Behavior of tetrahydrolipstatin in biological model membranes and emulsions

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Abstract Tetrahydrolipstatin (orlistat) (S)-1-{(2S,3S)-3-hexyl-4-oxooxetan-2-yl]methyl}dodecyl N-formyl-L-leucinate, a potent inhibitor of pancreatic lipase, is hydrophobic, amphipathic, and water-insoluble. It binds irreversibly to pancreatic lipases and inhibits fat absorption. The focus of this investigation is on the distribution of orlistat in emulsified fat and vesicular membranes such as might be present in the intestine during fat absorption. The models used were unilamellar vesicles and microemulsion particles. [^{13}C]orlistat was synthesized containing 99% ^{13}C in the leucine carbonyl. Spectra were collected on a Bruker DMX 500 Spectrometer. The chemical shift of the [^{13}C]leucinate carbon was recorded in solvents with increasing hydrogen bonding capacity. The chemical shift moved downfield as H-bonding increased. [^{13}C]orlistat was incorporated into triolein in the presence or absence of water, into sonocated unilamellar egg yolk phosphotidylcholine (EYPC) vesicles, and into microemulsions approximately 300 Å in diameter containing triolein and phospholipid in roughly equal molar proportions. [^{13}C]orlistat was soluble in triolein and had a chemical shift at 20°C of 171.46 ppm. When a small amount of water was added, the chemical shift moved down field to 171.69 ppm. When [^{13}C]orlistat was incorporated into EYPC unilamellar vesicles, the chemical shift increased to ~172.0 ppm at 25°C, indicating an orientation of [^{13}C]leucinate in orlistat closer to the aqueous interface of vesicles, i.e., more surface oriented. In all systems there was a modest downfield increase in chemical shift as the temperature was raised from 5° to 46°C. When small amounts of [^{13}C]orlistat (1% relative to the emulsion mass) were incorporated into microemulsions, the chemical shift was identical to that in the unilamellar vesicles indicating a surface-like orientation of [^{13}C]orlistat. However, when 3% was incorporated, two peaks appeared, one related to the surface at about 172 ppm, and one related to the core at about 171.65 ppm. Thus, orlistat first partitions into the surface and then when the surface is saturated, it moves into the more hydrophobic core. The fact that the two pools can be resolved using ^{13}C NMR spectroscopy indicates a modestly slow exchange between the core and surface pools. Thus, the potent lipase inhibitor orlistat is ideally situated in the surface layer of emulsion particles and membranes for interaction with enzymes that superficially bind to such surfaces.—**Ko, J., and D. M. Small.** Behavior of tetrahydrolipstatin in biological model membranes and emulsions. *J. Lipid Res.* 1997. **38:** 1544–1552.

Supplementary key words lipid bilayers • microemulsions • fat absorption lipase inhibitors • carbon-13 NMR • pancreatic lipase • lipstatin

Orlistat (tetrahydrolipstatin, THL), a potent inhibitor of pancreatic lipase, is a derivative of the naturally occurring lipase inhibitor lipstatin produced from *Streptomyces toxytricini* (1, 2). Orlistat, (S)-1-{(2S, 3S)-3-hexyl-4-oxooxetan-2-yl}methyl}dodecyl N-formyl-L-leucinate (Fig. 1), a rather hydrophobic molecule, poorly soluble in water, binds irrevesibly to pancreatic lipases. The role of pancreatic lipase in the human body is to permit the digestion of lipids (3). This lipase is produced in the pars exocrine of the pancreas and secreted through the pancreatic duct into the duodenum. There, the lipase breaks down triglycerides and diacylglycerides at the surfaces of emulsion particles and mixed micelles into free fatty acids and 2-monoglycerides for absorption. Because of its ability to inhibit pancreatic lipase, orlistat may have a role in the treatment of obesity (4–7).

The β-lactone group of orlistat is believed to play a vital role in the inhibition of pancreatic lipases. The mechanism of inhibition by orlistat is through a covalent bond to the active-site serine of the lipase (8, 9). This inhibition in the analog core of the carboxyl ester lipase has a characteristic irreversible inactivation of the uncompetitive type, and thus an enzyme-substrate-inhibitor complex is formed (5). This reaction between orlistat and the lipase probably takes place at the aqueous/oil interface of the substrate.

The inhibition by orlistat has been documented in both human and pig pancreatic lipase (4, 5). Experiments were conducted on humans and experimental animals to test the effectiveness of orlistat in blocking

Abbreviations: EYPC, egg yolk phosphatidylcholine; TO, triolein; PC, phosphatidylcholine; NMR, nuclear magnetic resonance.

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lipid absorption (7, 10). Further results indicate that orlistat remains in the GI tract without being absorbed (9). Whereas in animals a nearly complete inhibition of fat absorption can be achieved (6, 10), a more modest inhibition would be a reasonable goal in humans (11, 12).

The focus of this investigation is on the behavior of orlistat in emulsified fat and biological model vesicular membranes such as might be present in the intestine during fat absorption (3). The models used were unilamellar vesicles and microemulsion particles. The goal of this investigation was to better understand the partitioning behavior of orlistat between surface and core for both unilamellar PC vesicles and TO/PC microemulsion particles. The technique described by Hamilton and Small (13) to estimate the distribution of weakly polar lipids between hydrophilic surface orientation and hydrophobic core locations by ^{13}C NMR spectroscopy was used in this study.

MATERIALS AND METHODS

[^{13}C]Tetrahydrolipstatin

[^{13}C]orlistat was synthesized by Dr. Philip F. Huguenin at Hoffmann-La Roche Ltd., Basel, Switzerland. Its melting point is 46°C. Leucine 99% ^{13}C in carbonyl carbon was used to make [^{13}C]orlistat. The position of the enriched carbon is shown in Fig. 1.

Other reagents

Triolein (TO) was purchased from Nu-Chek Prep, Inc. (Elysian, MN). Egg phosphatidylcholine (PC) of 99% purity was purchased from Avanti Polar Lipids (Alabaster, AL). Both were quite pure by chromatography and used without further purification. Deuterium oxide (D_2O), deuterated methanol (CD_3OD), and deuterated chloroform ($CDCl_3$) were purchased from Wilmad Glass Co. (Buena, NJ). Carbontetrachloride (CCl_4) was purchased from J.T. Baker (Phillipsburg, NJ). Tetramethylsilane (TMS) was purchased from Stohler Isotope Chemicals (Rutherford, NJ).

Nuclear magnetic resonance

^{13}C NMR spectra were obtained on a Bruker DMX-500 spectrometer (Billerica, MA). The machine was fitted with a 10-mm broad band probe to detect the ^{13}C nuclei. All aqueous samples were placed in either 5-mm or 10-mm ^{13}C NMR tubes with D_2O added to provide an internal lock, unless otherwise reported.

Sample loading and collection of spectra. The NMR sample tubes were suspended on a stream of air before lowering them into the broad band probe. Once in, the samples were spun at 20 revolutions/min. Spectra taken with the Bruker DMX-500 spectrometer were obtained with a pulse interval of 5 seconds. The temperature varied with each experiment.

Chemical shift reference. For the [^{13}C]orlistat in TO ± H_2O, PC vesicles, and PC/TO microemulsion samples, the terminal methyl groups of the PC acyl chains at 14.10 ppm served as an internal chemical shift reference (13). For the organic solvent samples, TMS at 0 ppm was added and used as an internal chemical shift reference.

Signal locking. The signals for [^{13}C]orlistat were locked with internal D_2O for all samples with two exceptions. For both [^{13}C]orlistat in CCl_4 and [^{13}C]orlistat in TO ± H_2O, an external D_2O insert was placed into the tube for signal locking.

Sample preparation

[^{13}C]orlistat in organic solvents. Between 2.00 mg and 2.31 mg of [^{13}C]orlistat was dissolved into 0.5 ml of CCl_4, $CDCl_3$, CD_3OD, and $CDCl_3$: CD_3OD (1:1 by wt.) in 5-mm NMR tubes. The final concentrations of [^{13}C]orlistat were as follows: 9.07 mM in CCl_4, 9.23 mM in $CDCl_3$, 8.25 mM in CD_3OD, and 8.13 mM in $CDCl_3$: CD_3OD. For CCl_4, $CDCl_3$, and CD_3OD samples, spectra were taken at 10°C, 20°C, 25°C, 30°C, 40°C, and 50°C. For $CDCl_3$: CD_3OD samples, spectra were taken at 20°C and 25°C.

[^{13}C]orlistat in TO ± H_2O. Varying concentration of [^{13}C]orlistat were prepared in TO without and with a large molar excess of water.

0.5% [^{13}C]orlistat in TO. [^{13}C]orlistat (2.11 mg) was dissolved in 0.5 ml of TO in a 5-mm NMR tube. The tube was sealed and incubated at 50°C (i.e., 4°C above the melting point of orlistat) for 15 min., then lightly vortexed. The final concentration of [^{13}C]orlistat was 0.46% by weight. Spectra were taken at 20°C and 25°C.

0.5% [^{13}C]orlistat in TO + 50 µl H_2O. [^{13}C]orlistat (2.31 mg) was dissolved in 0.5 ml of TO in a 5-mm NMR tube. Fifty µl of H_2O was added. The tube was sealed and incubated at 50°C for 15 min., then lightly vortexed. The final concentration of [^{13}C]orlistat was 0.46% by weight. Spectra were taken at 10°C, 20°C, 25°C, 30°C, 40°C, and 50°C.

2% [^{13}C]orlistat in TO + 100 µl H_2O. [^{13}C]orlistat (9.57 mg) was dissolved in 0.5 ml of TO in a 5-mm NMR tube. One hundred µl of H_2O was added. The tube was sealed and incubated at 50°C for 15 min., then lightly vortexed. The final concentration of [^{13}C]orlistat was 1.7% by weight. Spectra were taken at 10°C, 20°C, 30°C, 40°C, and 50°C.

5% [^{13}C]orlistat in TO + 100 µl H_2O. [^{13}C]orlistat (30.23 mg) was dissolved in 0.5 ml of TO in a 5-mm NMR tube. One hundred µl of H_2O was added. The tube was sealed and incubated at 50°C for 15 min., then lightly vortexed. The final concentration of [^{13}C]orlistat was 5.17% by weight. Spectra were taken at 10°C, 20°C, 30°C, 40°C, and 50°C.

[^{13}C]orlistat in egg PC. Four samples of unilamellar vesicles with varying amounts of [^{13}C]orlistat (1%, 2%, 5%, and 10%) were prepared by weighing out 1.00 mg, 2.15 mg, 5.08 mg, and 10.07 mg of [^{13}C]orlistat, adding them to aliquots of egg PC in chloroform (dry weight of 97 mg), and evaporating the solvent under N_2. Each sample was hydrated with 1.6 ml of a 0.56% w/w KCl solution in H_2O and 0.2 ml D_2O, incubated at 47°C for 15 min, and ultrasonically irradiated using a Branson W-350 sonifier in a pulsed mode at 25–30°C. Sonification was carried out for 60 min and the titanium debris from the sonifier was removed by spinning the samples for 15 min using a low speed table-top centrifuge. The translucent supernatant was transferred to an NMR tube. The final concentration of PC and the mol ratio of orlistat to PC in each sample are as follows: for 1%, 55.1 mg PC/ml and 1:63.7; for 2%, 59.6 mg PC/ml and 1:32.1; for 5%, 53.6 mg PC/ml and 1:12.2; and for 10%, 50.1 mg PC/ml and 1:5.8. For the 1% and 10% samples, spectra were taken at 5°C, 15°C, 25°C, 35°C, and 46°C. For the 2% and 5% samples, spectra were taken at 46°C.

[^{13}C]orlistat in TO/PC microemulsions. Three samples of microemulsion particles with varying amounts of [^{13}C]orlistat (1%, 3%, and 6%) were prepared by weighing out 1.52 mg, 5.08 mg, and 10.00 mg of [^{13}C]orlistat and adding them to aliquots of 80 mg of TO and 70 mg of egg PC in chloroform and evaporating the solvent under N_2. The samples were lyophilized in a Labconco Lyph-Lock 6 lyophilizer (Kansas City, MO) for 20 min and hydrated with 10 ml of Tris buffer (0.1 M KCl, 0.01 M Tris-HCl at pH 8.0, and 0.025% NaN_3). They were then ultrasonically irradiated using a Branson W-350 sonifier in a continuous mode at 25–30°C for 1 hr and transferred to Beckman 50-3Ti ultracentrifuge tubes. A small layer of H_2O was placed on top of each sample and spun at 25,000 rpm for 15 min without braking on a Beckman L8-70 ultracentrifuge (Palo Alto, CA). The top 1 ml was lifted off and 3.31 g of KBr was added to the samples. They were returned to the ultracentrifuge and spun at 30,000 rpm for 1 h without braking. The top 2 ml was lifted off and placed into Amicon Centricon concentrators (30,000 MW) (Beverly, MA). The concentrators were spun in a Sorvall RC-5B centrifuge at 5000 rpm for 75 min. Tris buffer was added to the remaining concentrates to bring the volume to 2 ml. From each sample, an aliquot of 50 µl was taken for negative staining electron microscopy and 14 µl was taken for lipid analysis. The samples were transferred to 10-mm NMR tubes and 0.2 ml of D_2O was added to each. The mol ratios of orlistat to TO to PC in each sample are as follows: for 1%, 1:28.5:29.6; for 3%, 1:8.5:8.9; and for 6%, 1:4.3:4.5. For 1% and 6% samples, spectra were taken at 5°C, 15°C, 25°C, 35°C, and 46°C. For 3% samples, spectra were taken at 25°C and 46°C.

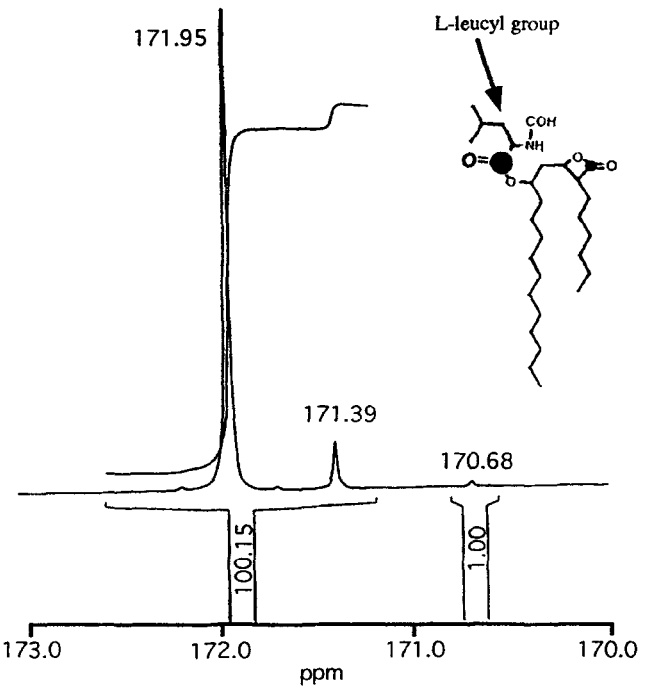

Fig. 1. Integration spectra analysis of [^{13}C]orlistat. The chemical structure of orlistat is shown on the right. The leucinyl carbonyl was enriched by using [^{13}C]leucine enriched by 99% at the carbonate so it would be easily seen in low concentrations. It is marked with a large black dot. The small black dot represents the β-lactone carbonyl. The carbonyl peaks of [^{13}C]orlistat were integrated and compared to one another to determine the ^{13}C enrichment of orlistat. From the area, the enrichment appears to be 99% pure.

RESULTS

Purity of [^{13}C]orlistat

The ^{13}C enrichment of orlistat was determined by integration spectra analysis. The spectrum of [^{13}C]orlistat in $CDCl_3$ at 25°C was used for this calculation. In the carbonyl region of the spectrum, a major peak at 171.95 ppm and two very minor peaks at 171.39 ppm and 170.68 ppm are seen. The peak at 171.95 ppm belongs to the ^{13}C-enriched carbonyl. The peak at 170.68 ppm is from the carbonyl in the β-lactone group of orlistat. The third peak (171.39 ppm) is from the ^{13}C-enriched

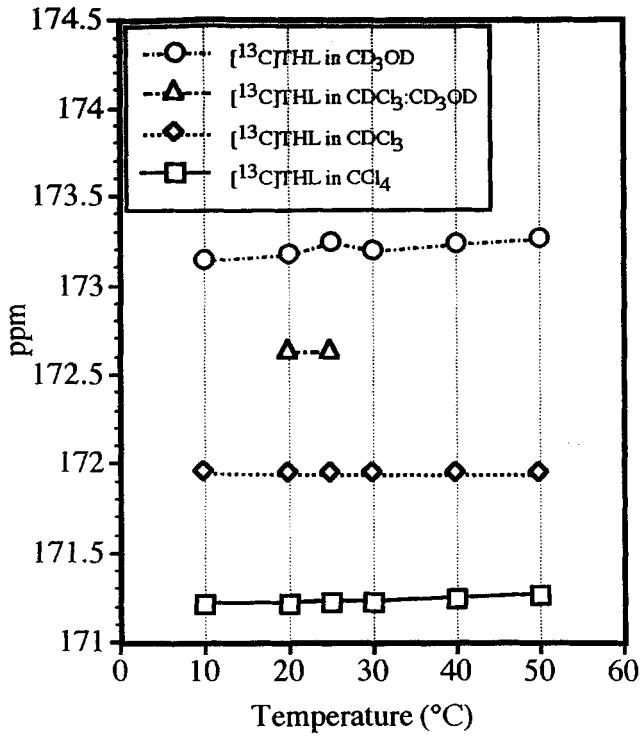

Fig. 2. The temperature dependence of the chemical shifts of [^{13}C]orlistat in CCl_4, $CDCl_3$, CD_3OD, and $CDCl_3$:CD_3OD (1:1 by wt.).

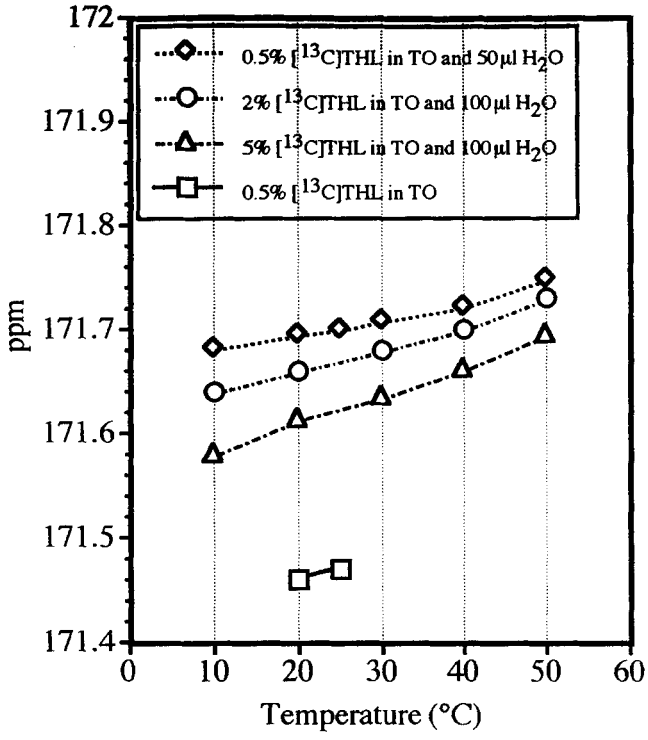

Fig. 3. Chemical shifts of [^{13}C]orlistat in TO ± H_2O. The upfield shift from the 0.5% orlistat in TO with water to the 0.5% orlistat in pure TO is due to the net decrease in hydrogen bonding of the orlistat.

carbonyl carbon of an isomer form of orlistat. The [^{13}C]orlistat signals from the two ^{13}C-enriched carbonyls were integrated and added together. Their combined area was compared to that of the integrated β-lactone carbonyl. The results indicate that the ^{13}C enrichment of orlistat was 99% pure. **Figure 1** illustrates the three carbonyl peaks of ^{13}C orlistat with their integration.

[^{13}C]orlistat in different organic solvents

The chemical shifts of [^{13}C]orlistat in four different solvents (CCl_4, $CDCl_3$, CD_3OD, and 50% $CDCl_3$:50% CD_3OD) are plotted as a function of temperature and illustrated in **Fig. 2.** The peaks do not shift more than 0.12 ppm for any of the solvents throughout the temperature range. The line widths remain at 1.4 Hz for [^{13}C]orlistat in $CDCl_3$, CD_3OD, and 50% $CDCl_3$:50% CD_3OD throughout the entire temperature range. The line widths for [^{13}C]orlistat in CCl_4 start out at 1.4 Hz (10°C) and increase to 3 Hz by 50°C. The chemical shift values are in good agreement with the observation that upfield shifts are proportional to a decrease in hydrogen bonding. Moving from CD_3OD to CCl_4, the peaks progressively shift upfield.

[^{13}C]orlistat in triolein

The chemical shifts of different percentages of [^{13}C]orlistat in TO ± H_2O are combined and plotted in **Fig. 3** as a function of temperature. These frequencies are in order of amount of hydration. Small, nearly linear increases in chemical shift are evident as the temperature is increased. The presence of water in large molar excess to orlistat causes significant shift to higher values. In excess water the most downfield set of frequencies belongs to 0.5% [^{13}C]orlistat in TO + 50 µl H_2O.

Chemical shifts versus dielectric constants

The chemical shifts at 25°C of [^{13}C]orlistat in CCl_4, $CDCl_3$, CD_3OD, $CDCl_3$:CD_3OD (1:1 by wt.), and TO are combined and plotted against the log of the dielectric constants of the solvents listed (**Fig. 4**). Linear curve fitting of the chemical shifts produces a line ($y = 1.628x + 170.71$) with an r value of 0.992. This line is used as a guide for the chemical shifts of orlistat in model membranes to estimate its ionic environment.

[^{13}C]orlistat in egg PC vesicles—model cellular membrane

The effect of temperature on the chemical shifts of 1%, 2%, 5%, and 10% [^{13}C]orlistat in egg PC vesicles

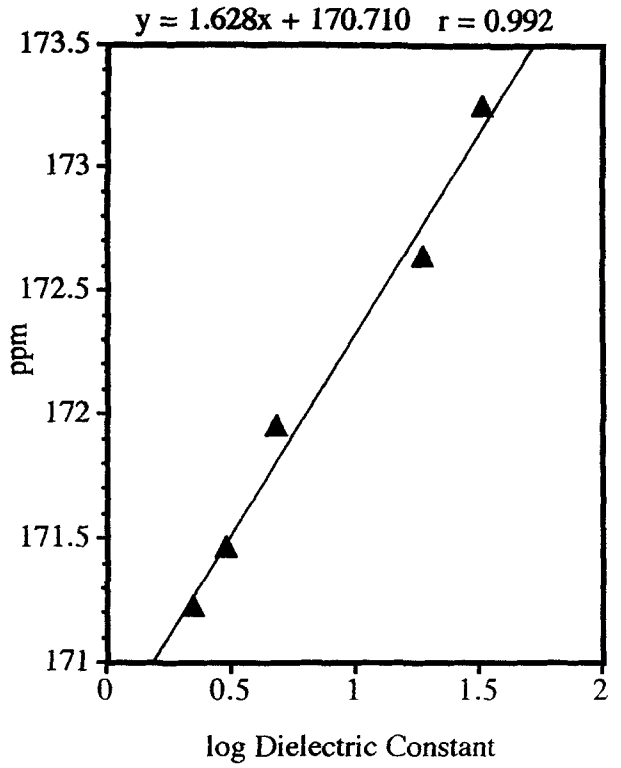

Fig. 4. Chemical shifts of orlistat as a function of the log of the dielectric constant of the solvent. The chemical shifts at 25°C of [^{13}C]orlistat from various solvents are plotted with respect to the log of the dielectric constants of those solvents. The five solvents from left to right are as follows: CCl_4, TO, $CDCl_3$, $CDCl_3$:CD_3OD, and CD_3OD. The linear curve fit produces the line y = 1.684x + 170.67.

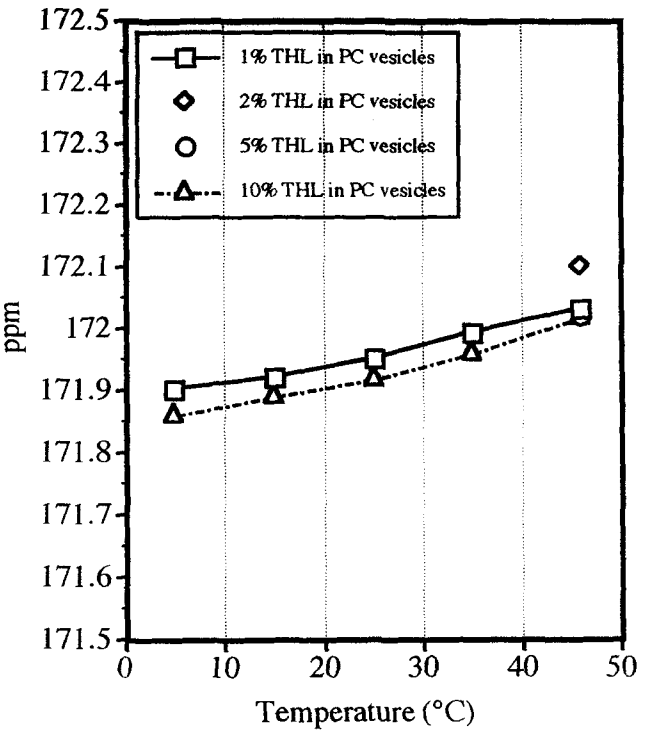

Fig. 5. The chemical shifts of 1%, 2%, 5%, and 10% [^{13}C]orlistat in egg PC vesicles. The line widths broaden with decreasing temperature. The points at 5% and 10% [^{13}C]orlistat at 46°C are hidden behind the 1% point.

is shown in **Fig. 5.** The greatest change in chemical shift within any one sample is 0.16 ppm. In all vesicle samples, the line widths broaden with decreasing temperature. This is probably due to the decrease in exchange of orlistat between the inner and outer layers of the vesicle. As an example, **Fig. 6** illustrates the spectra of 1% [^{13}C]orlistat in PC vesicles at different temperatures. It shows the line widths broadening from 8 Hz (46°C) to 19 Hz (5°C). The line widths for 10% [^{13}C]orlistat in PC vesicles start at 6 Hz (46°C) and broaden to 24 Hz (5°C).

[^{13}C]orlistat in triolein/egg PC microemulsions

The results from lipid analysis of the 1%, 3%, and 6% [^{13}C]orlistat in TO/PC microemulsion samples indicate that the diameters of the particles are between 240 Å and 380 Å. These microemulsions were also imaged with negative staining electron microscopy. The measurements were in agreement with the calculated diameters based on lipid composition from above. The chemical shifts of all three samples are shown in **Fig. 7.** The 1% and 6% [^{13}C]orlistat samples have one peak whereas the 3% sample has two peaks. The line widths for the 1% and 3% samples are difficult to calculate because the peaks are partly overlapped by TO peaks. However, the line widths for the 6% sample are 13 to 14 Hz throughout the experimental temperature range.

Partitioning between surface and core

Table 1 lists the chemical shifts of all the samples. They are plotted as a function of temperature and are shown in **Fig. 8.** The chemical shifts are clustered in two groups. The upfield group consists of all of the orlistat in TO + H_2O samples, the 6% orlistat microemulsion sample, and one of the peaks of the 3% orlistat microemulsion sample. The downfield group consists of all of the vesicle samples, the 1% orlistat microemulsion sample, and the other peak of the 3% orlistat microemulsion sample. It is proposed that the upfield chemical shifts represent orlistat in an oily core environment, whereas the downfield chemical shifts represent orlistat that exists on the surface of vesicles and microemulsion particles (see Discussion section). Therefore, [^{13}C]orlistat in the 1% microemulsions resides mainly on the surface. In the 3% emulsions, orlistat partitions between the core and surface, and two peaks are present. At 6%, partitioning is mainly to the core and even though a

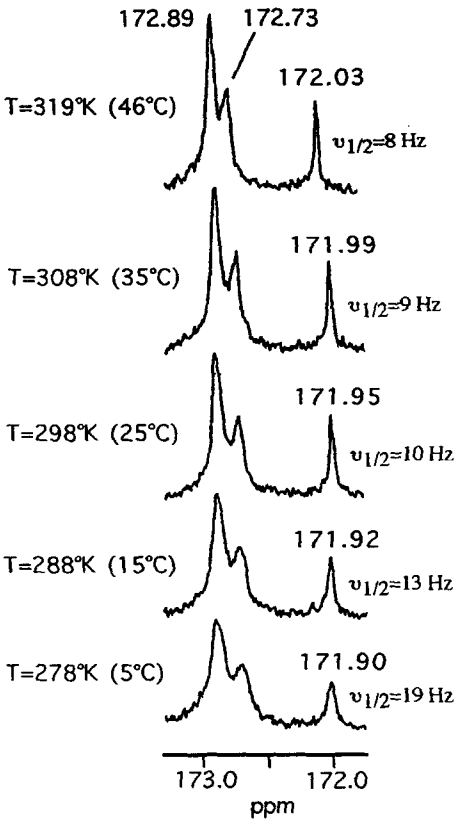

Fig. 6. The ^{13}C-NMR spectra of 1% [^{13}C]orlistat in egg PC vesicles. The spectra shows the broadening of the line widths from 8 Hz (46°C) to 19 Hz (5°C). The two peaks at 172.73 and 172.89 ppm are from the inner and outer PC carbonyl carbons, respectively.

small fraction (~1/6) is present in the surface, it cannot be definitely separated from the large peak.

DISCUSSION

The chemical shifts of orlistat in pure TO well represent the behavior of orlistat in the middle of a pure oil droplet with no water present. Each orlistat molecule is completely surrounded by TO, at 25°C, the chemical shift is less than 171.5 ppm. This purely hydrophobic environment, however, is unrealistic as a model of biological emulsion particles and membranes as an aqueous environment bathes these structures. It only illustrates one extreme of the possible types of environments orlistat could encounter. The samples of orlistat in hydrated TO are more realistic. These appear to mimic the environment found in the lipid core of emulsion particles. While still immersed in triglycerides, there are some interactions of orlistat with water, and hence, there is a small downfield shift in frequency for

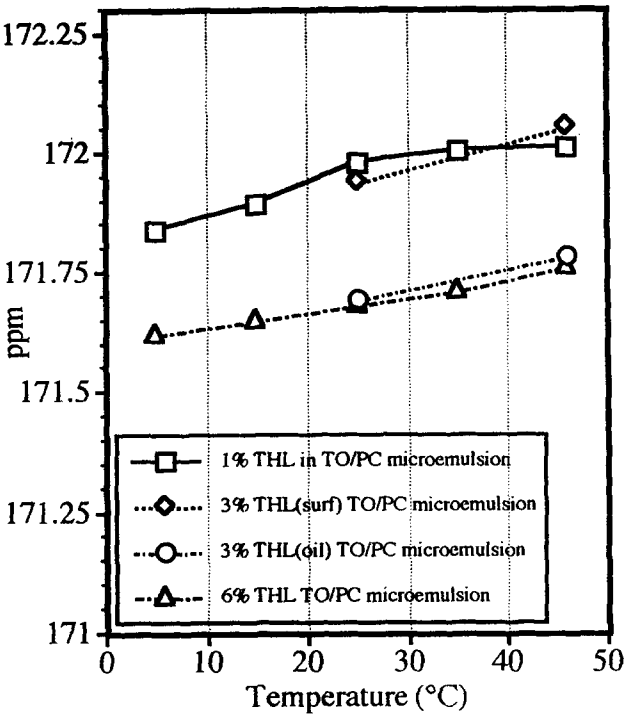

Fig. 7. Chemical shifts of 1%, 3%, and 6% [^{13}C]orlistat in TO/PC microemulsion particles. The line widths for 1% and 3% are not estimated. The line widths for 6% fluctuate between 13 and 14 Hz.

orlistat. At 25°C, these samples range from 171.6 ppm to 171.7 ppm, depending on the amount of water saturation.

In unilamellar vesicles, orlistat can be found in either the outer layer or the inner layer of the lipid bilayer. This is suggested by the broadening of the orlistat peaks at low temperatures. The exchange between the two layers slows down enough (in the NMR time scale) to be registered by the NMR as peak broadening but it does not slow enough to allow two separate peaks representing the inside and outside pool to be distinguished. A decreased temperature does bring out splitting of inner and outer pools of some other amphipaths such as bile acids (14, 15) and diacylglycerols (16) so that exchange rates between pools can be measured. In Fig. 8, the chemical shifts for all orlistat samples in vesicles cluster together. At 25°C, they ranged from 171.9 ppm to 172.0 ppm. This tight grouping suggests that all of the orlistat in vesicles were experiencing very similar environments.

Using the log Dielectric Constant plot, the average theoretical dielectric constant for vesicle aqueous environments at 25°C is 5.8 ϵ. This is similar to the behavior of orlistat in $CDCl_3$, a rather hydrophobic environment. This low value suggests that while the leucine carbonyl of orlistat is exposed to the aqueous environment, it is still very shielded by hydrophobic groups.

For samples of orlistat in TO/H_2O and 6% orlistat

TABLE 1. Chemical shift values of [^{13}C] orlistat in various environments

Organic Solvents and Pure Triolein	Chemical Shift	PC Vesicles and TO/PC Microemulsions	Chemical Shift	Triolein and Water	Chemical Shift
Orlistat in CCl_4		Orlistat (1.0%) in PC vesicles		Orlistat (0.5%) in TO/50 μl H_2O	
283°K (10°C)	171.22	278°K (5°C)	171.90	283°K (10°C)	171.68
293°K (20°C)	171.22	288°K (15°C)	171.92	293°K (20°C)	171.69
298°K (25°C)	171.23	298°K (25°C)	171.95	298°K (25°C)	171.70
303°K (30°C)	171.23	308°K (35°C)	171.99	303°K (30°C)	171.71
313°K (40°C)	171.25	319°K (46°C)	172.03	313°K (40°C)	171.72
323°K (50°C)	171.27			323°K (50°C)	171.75
Orlistat in $CDCl_3$		Orlistat (2.0%) in PC vesicles		Orlistat (2.0%) in TO/100 μl H_2O	
282°K (10°C)	171.96	319°K (46°C)	172.10	283°K (10°C)	171.64
293°K (20°C)	171.95			293°K (20°C)	171.66
298°K (25°C)	171.95			303°K (30°C)	171.68
303°K (30°C)	171.95	Orlistat (5.0%) in PC vesicles		313°K (40°C)	171.70
313°K (40°C)	171.95			323°K (50°C)	171.73
323°K (50°C)	171.95	319°K (46°C)	172.02		
Orlistat in CD_3OD		Orlistat (10.0%) in PC vesicles		Orlistat (5.0%) in TO/100 μl H_2O	
283°K (10°C)	173.15	278°K (5°C)	171.86	283°K (10°C)	171.58
293°K (20°C)	173.18	288°K (15°C)	171.89	293°K (20°C)	171.61
298°K (25°C)	173.25	298°K (25°C)	171.92	303°K (30°C)	171.63
303°K (30°C)	173.20	308°K (35°C)	171.96	313°K (40°C)	171.66
313°K (40°C)	173.24	319°K (45°C)	172.02	323°K (50°C)	171.69
323°K (50°C)	173.27				
Orlistat in TO		Orlistat (1.0%) in Microemulsions			
293°K (20°C)	171.46	278°K (5°C)	171.84		
298°K (25°C)	171.47	288°K (15°C)	171.89		
		298°K (25°C)	171.98		
		308°K (35°C)	172.00		
		310°K (46°C)	172.01		
Orlistat in $CDCl_3$:CD_3OD		Orlistat (6.0%) in Microemulsions			
293°K (20°C)	172.64	278°K (5°C)	171.62		
298°K (25°C)	172.64	288°K (15°C)	171.65		
		298°K (25°C)	171.68		
		308°K (35°C)	171.71		
		319°K (46°C)	171.76		
CD_3OD/H_2O 5:1	173.52	Orlistat (3.0%) in Microemulsions	Surface Oil		
		298°K (25°C)	171.94 171.69		
		319°K (46°C)	172.05 171.78		

microemulsion particles, a similar grouping developed in the chemical shifts. At 25°C, they were all within the 171.6–171.7 ppm range. This indicates that orlistat in those microemulsions behaves as if it were in an oily environment. By placing the chemical shift values on the plot of the log dielectric constant, the theoretical average dielectric constant at 25°C for this oily environment is 3.8 ϵ., like TO in the presence of water. This is slightly higher than for orlistat in a pure (dry) TO environment. The chemical shifts for the 1% orlistat microemulsion particles indicate that the orlistat molecules are more surface located, similar to those in vesicles. The values of these chemical shifts are indistinguishable from those of the vesicles (Fig. 8).

An interesting finding results from the 3% microemulsion samples. Not only did this sample produce the "oily" chemical shift, it also produced a second peak that grouped with the chemical shifts of the orlistat vesicles (Fig. 8). It is speculated that at 3%, orlistat partitions to both the core and surfaces of microemulsion particles and that the exchange is slow enough to observe the two separate pools. As emulsion particles have two different regions (the hydrophobic core and the hydrophilic surface) and orlistat is an amphiphilic molecule, it is reasonable to conclude that orlistat would be found in both. Hence, there are two populations of orlistat with different chemical shifts. The presence of only one peak in the 1% sample indicates that the distribution of orlistat is not random. It appears that orlistat partitions to the surface first. Once the surface is saturated, then orlistat starts to enter the lipid core. The saturation point for these microemulsions is less than

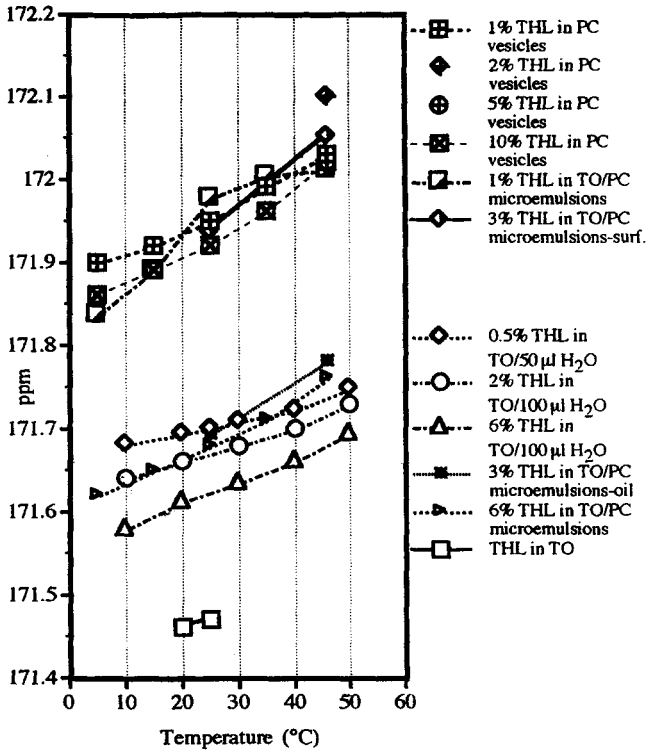

Fig. 8. Chemical shifts of [^{13}C]orlistat in various environments as a function of temperature.

3% orlistat (less than 11 mol% relative to surface phospholipid). Even though only one peak is seen at 6%, it is probable that there are actually two. Because the chemical shift difference between core and surface peaks is small (~0.3 ppm) and because the core peak becomes so large, it probably overlaps and masks the smaller surface peak.

The presence of orlistat in the surface of emulsion particles would allow easy access of orlistat to lipases. Thus as each lipase is adsorbed, it would rapidly interact with orlistat, forming an irreversible covalent bond to the active-site serine of the lipase at the aqueous/oil interface and becoming inactive (8, 9). The functional group of the orlistat is the β-lactone group. By altering this group, the action of orlistat as a lipase inhibitor is abolished (1).

Bioavailability is an important issue in the formulation of orlistat; as its melting point (46°C) is above body temperatures, it is quite insoluble in water and its desired activity is restricted to the gut lumen (12). The goal is to define the point where the minimum amount of drug can produce the desired effects. It is known that pancreatic lipase and other triglyceride lipases bind loosely to the surface of emulsions and probably utilize substrate present in the interface. Thus to effectively come in contact with lipase, orlistat should be on the surface. A hypothetical model of orlistat in the monolayer, a membrane, or an emulsion is represented in **Fig. 9.** In this model, the two hydrocarbon chains fold together and insert into the hydrophobic bilayer core. The β-lactone group and N-formyl are partly exposed to the aqueous environment. The L-leucyl side chain could either be free to interact with the aqueous environment or buried within the bilayer. The microemulsion particles studied here suggest that orlistat at low concentrations prefers a surface location, but as the concentration increases it partitions between core and surface. At 3% orlistat, peaks for both core and surface are present and this suggests that orlistat is exchanging relatively slowly between the core and surface. The triolein core of these particles could act as a reservoir to continually supply orlistat to the surface so that new enzymes would always find an orlistat to bind.

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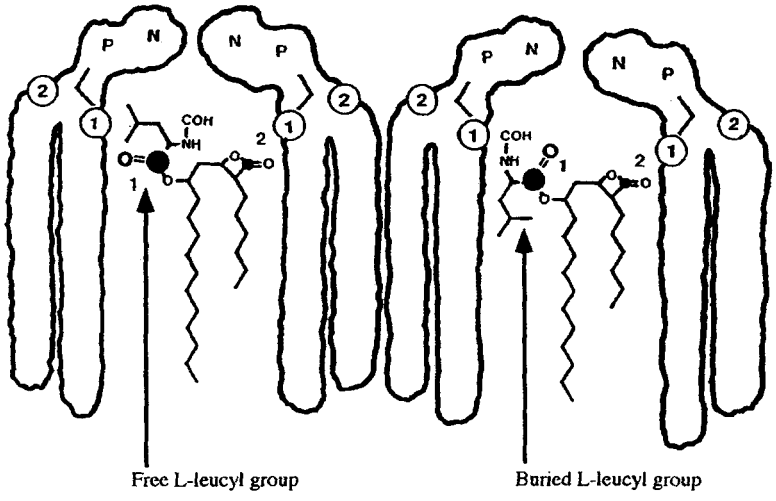

Fig. 9. Model of orlistat in membranes or the surface phospholipid layer of emulsions. In this model, the two hydrocarbon chains of orlistat fold together and insert into the hydrophobic bilayer core. The β-lactone group and N-formyl group are exposed to the aqueous environment. The L-leucyl side chain may either be free (left) to interact with the aqueous environment or buried (right) within the molecule.

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