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Quantitation of model digestive mixtures by ^{13}C NMR

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Abstract ^{13}C nuclear magnetic resonance (NMR) spectra were obtained at 50.3 and 100.5 MHz for methanolic and aqueous mixtures of sodium taurocholate, 1-monocapryloyl-*rac*-glycerol, and caprylic acid. Distortionless Enhancement by Polarization Transfer (DEPT) was used to improve spectral sensitivity and resolution, and to generate calibration curves for quantitative determinations of each lipid in methanol. Alternatively, the heights for nonoverlapping peaks in a ^{13}C NMR spectrum acquired with inverse-gated decoupling provide reliable quantitative estimates for each component of the mixture, particularly when the data are obtained in methanol. These experiments also demonstrate the feasibility of detailed NMR structural investigations in model systems for glyceride digestion.—**Wang, D., N. L. Hadipour, E. A. Jerlin, and R. E. Stark.** Quantitation of model digestive mixtures by ^{13}C NMR. *J. Lipid Res.* 1992. **33:** 431–439.

Supplementary key words glyceride digestion • lipid aggregation • distortionless enhancement by polarization transfer

The formation of organized molecular assemblies is known to play a critical role in both the hydrolysis of dietary lipids and the transport of digestive products to the absorptive mucosa of the small intestine (1, 2). For instance, the pancreatic lipase-colipase system is activated toward acylglycerols by the presence of substrate interfaces, which are associated in turn with emulsions, vesicles, and micellar aggregates. Both theoretical and experimental studies of lipolysis have addressed explicitly the impact of substrate surfaces on catalytic rates (3–6), though many questions remain unresolved in this area.

Our own and related investigations have used multinuclear NMR, quasielastic light scattering (QLS), and enzyme kinetics to characterize mixed lipid micelles and unilamellar vesicles that are substrates for phospholipases (7–12). In particular, these studies have provided valuable information about particle size and size distribution, phospholipid packing in contrasting aggregation states, and particle reorganization brought on by changes in lipid composition or overall concentration. For lipid mixtures relevant to digestion and absorption of fats, the available spectroscopic data are more rudimentary, largely because these are often multicomponent and/or two-phase systems. Nevertheless, the importance of micellar, vesicular, and liquid-crystalline assemblies has been established by microscopy, QLS, and ultracentrifugation (13, 14) (R. E. Stark, H. N. Halladay, and A. Al-Bashir, unpublished observations).

Quantitative assessments of the various constituents of biliary and intestinal mixtures have relied traditionally on enzyme assays for the bile salts (15) or thin-layer chromatography for the glycerides (16). Such methods are quite sensitive but somewhat indirect in their application; moreover, they reveal no molecular details regarding the arrangement of lipid species within catalytically relevant assemblies. Based on prior NMR investigations of triglycerides, monoglycerides, bile salts, and phospholipids at concentrations close to those found physiologically (8, 17–24), the latter spectroscopic methods appeared to offer an attractive alternative for both quantitative determinations of two-phase partitioning and intensive studies of aggregate organization and dynamics.

Abbreviations: NMR, nuclear magnetic resonance; QLS, quasielastic light scattering; DEPT, distortionless enhancement by polarization transfer; NOE, nuclear Overhauser effect; T_1, spin-lattice relaxation time; TMS, tetramethylsilane; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TC, sodium taurocholate; MC, 1-monocapryloyl-*rac*-glycerol; CA, caprylic acid.

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In the work reported herein, simple model digestive mixtures have been examined in two solvents using ^{13}C NMR with Distortionless Enhancement by Polarization Transfer (DEPT) (25) as well as with inverse-gated ^{1}H decoupling (26). The test mixtures contained medium-chain species that typify partial hydrolysis products of butter fat or therapeutic nutrient cocktails (2, 27). Spin-lattice relaxation times and nuclear Overhauser effects help to confirm the spectral assignments and to delineate protocols for quantitation. Assessments of spectral resolution, sensitivity, and quantitative reliability have been made in order to evaluate the potential of ^{13}C NMR for detailed structural studies of these multicomponent assemblies. Finally, critical comparisons have been made with other physical methods available for the quantitation of lipid mixtures.

MATERIALS AND METHODS

Sodium taurocholate and 1-monocapryloyl-*rac*-glycerol were purchased from Sigma Chemical Co. (St. Louis, MO). Octanoic (caprylic) acid and 99.8% D_2O were obtained from Aldrich Chemical Co. (Milwaukee, WI). Spectrograde methanol was purchased from Fisher Scientific (Pittsburgh, PA) or as 99.5% CD_3OD from Cambridge Isotope Laboratories (Woburn, MA). To prepare aqueous mixtures, the lipids were first cosolubilized in methanol, dried under a stream of nitrogen and at reduced pressure, and then hydrated with D_2O (containing 150 mM NaCl, except as noted) (9, 28). The resulting solutions (56 mM taurocholate, 66 mM monoglyceride, 198 mM caprylic acid, 7.3 g/dl total lipids, pH 5) were incubated for at least 7 days and vortex-mixed prior to the NMR experiments.

For ^{13}C NMR spectra, measurements were carried out at 50.3 MHz and 26°C with an IBM Instruments WP-200 spectrometer under conditions of broadband ^{1}H decoupling. Spectra were acquired with a relaxation delay of 10 sec, requiring 2–12 h depending on the solution concentration and tube size (5 or 10 mm). Chemical shifts were referenced to an internal closed capillary of tetramethylsilane (TMS) or via sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an external substitution reference.

The DEPT experiment was implemented at 50.3 MHz with the following pulse sequence (25): $[\text{wait}-90°(H,y)-\tau-180°(H),90°(C,x)-\tau-\vartheta(H,\pm x),180°(C)-\text{decouple}(H),\text{acquire}(C)]_n$. A waiting time of several ^{1}H spin-lattice relaxation times (10 sec) was used along with a free-precession time τ equal to $(2J_{CH})^{-1}$, where the scalar coupling constant J was estimated at 125 Hz. A value of ϑ=135° was chosen for the last ^{1}H pulse, yielding spectra for which CHs and CH_3s are 180° out of phase with respect to CH_2s.

$^{13}C\{^{1}H\}$ nuclear Overhauser effects (NOEs) were determined at 100.5 MHz and 35°C with a JEOL GX-400 spectrometer. Heights were compared for non-overlapping peaks in sets of ^{13}C spectra obtained alternately with full ^{1}H decoupling and with decoupling gated on only during the signal-acquisition period (26). A pulse interval of at least 10 ^{1}H spin-lattice relaxation times (60 sec) was used, and a typical sampling density of 53 points/peak was obtained through data acquisition (8K real points/8000 Hz) and artificial line broadening (7 Hz). The reported NOEs, which are derived from two data sets differing by less than 2%, are average peak-height ratios obtained with line-broadening factors of 7, 10, and 15 Hz. Identical processing of an ethylene glycol reference sample yielded the expected NOEs of 2.99 ± 0.01.

For quantitative analysis of carbon signals from a given lipid component or among the various components of a model digestive mixture, several methods of measurement were applied to spectra acquired with inverse-gated decoupling, as described above (26, 29). Using the well-resolved resonances from taurocholate (TC), monocapryloylglycerol (MC), and caprylic acid (CA), *a*) peak heights were measured by hand; *b*) integrals were computed for individual peaks; and *c*) curve-fitting of the resolved peaks was used to derive integrals. Procedures *b*) and *c*) were carried out using software from New Methods Research (East Syracuse, NY) running on a SUN 3/110 computer (SUN Microsystems, Mountain View, CA). All height determinations have an estimated precision of 1.6%.

^{13}C spin-lattice relaxation times $T_1(C)$ were measured at 100.5 MHz and 35 ± 1°C with the fast inversion-recovery pulse sequence (30): $[180°-\tau-90°-\text{acquire}(C)-\text{wait}]_n$ and ^{1}H decoupling throughout. A waiting time of 3 sec was inserted between successive acquisitions, and a data set was comprised of 15 τ values between 0.01 and 5 times T_1. The reported $T_1(C)$ values, which are derived from duplicate experiments, were found from a three-parameter exponential fit using software from New Methods Research. They have an estimated precision of 10%.

RESULTS

Displayed in **Fig. 1** are typical ^{13}C NMR results for TC, including a comparison of the crowded aliphatic region under normal acquisition conditions and with DEPT spectral editing (25). Peak assignments follow the guidelines of Campredon et al. (23), who used 2D methods to refine earlier work (20–22, 31, 32). Either the attached proton test (22, 33) or the DEPT sequence (25) serve to distinguish methyl and methine from methylene and quaternary carbon signals (Fig.

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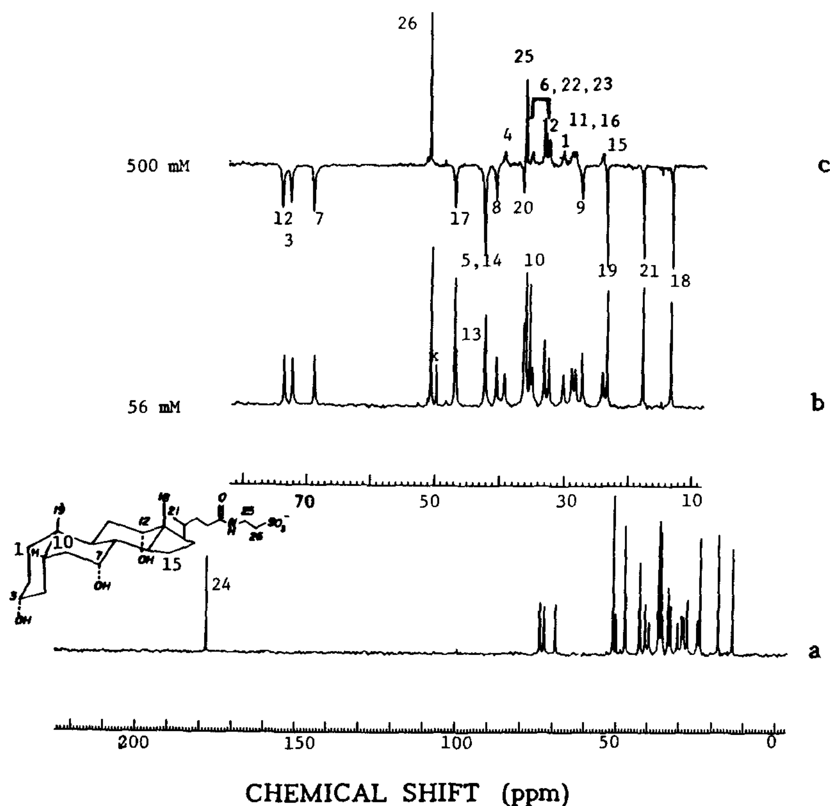

Fig. 1. Natural-abundance ^{13}C NMR of aqueous sodium taurocholate with single-pulse acquisition (a,b) and DEPT spectral editing (c). Phase correction in (c) has been performed to display CH_2s as positive-going signals. A relaxation delay of 10 sec was used in both experiments. All solutions were prepared in 150 mM NaCl with D_2O as solvent and taurocholate concentrations as noted in each plot.

1c). For instance, the CH at position 20 may be resolved from the CH_2s at positions 1 and 2; a methylene (at position 4) which absorbs at a frequency typical of methines is also easily identified. In studies of complex dilute multicomponent mixtures (vide infra), DEPT offers the added advantage of sensitivity enhancement. The removal of NMR signals from nonprotonated carbons (including deuterated solvents) by this latter technique may also result in spectral simplication, as illustrated with carbons at sites 10 and 13.

The relative peak intensities in a given DEPT spectrum do not correspond strictly to the numbers of each carbon type: polarization transfer is sensitive to the number of attached protons, scalar coupling constant, and pulse angle (34); the ^{13}C NMR spectra must also be acquired with repetition rates slow enough to permit repolarization of ^{1}H nuclei for monomers and micelles (35) in the respective mixtures. Nonetheless, one reliable approach to determining lipid concentrations in a sample of interest uses DEPT-based calibration curves, as illustrated in **Fig. 2** for taurocholate dissolved in a nonaggregating solvent.

^{13}C NMR data are summarized in **Fig. 3** and **Table 1** for a bile salt–monoglyceride–fatty acid mixture, with proportions roughly corresponding to those found physiologically in the small intestine after ingestion of a fatty mixture rich in medium-chain triglycerides (27, 36, 37). When such a lipid mixture is prepared in a nonaggregating solvent (CD_3OD), we expect and find that chemical shifts are unaltered (see Table 1 for the MC component). This chemical shift invariance makes it straightforward to identify peaks for key carbon sites of each lipid molecule, though acyl-chain resonances do coincide for the MC and CA components. The acyl-chain assignments are corroborated by the progression of $T_1(C)$ values observed for these resonances (**Table 2**).

The entries of Table 1 show that relative ^{13}C peak heights measured at 50.3 MHz compare favorably for the backbone sites of MC examined alone or in a lipid mixture. These three single-carbon resonances also display nearly the same signal size in ^{1}H-decoupled spectra, an expected result given their similar values of $T_1(C)$ and NOE (Table 2). By contrast, aliphatic carbons within the same molecule show significant height anomalies in both solutions, which may be attributed to long and unequal values of T_1 (Table 2), minor variations in NOE, some overlap with TC resonances,

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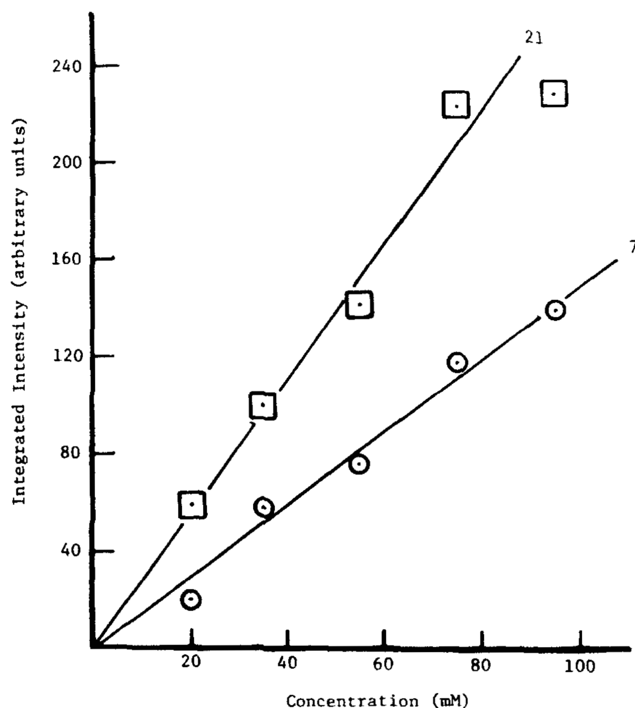

Fig. 2. Integrated intensities as a function of taurocholate concentration in CD_3OD, for several carbon peaks in ^{13}C NMR spectra acquired with the DEPT pulse sequence. The carbons are numbered as shown in Fig. 1. Similar correlations are found for a concentration series of MC or CA spectra.

and uncertain contributions from the CA component. The long values of $T_1(C)$ displayed by many chain carbons at 100.5 MHz (Table 2) suggest that ^{13}C peak heights measured under full ^1H decoupling conditions at this latter field strength would also be an unreliable source of quantitative information.

Despite more demanding time requirements, inverse-gated ^1H decoupling has been used with considerable success to provide quantitative ^{13}C NMR data that are insensitive to $T_1(C)$ and NOE considerations (26, 29). The application of this protocol to a model digestive mixture in methanol is demonstrated in **Fig.4; Table 3** reveals that the measured peak heights are self-consistent for single-carbon resonances of a given chemical component. If heights are measured for those carbon peaks that are free of spectral overlap, it is also possible to derive values for the concentrations [TC], [MC], and [MC + CA]. The results summarized in Table 3 are fully in accord with the known compositions of each component in the mixture, with deviations from the true molarities that fall beyond the precision of our measurements but within the practical limits reported for our signal-to-noise ratio (29). (Since all spectral peaks have real linewidths close to 4 Hz, peak-*height* measurements offer an expedient and entirely rigorous alternative to peak *areas*.)

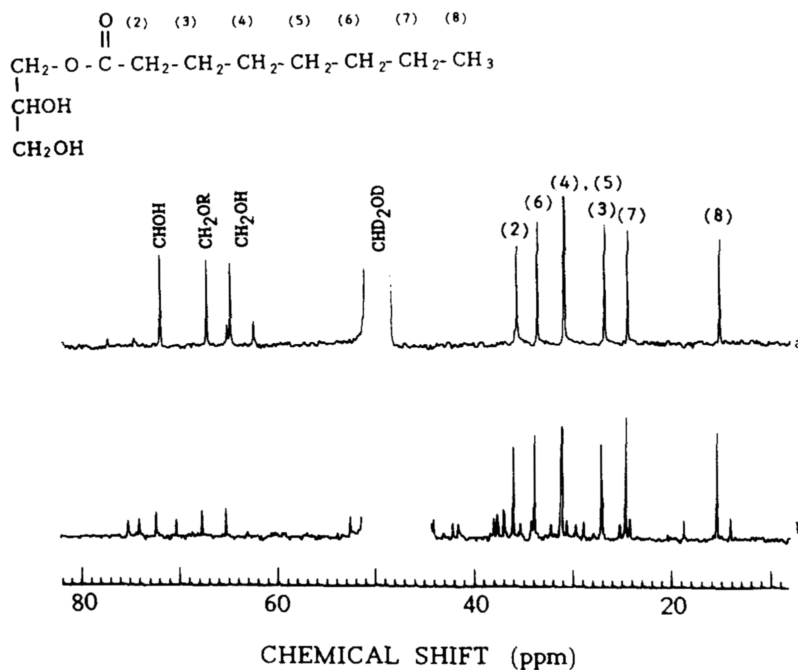

Fig. 3. ^{13}C NMR spectra at 50.3 MHz for lipids in methanol-d4, comparing the crowded upfield region for 1-monocapryloyl-*rac*-glycerol (MC) alone (a); a mixture of TC, MC, and caprylic acid (CA) (b). The downfield carbonyl region (not shown) includes peaks from MC (176.4 ppm), TC (177.5 ppm), and CA (178.6 ppm). The concentration of MC is 66 mM in both solutions.

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TABLE 1. ^{13}C NMR of 1-monocapryloyl-*rac*-glycerol in methanolic solutions

Carbon Position[a]	Chemical Shift (ppm)[b]		Relative Height[c]	
	MC	Mixture	MC	Mixture[d]
C=O	176.4	176.4	0.46	0.52
CHOH	72.0	72.0	1.05	1.01
CH_2OR	67.3	67.3	(1.00)	(1.00)
CH_2OH	64.9	64.9	1.02	1.06
(2)	35.8	35.8	1.13	1.08
(3)	26.8	27.0	1.36	1.07
(4), (5)	31.0	31.0	3.30	2.61
(6)	33.7	33.7	1.38	1.21
(7)	24.5	24.5	1.30	1.37
(8)	15.2	15.3	1.18	1.22

[a]Numbering scheme and molecular structure are as shown in Fig. 3.
[b]Measured at 50.3 MHz and referenced externally to a TMS capillary. Peak assignments follow references 8 and 18 and expected trends for carbon spin-lattice relaxation times.
[c]The height of the CH_2OR peak is defined as 1.00 in each spectrum. Both spectra were acquired at 50.3 MHz with full ^{1}H decoupling.
[d]Heights of the acyl chain resonances have been scaled to remove contributions from the CA component.

TABLE 2. Spin-relaxation parameters for 1-monocapryloyl-*rac*-glycerol in a methanolic model digestive mixture

Carbon Position[a]	T_1(C) (sec)		NOE[b]	
	50 MHz	100 MHz	50 MHz	100 MHz
CHOH	2.8	2.6	2.5	2.9
CH_2OR	1.5	1.5	2.5	3.0
CH_2OH	1.5	1.5	2.5	3.0
(2)	3.0	2.8	2.7	3.0
(3)	3.9	3.5	2.7	3.0
(4), (5)	4.6	4.0	2.8	3.0
(6)	6.4	5.5	2.8	3.0
(7)	8.6	7.0	2.9	3.0
(8)	7.3	7.5	2.9	3.0

[a]Numbering scheme and molecular structure are as shown in Fig. 3. The acyl chain peaks (2)–(8) are attributed to both MC and CA components of the mixture.
[b]$1 + \eta$, where η is the Overhauser enhancement of the carbon signal.

Analogous ^{13}C data for the TC–MC–CA mixture in water are presented in **Fig. 5** and **Fig. 6** and **Table 4**. Although most carbon signals in the normal ^{13}C spectrum may be assigned readily, DEPT acquisition clearly improves both spectral resolution and sensitivity (Fig. 5c). Chemical shifts for the mixture are generally in close agreement with those of the individual constituents (if one accounts for a methanol-to-water solvent shift of the monoglyceride). Carbons at positions 1 and 2 of CA do exhibit 4–6 ppm upfield shifts in the lipid mixture; possible explanations include changes in the solution pH, the pK of the carboxyl group, and the degree of aqueous exposure (17, 21, 38).

Even when inverse-gated decoupling is used (Fig. 6), the quantitative results are less satisfactory in D_2O than in CD_3OD. The known MC/CA *ratio* is verified in the ^{13}C NMR spectra, but the *absolute* concentrations of both these compounds are seriously underestimated with respect to TC (Table 4). Using peak integrals rather than heights produces much more scatter in the quantitative estimates, and it does no better job in determining the molarities of constituents in the model digestive mixture (**Table 5**). This surprising result is discussed further below.

DISCUSSION

The spectroscopic results presented herein for TC–MC–CA lipid mixtures indicate the potential usefulness of ^{13}C NMR for investigations of both analytical and physical chemistry of glyceride digestion. Our

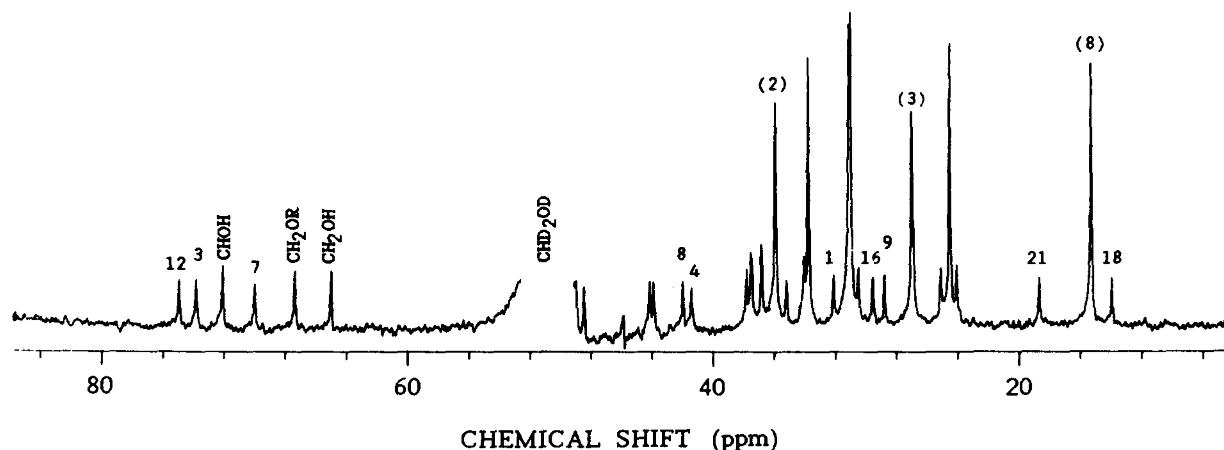

Fig. 4. Upfield portion of the ^{13}C NMR spectrum at 100.5 MHz for lipids in methanol-d4, acquired with inverse-gated decoupling and at 35°C. Assignments are noted only for those peaks used in the quantitative analysis.

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TABLE 3. ^{13}C NMR for quantitation of a model digestive mixture in methanol[a]

Carbon Position[b]	Chemical Shift (ppm)[c]	Relative Height[d]	Apparent Molarity	Molarity Deviation
			mM[e]	%[f]
18	13.9	1.03		
21	18.6	1.02		
9	28.7	1.06		
16	29.5	1.05		
1	32.0	1.00	(56)	
4	41.3	0.97		
8	41.9	1.03		
7	69.9	0.94		
3	73.7	0.95		
12	74.9	0.94		
(8)	15.3	5.11		
(3)	27.0	4.13	253	−4.3
(2)	35.8	4.29		
CH_2OH	64.9	1.16		
CH_2OR	67.3	1.12	66	0.0
CHOH	72.0	1.25		

[a]All spectra were acquired with inverse-gated decoupling and at 100.5 MHz.
[b]Numbering scheme and molecular structures are as shown in Figs. 1 and 3. Data are presented only for peaks which do not overlap, as designated in Fig. 4. Coincident signals from MC and CA are denoted as (2)–(8).
[c]Referenced to external DSS.
[d]Average of duplicate measurements. Relative heights are calculated with respect to the TC average (10 peaks).
[e]The concentration of TC has been fixed at 56 mM. Other values are derived from the average heights of (MC + CA), MC, and TC resonances.
[f]Molarity deviations are calculated for the physiological proportions of TC, MC, and (MC + CA) (36, 37).

water-soluble model system uses the medium-chain fatty-acid and monoglyceride species and bile-salt emulsifier typical of therapeutic lipid fuels (27); the model is also related to lipid mixtures present in the upper small intestine following a fatty meal (13).

DEPT editing of the ^{13}C data has been shown to improve spectral sensitivity and resolution for TC–MC–CA mixtures, and the method may be used for quantitative determinations if accompanied by calibration curves derived from samples of known concentration. Alternatively, reliable quantitative information for lipid mixtures in nonaggregating solvents (molarities, lipid ratios) is available from peak heights observed in ^{13}C NMR spectra acquired with inverse-gated decoupling. For aqueous TC–MC–CA assemblies, however, quantitative analysis using inverse-gated decoupling results in underestimation of MC and CA concentrations. Thus, NMR methods will be most useful for estimating the composition of interesting digestive phases when they are applied to dried samples reconstituted in organic solvents.

It should be noted that although inverse-gated decoupling is a standard technique in quantitative ^{13}C NMR, the use of peak heights is not (29). The accuracy of the heights themselves is not in question, since our experimental protocol includes high sampling densities and NOE calibration tests with ethylene glycol. Since the MC peak intensities, for instance, are confined within somewhat smaller linewidths than are TC peaks (Table 4), using heights for quantitation should overestimate MC concentration. The observed errors are in fact underestimates, so it is not reasonable to attribute them to the use of heights.

Integrated areas are preferable in principle for quantitative NMR assessments, but integration limits of ±6.3 linewidths would be required to include even 90% of the signal intensity for each peak of interest (29). This requirement is not practical for analysis of ^{13}C NMR spectra of the complexity found in model digestive mixtures. Even when partially overlapped peaks were deconvolved using an advanced curve-fitting protocol, the quantitative results for TC–MC–CA mixtures were unsatisfactory. Given the modest signal-

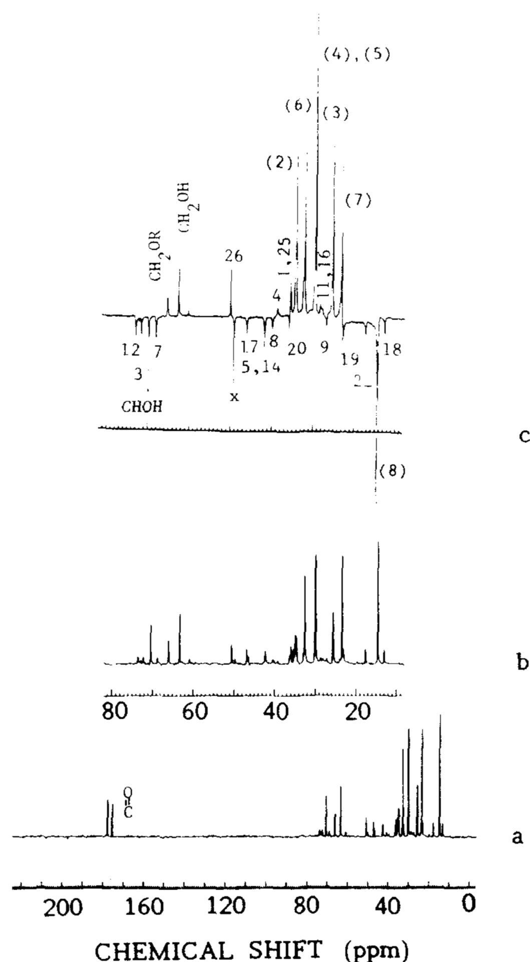

Fig. 5. ^{13}C NMR of a model digestive mixture in D_2O, with single-pulse acquisition (a,b) and DEPT spectral editing (c). A relaxation delay of 10 sec was used in both experiments. The carbon numbering schemes are shown in Figs. 1 and 3; MC and CA resonances are indicated as (n) in cases for which identification by number alone is ambiguous.

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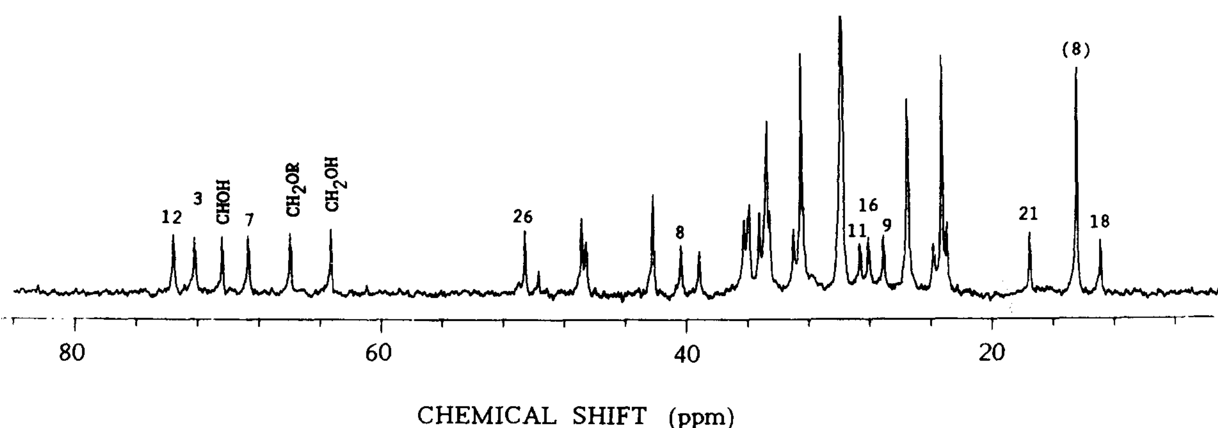

Fig. 6. Upfield portion of the ^{13}C NMR spectrum at 100.5 MHz for lipids in D_2O, acquired with inverse-gated decoupling and at 35°C. Assignments are noted only for those peaks used in the quantitative analysis.

to-noise ratio for many peaks in the spectrum, these curve-fitting methods are in fact expected to suffer from large errors (29). Thus, no analytical advantage is to be gained from integrated areas if the mixture is dilute and chemically complex. As concluded above, the best quantitative results are likely to be obtained from organic solutions in which the NMR linewidths are constant and peak heights reflect ^{13}C signal intensity reliably.

The quality of our quantitative determinations in CD_3OD is quite good, meeting the expectations of prior ^{13}C NMR reports (29) and comparing favorably with a ^{31}P study of concentrated phospholipid mixtures (24). If model digestive mixtures are prepared with concentrations exceeding 20 mM, it should be possible to determine ratios of any component to within a few percent. Absolute concentrations may be found if a reference spectrum is available for one component.

Despite the inherent insensitivity of NMR methods in general and ^{13}C NMR in particular, the described procedures are rapid, direct, and straightforward. The concentration of each compound is typically determined by several independent measurements of peak height; the large chemical shift dispersion of the ^{13}C nucleus makes it feasible to examine multicomponent mixtures *without prior separation.* By contrast, a number of investigators (19, 24) have noted the time-consuming nature and methodological difficulties of quantitative TLC and HPLC as applied to glycerides (16). The customary enzymatic or HPLC analyses of bile salts are sensitive and precise, but they require pretreatment and calibration (15, 39).

TABLE 4. ^{13}C NMR for quantitation of a model digestive mixture in water[a]

Carbon Position	Chemical Shift (ppm)	Relative Height	Apparent Molarity	Molarity Deviation	Line width
			mM	%	*Hz*[b]
18	13.0	1.01			
21	17.6	1.10			
9	27.2	1.03			
16	28.3	0.99			
11	28.8	0.94	(56)		14.6
8	40.5	0.92			
26	50.6	1.08			
7	68.8	0.96			
3	72.3	0.95			
12	73.6	1.01			
(8)	14.6	3.99	223	–16	13.0
CH_2OH	63.4	1.09			
CH_2OR	66.0	1.01	57	–14	10.3
CHOH	70.5	0.96			

[a]Notes as for Table 3. Peaks used in this analysis are designated in Fig. 6.
[b]Full width at half height for measured peaks, including a 7-Hz contribution from digital line broadening.

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TABLE 5. Quantitation methods for an aqueous model digestive mixture[a]

Carbon Position	Relative Height[b]	Relative Integral[c]	Curve-Fit Integral[d]
18	1.01	1.07	1.12
21	1.10	1.00	0.98
9	1.03	1.20	0.88
16	0.99		1.06
11	0.94		0.87
8	0.92	0.59	0.83
26	1.08	1.20	1.21
7	0.96	0.94	1.01
3	0.95	1.21	1.11
12	1.01	0.79	0.93
Std. dev. for TC	0.06	0.22	0.12
(8)	3.99	3.84	3.94
CH_2OH	1.09	0.93	0.97
CH_2OR	1.01	0.88	0.90
CHOH	0.96	0.90	0.90

[a]From 100.5 MHz spectra acquired with inverse-gated decoupling (see Fig. 6). Numbering scheme and molecular structures are as shown in Figs. 1 and 3.
[b]Measured manually and summarized in Table 4.
[c]Computed integrals for nonoverlapping peaks of a typical spectrum.
[d]Computed integrals, using limits of ± 6.31 linewidths, after curve fitting of nonoverlapping peaks.

Our quantitative results for TC–MC–CA in water were somewhat disappointing, regardless of whether ^{13}C peak heights or integrated areas were used for the analysis. It is possible that MC and CA signals have been undercounted because they are tied up in large molecular aggregates, though the optical clarity of the solutions argues against this hypothesis. Nonetheless, the excellent ^{13}C NMR spectra obtained for aqueous lipid mixtures have set the stage for more challenging spin-relaxation investigations of both molecular dynamics and organization in their micellar and bilayer assemblies. It is hoped that such information will ultimately enhance our understanding of glyceride digestion and product transport. ■

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