonds, thus producing specifically deuteriated polyunsaturated compounds. In this paper we report a procedure for the biosynthesis and subsequent isolation of a series of specifically deuteriated *cis,cis*-octadeca-6,9-dienoic acids (hereafter referred to as isolinoleic acid) using an unsaturated fatty acid auxotroph of *Tetrahymena*. The initial deuterium NMR analysis of model membranes composed of lecithins containing an isolinoleoyl chain deuteriated at two positions will also be presented.

Materials and Methods

Culture Conditions. Eleven-liter batches of media consisting of bacteriological peptone (1.0% w/v; Oxoid L37), yeast extract (0.1% w/v; Oxoid L21), and $FeCl_3$ added to a final concentration of 36 μM were autoclaved in fourteen-liter New Brunswick Microferm fermenters. After cooling, each fermenter was supplemented with 100 mg of α-tocopherol, 4 g of fatty acid free BSA, and 400 mg of both penicillin and streptomycin. The oleic acid and α-tocopherol were added in solutions of 95% ethanol whereas the BSA and antibiotics were added as aqueous solutions after sterile filtration. The oleic acid was then complexed to the BSA by rapid stirring for at least 1 h, and the medium was subsequently innoculated with the *Tetrahymena* mutant RH179E1 (Hill et al., 1983).

Cultures were grown to late log phase at 30 °C with aeration at 3.5 L·min^{-1} and vane rotation at 30 rpm. The cells were cooled on ice and harvested by continuous-flow centrifugation, and the resultant cell pellets were transferred to a round-bottomed flask and lyophilized.

Purification of 18:2$^{\Delta 6,9}$. The deuteriated 18:2$^{\Delta 6,9}$ was isolated from the other cellular fatty acids by a protocol based on that of Conner et al. (1984). The lipid was extracted from the freeze-dried cell material with $CHCl_3/CH_3OH$ (2:1 v/v) and was saponified with KOH (10% w/v) in CH_3OH/H_2O (2:1 v/v) at 75 °C for 45 min. The saponification mixture was then acidified with HCl, and the free fatty acids were extracted with hexane.

The fatty acids were passed through a small Bio-Sil column (Bio-Rad) to remove polar contaminants and then were applied to a Bio-Sil column impregnated with 25% $AgNO_3$ (w/w). The $AgNO_3$/Bio-Sil was prepared a few days in advance by evaporating an aqueous solution of the two components and was activated in the oven at 110 °C before use. Saturated and monounsaturated fatty acids were first eluted from the column with hexane, and the 18:2$^{\Delta 6,9}$ (70–80% pure) was subsequently eluted with diethyl ether. Both solvents contained acetic acid at a level of 0.5% (v/v).

The final purification step was performed by HPLC using a 19 mm × 150 mm μBondapak C_{18} reverse-phase column (part 088500, Waters Scientific). The column was eluted isocratically with $CH_3CN/H_2O/H_3PO_4$ (75:25:0.001 v/v), and several injections were performed to purify the entire sample. The appropriate fraction, from each injection, was collected in a flask gassed with argon, and after the bulk of the solvent was evaporated, the pure product was extracted with hexane.

Characterization. The isolated fatty acid was analyzed by thin-layer chromatography (TLC) on silica gel 60 plates (BDH). TLC was performed using hexane/diethyl ether/acetic acid (90:10:1 v/v) and $CHCl_3/CH_3OH/H_2O$ (65:25:4 v/v) as solvents, and the chromatograms were charred with a solution of 10% $CuSO_4$ (w/v) in H_3PO_4 (8% v/v; Bitman & Wood, 1982). Both the methyl and ethyl esters of the fatty acid were also analyzed by gas chromatography (GC). The methyl esters were prepared by the method of Morrison and Smith (1964) except that the reaction was carried out in methanol/hexane (2:1) rather than in pure methanol. The ethyl esters were prepared by refluxing the fatty acid for 1 h in ethanolic HCl (acetyl chloride/ethanol, 1:16 v/v). The samples were then analyzed by GC on a 30 m × 0.75 mm i.d. Supelcowax 10 column (Supelco) using flame ionization detection.

GC–MS analysis of the *O*-TMS derivatives of both the methyl and ethyl esters was performed to identify the number and location of the double bonds and to localize the sites and extent of deuteriation. The *O*-TMS derivatives were prepared by the method of Schmitz and Egge (1979) except that the silylation step was performed with a premixed silylation reagent, Tri-Sil (Pierce). Both derivatives were analyzed with an HP5985 GC–MS system, operating in the electron impact mode at 15 eV, equipped with a 25 m × 0.75 mm i.d. OV-17 capillary column.

The specific sites and extent of deuteriation were confirmed by ^{13}C NMR analysis of the fatty acid. The sample was run on a Bruker MSL-300 spectrometer operating at 75.5 MHz. Spectra were acquired using the WALTZ-16 decoupling sequence at a power level of 1.3 W (Shaka et al., 1983), a $\pi/2$ pulse length of 9.5 μs, and a recycle time of 5 s.

Esterification to Lyso-PC. The deuteriated 18:2$^{\Delta 6,9}$ was esterified to 1-palmitoyl-lyso-PC by the method of Perly et al. (1984). The product was purified on a Bio-Sil column with elution of the unesterified material with 100 mL of $CHCl_3$ and the pure product with $CHCl_3/CH_3OH/H_2O$ (45:25:2.5 v/v).

[1] Abbreviations: NMR, nuclear magnetic resonance; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; GC, gas chromatography; GC–MS, gas chromatography coupled to mass spectrometry; *O*-TMS, *O*-(trimethylsilyl); BHT, butylated hydroxytoluene; PC, *sn*-glycero-3-phosphocholine; PiLPC, 1-hexadecanoyl-2-*cis,cis*-octadeca-6,9-dienoyl-*sn*-glycero-3-phosphocholine; POPC, 1-hexadecanoyl-2-*cis*-octadec-9-enoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine.

The purity of the synthetic phospholipid was verified by TLC using 10% $CuSO_4$ (w/v) in H_3PO_4 (8% v/v) and Dragendorff's reagent (Kates, 1969) to visualize and identify the spot. The product was dried under a stream of argon, lyophilized, and then dispersed in deuterium-depleted H_2O to form multilamellar vesicles. The sample was subjected to, on average, five freeze–thaw–vortex cycles to ensure complete dispersion of the lipid.

A portion of one sample was also checked for acyl chain migration. The phospholipid was treated with phospholipase A_2 (*Crotalus adamanteus*; Keough & Davis, 1979), and the fatty acid liberated from the *sn*-2 position was analyzed by GC.

Deuterium NMR Spectroscopy. The deuterium NMR spectra were recorded on a 200-MHz custom-built spectrometer operating at 30.7 MHz, using a modified quadrupolar echo pulse sequence (Rance et al., 1980). The $\pi/2$ pulses were approximately 2.3 μs in length (5-mm solenoid coil), and an echo pulse spacing of 60 μs was used. The pulse sequence was repeated every 100 ms, and the spectra were acquired by using a spectral width of 100 kHz. The temperature was regulated electronically to within ±0.5 °C with a custom-built variable-temperature unit.

Spectra were analyzed on a Nicolet 1180E data processor. The free induction decays were left shifted to the top of the echo before Fourier transformation of the trailing edge. NMR spectra were de-Paked by the method of Bloom et al. (1981) to give the 90° oriented sample spectra from which the quadrupolar splittings were obtained.

Oxidation. Several precautions were taken to prevent oxidation of the fatty acids. Wherever possible, manipulations were carried out under argon. Solvents were also bubbled with argon before use and contained the antioxidant, butylated hydroxytoluene (BHT), at a level of approximately 0.01% (w/v). Column fractions were collected in Teflon-capped vials which were presaturated with argon, and samples were stored under argon in the freezer at −20 °C.

The final column, which was used to purify the deuteriated phospholipid, was run with solvents that did not contain BHT in order to prevent contamination of the NMR samples with the antioxidant. The dispersed lipid was placed in 5-mm NMR tubes which were gassed with argon and sealed with epoxy resin. Samples prepared with these precautions showed no sign of degradation and were stable for several months.

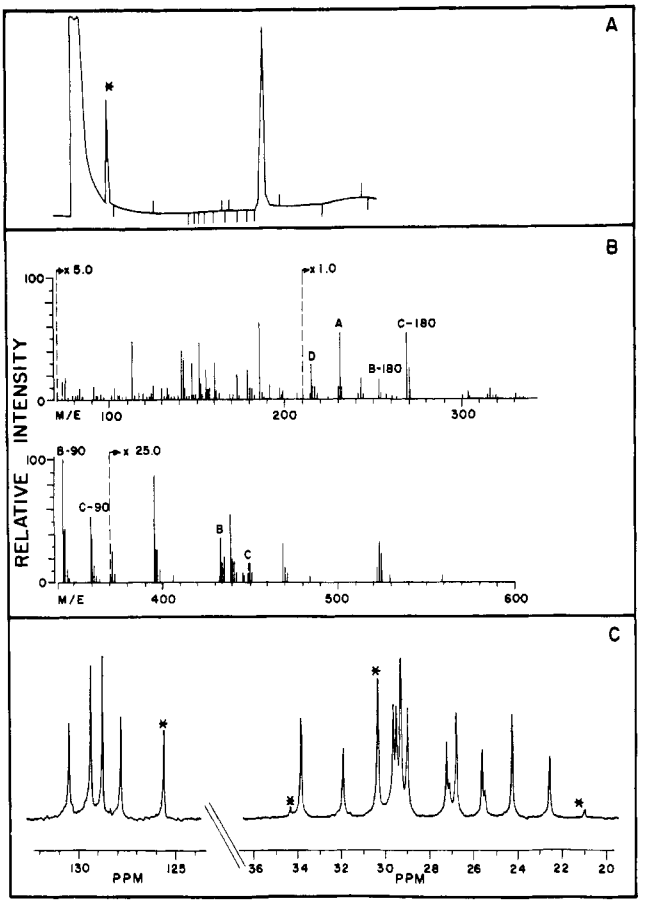

FIGURE 1: Analysis of purified, specifically deuteriated isolinoleic acid. (A) GC analysis of the fatty acid methyl ester, which was found to be greater than 99% pure. The retention times of BHT and the 18:2$^{\Delta 6,9}$ methyl ester are 3.6 and 13.8 min, respectively. (B) GC–MS analysis of the *O*-TMS derivative of the fatty acid ethyl ester. Diagnostic fragments are labeled by the convention of Schmitz and Egge (1979): A, $[CH_3CH_2OOC(CH_2)_4CHO(TMS)]^+$; B, $^+[CHO(TMS)CH_2CHO(TMS)CHO(TMS)(CH_2)_7CH_3]$; C, $[CH_3CH_2OOC(CH_2)_4CHO(TMS)CHO(TMS)CH_2CHO(TMS)]^+$; D, $^+[CHO(TMS)(CH_2)_7CH_3]$. B-90 and C-180 refer to fragments B and C with the additional loss of one and two HO(TMS) groups, respectively. (C) A portion of the ^{13}C NMR spectrum of the isolated fatty acid showing the alkene (127–132 ppm) and methylene resonances (20–36 ppm; see text for discussion). The "*" labels peaks that correspond to the antioxidant, BHT. For experimental details on the acquisition of the three diagrams, see Materials and Methods.

RESULTS

Two separate 11-L cultures of *Tetrahymena*, strain RH179E1, were supplemented with oleic acid deuteriated at both the 9- and 10-positions. Each culture was harvested, and the deuteriated isolinoleic acid (18:2$^{\Delta 6,9}$) was isolated and purified as described under Materials and Methods. The purity of each preparation was established by thin-layer and gas chromatography (TLC and GC, respectively). TLC was performed in two solvents of different polarity and in each case resulted in only one spot having the same R_f as a free fatty acid standard. GC analysis of the methyl ester of the fatty acid also showed that both preparations of isolinoleic acid were greater than 99% pure (Figure 1A). The *O*-TMS derivatives of both fatty acid esters (see below), however, were resolved into two peaks on the GC–MS capillary column. In each case, the two peaks had identical mass spectra and therefore could be attributed to the two diasteriomers formed, during the derivatization, by addition of osmium tetroxide to the double bonds. The addition to each individual double bond is syn, but with respect to each other both syn and anti addition are possible. Although the formation of such diasteriomers has not previously been reported, most of the early GC–MS work was performed with packed columns, and we found that our packed column (6 ft × $^1/_{16}$ in. i.d., SP2300/2310, Supelco) was unable to resolve the two peaks (data not shown).

The structure of the isolated fatty acid was confirmed by GC–MS of its *O*-TMS derivative. Under electron impact, a relatively simple fragmentation occurs which allows determination of the number and location of the double bonds [for details see Dommes et al. (1976)]. The mass spectrum of the methyl ester derivative was difficult to interpret due to the many overlapping diagnostic fragments; the ethyl ester derivative was therefore examined (Figure 1B). All the major diagnostic peaks expected for a derivatized, Δ6,9-diunsaturated, 18-carbon fatty acid ethyl ester are found; however, one peak of high molecular weight [fragment "C", Schmitz and Egge (1982)] is only present at low intensity due to excessive breakdown to smaller fragments. The fragmentation patterns for all other 18-carbon, straight-chain, diunsaturated ethyl esters were also predicted, and none are compatible with the observed spectrum. Only the proposed structure of the fatty acid (18:2$^{\Delta 6,9}$) is therefore compatible with our results.

Analysis of the mass spectrum for isotopic shifts also allowed localization of the sites and an estimation of the extent of deuteriation. The spectrum indicated that the fatty acid is deuteriated at two sites with one deuterium located on the fragment from carbons 10–18 and the other on the fragment from carbons 7–9. By comparing the intensities of the deuteriated and nondeuteriated fragments (after taking into account the natural abundance of the various elements), we were able to estimate the level of deuteriation to be between 32% and 35%. In addition, fragments containing both deuterium sites (fragments B, B-90, B-180) had isotopic shifts of only 2 mass units and therefore the labels appear simultaneously in the same molecule.

The ^{13}C NMR data also support the proposed structure of the isolated fatty acid (Figure 1C). The presence of two double bonds is indicated by the four alkene resonances (127–132 ppm), and the reduction in intensity of two of these indicates that the corresponding carbon atoms are deuteriated. Comparison of the peak intensities of the deuteriated and nondeuteriated alkene resonances indicates, in agreement with the GC–MS data, that the level of deuteriation is 35%.

Resonances from two methylene carbons are shifted by 0.1 ppm (25.5 and 27.1 ppm) due to the location of these carbons adjacent to deuteriated carbons (only 35% of the intensity is shifted because 35% of the sample is deuteriated). Two of the fatty acid's carbon atoms are deuteriated (GC–MS), and therefore, since only two methylene groups show this β effect, the sites of deuteriation must be located either on adjacent carbons or on the terminal carbons of the chain (the methyl and carboxyl carbons). The only possibility is that the two sites are adjacent and are therefore located on one of the two double bonds. When this information is combined with the GC–MS data, it can be concluded that the fatty acid is deuteriated at positions 9 and 10.

Both preparations of deuteriated isolinoleic acid were esterified to 1-palmitoyl-lyso-PC, and aqueous dispersions of each were analyzed by deuterium NMR. The spectra from both samples have line shapes typical of lipids undergoing axially symmetric motions and consist of two overlapping powder patterns (Figure 2).

The variation of the two quadrupolar splittings with temperature was virtually identical, for both samples (Figure 3). Over the temperature range 0–40 °C, one splitting decreases from 6.6 to 1.8 kHz while the other increases from 4.5 to 5.3 kHz, and the magnitudes of the two are equivalent between 10 and 15 °C. This temperature dependence indicates that the 9–10 double bond of isolinoleic acid changes its orientation with respect to the axis of motional averaging, in response to changing temperature, and suggests that isolinoleic acid has structural properties very different from the monounsaturate, oleic acid (see Discussion).

Discussion

Biosynthesis of Deuteriated Isolinoleic Acid (*18:2*$^{\Delta 6,9}$). The protozoan *Tetrahymena* shares with bacteria an ease of culture but as a eukaryote has the capacity to form highly unsaturated lipids. The desaturation of 18-carbon fatty acids in *Tetrahymena* (Figure 4) involves three separate desaturase enzymes and together with chain elongation leads to the production of a variety of polyunsaturates.

An unsaturated fatty acid auxotroph of the eukaryote, strain RH179E1, has limited capacity to form unsaturated fatty acids because it lacks $\Delta 9$ and $\Delta 12$ desaturase activity [Figure 4 and Hill et al. (1983)]. When supplemented with oleic acid, RH179E1 incorporates and converts the fatty acid to the $\Delta 6,9$ isomer of linoleic acid (isolinoleic acid, 18:2$^{\Delta 6,9}$; Hill et al., 1983; Conner et al., 1984). We proposed that by supplementing the auxotroph with a series of deuteriated oleic acids we could produce the corresponding series of specifically deuteriated isolinoleic acids and have demonstrated the fea-

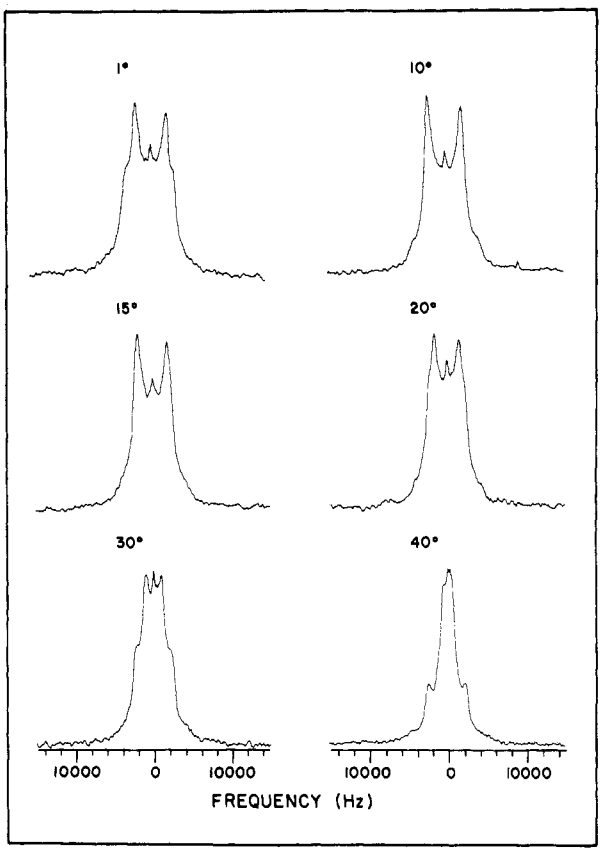

FIGURE 2: Deuterium NMR spectra of bilayers composed of PiLPC deuteriated at the 9–10 double bond of the isolinoleoyl chain. Spectra were acquired and processed as described under Materials and Methods.

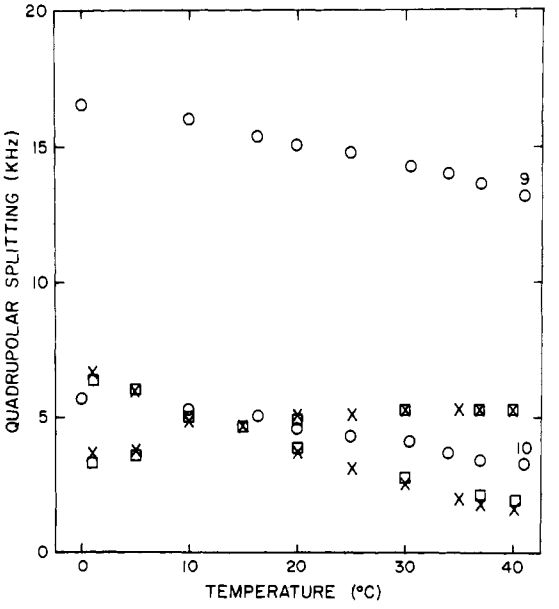

FIGURE 3: Summary of quadrupolar splittings and their response to temperature for [9,10-^{2}H]isolinoleic acid in membranes of PiLPC and [9,10-^{2}H]oleic acid in membranes of *A. laidlawii* [data taken from Rance et al. (1980)]: □ and ×, splittings for the two preparations of deuteriated isolinoleic acid each deuteriated at the 9- and 10-positions; ○, oleic acid deuteriated at the 9- and 10-positions. The numbers in the figure represent the deuteriated carbons of oleic acid to which the quadrupolar splittings have been assigned.

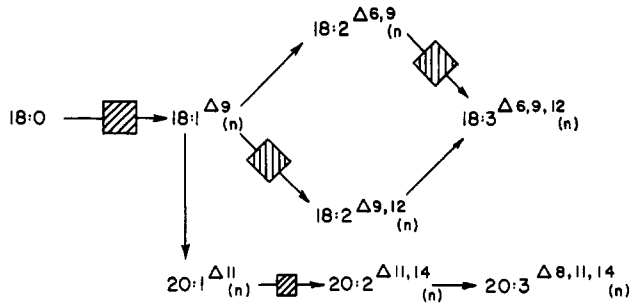

FIGURE 4: Desaturation pathway of 18-carbon fatty acids in *Tetrahymena*. Hatched boxes represent steps in the pathway that are blocked in the mutant RH179E1, due to deficiencies in the Δ9 and Δ12 desaturase enzymes.

sibility of the procedure by biosynthesizing isolinoleic acid deuteriated at the 9- and 10-positions.

The isolinoleic acid isolated from two cultures of RH179E1 supplemented with oleic acid deuteriated at the 9- and 10-positions was found, in each case, to be greater than 99% pure. It was identified as the Δ6,9 isomer and was found to be deuteriated at both the 9- and 10-positions to a level of 35% (see Results). The low level of deuteriation is surprising because in defined media RH179E1 cannot synthesize unsaturated fatty acids (Hill et al., 1983). We expected that the organism's ability to synthesize isolinoleic acid would be entirely dependent upon the supplement, which was deuteriated to a level greater than 95% (unpublished results). The cells, however, were grown in a peptone medium from which they may acquire a precursor for the synthesis of unlabeled 18:2$^{\Delta 6,9}$. This is supported by the fact that RH179E1 can grow in peptone without a supplement and acquires precursors, from the media, for the synthesis of both 18:2$^{\Delta 6,9}$ and 18:3$^{\Delta 6,9,12}$ (unpublished results).

Approximately 100 mg of isolinoleic acid (estimated by GC using 17:0 as an internal standard) was isolated from each 11-L culture, and thus, in each case, 35 mg of specifically deuteriated fatty acid was produced. Despite the low level, 35 mg is sufficient to allow extensive NMR studies (see below). Should higher levels of deuteriation be required, the cultures could be grown in a defined medium which should both increase the level of deuteriation and increase the yields of the product.

Deuterium NMR. The deuterium NMR spectra of PiLPC deuteriated at the 9–10 double bond of the isolinoleoyl chain consist of two overlapping powder patterns and therefore yield two quadrupolar splittings. Approximately 15% of the sample, however, contained the 1-isolinoleoyl-2-palmitoyl isomer of PiLPC, formed by acyl chain migration during the synthetic procedure. Examination of the de-Paked spectra indicates that only two powder patterns exist, and therefore, the structural properties of the 9–10 double bond of the isolinoleoyl chain appear to be identical in the two molecules.

At a given temperature the magnitudes of the splittings for the 9–10 double bond are much smaller than the magnitudes of the splittings obtained from spectra of palmitoyl chains (deuteriated at positions 9 and 10) in microbial membranes of *Acholeplasma laidlawii* (Stockton et al., 1977) and in bilayers of DPPC (Seelig & Seelig, 1974). The two splittings are also much smaller than the splittings obtained from spectra of oleoyl chains in microbial membranes and in bilayers of POPC when deuteriated at the 9-positions but are of similar magnitude to the splittings for the 10-positions (Rance et al., 1980; Seelig & Waespe-Šarčevič, 1978). Both splittings can be directly related to the order parameter S_{CD}, but due to the geometry of the cis double bond additional parameters are required to calculate the molecular order parameter S_{mol} and to determine the double bond orientation with respect to the bilayer normal (Seelig & Waespe-Šarčevič). A direct comparison of the ordering at the 9- and 10-positions of the isolinoleoyl, oleoyl, and palmitoyl chains can only be made by using the molecular order parameter, and therefore a comparison cannot be made at this time.

The temperature dependence of the splittings does indicate that the 9–10 double bond of isolinoleic acid changes its orientation with respect to the axis of motional averaging and that its orientation is parallel to this axis between 10 and 15 °C. The same splittings for oleic acid in model and microbial membranes both show a gradual decrease with increasing temperature (Seelig & Waespe-Šarčevič, 1978; Rance et al., 1980). The orientation of the double bond therefore remains constant and is tilted 7–8° from the axis of motional averaging (generally assumed to be the bilayer normal; Seelig & Waespe-Šarčevič, 1978; Rance et al., 1980). Although a rigorous mathematical analysis cannot as yet be made, the data show that isolinoleic acid has structural properties very different from the corresponding chain with one double bond.

CONCLUSIONS

The work presented in this paper demonstrates the viability of a biological approach to the synthesis of specifically deuteriated polyunsaturated fatty acids. We have developed the techniques to convert specifically deuteriated oleic acid (18:1$^{\Delta 9}$) into the corresponding specifically deuteriated isolinoleic acid (18:2$^{\Delta 6,9}$) and have initiated the deuterium NMR analysis of membranes containing this lipid. This procedure is unique and is the only route available, at present, for the synthesis of a specifically deuteriated polyunsaturated fatty acid.

Similar biosynthetic procedures could also be used to produce an extensive series of specifically deuteriated polyunsaturated fatty acids. For example, the deuteriated isolinoleic acids could be introduced into the normal strain of *Tetrahymena* and would be converted to the corresponding deuteriated γ-linolenic acids (18:3$^{\Delta 6,9,12}$). In addition, the deuteriated isolinoleic or γ-linolenic acids could be reintroduced into the mutant and specifically deuteriated ciliary membrane vesicles could be isolated. The deuterium NMR analysis of the physicochemical properties of a wide range of polyunsaturated fatty acids in both model and eukaryotic membranes is therefore possible.

The initial deuterium NMR analyses of PiLPC show that the presence of a second double bond, at the 6-position, strongly influences the structural properties of the 9–10 double bond of isolinoleic acid and supports the postulate that polyunsaturates have unique structural properties. More detailed experiments will give the parameters necessary for the calculation of the order parameter, S_{mol}, and the double bond orientation, while T_1 measurements will give an indication of the dynamics of the double bond. The extension of these experiments to isolinoleic acid deuteriated at several positions along the chain will thus lead to a complete analysis of the physicochemical properties of the diunsaturate and should give great insight into the special role of polyunsaturated lipids in nature.

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Registry No. [9,10-$^2H_2$]-18:2$^{\Delta 6,9}$, 111210-15-2; [$^2H_2$]-PiLPC, 111210-16-3; [9,10-$^2H_2$]-18:1$^{\Delta 9}$, 5711-29-5; [9,10-$^2H_2$]-18:2$^{\Delta 6,9}$ methyl ester, 111210-17-4; [9,10-$^2H_2$]-18:2$^{\Delta 6,9}$ ethyl ester *O*-TMS derivative, 111210-18-5.

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Kinetic Mechanism of DNA Polymerase I (Klenow)†

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ABSTRACT: The minimal kinetic scheme for DNA polymerization catalyzed by the Klenow fragment of DNA polymerase I (KF) from *Escherichia coli* has been determined with short DNA oligomers of defined sequence. A key feature of this scheme is a minimal two-step sequence that interconverts the ternary KF·DNA$_n$·dNTP and KF·DNA$_{n+1}$·PP$_i$ complexes. The rate is not limited by the actual polymerization but by a separate step, possibly important in ensuring fidelity [Mizrahi, V., Henrie, R. N., Marlier, J. F., Johnson, K. A., & Benkovic, S. J. (1985) *Biochemistry 24*, 4010–4018]. Evidence for this sequence is supplied by the observation of biphasic kinetics in single-turnover pyrophosphorolysis experiments (the microscopic reverse of polymerization). Data analysis then provides an estimate of the internal equilibrium constant. The dissociations of DNA, dNTP, and PP$_i$ from the various binary and ternary complexes were measured by partitioning (isotope-trapping) experiments. The rate constant for DNA dissociation from KF is sequence dependent and is rate limiting during nonprocessive DNA synthesis. The combination of single-turnover (both directions) and isotope-trapping experiments provides sufficient information to permit a quantitative evaluation of the kinetic scheme for specific DNA sequences.

Escherichia coli DNA polymerase I (Pol I)[1] is a multifunctional enzyme involved in the repair and replication of DNA in vivo (Kornberg, 1980). In addition to its reversible polymerase activity that catalyzes the template-directed extension of a primer DNA strand, the enzyme also possesses distinct 3′ → 5′ and 5′ → 3′ exonuclease activities residing on a single 103-kDa polypeptide chain (Jovin et al., 1969). In view of its extensive catalytic repertoire, Pol I has served as a useful model for describing certain enzymatic processes

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[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; KF, Klenow fragment; kDa, kilodalton(s); PP$_i$, inorganic pyrophosphate; dNTP, deoxynucleoside 5′-triphosphate; HPLC, high-pressure liquid chromatography; TEAB, triethylammonium bicarbonate; TEAA, triethylammonium acetate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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