Structures of the subgel phases of *n*-saturated diacyl phosphatidylcholine bilayers: FTIR spectroscopic studies of $^{13}C{=}O$ and ^{2}H labeled lipids

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ABSTRACT The subgel phases of unlabeled, specifically chain perdeuterated and specifically $^{13}C{=}O$ labeled representative samples of the *n*-saturated diacylphosphatidylcholines were studied by Fourier-transform infrared spectroscopy. Our results indicate that the spectroscopic properties exhibited by the subgel phases of the longer chain homologues are not consistent with that of a pure phase and we suggest that this is because the observed spectrum is a summation of spectroscopic features arising from both their subgel and L_β gel phases. Using spectral subtraction techniques, we obtained a spectrum which we believe is more representative of the pure subgel phase and from it we suggest that the subgel phase of the long chain phosphatidylcholines is an ordered crystallike structure containing two vibrationally inequivalent populations of lipid molecules arranged with the zigzag planes of their hydrocarbon chains parallel. For dipalmitoylphosphatidylcholine, our data indicate that its stable subgel phase is generally similar to that of the longer chain homologues but it is a more ordered structure in which the polar/apolar interfacial region is probably less hydrated. With the medium chain ($N = 13 - 15$) compounds, two populations of vibrationally equivalent molecules are also present in the subgel phase, but unlike DPPC and the longer chain homologues, the zigzag planes of their *sn*1- and *sn*2- acyl chains are perpendicular to each other, and a *sn*1-ester C=O group of one of the populations is in relatively close contact with an *sn*2-ester C=O group of the other population. With the shorter chain ($N = 10 - 12$) compounds, our data is indicative of a very complex quasi-crystalline assembly in which there may be at least three vibrationally inequivalent populations of lipid molecules with rotationally disordered hydrocarbon chains. Moreover, the conformation of the glycerol backbone may well be very different from that usually expected of this class of phospholipids. With all of these lipids, the structural pictures which emerge from our studies of the various subgel phases are in many aspects incompatible with that deduced from the single crystal x-ray studies of dimyristoylphosphatidylcholine. We suggest that this is because under our experimental conditions, these lipids have effectively been crystallized from water, whereas the sample used for the single-crystal x-ray study was crystallized from organic solvents.

INTRODUCTION

Aqueous dispersions of dipalmitoylphosphatidylcholine (DPPC)[1] are the most thoroughly studied of all model lipid bilayers, and as a result the thermotropic phase behavior, and the organization and dynamics of DPPC molecules in their various gel (L_β and P'_β) phases and in the liquid-crystalline (L_α) phase, are relatively well understood (see Cevc and Marsh, 1987 and references cited therein). However, the formation and structure of the subgel (L_c) phases formed by aqueous dispersions of this and other linear saturated PCs are less well understood and it is apparent that those processes are more complex than first described by Chen et al. (1980). For instance, DSC has shown that the formation of the stable L_c phase of most PCs is a multistage process which proceeds via a number of L_c-like intermediates (Lewis et al., 1987; Silvius et al., 1985; Tristram-Nagel et al., 1987; Finegold and Singer, 1984; 1986), and that the rate of L_c phase formation decreases markedly with increases in acyl chain length (Lewis et al., 1987). In a previous FTIR spectroscopic study we demonstrated that the above results are attributable to the fact that one or more spectroscopically distinct L_c phases are sequentially formed upon incubation of aqueous dispersions of these PCs at low temperatures (Lewis and McElhaney, 1990). Moreover, that study also showed that the structures of the stable L_c phases formed by this homologous series of PCs also differ significantly as the length of the hydrocarbon chain is varied. Specifically, we demonstrated that the stable L_c phases of the longer chain PCs are characterized by less extensive hydrogen-bonding interactions at the polar/apolar interface but by tighter packing of the hydrocarbon chains, whereas the stable L_c phases of the short chain homologues are characterized

[1] *Abbreviations used in this paper:* C=O, carbonyl; DCCD, *N,N*-dicyclohexylcarbodiimide; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphorylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; DSC, differential scanning calorimetry; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine; FTIR, Fourier transform infrared; ΔH_{cal}, calorimetric enthalpy; Lyso-PC, 1-O-acyl-lysophosphatidylcholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; T_m, gel to liquid-crystalline phase transition temperature.

0006-3495/92/01/63/15 $2.00

by more extensive hydrogen bonding at the polar/apolar interfacial region but by suboptimal packing of the acyl chains. We therefore suggested that the structure of the L_c phase formed by any particular PC is determined by an optimization of the partially incompatible requirements for maximal van der Waal's contacts between the hydrocarbon chains and for maximal polar interactions at the headgroup and polar/apolar interfacial regions of the lipid bilayer. Because L_c phases are relatively dehydrated structures characterized principally by strongly interacting and fairly immobilized polar headgroups (Cevc and Marsh, 1987), one can thus rationalize the faster kinetics of L_c phase formation and the more extensive polar headgroup and interfacial hydrogen-bonding interactions observed in the stable L_c phase of the shorter chain PCs.

We describe here a follow-up FTIR spectroscopic study designed to further elucidate the structural basis of the spectroscopic differences previously observed between the stable L_c phases of the homologous series of linear saturated diacyl PCs. In this study we utilized PC molecules specifically labeled with either ^{13}C at the carbonyl group of the acyl chain, or those in which one of the acyl chains has been perdeuterated. With the specifically $^{13}C{=}O$ labeled PCs, the interpretation of the complex C=O stretching band contours which typify the subgel phases of these lipids are considerably simplified, because one can unambiguously assign the infrared absorption bands arising from the *sn*1- and the *sn*2-ester carbonyl groups. In addition, our studies with the specifically chain-perdeuterated PCs enable us to determine whether there are any intramolecular interchain interaction components to the complex CH_2 scissoring bands exhibited by the L_c phases of some samples. Interchain interaction is usually inferred from the presence of the factor group splitting, which arises from interchain coupling of the CH_2 scissoring (and rocking) vibrations of orientationally inequivalent chains in the hydrocarbon subcell (see Snyder, 1979, and references cited therein). However, with normal unlabeled lipids, it is often difficult to determine whether the observed splitting is real or an artefact of the coexistence of two different phases. This problem can be effectively overcome by a comparison of the infrared spectra of the specifically chain-perdeuterated lipids with those of the unlabeled lipids. This approach exploits the fact that the CD_2 group has the greater reduced mass and as a result the frequencies of all of its vibrational modes are considerably lower than those of the corresponding modes of the CH_2 group. Thus, if there is any interchain vibrational coupling between *sn*1 and *sn*2 chains, perdeuteration of any one of those chains would effectively uncouple such interactions with the result that there would be a collapse of the band splitting observed with the fully proteated sample. These experiments have enabled us to deduce more structural information from our FTIR spectroscopic data than was previously possible. Significantly, we show that the structures of the subgel phases which are consistent with these FTIR spectroscopic studies are in many respects different from that deduced by Pearson and Pascher (1979) from the single crystal x-ray study of DMPC.

MATERIALS AND METHODS

The *sn*2-$^{13}C{=}O$ labeled samples of DL-, DM-, DP- and DSPC and those of *sn*2-^2H DPPC and DMPC were synthesized by the 4-pyrrolidinopyridine-catalyzed acylation of the respective 1-O-acyl lyso-PCs (Avanti Polar Lipids Inc., Birmingham, AL) using an excess of the respective 1-^{13}C or perdeuterated fatty acid anhydride. Typically, 700 mg of the labeled fatty acid (^{13}C enrichment ≅ 99%; ^2H enrichment ≅98%, MSD Isotopes, Montreal, Quebec, Canada) were dissolved in carbon tetrachloride and 1 gram of DCCD was added. The mixture was then shaken at room temperature for 1 h after which time the precipitated *N,N*-dicyclohexyl urea was removed by filtration. After removal of the solvent in a stream of nitrogen, the residue, which consists of the fatty acid anhydride and excess DCCD, was stirred at 45°C and the catalyst (0.1 moles per mole of fatty acid) was added in 0.5 ml of benzene, followed by a slurry of 100 mg of the lyso-PC in 2 ml of chloroform. The final mixture was sealed and stirred at 45°C for 3 h, after which time TLC analysis of the reaction mixture indicated that conversion of the lyso-PC to PC was virtually quantitative. The reaction mixture was then diluted with chloroform and applied to a column of silicic acid (Biorad Biosil A) in chloroform and purified using methods which we routinely use for the preparation of highly purified PC samples (Lewis and McElhaney, 1985). From our ^{13}C-NMR spectroscopic analysis of the ^{13}C labeled samples prepared we find that no more than 3% acyl chain migration occurs under our reaction conditions. The *sn*1-$^{13}C{=}O$ and the *sn*1-^2H labeled samples were prepared by the 4-pyrrolidinopyridine-catalyzed acylation of the respective labeled Lyso-PC using an excess of the appropriate unlabeled fatty acid anhydride as described above. The labeled Lyso-PC was prepared from its respective doubly labeled PC by the procedure described by Mason et al., (1980). The doubly labeled PCs used for the synthesis of the labeled Lyso-PCs were themselves synthesized from appropriately labeled fatty acids and purified by methods previously used in this laboratory (Lewis and McElhaney, 1985). The unlabeled PCs were from a stock of highly purified PCs previously prepared for DSC studies (Lewis et al., 1987).

Sample preparation for infrared spectroscopy was as follows. 3–4 mg samples of the dry lipid were hydrated by the addition of 50 μl of D_2O followed by vigorous vortexing at temperatures near 70°C. The hydrated sample was then squeezed between the BaF_2 windows of a heatable liquid cell to form a 25-micron film and mounted in a cell holder attached to a computer-controlled circulating water bath that was used to regulate the temperature. Infrared spectra were recorded with a Digilab FTS-40 Fourier-Transform Infrared Spectrometer using the standard methodology for these types of samples (Mantsch et al., 1985). Initial data were acquired in the cooling mode from temperatures some 10°C above the T_m of each lipid, and once the sample was cooled to temperatures well below its T_m, it was converted to the L_c phase in situ by following the protocol recommended by Lewis et al., (1987). Once the nucleation of the subgel phase was initiated, the formation of the L_c phase could then be monitored as a function of time at an appropriate temperature. The data acquired was processed using software supplied by Digilab Inc. (Cambridge, MA),

and other computer programs developed by the National Research Council of Canada. In cases where the spectra obtained consists of broad overlapping bands, data processing usually involved the use of Fourier deconvolution to obtain fairly accurate estimates of the frequencies of the component bands, followed by curve-fitting procedures to obtain estimates of band width and intensity. Typically band narrowing factors of 1.8–2.0 were used during deconvolution. Under our conditions, band narrowing factors of 2.5 could be used without introducing significant distortions to the spectra. In cases where a mixture of labeled and unlabeled lipids was required, the two lipids were codissolved in chloroform in the required proportions and the solvent removed with a stream of nitrogen. The resultant mixture was subsequently lyophylized from benzene and the solid obtained was then hydrated and prepared for the infrared spectroscopic measurements as described above.

RESULTS

Illustrated in Fig. 1 are the C=O stretching and CH_2 bending regions of the infrared spectra exhibited by the four types of subgel phases which we have previously characterized for the *n*-saturated diacyl PCs (Lewis and McElhaney, 1990). These L_c phases exhibit distinct spectroscopic features which are qualitatively interpretable in terms of the structure and organization of the bilayer polar/apolar interface and the packing of the hydrocarbon chains, respectively. However, as shown in Fig. 1, the contours of the C=O stretching bands of all of the samples, and those of the CH_2 bending bands of the DMPC and the longer chain compounds ($N \geq 17$), are fairly complex, thus limiting one's ability to extract structural information from this data. Thus, to simplify the interpretation of the spectra and to make any structural deductions less ambiguous, we have also examined the FTIR spectra of the L_c phases of specifically chain perdeuterated or $^{13}C{=}O$ labeled samples of four representative PCs. The stable L_c phase of DLPC (designated L_c3 by Lewis and McElhaney, 1990) was studied as the representative of those formed by the short-chain PCs ($N \leq 12$); the stable L_c phase of DMPC (designated L_c2 by Lewis and McElhaney, 1990) was studied as a representative of the L_c phase formed by medium chain PCs ($N = 13$–15), and the L_c phase of DSPC (designated L_c1 or L_c1' by Lewis and McElhaney, 1990) was studied as a representative of the L_c phase formed by the long chain PCs ($N \geq 17$). In our previous FTIR spectroscopic study of unlabeled lipids (Lewis and McElhaney, 1990), we also demonstrated that when an aqueous dispersion of DPPC is incubated at 0–4°C, it first forms a L_c1 type of subgel phase ($\cong$2–4 days incubation), which upon prolonged low temperature incubation eventually transforms to a more stable type of L_c phase (designated L_c4 by Lewis and McElhaney, 1990). This, the stable L_c phase of DPPC, is spectroscopically distinct from the L_c phases of all other members of the homologous series. Thus both the metastable and the stable L_c phases of DPPC were studied as representative L_c1 and L_c4 phases, respectively. The facility of using DPPC to study the L_c1 type of L_c phase was exploited in this study primarily as a cost-saving measure, because it enabled us to avoid the added cost of preparing specifically chain perdueterated samples of the long chain lipids without any significant sacrifice of information. Thus, in studies of the L_c1 type of subgel phase, the spectra of the metastable phases of specifically chain perdeuterated samples of DPPC were examined, whereas in the case of the specifically $^{13}C{=}O$ labeled lipids spectra were acquired for the L_c1 phases formed by both DPPC and DSPC. In addition, because our previous studies of the short chain PCs (Lewis and McElhaney, 1990) indicated that the molecules pack with rotationally disordered hydrocarbon chains and thus do not exhibit significant interchain vibrational coupling, we did not prepare a specifically chain perdeuterated sample of DLPC, because it is unlikely that any new information would be obtained from its study.

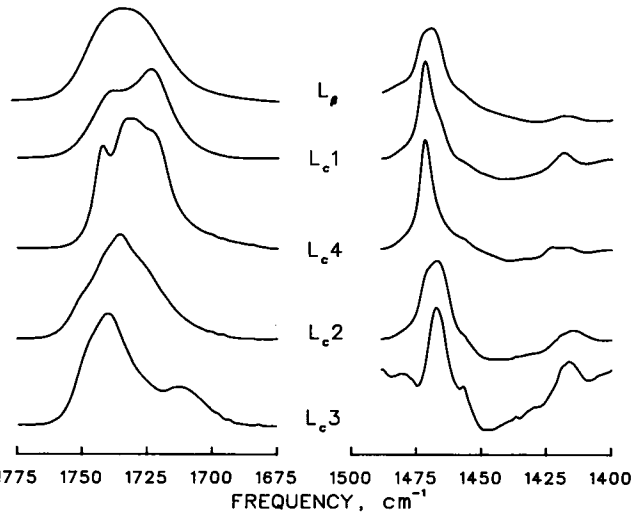

FIGURE 1 Infrared spectra of the L_β phase and the four L_c phases formed by the *n*-saturated diacyl PCs. The spectra are presented in the absorbance mode showing the C=O stretching region on the left and the CH_2 deformation region on the right. The L_c phases are designated according to the nomenclature defined by Lewis and McElhaney, 1990.

L_c1 Phase

Illustrated in Fig. 2 are the hydrocarbon chain CH_2 bending regions of the FTIR spectra exhibited by the metastable L_c phase of unlabeled DPPC and of its *sn*2- and *sn*1-chain perdeuterated analogues. A comparison of the spectra reveals two important points. The first pertains to the weak band near 1,418 cm^{-1}, which has

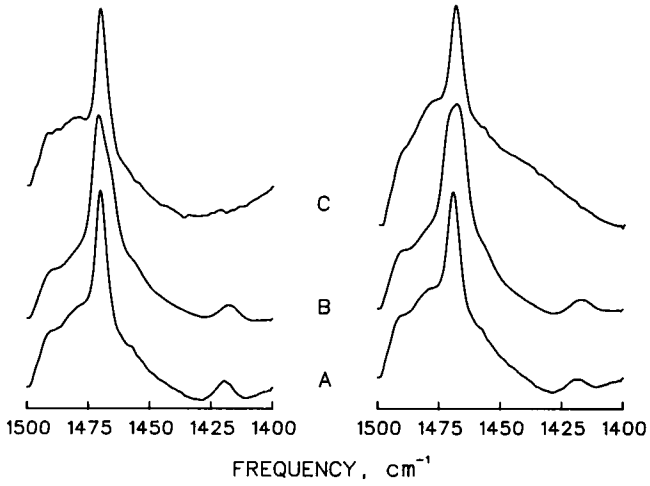

FIGURE 2 The CH_2 deformation region of the infrared spectra of the L_β and L_c1 phases of DPPC. The data shown are spectra of: (*A*) *sn*2-chain perdeuterated DPPC; (*B*) unlabeled DPPC; and (*C*) *sn*1-chain perdeuterated DPPC. The spectra are shown in the absorbance mode with those of the L_c1 phase on the left and those of the L_β phase on the right. The L_c1 phase is formed after 2–4 d incubation at 0–4°C.

previously been assigned to the scissoring vibration of the methylene groups next to the carboxylic ester function (i.e., to the α-methylene group, see Mendelsohn and Mantsch, 1986). We find that perdeuteration of the *sn*2-chain has no discernible effect on the contours of this band, whereas perdeuteration of the *sn*1-chain results in the virtual suppression of infrared absorption near 1,418 cm^{-1}. Moreover, an examination of the integrated intensities of the 1,418 band relative to that of the main CH_2 scissoring band near 1,468 cm^{-1} indicates that with the *sn*2-chain perdeuterated sample, the integrated intensity of the α-methylene band (relative to that of the main CH_2 scissoring band) is $\sim 2 \times (\cong 1.9)$ that of the unlabeled sample. These observations are consistent with the suggestion that the band near 1,418 cm^{-1} arises exclusively from the scissoring vibrations the α-methylene groups of proteated *sn*1 fatty acyl chains, whereas with unlabeled samples the main CH_2 scissoring band near 1,468 cm^{-1} is a summation of comparable contributions from both *sn*1 and *sn*2 fatty acyl chains. Similar conclusions were made from our studies of the other PCs studied (see below). The fact that the α-methylene band of the *sn*2 fatty acyl chain does not contribute to the infrared absorption near 1,418 cm^{-1} is structurally significant, because it is probably a reflection of conformational differences between this region of the *sn*1 and *sn*2 fatty acyl chains.

The other significant observation here is that the main CH_2 scissoring band of the unlabeled sample consists of two components with maxima near 1,472 and 1,466 cm^{-1}, whereas both the *sn*1 and *sn*2 chain perdeuterated samples seems to consist of a single band near 1,471 cm^{-1}. The two components present in the infrared spectrum of the unlabeled sample were assumed to arise from factor group splitting of the methylene band and were taken to indicate orthorhombic packing of the hydrocarbon chains in the L_c phase (see Lewis and McElhaney, 1990). However, if such were the case, perdeuteration of either the *sn*1 or *sn*2 fatty acyl chains should have resulted in a collapse of the factor group splitting, with the result that a single absorption band at 1,468–1,469 cm^{-1} would have been observed (see Snyder, 1960; Casal et al., 1983). Instead, we find that with the perdeuteration of either the *sn*2 or *sn*1 fatty acyl chain, the low frequency component near 1,466 disappears whereas the high frequency component persists, albeit at a slightly reduced frequency ($\cong$1,471 cm^{-1}). From this we conclude that the data is incompatible with factor group splitting of the main CH_2 scissoring band of this particular type of L_c phase. Instead, the data could be explained by considering the possibility that the doubling of the CH_2 scissoring band as observed with the unlabeled sample is the result of the coexistence of the L_c and L_β gel phases. We believe this interpretation of the data is correct for the following reasons. Firstly, it is well known that the formation of the L_c phase of the longer chain PCs is a very sluggish process and at this time there is no reliable means of assessing whether or not it has gone to completion (see Lewis et al., 1987). Secondly, factor group splitting of the main CH_2 scissoring band of the L_β phase has been inferred from FTIR spectroscopic studies of DPPC and the longer chain *n*-saturated diacyl PCs (Cameron and Mantsch, 1982; Lewis and McElhaney, 1990). Such studies have shown that once the reorientational fluctuations of the acyl chains are damped by cooling to temperatures well below T_m, the main CH_2 scissoring band of the L_β phase splits into two components. As illustrated in Fig. 2, these components are of comparable integrated intensity and exhibit their maxima at frequencies near 1,472 and 1,466 cm^{-1}. However, with the perdeuteration of either the *sn*1 or *sn*2 fatty acyl chain there is a complete collapse of this splitting, with a result that a single band near 1,469 cm^{-1} is observed (see Fig. 2). We therefore conclude that unlike the L_c phase, there is genuine factor group splitting of the CH_2 scissoring band of the L_β phase. Given these observations and the fact that the formation of this type of L_c phase results in a progressive increase in the relative integrated intensity of the high frequency band at the expense of the band near 1,466 cm^{-1} (Cameron and Mantsch, 1982; Lewis and McElhaney, 1990), it seems more likely that the low frequency

component of the CH_2 scissoring band of the unlabeled lipid arises from unconverted domains of the L_β phase, whereas the high frequency component is a summation of contributions from the L_c phase and residual domains of the L_β phase (i.e., factor group splitting of the CH_2 scissoring band occurs in the L_β phase but not in the L_c phase). In the case of the specifically chain perdeuterated samples, the observed CH_2 scissoring band would also be a summation of contributions from the L_c phase and unconverted domains of the L_β phase. However, in this case, the L_c phase would still give rise to a high frequency band near 1,472 cm^{-1}, but the factor group splitting of the CH_2 scissoring band of the L_β phase would have collapsed (because of the perdeuteration of one of the acyl chains) to produce a single absorption band near 1,469 cm^{-1}. Unlike the unlabeled samples, however, the resolution of these component bands is probably impractical because the separation between the bands ($\cong$3 cm^{-1}) is considerably smaller than their intrinsic bandwidths ($\cong$6–7 cm^{-1}). (Using simple computer simulations we find that resolution between such bands using Fourier deconvolution and other techniques, though theoretically possible for ideal data, is impractical for 'real' spectroscopic data which are always influenced by spectral noise and background water vapor.) As a result, the CH_2 scissoring band of the L_β phase of the specifically chain perdeuterated sample should appear as a single band which progressively approaches 1,472 cm^{-1} (the higher frequency limit) as the formation of the L_c phase proceeds.

From the above data, we now believe that it is unlikely that a FTIR spectroscopic characterization (or indeed any other physical characterization) of a pure sample of this particular type of L_c phase (i.e., the L_c1 phase) has ever been done. Moreover, on account of the exceedingly slow kinetics of formation of the L_c phases of the longer chain compounds (see Lewis et al., 1987), such studies may not be practical unless some means are found to accelerate the process. Given this we have used spectral subtraction techniques to arrive at a probable spectrum of the pure L_c1 phase. Illustrated in Fig. 3 are the experimental spectra obtained in our studies of the L_c phase of DSPC (which we suggest is a mixture of L_β and L_c1 phases) and that which we believe represents the spectrum of the pure L_c1 phase. (This spectrum was obtained by subtraction of the contours of the L_β phase scaled at approximately 30% of its total integrated intensity.) Our results suggest that the pure L_c1 phase should exhibit a single CH_2 scissoring band near 1,472 cm^{-1} and from this observation we would conclude that in this type of L_c phase the hydrocarbon chains pack in a subcell with parallel zigzag planes (Snyder, 1961). This conclusion is the opposite of that which appears to

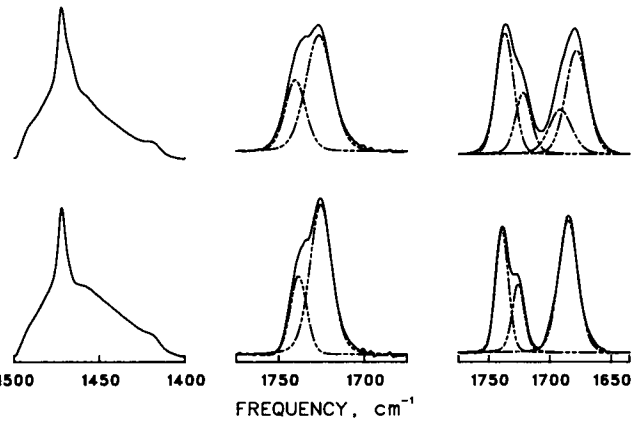

FIGURE 3 Infrared spectra of the L_c phase of DSPC. The top panel shows the spectra actually acquired whereas the bottom panel shows the spectra which we propose are more representative of pure samples of the L_c1 phase. The data shown on the bottom panel were obtained after spectral subtraction of some 30% of the band contours of the L_β phase. All spectra are shown in the absorbance mode with the solid lines representing the actual data and the dashed lines representing bands resolved by curve fitting techniques. (*Left*) CH_2 Deformation region; (*Middle*) C=O stretching region (unlabeled sample); and (*Right*) C=O stretching region (*sn*2-^{13}C=O labeled sample).

follow from the apparent splitting of the bands as observed in the actual spectra of the unlabeled lipids. In the C=O stretching region of the spectrum, spectral subtraction does not appear to drastically change the overall features of the band contour of the unlabeled sample. Overall, the band contour is narrower than that of the spectrum actually acquired (presumably because of the removal of the broader wings characteristic of the L_β phase), and there appears to be a relative increase in the relative proportions of the lower frequency component of the band. Also, Fourier deconvolution confirms that in both the observed spectrum and that proposed for the pure L_c1 phase, the band contour consists of a major component near 1,724 cm^{-1} and a smaller component near 1,738 cm^{-1}. However, a clearer picture emerges from an examination of the corresponding spectra of the isotopically labeled PC. Here, because of the specific *sn*2-^{13}C=O labeling, the absorption bands arising from the *sn*1- and the *sn*2- ester carbonyls are separately resolved and appear at ~1,735 and 1,695 cm^{-1}, respectively. In addition, it is clear that both the *sn*1- and the *sn*2- components are in turn each resolvable into two components with maxima near 1,738 and 1,725 cm^{-1} (*sn*1- carbonyls) and 1,697 and 1,683 cm^{-1} (*sn*2- carbonyls). Thus, once allowances are made for the magnitude of the isotopic shift ($\cong$42 cm^{-1}), it becomes clear that the two components observed in the C=O stretching band contour of the unlabeled sample contain contribu-

tions from both the *sn*1- and the *sn*2- ester carbonyl groups. However, the situation is somewhat different in the spectrum proposed for the pure L_c1 phase (i.e., that obtained after spectral subtraction of the L_β component). Whereas there are two components to the *sn*1 ester carbonyl band ($\cong$1,738 and 1,725 cm^{-1}), the *sn*2-band consists of only one component ($\cong$1,683 cm^{-1}). Thus the proposed spectrum for the pure L_c1 phase of the unlabeled sample indicates that the high frequency component near 1,738 cm^{-1} arises exclusively from the stretching vibrations of a population of *sn*1- ester groups. Moreover, although the lower frequency component near 1,724 cm^{-1} contains a contribution from a subpopulation of *sn*1 ester carbonyls ($\cong$30% of the total integrated intensity; our estimates), it arises predominantly from the stretching vibrations of *sn*2 carbonyl groups. We do stress, however, that despite prolonged low-temperature incubation of samples of the longer chain lipids, we have not yet observed what we believe is a spectroscopically pure sample of their L_c phase. However, we believe that our projections of the spectroscopic properties of this type of L_c phase are essentially accurate, especially because we have observed a close approach to such a spectrum in a sample of *sn*2-^{13}C=O labeled DPPC before the onset of the formation of its stable L_c phase (see Fig. 4).

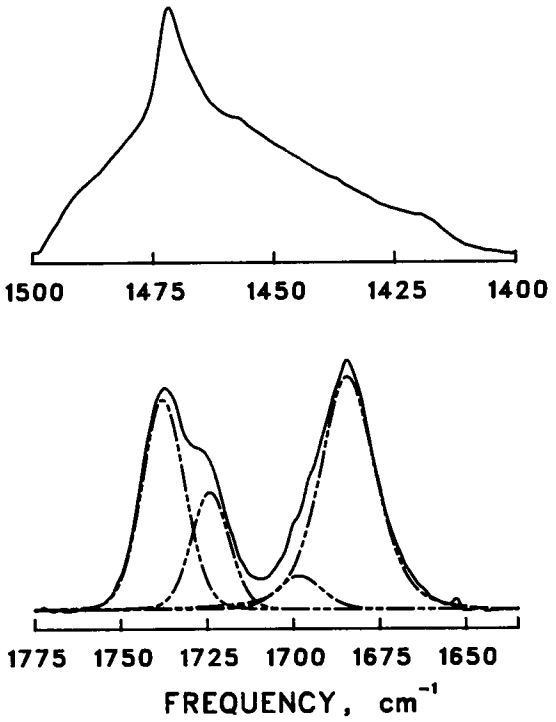

FIGURE 4 The C=O stretching and CH_2 deformation region of a sample of sn2-^{13}C=O DPPC. This particular spectrum was acquired after 10 d of low-temperature incubation of the sample and describes the spectroscopic properties of its metastable L_c phase just before the nucleation and growth of its stable L_c phase. The spectra are shown in the absorbance mode and the component C=O stretching bands are indicated by the dashed lines. Note the relatively low integrated intensity of the *sn*2-^{13}C=O band near 1,695 cm^{-1}.

L_c4 Phase

Fig. 5 compares the C=O stretching and CH_2 bending regions of the infrared spectra exhibited by the stable L_c phase formed by the unlabeled and the specifically labeled samples of DPPC. As with the longer chain PCs, the perdeuteration of the *sn*2 chain does not affect the contours of the α-methylene scissoring bands near 1,420 cm^{-1}, whereas perdeuteration of the *sn*1 chain virtually suppresses all infrared absorption in that region. With both the unlabeled and the *sn*2-chain perdeuterated samples, the α-methylene scissoring band contour resolves into two bands with maxima near 1,417 and 1,424 cm^{-1}. Because these bands obviously arise from the scissoring vibrations of the fully proteated *sn*1-α-methylene groups, there clearly must be two populations of such groups present. The presence of two spectroscopically distinct populations of *sn*1-α-methylene groups in turn suggests that there are two vibrationally inequivalent populations of lipid molecules present in the stable L_c phase of DPPC. Another significant feature of the data shown in Fig. 5 is the fact that the unlabeled and both chain-perdeuterated samples exhibit a sharp CH_2 scissoring band near 1,472 cm^{-1}. This clearly indicates that interchain vibrational coupling does not contribute to the relatively high frequency of this band. The appearance of single band in this frequency range has been observed in solid paraffins with triclinic subcellular packing (Snyder, 1961). Thus, at the very least, our data suggest that also in its stable L_c phase the hydrocarbon chains of DPPC form a subcell in which the zigzag planes of the acyl chains are parallel.

Fig. 5 also shows that in the C=O stretching region of the infrared spectrum, the stable L_c phase of the unlabeled DPPC exhibits a complex band contour which can be resolved into four component bands with maxima near 1,743, 1,735, 1,728 and 1,721 cm^{-1} using Fourier deconvolution techniques. It is also clear that the spectrum exhibited by the *sn*2-^{13}C=O-labeled sample contains four relatively sharp, easily resolved bands, of which the *sn*1-carbonyls exhibit their maxima near 1,743 and 1,729 cm^{-1} whereas the *sn*2-carbonyls exhibit their maxima near 1,694 and 1,680 cm^{-1}. Given this, it is clear that there must be at least two subpopulations of *sn*1 and the *sn*2 ester carbonyl groups present. In addition, the above data also enables a relatively straightforward

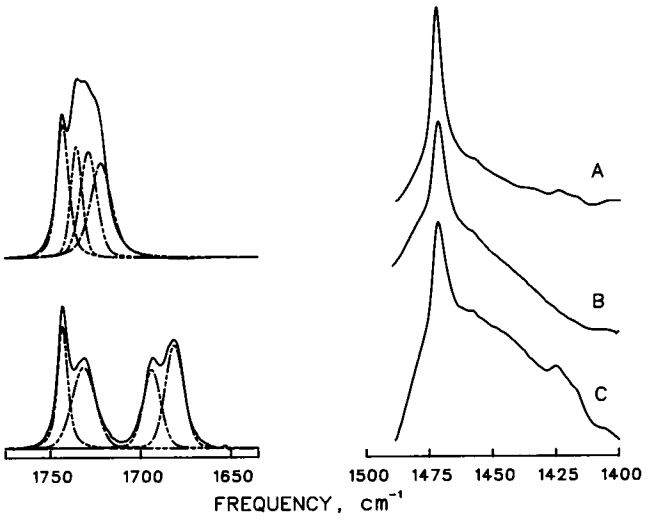

FIGURE 5 The C=O stretching (*left*) and CH_2 deformation (*right*) regions of the infrared spectra of the stable L_c phases formed by unlabeled and specifically labeled samples of DPPC. In the left panel, the top spectrum is that of the unlabeled sample, and the bottom spectrum is that of the *sn*2-^{13}C=O labeled sample. In the right panel, spectra are shown of: (*A*) unlabeled DPPC; (*B*) *sn*1-chain perdeuterated DPPC; and (*C*) *sn*2-chain perdeuterated DPPC. All spectra are shown in the absorbance mode with the solid lines representing the actual data acquired whereas the dashed lines represent component bands estimated by a combination of Fourier deconvolution and band fitting.

assignment of the components of the complex band contour of the unlabeled sample to be made. Here, one can confidently assign the bands near 1,743 and 1,728 cm^{-1} to the infrared absorptions of subpopulations of *sn*1-carbonyl groups and those occurring near 1,735 and 1,721 cm^{-1} to subpopulations of *sn*2-carbonyl groups. The assignment of the bands near 1,735 and 1,721 cm^{-1} as *sn*2-carbonyl bands is clearly supported by the fact that the frequencies of the *sn*2-^{13}C=O bands of the labeled sample are very close to what would be theoretically predicted solely on the basis of the greater reduced mass of the ^{13}C=O group. Moreover, further support for this assignment is given by the fact that we can easily reconstruct the band contour of the unlabeled sample by a simple spectral addition of the *sn*1- and *sn*2-bands of the *sn*2-^{13}C=O-labeled sample, provided that there is an upward adjustment of the frequencies of the *sn*2-^{13}C=O component bands by a value equivalent to that theoretically predicted on the basis of the difference in the reduced masses of the ^{12}C=O and ^{13}C=O groups. Given all of the above data, and the clear suggestion that there are two populations of *sn*1-α-methylene groups present (see data on the chain perdeuterated samples), it is logical to conclude that the stable L_c phase of DPPC is a complex, crystallike structure containing two vibrationally inequivalent populations of DPPC molecules.

L_c2 Phase

Fig. 6 shows the CH_2 bending band contours of the infrared spectra of the stable L_c phases formed by the labeled and chain perdeuterated samples of DMPC. One of the significant features of the data shown is that similar to the L_c phases described above, the α-methylene scissoring band near 1,418 cm^{-1} is unaffected by the perdeuteration of the *sn*2 chain, whereas the perdeuteration of the *sn*1 chain virtually eliminates all infrared absorption in this region. Moreover, in the stable L_c phase of these lipids the α-methylene scissoring band resolves into two components with maxima near 1,420 and 1,413 cm^{-1}. Because these bands evidently arise from the scissoring vibrations of the α-methylene group of the *sn*1-chain, the appearance of two such bands clearly suggests that there must be two subpopulations of vibrationally inequivalent *sn*1-chains present. This in turn is consistent with the formation of a quasicrystalline structure which contains two vibrationally inequivalent phospholipid molecules. Another feature of the data is

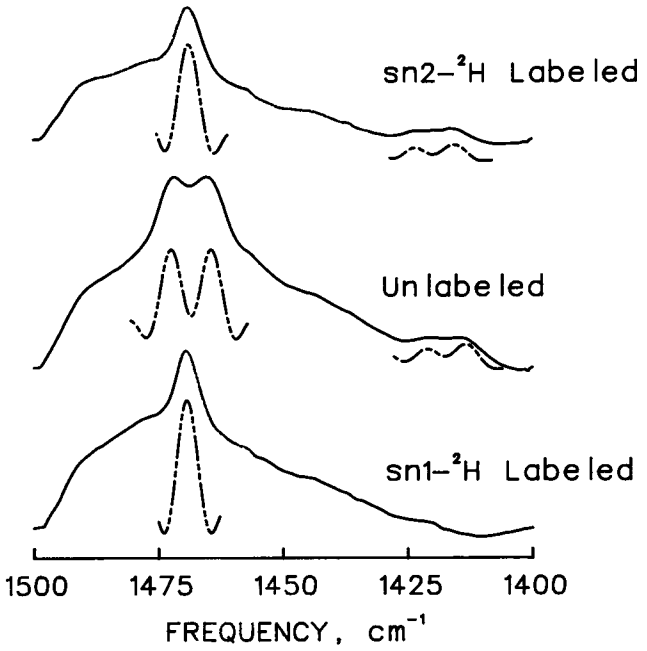

FIGURE 6 The CH_2 deformation region of the infrared spectra of the stable L_c phases formed by the unlabeled and the specifically chain perdeuterated samples of DMPC. The spectra are shown in the absorbance mode with the solid lines representing the actual data acquired and the dashed lines representing our estimates of the positions and characteristics of the component bands.

that in the CH_2 bending region of the spectrum of the unlabeled sample, the main CH_2 scissoring band near 1,468 cm^{-1} is clearly a composite of two components with maxima near 1,472 and 1,466 cm^{-1} (see Fourier deconvolved spectra in Fig. 6). However, it is also clear that the main CH_2 bending band of the specifically chain-perdeuterated samples consists of a single narrow band centered at 1,469 cm^{-1}. Evidently, the two bands present in the spectrum of the unlabeled sample are the result of real factor group splitting (i.e., they are the product of interchain vibrational coupling), and with the perdeuteration of the either the *sn*1- or the *sn*2-chains this splitting has completely collapsed. This observation clearly establishes several important points which have significant structural implications. Firstly, the unambiguous demonstration of factor group splitting of the CH_2 scissoring band of the unlabeled sample indicates that the hydrocarbon chains must be arranged in a subcell in which the zigzag planes are not parallel. Of the hydrocarbon subcellular packing arrangements that have been observed so far (Segerman, 1965; Abrahamsson et al., 1978), the orthorhombic perpendicular (both $\perp$ and $\perp'$) and the so-called hybrid HS2 and HS1 type subcells are the only ones which are compatible with these data (note that in these subcells the zigzag planes of all or at least a significant fraction of the chains are more or less perpendicular to each other). Secondly, the fact that perdeuteration of any one of the acyl chains completely decouples the interchain vibrational interactions responsible for the factor group splitting of the CH_2 scissoring also indicates that such splitting arises exclusively from interactions between the *sn*1- and the *sn*2-acyl chains. Given this, one is led to the conclusion that in the stable L_c phases of these medium chain PCs, the acyl chains of each phospholipid molecule must be more or less perpendicular to each other. This finding effectively eliminates the possibility that the hydrocarbon chains of these molecules pack in HS1 and HS2 type subcells, because the requirement that interchain vibrational coupling be exclusively between the *sn*1- and *sn*2-chains cannot be fulfilled within those subcells. Moreover, the subcellular packing which is suggested by our data is completely different from that deduced from the single crystal x-ray study of DMPC, which showed that in that particular crystalline form, there are two crystallographically inequivalent molecules in which the fatty acyl chains are alternately parallel and perpendicular to each other (Pearson and Pascher, 1979).

The C=O stretching region of the infrared spectrum of this type of L_c phase (see Fig. 7) reveals additional interesting points with significant structural implications. As is evident from the spectra *A* and *B* in Fig. 7, the complex band contour of the unlabeled sample is resolvable into four component bands with maxima near

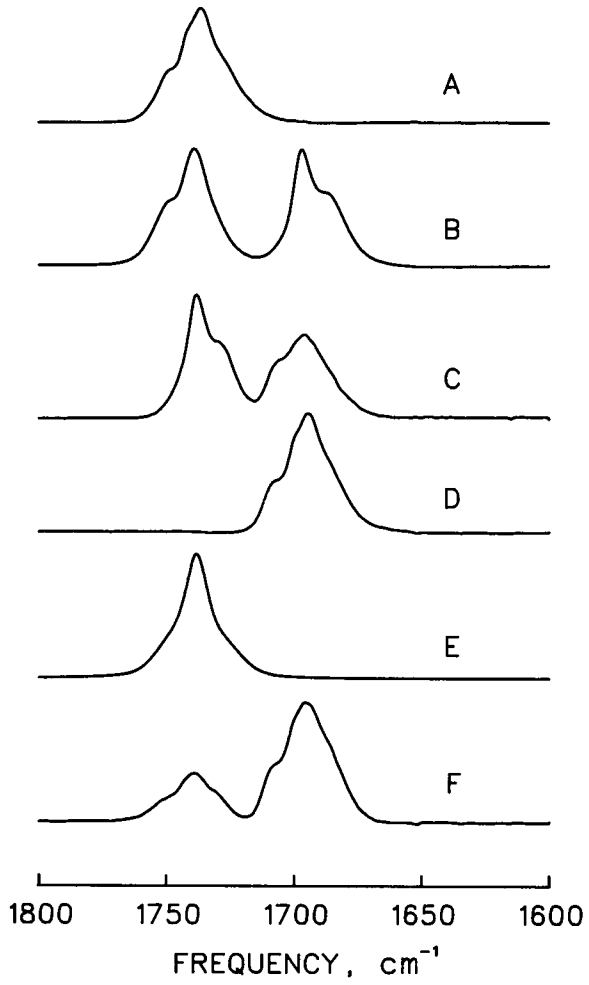

FIGURE 7 The C=O stretching band contours of the stable L_c phase of DMPC. Data is presented for: (*A*) unlabeled DMPC; (*B*) *sn*2-^{13}C=O labeled DMPC; (*C*) *sn*1-^{13}C=O labeled DMPC; (*D*) doubly ^{13}C=O labeled DMPC; (*E*) a simulation of the unperturbed band contours of unlabeled DMPC as would be expected in the absence of vibrational coupling (to obtain this spectrum the frequencies of the *sn*2-^{13}C=O bands in *B* were upward adjusted by 42 cm^{-1} and the resulting contours added to that of the *sn*1-^{12}C=O bands); and (*F*) a 1:4 mixture of unlabeled and doubly ^{13}C=O labeled DMPC.

1,750, 1,742, 1,735 and 1,728 cm^{-1}, whereas the C=O stretching region of the spectrum of the *sn*2-^{13}C=O-labeled sample contains four bands of which the *sn*1-components (i.e., those in the higher frequency range) have maxima near 1,750 and 1,739 cm^{-1} and the *sn*2-components exhibit their absorption maxima near 1,697 and 1,686 cm^{-1}. The spectrum exhibited by the *sn*2-^{13}C=O-labeled sample suggests that in this particular type of L_c phase the *sn*1 and *sn*2 ester carbonyl groups are each subdivided into two vibrationally inequivalent populations. These results are clearly consistent with the conclusion that this particular type of L_c phase is a

quasicrystalline structure in which there are two vibrationally inequivalent phospholipid molecules (see above). From these data one can also be confident that in the spectrum of the unlabeled sample, the components with maxima near 1,750 and 1,728 cm^{-1} can be assigned to the stretching vibrations of subpopulations of *sn*1 and *sn*2 ester carbonyl groups, respectively (Note that the band near 1,728 cm^{-1} corresponds with the *sn*2-$^{13}C{=}O$ band near 1,686 cm^{-1}). However, it is also apparent that the spectrum of the unlabeled sample contains two bands (i.e., those at 1,743 and 1,735 cm^{-1}) which cannot be matched with any of the bands in the spectrum of the *sn*2-$^{13}C{=}O$ labeled sample. Moreover, we find that the spectrum of the unlabeled sample cannot be reconstructed by the type of simple spectral addition which was successfully employed with DPPC. Instead, we find that if one were to 'correct' the frequencies of the *sn*2-$^{13}C{=}O$ bands for the difference between the reduced masses of the $^{12}C{=}O$ and the $^{13}C{=}O$ groups and then add the resultant contours to contours of the *sn*1-$^{12}C{=}O$ bands, the resulting spectrum would contain a major band near 1,739 cm^{-1} (the summation of contributions from populations of *sn*1 and *sn*2 ester carbonyl groups; see spectrum *E* in Fig. 7). From this observation we conclude that simple isotopic shift effects alone cannot account for the differences in the contours of the C=O stretching regions of the spectra of the unlabeled and of the *sn*2-$^{13}C{=}O$ labeled samples. This conclusion leads us to suspect that the absorption bands arising from some subpopulations of *sn*1 and *sn*2 ester carbonyl groups may be perturbed, possibly by the coupled interactions of the oscillators concerned. The possibility that there may be vibrational coupling between subpopulations of *sn*1 and *sn*2 ester carbonyl groups has very important structural implications for this type of L_c phase and was explored further by studies with the reversed labeled and the doubly $^{13}C{=}O$ labeled samples of DMPC.

The spectrum *D* in Fig. 7 shows the C=O stretching band contours exhibited by the L_c phase of the doubly $^{13}C{=}O$ labeled sample of DMPC. Here, we find that the contours of this spectrum are essentially similar to those exhibited by the unlabeled sample and that differences between this spectrum and the spectrum A in Fig. 7 can be entirely accounted for by an isotopic shift of some 42 cm^{-1}. As is the case with the unlabeled sample, the C=O stretching band contours of the doubly labeled sample can be resolved into four bands, but in this case their maxima occur near 1,707, 1,700, 1,693, and 1,686 cm^{-1}, respectively. Thus, when due corrections are made for the magnitude of the isotopic shift, the band contours of the unlabeled sample and doubly labeled sample are well matched. With the *sn*1-$^{13}C{=}O$ labeled sample, however, the two bands arising from the *sn*1-ester groups are observed near 1,707 and 1,697 cm^{-1}, whereas those arising from the *sn*2-ester groups are observed near 1,739 and 1,728 cm^{-1} (Fig. 7, spectrum *C*). Thus, given these data and that obtained with the *sn*2-$^{13}C{=}O$ labeled lipids, one would logically expect that in the absence of any perturbing factors, the spectrum of the unlabeled lipid should contain a major band near 1,739 cm^{-1}, which would be the summation of equivalent contributions from subpopulations of *sn*1 and *sn*2 ester carbonyl groups. Evidently such a band (the corresponding band in the doubly-labeled sample should occur at 1,697 cm^{-1}) is not present in the infrared spectrum of the unlabeled sample, and it would appear that it has been replaced by two bands which exhibit their maxima near 1,742 and 1,735 cm^{-1}. Thus, we conclude the latter two bands (the corresponding bands in the doubly-labeled samples occur at 1,700 and 1,693 cm^{-1}) could not have arisen from the unperturbed stretching vibrations of the given *sn*1 and *sn*2 carbonyl groups. Instead we propose that they are the result of the interaction and coupling of those oscillators and most probably arise from the in-phase and out-of-phase interactions between the vibrating groups. These results also indicate that such interactions only occur with the unlabeled and doubly labeled samples. This should be expected with those particular samples because the unperturbed frequencies of the given populations of *sn*1 and *sn*2 ester carbonyl groups would be the same ($\cong$1,739 cm^{-1} for the unlabeled sample and $\cong$1,697 cm^{-1} for the doubly labeled sample). However, in the case of the singly labeled samples, the natural frequencies of the *sn*1 and the *sn*2 carbonyl groups would differ by some 42 cm^{-1}, and this will effectively uncouple the interaction of the stretching vibrations of the groups concerned. These data are thus consistent with the interaction and coupling of the stretching vibrations of subpopulations of *sn*1 and *sn*2 ester carbonyl groups and are of major structural significance because such interactions could only occur if there is close contact between the groups concerned. Interestingly, an examination of molecular models indicates that if the usual conformations of the *sn*1 and *sn*2 fatty acyl chains are assumed (see Hauser et al., 1988 and references cited therein), intramolecular close contact between *sn*1 and *sn*2 ester groups is extremely unlikely. As a result one could further suggest that the close contacts between the populations of *sn*1 and the *sn*2 ester groups must be intermolecular. However, if the interaction and coupling of the stretching vibrations described above arise from intermolecular close contacts between *sn*1 and *sn*2 ester carbonyl groups, one should also be able to uncouple these interactions by diluting a sample of the unlabeled lipid with a large excess of the doubly labeled analogue (or vice versa). This has been verified by such an

experiment, the results of which are presented in Fig. 7 (see Spectrum *F*). The spectrum presented is that exhibited by the L_c phase of a DMPC sample in which the unlabeled species has been diluted with a four-fold excess of the doubly $^{13}C{=}O$ labeled analogue. The data clearly show that the band contours arising from the unlabeled species of the mixture are markedly different from those exhibited by the pure unlabeled sample. The significant feature here is that when compared with the contours exhibited by the pure unlabeled sample, infrared absorption bands with maxima near 1,743 and 1,735 cm^{-1} are suppressed whereas infrared absorption centered near 1,739 cm^{-1} is substantially enhanced. In fact the contours of the $^{12}C{=}O$ region of spectrum *F* closely resemble those shown in spectrum *E* and this is precisely what would be expected if the intermolecular vibrational interactions between subpopulations of *sn*1-$^{12}C{=}O$ and *sn*2-$^{12}C{=}O$ have been effectively uncoupled by dilution with an excess of the doubly $^{13}C{=}O$ labeled species. Obviously, these data demonstrate that the vibrational interactions between subpopulations of *sn*1-$^{12}C{=}O$ and *sn*2-$^{12}C{=}O$ groups were uncoupled by isotopic dilution, thereby strongly supporting the conclusion that there are intermolecular close contacts between subpopulations of *sn*1- and *sn*2-carbonyl groups.

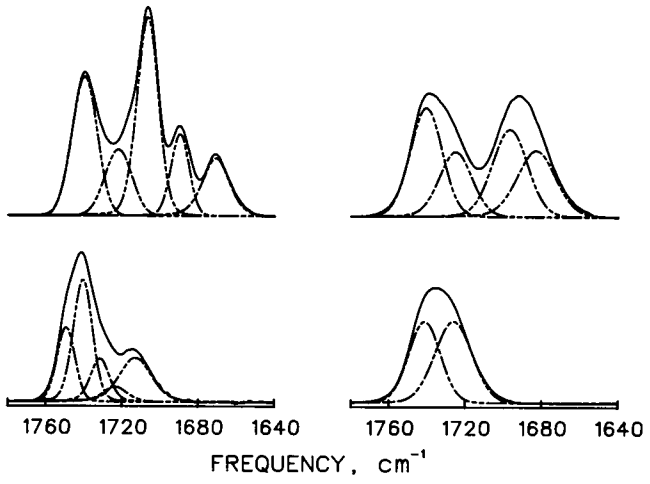

FIGURE 8 The C=O stretching region of the infrared spectra of unlabeled (*bottom*) and *sn*2-$^{13}C{=}O$ labeled (*top*) samples of DLPC. Spectra of the L_c3 phase are shown on the left and for comparative purposes spectra of the L_β phase are shown on the right. The contours of spectra of the L_β phase of the lipids shown are similar to those of all other homologues. The spectra are shown in the absorbance mode with the solid line showing the observed band contour, and the dashed line showing our estimates of the positions and characteristics of the component bands.

L_c3 Phase

Illustrated in Fig. 8 are the C=O stretching band contours of the stable L_c phases formed by unlabeled and *sn*2-$^{13}C{=}O$ labeled samples of DLPC. It is immediately apparent that the situation with this particular type of L_c phase is more complex than is the case with any of the others studied. As we have previously shown (Lewis and McElhaney, 1990), the unlabeled sample exhibits a complex C=O stretching band contour which seems to consist of bands which have their maxima near 1,748, 1,740, 1,724 and 1,714 cm^{-1}. However, an examination of the band contours of the *sn*2-$^{13}C{=}O$ labeled sample indicates that the system must be more complex than was suggested by our previously published analysis (Lewis and McElhaney, 1990). It is clear from Fig. 8 that at the very least there are two populations of *sn*1-ester carbonyl groups which have their maxima near 1,740 and 1,723 cm^{-1}, and a minimum of three populations of *sn*2-^{13}C labeled ester carbonyl groups with maxima near 1,706, 1,688, and 1,670 cm^{-1}. Evidently these data are indicative of a very complex, quasicrystalline structure in which there are at least three vibrationally inequivalent populations of lipid molecules. Moreover, from these data, and taking into account the effects expected of the differences in the reduced masses of the $^{12}C{=}O$ and $^{13}C{=}O$ groups, we can examine the C=O stretching contour of the unlabeled sample and assign the components near 1,740 and 1,723 cm^{-1} to the stretching vibrations of *sn*1-C=O groups and those with maxima near 1,748, 1,730, and 1,712 cm^{-1} to the stretching vibrations of the *sn*2-C=O groups. We find that these assignments are supported by our ability to reconstruct the band contour of the unlabeled sample using a linear combination of the bands resolved in the spectrum of the *sn*2-$^{13}C{=}O$ labeled sample after appropriate corrections for the differences in the reduced masses of the ^{12}C and ^{13}C carbonyl groups. However, an unexpected finding of this new analysis is that the high frequency component ($\cong$1,748 cm^{-1}) of the C=O band contour of the unlabeled sample arises from a subpopulation of *sn*2-carbonyl groups. This observation is striking since the high frequency of this band suggests that there exists a subpopulation of *sn*2-ester carbonyl groups which are in a dehydrated, nonpolar environment (Mushayakarara et al., 1986; Wong and Mantsch, 1988). At this time it is difficult to reconcile this particular observation with commonly held views of the conformation of the glycerol backbone of these lipids and the conformational inequivalence of the *sn*1- and *sn*2-carbonyl groups which have been deduced from single crystal x-ray studies of phospholipids (Hitchcock et al., 1974; Pearson and Pascher, 1979). In fact, the data presented is all the more intriguing because the low frequency of one of the other *sn*2-carbonyl groups ($\cong$1,712 cm^{-1}) suggests that the stable L_c phase of these short chain lipids also contains a

subpopulation of *sn*2-ester carbonyls that are in a very polar environment and may even be strongly hydrogen bonded. These results thus point to a very complex crystalline structure which is very difficult to reconcile with the published single crystal x-ray studies. These and other structural implications of our data will be further explored below in the discussion.

DISCUSSION

Several useful conclusions can be drawn from the data presented here. Firstly, although peripheral to the thrust of this paper, this study provides data which should eventually be useful in addressing the question of the physical basis of the component bands resolved in the C=O stretching band contours of hydrated lipid bilayers. With virtually all hydrated 1,2-diacyl lipid bilayers studied so far, it is well known that in both the gel and liquid-crystalline states the C=O stretching band consists of two components which exhibit their maxima near 1,743 and 1,728 cm^{-1}. Moreover, on the basis of previous vibrational spectroscopic studies of solid phospholipids (Levin et al., 1982; Mushayakarara and Levin, 1982; Mushayakarara et al., 1982) and the conformational inequivalence of the *sn*1- and *sn*2-carbonyl groups as deduced from single crystal x-ray diffraction data (Hitchcock et al., 1974; Pearson and Pascher, 1979), it seemed logical to assign the bands near 1,743 and 1,728 cm^{-1} to the *sn*1- and *sn*2-carbonyl groups, respectively. Indeed, given evidence that the conformational inequivalence of the *sn*1- and *sn*2-carbonyl groups is largely maintained in hydrated lipid bilayers (Hauser et al., 1981, 1988), the assignment of the lower frequency band to the *sn*2-group seemed very reasonable, because this conformational inequivalence would inevitably result in the location of the *sn*2-ester group in a more polar environment than its *sn*1-counterpart. However, a recent study of hydrated *sn*2-^{13}C=O and *sn*1-^{13}C=O labeled phospholipids (Blume et al., 1988) has shown that with hydrated lipids, the effect of the conformational inequivalence between *sn*1- and *sn*2-ester groups can only account for a 3–4 cm^{-1} difference in the frequencies of the *sn*1- and *sn*2-carbonyl bands, as opposed to the 13–15 cm^{-1} difference which is observed experimentally. Moreover, these authors also showed that each of the two component bands present in the C=O stretching band contour of the unlabeled lipids is in fact the summation of comparable contributions from both the *sn*1- and *sn*2-carbonyl groups. Thus, given the fast time scale of the vibrational spectroscopic experiment, they proposed that in hydrated lipid bilayers the two-component C=O stretching bands are a reflection of instantaneous subpopulations of hydrated and nonhydrated (or free and hydrogen-bonded) carbonyl groups. Although such data was not explicitly presented here, our studies of *sn*1-^{13}C=O and *sn*2-^{13}C=O labeled DMPC samples have verified many of the experimental observations of Blume and co-workers (Blume et al., 1988). Moreover, our data have shown that high-frequency and low-frequency populations of both the *sn*1 and *sn*2 ester carbonyl also exist even when many of these lipids are effectively immobilized in their quasicrystalline L_c phases. Given this and the observation that such subpopulations are not observed when the anhydrous samples of these lipids are dissolved in a nonpolar solvent (Blume et al., 1988), we are inclined to support the proposal that in hydrated lipid bilayers the two components which define the contours of the C=O stretching band are predominantly the result of differences in the hydration of (and/or hydrogen bonding to) both the *sn*1- and *sn*2-ester carbonyl groups, instead of the conformational inequivalence of the *sn*1- and *sn*2-carbonyl groups.

Secondly, it is also clear that FTIR spectroscopy, when used in concert with specific isotopic labeling, can be a very powerful tool for structural studies of lipids. Here, with the aid of the specifically labeled materials, we have clarified several aspects of the infrared spectra of the L_c phases of the *n*-saturated diacyl PCs and have simplified the structural interpretation of much of the data. In particular, our experiments with the ^{13}C=O labeled samples simplified (though not completely solved) the task of assigning the *sn*1- and *sn*2-components of the complex C=O stretching contours characteristic of the L_c phases of these lipids. This is a very useful facility and has been exploited in other studies of phospholipids (Green et al., 1987; Blume et al., 1988), though not to address the question of the structure of their subgel phases. Our studies have enabled us to unambiguously determine that there are discrete subpopulations of vibrationally inequivalent molecules in the stable quasicrystalline L_c phases of these lipids. Thus, at the very least, our data are indicative of a very complex crystalline structure, especially in the case of the short chain homologues. In fact with these data we can also draw some tentative conclusions about the polarity of the interfacial region of the lipid molecule near to the carbonyl ester groups. With the L_c1 type of L_c phase (i.e., those exhibited by the longer chain lipids), our data suggest that the entire population of *sn*2-ester carbonyls and a nontrivial population of *sn*1-ester carbonyls are in a relatively polar environment and may thus be hydrated or hydrogen bonded. Because, with these lipids, interfacial water is the sole source of hydrogen bonding donors, these data could be interpreted in terms of a fairly hydrated environment in the region of the carbonyl ester groups of the lipid. In the case of the stable L_c phase of DPPC (i.e., the L_c4 phase), the appearance of consider-

ably sharper components in the C=O stretching band is indicative of a considerable reduction in the mobility of the carbonyl groups, and the increase in the relative integrated intensities of the populations in the higher frequency range suggests that the environment around the ester groups is considerably less polar than is the case with the longer chain lipids. We further suggest that this is the result of a less hydrated polar/apolar interface. In the case of the L_c2 type of L_c phase (i.e., the stable L_c phase of DMPC and the other medium chain lipids), we note that the component bands are very sharp and that a significant population of *sn*1-ester carbonyl groups are in a frequency range which approaches the limits expected of anhydrous esters dissolved in nonpolar, nonbonding solvents. This observation, along with the fact that there is further reduction in the relative integrated intensities of all of the bands in the lower frequency range (with these lipids only one of the component bands was in the lower frequency range), indicates that the environment of C=O ester groups must be even less polar and we believe even less hydrated than is the case with DPPC. With the L_c3 type of subgel phase (i.e., the stable L_c phase of the short chain lipids), our data are indicative of a very complex situation indeed. There is a population of *sn*2-ester carbonyls of unusually low frequency ($\cong$1,712 cm^{-1}), and in total the various populations of low frequency ester carbonyls are the dominant bands present (Note that both *sn*1- and *sn*2-ester groups contribute to these populations). Whereas this observation may logically suggest that the polar/apolar interfacial region near the ester carbonyls is fairly polar (and we believe fairly hydrated), the situation is made all the more confusing by the evidence for a nontrivial population of *sn*2-ester carbonyls which, judging from its frequency (1,748 cm^{-1}), must be in an anhydrous, nonpolar environment. We find it difficult to reconcile these two conflicting sets of data into a single structure that is consistent with all available data, without invoking the possibility that the conformations of the constituent moieties at the polar/apolar interfacial region of these lipids are fundamentally different from those of their longer chain homologues, and the possibility that two different types of micro crystalline structures may in fact be present. Additional work is clearly needed before a clearer picture of the structure of the stable L_c phases of the short chain PCs emerges.

Our studies of *sn*2-^{13}C=O-labeled DMPC also reveal an unexpected result from which very valuable structural information was deduced. The key finding here is our evidence for intermolecular coupling of the stretching vibrations of subpopulations of *sn*1 and *sn*2 ester carbonyl groups. (Although not presented here, the same results were obtained in our studies of the metastable subgel phase of DLPC. DLPC and the other short-chain lipids form a similar type of L_c phase as a metastable intermediate en route to the formation of their subgel phases [Lewis and McElhaney, 1990.]) Similar phenomena have been observed in solids containing carbon-oxygen double bonds and is believed to be the result of either steric or electrostatic interactions between the vibrating groups (Hollingsworth and McBride, 1986, 1990). In fact such are usually detected when the vibrational interactions are uncoupled by the use of specifically labeled materials, and much structural information is usually gleaned from such studies (Hollingsworth and McBride, 1990). From the purely structural perspective, however, the important conclusion from this observation is that such interactions require a close approach of the two vibrating groups, and our data clearly support the conclusion that those groups are located on different molecules. The evidence for intermolecular close contact between populations of *sn*1 and *sn*2 ester carbonyl groups is especially interesting, because if one assumes that the conformations of the various moieties at the polar/apolar interfacial regions are anything close to that which is generally accepted (see Hauser et al., 1981; 1988 and references cited therein), one would also have to conclude that intermolecular close contacts between subpopulations of *sn*1- and *sn*2-ester carbonyl groups can only occur if there are two inequivalent molecules present and provided that one of those molecules is vertically displaced in the bilayer plane relative to the other. Interestingly, this type of structural arrangement has been deduced from a single crystal x-ray study of DMPC (Pearson and Pascher, 1979). However, as will be shown later, this is the only aspect of the structural picture of the stable subgel phase of DMPC (and the other medium chain lipids) that is consistent with our spectroscopic data and the single crystal x-ray structure published by Pearson and Pascher (1979).

The studies with the *sn*2-perdeuterated DPPC have also clarified one of the more confusing aspects of the behavior of the L_c phase of the longer chain compounds and for the first time has enabled at least a tentative characterization of the spectroscopic properties of the L_c phase that they form. The problem here is that for virtually all of the lipids that form this type of L_c phase, the process is extremely sluggish and this has made it too difficult to determine if and/or when the process is complete. Our new data have suggested that the infrared spectra previously reported for the subgel phase of the longer chain compounds (Lewis and McElhaney, 1990), and for the metastable L_c phase subgel phase of DPPC (Cameron and Mantsch, 1982; Lewis and McElhaney, 1990), are in fact those of a mixture of their gel (L_β) and subgel (L_c) phases. Once this was determined, it was

feasible for us to obtain reasonable estimates of the real spectroscopic properties of the subgel phase of these longer chain lipids (Fig. 3) and interpret the data accordingly. In this particular case, the availability of the isotopically labeled sample enabled us to correctly determine that these long chain lipids pack into hydrocarbon subcells with parallel zigzag planes, and not with nonparallel planes as suggested from data obtained solely from studies of unlabeled samples.

Our studies with the *sn*2-chain perdeuterated samples have also provided other structurally relevant data. We find that with these lipids the α-methylene scissoring band which occurs near 1,418 cm^{-1} arises exclusively from the *sn*1-chain. This is a surprising result which implies that the *sn*2-α-methylene groups are not active (or are considerably weaker infrared absorbers) in this region of the infrared spectrum. At this time there is no obvious rationale for such observations other than to suggest that they are the result of conformational inequivalence between the *sn*1- and *sn*2-α-methylene groups. However, this discovery did simplify the interpretation of these particular data and supported our contention that the L_c phases of many of these lipids contain at least two vibrationally inequivalent populations of lipid molecules.

Perhaps the most interesting result of our studies of the *sn*2-chain perdeuterated PCs concerned the main CH_2 scissoring bands of the medium chain compounds as exemplified in these studies by DMPC. Our studies show unequivocally that the splitting of the main CH_2 scissoring band of these lipids arises exclusively from interactions between the *sn*1- and the *sn*2-fatty acyl chains and from this result we were able to conclude that, in marked contrast to the results of the single crystal x-ray study (Pearson and Pascher, 1979), the lipid molecules are arranged in an orthorhombic perpendicular type of subcell with their *sn*1- and *sn*2-chains more or less perpendicular to each other. This is a very significant result, and it is one which could not have been deduced from FTIR spectroscopic studies of the unlabeled sample alone. Although our studies of the stable L_c phases of unlabeled samples of DMPC and the other medium chain lipids provides strong evidence for factor group splitting of the main CH_2 scissoring band (this work, Lewis and McElhaney, 1990), such data are not sufficient for one to assume that the *sn*1- and *sn*2-chains of each molecule are perpendicular to each other. In fact as illustrated in Fig. 9 *A*, it is quite possible to construct an orthorhombic perpendicular type of lattice with lipid molecules in which the *sn*1- and *sn*2-chains are parallel to each other but are perpendicular to the hydrocarbon chains of the neighboring molecules in the lattice. In these studies, the specific perdeuteration of one of the lipid chains enabled us to demonstrate that the factor

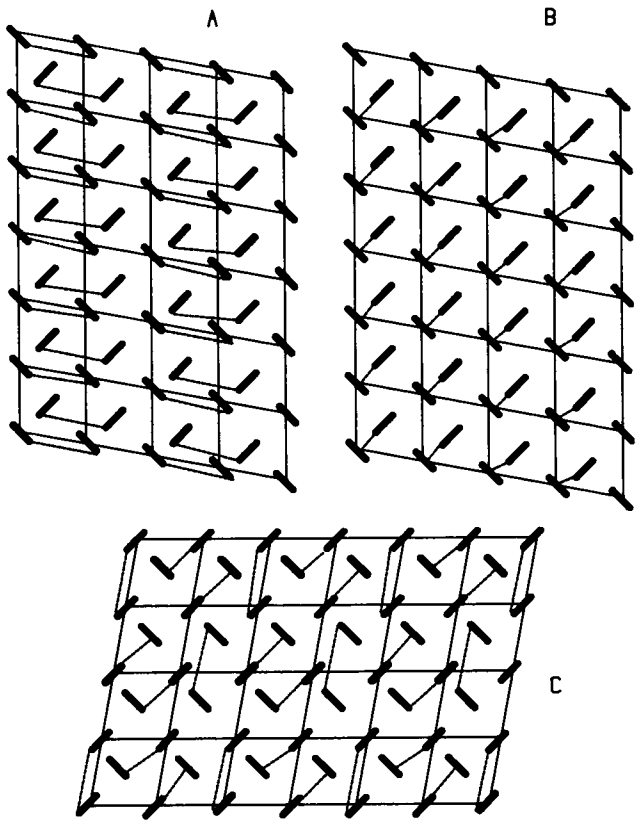

FIGURE 9 Diagram illustrating some of the possible arrangements of lipid molecules in a bulk subcellular lattice with the hydrocarbon chains perpendicular to each other. As drawn the *ab* plane of the lattice is in the plane of the page, the solid lines represent the zigzag planes of the hydrocarbon chains and the dashed lines represent the connectivity between the two hydrocarbon chains which form each lipid molecule. (*A*) Hydrocarbon chains in each molecule are parallel to each other. (*B*) Hydrocarbon chains in each molecule perpendicular to each other. (*C*) Hybrid lattice containing both types of the lipid molecules described in *A* and *B*.

group splitting of the CH_2 scissoring band arises exclusively from interactions between *sn*1- and *sn*2-chains, and so provide the necessary evidence to make our conclusions about the inter- and intramolecular orientation of the acyl chains unambiguous. To date, whenever infrared spectroscopy has been used to study hydrocarbon chain packing of diacylglycerolipids such as these, there has been little consideration given to the fact that the hydrocarbon chains of the lipid molecules are linked pairs of chains which are not independent of each other. However, if due consideration is given to this fact, our experimental observations will impose very severe restrictions on the types of structural models needed to maintain compatibility with the data. Thus, for example, although the three molecular arrangements illustrated in Fig. 9 each define a bulk lattice of perpendicular

hydrocarbon chains, only the arrangement illustrated in Fig. 9 *B* is compatible with our data, whereas the arrangements represented by Figs. 9 *A* and 9 *C* are incompatible because they cannot meet the requirement that the interchain interactions likely to cause factor group splitting of the CH_2 scissoring band must arise exclusively from interactions between *sn*1- and *sn*2-chains. The fact that so much structural information can be gleaned from this type of spectroscopic data underscores the currently under-utilized potential of infrared spectroscopy as a probe of the microscopic structure of lipid bilayers.

Finally, it is clear that for each of the L_c phases formed by these lipids, the 'minimal structural picture' which emerges from this type of data is different. Such differences probably reflect differences in the overall effect of chain length on the balance between the polar and hydrophobic forces which drive the crystallization of these lipids (see discussion in Lewis and McElhaney, 1990). To facilitate comparison of these structures, we have described the stable L_c phases of these lipids in terms of the minimal structural features that are consistent with the spectroscopic data (see Table 1). In addition, where feasible, we have included in Table 1 the corresponding structural features deduced from the only available single crystal x-ray structure of a phosphatidylcholine (Pearson and Pascher, 1979). From an examination of Table 1, one can suggest that there may be overall structural similarities between the stable L_c phase of DPPC and those of the longer chain lipids. In fact our data seems to suggest that these two subgel phases may differ only in terms of the details of the hydration and possibly conformation at the polar interfacial region. However, it is also clear that these are the only instances of general similarity between the various proposed structures. Moreover, it is also obvious that the overall structural features of the subgel phases of these lipids are dissimilar from that deduced from single crystal x-ray studies of DMPC. With amphipathic molecules such as these such a result should not be too surprising, because it is well known that the solid phase structures adopted by such molecules are strongly dependent upon the solvent from which they were isolated (for an example, see Mantsch et al., 1983). Thus, the obvious differences between our proposed structures and the published x-ray structure may be a reflection of the fact that here we were dealing with quasicrystalline structures formed from aqueous dispersions, whereas the crystals used for the single-crystal x-ray study were formed from a mixture of organic solvents (i.e., the solid-phase structure(s) of lipids crystallized from organic solvents may have little in common with the structures which the same molecules form in water). However, from the biochemical/biophysical standpoint, the most relevant of the solid phase structures of lipids such as these are those formed from aqueous dispersion, and this fact presents a dilemma to researchers working in this area. This is because these types of lipid molecules are insoluble in water and as a result it is extremely unlikely that crystalline specimens suitable for single crystal x-ray studies could be obtained from such a medium. Thus, despite the fact that single crystal x-ray studies can provide very definitive structural information, the technique may not be useful for the determination of the structure of hydrated lipid phases because it may not be possible to prepare the types of crystalline specimens needed for such studies. Consequently researchers may have to rely on the structural information gleaned from noninvasive techniques such as NMR and vibrational spectroscopy (infrared and Raman) to provide the vital clues needed to determine the microscopic structure of hydrated lipid bilayers.

TABLE 1 Comparison of the structural features of the subgel phases of the *N*-saturated diacyl phosphatidylcholines with the single crystal x-ray structure of DMPC

L_c1 phase Long chain PCs	L_c4 phase DPPC	L_c2 phase DMPC	L_c3 phase Short chain PCs	(X-ray) DMPC
Two inequivalent molecules	Two inequivalent molecules	Two inequivalent molecules	Three inequivalent molecules (min)	Two inequivalent molecules
Parallel chains	Parallel chains	Perpendicular chains	Rotationally disordered chains	Both parallel and perpendicular chains
Interfacial hydration (fair)	Interfacial hydration (moderate)	Interfacial hydration (poor)	Uncertain	Not applicable
Noninteracting ester carbonyls	Noninteracting ester carbonyls	Intermolecular interacting ester carbonyls (*sn*1- vs *sn*2-)	Noninteracting ester carbonyls	Unknown
Not known	Not known	Molecules offset vertically in bilayer plane	Not known	Molecules offset vertically in bilayer plane

We are indebted to Dr. Mark Hollingsworth of the Department of Chemistry of the University of Alberta for his many helpful and insightful discussions.

This work was supported by operating and major equipment grants from the Medical Research Council of Canada, and by major equipment grants from the Alberta Heritage Foundation for Medical Research.

Received for publication 15 April 1991 and in final form 19 August 1991.

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