Glycerol Conformation and the Crystal Structure of Lipids. I. An Electron Diffraction Study of Tripalmitin and Conformationally Fixed Analogs

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Triglycerides, Crystal Structure, Electron Diffraction

Transmission electron diffraction is used as a qualitative analytical probe of polymethylene chain packing in three conformationally-restricted analogs of tripalmitin derived from configurational isomers of cyclopentane-1,2,3-triol. The analysis demonstrates that the all *trans* "tuning-fork" conformation seen in X-ray crystal structure analyses on homologous compounds is also found in microcrystals, in contrast to a proposal by Fringeli *et al.* (Z. Naturforsch. **27 b**, 780 [1972]) that an all *cis* conformation of the triglyceride exists in solvent-grown microcrystals and in multilayers. The presence of mixed polymorphs is also shown for bulk samples previously examined by X-ray powder diffraction techniques by other workers.

Introduction

Crystallographic investigations of biologically interesting lipids seek to derive some notion of what overall molecular conformation(s) is (are) energetically favored in order to explain their functions *in vivo*. From the standpoint of the divers milieus which may be encountered in physiological situations (*e. g.* the matrix of a membrane, the interior of a lipoprotein, the active site of an enzyme, an aqueous interface) this task is readily seen to be quite imposing and may beg too much interpretation from a single crystal structure determination on a natural compound.

Although the crystal packing in glycerol-containing lipids is largely determined by the nature of the paraffinic chains affixed to the glycerol moiety in ester or ether linkage, the potential rotational freedom about the glycerol carbon-carbon single bonds can allow an amphiphilic molecule to present the surface which is energetically most consistent with its environment. Thus a totally non-polar environment may favor extended chain conformations, whereas a polar/nonpolar interface will cause all long chains to fold in the same direction.

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In order to study the crystal packing of glyceride rotamers *and* to determine which are biologically most active, analogs of several lipid classes, based on the three diastereoisomeric cyclopentane-1,2,3-triols (Fig. 1), have been synthesized [1–4]. In this paper we consider the crystal packing of three "pseudotriglyceride" analogs related to tripalmitin, and the natural glyceride itself, as determined by electron diffraction examination of single thin solvent-grown microcrystals. Our findings are correlated to earlier studies on single crystals or bulk samples and on monolayers on a water surface.

Experimental

Sample preparation

Cyclopentane-1,2,3-triol analogs of tripalmitin were prepared and purified by one of us (AJH) as described in an earlier paper [1]. The three diastereoisomers studied (Fig. 1) mimic the glyceride conformers with acyl groups all *trans* (1,3/2), all *cis* (1,2,3/0) or *cis-trans* (1,2/3) to the cyclopentane ring. Cyclic compounds described in this paper are

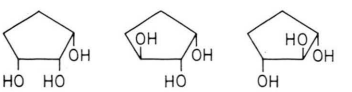

Fig. 1. Configurations of diastereoisomeric cyclopentane-1,2,3-triols used to form tripalmitin analogs. Notation: 1,2,3/0, all *cis*; 1,2/3, *cis-trans*; 1,3/2, all *trans*.

named according to tentative rules for the nomenclature of cyclitols [5]. Natural tripalmitin (99%) was purchased for this study from Sigma Chemical Co., St. Louis, Missouri and was stored desiccated at 0 °C before use. Solvents used for formation of microcrystals on Formvar-carbon covered 400-mesh electron microscope grids were *n*-hexane, ethanol, and *n*-pentanol. Warm dilute solutions were rapidly evaporated from the grid surface to effect crystallization.

Electron diffraction and microscopy

All electron diffraction and electron microscopy experiments were carried out at 100 kV on a JEOL JEM-100U electron microscope. As described elsewhere [6] an attenuated incident beam current and a fast photographic emulsion ensure recording of electron diffraction patterns and diffraction contrast bright field images of the crystals (initial magnification 2.5 K) well before the onset of discernible radiation damage to the sample. Bright field images of crystals were obtained by isolating the transmitted incident beam from the diffracted beams with the objective aperture. The effective selective area in diffraction work was approx. 16 μm diameter. Diffraction spacings were calibrated by a preliminary recording of orthorhombic *n*-hexatriacontane diffraction patterns [6] at the same camera length.

Powder X-ray diffraction

Bulk samples of the isomeric pseudotriglycerides, tripalmitin and *n*-hexatriacontane were packed in 0.7 mm diameter capillary tubes which, after mounting on a goniometer head, were placed on a Charles Supper Weissenberg camera. With the film holder stationary (radius = 28.65 mm) the sample was rotated in the beam for two hours, and spacings on the resultant powder diffraction pattern were derived in the standard fashion as for axial rotation diffraction patterns [7]. The X-ray generator used was an Enraf-Nonius Diffractis 601 with a copper X-ray tube (CuKα after filtering with Ni foil) operated at 40 kV electron accelerating voltage and 20 mA current.

Results

Tripalmitin

n-Pentanol proved to be a superior solvent over ethanol for the growth of large tripalmitin microcrystals. The lath-like habit (Fig. 2) is similar in appearance to the forms obtained from other solvents by Albon and Packer [8] and Okada [9] for the β-form. Fig. 3 shows an h k 0 electron diffraction pattern of the β-form which looks very similar to the analogous pattern for β_3 trilaurin published by Buchheim [10]. A comparison of triclinic lattice parameters for the (001) projection is given in Table I. Salient in the pattern of Fig. 3 is the intense row of diffraction spots along (110) with the spacing of 4.54 ± 0.02 Å. Other intense bands of reflections occur parallel to this line at $d = 1.40$ Å.

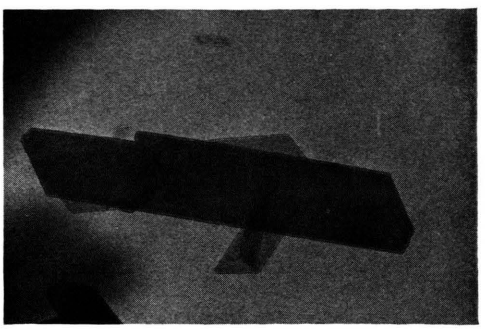

Fig. 2. Lath-like crystals of tripalmitin from *n*-pentanol.

The X-ray crystal structure of this β-form of tricaprin [11, 12], of trilaurin [13, 14], of 2,11-bromoundecanoyl-1,1′ dicaprin [15] and of tritetracosanoin [16] have been shown to be a variant of the "tuning-fork" conformation of the acyl chains about the glycerol moiety (Fig. 4). Since the chains are tilted to the methyl end plane such that alternate pairs of methylene groups are nearly eclipsed in the (001) projection (*e. g.* see ref. 14), the intense bands of reflections in Fig. 3 parallel to (110) represent an interference band pattern similar to those also seen in monoclinic fatty acid and wax (001) electron diffraction patterns [7, 18] and are roughly in accord with the reported chain tilt of 61° to the end plane [*i. e.* $\cos^{-1}(1.4\text{ Å}/2.5\text{ Å}) = 56°$].

As already reported, the subcell packing is $T_{\|}$. This is again verified by powder diffraction patterns from bulk material obtained from the manufacturer. Strong bands at 4.67, 3.89 and 3.70 Å, are in accord with other measurements on triglycerides [13, 16, 19].

Table I. Comparison of tripalmitin lattice parameters with those found by Buchheim [10] for β_3 trilaurin.

Parameter	Tripalmitin	Trilaurin
d_{100}	11.75 ± 0.04 Å	12.05 Å
d_{010}	5.36 ± 0.07 Å	5.38 Å
γ^*	80.8 ± 0.8°	79.4°

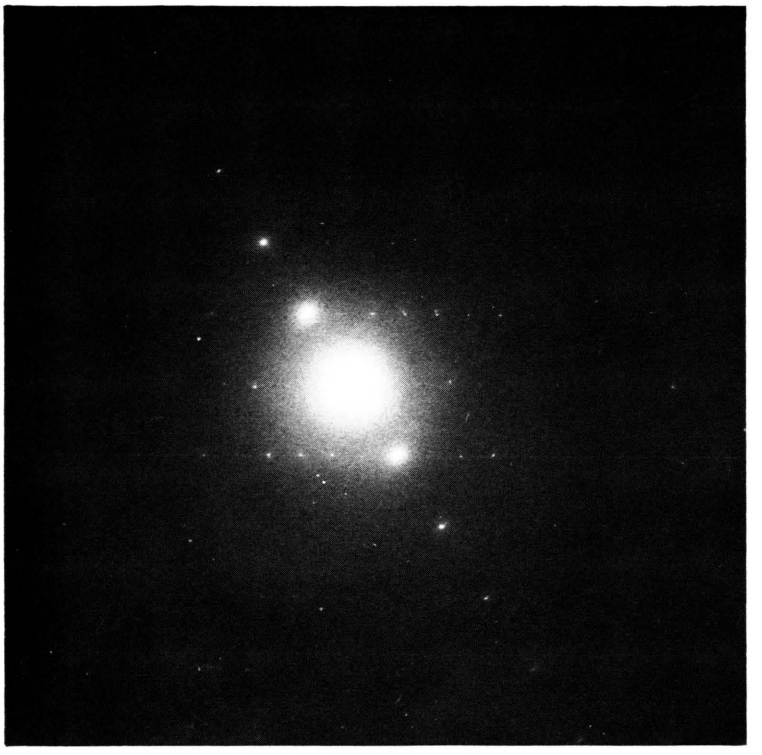

Fig. 3. Electron diffraction pattern (h k 0) from β-form of tripalmitin. Accelerating voltage: 100 kV.

Tripalmitate of (1,3/2) cyclopentane-1,2,3-triol

Earlier powder X-ray diffraction [4] and infrared spectroscopic [1, 4] studies on this compound, which models the conformer seen in X-ray crystal structures of natural triglycerides, were interpreted on the basis of identical crystal packings for the two materials. The reported strong powder diffraction spacings are similar to the ones found in this study *viz*: 4.71 Å (s), 4.29 Å (s), 3.96 Å (s), 3.76 Å (m). The CH_2 rocking mode [20], being a singlet (717 cm^{-1}), the subcell packing was proposed to be $T_{\|}$.

Large plate microcrystals of the pseudotriglyceride were obtained from *n*-pentanol or ethanol giving h k 0 diffraction patterns as shown in Fig. 5. Lattice parameters from the (001) projection are $d_{100} = 21.9 \pm 0.2$ Å, $d_{010} = 5.22 \pm 0.5$ Å, $\gamma^* = 82.9 \pm 0.8^\circ$. The crystal habit is shown in Fig. 6. Some needles are also observed.

The overall intensity distributions for diffraction patterns in Fig. 3 and Fig. 5 (*e. g.* the intense line of spots in Fig. 5) indicate a tilted chain structure and have a spacing $d = 4.50 \pm 0.05$ Å, but the crystal structures of the natural β-polymorph triglyceride and its analog must have some differences. For example the d_{100} spacing of the analog, which is not an exact doubling of the glyceride d_{100} spacing, reveals a superlattice structure. This implies that there is some perturbation due to the cyclopentane moiety. On the other hand, in comparison to other reported models of triglycerides

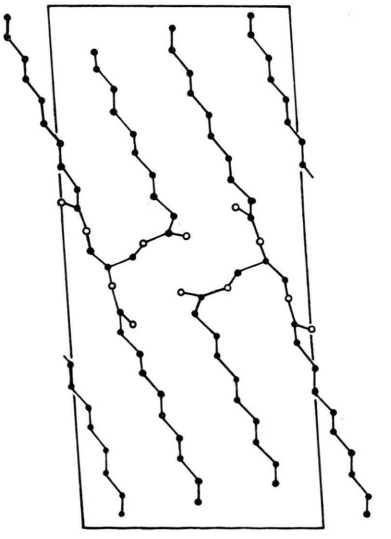

Fig. 4. Crystal packing of β-form of tricaprin (0 1 0) projection. Redrawn from Fig. 4 of ref. 12.

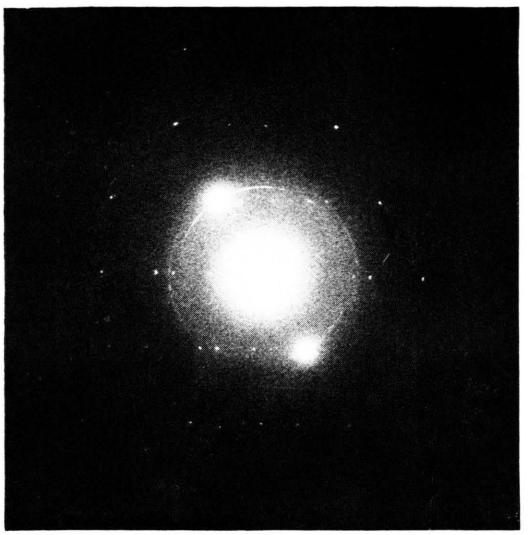

Fig. 5. Electron diffraction pattern (h k 0) from β-form, tripalmitate of (1,3/2) cyclopentane-1,2,3-triol.

Fig. 6. Crystal habit of 1,3/2 pseudotripalmitin, β-form.

based on tris(hydroxymethyl)-1,1,1 propane and tri(hydroxymethyl)-1,1,1 pentane instead of glycerol [18], the analogy to the natural compound is much closer (*vide infra*).

In addition to the prevalent β crystal form, an α-form is also obtained from solvent, especially nonpolar solvents like *n*-hexane, giving a hexagonal diffraction pattern as shown in Fig. 7. Although an α-form was not observed in the present study on tripalmitin, one was obtained for trilaurin from petroleum ether [10]. The crystal habit (Fig. 8) has the circular cross section found for several phospholipids with hexagonally-packed acyl chains [21, 23].

Tripalmitate of (1,2/3) cyclopentane-1,2,3-triol

In their description of the 1,2/3 isomer of the pseudotripalmitin Greenwald *et al.* [4] cite X-ray powder diffraction spacings at 4.38, 4.12 and 3.75 Å for the solvent-grown material along with doublet CH_2 rocking mode infrared absorption bands at 729 cm^{-1} and 719 cm^{-1}. A repeat of the powder diffraction work reproduces their results. Moreover, the 4.38 Å band is strongest whereas the 4.12 and 3.75 Å bands have only medium intensity. The infrared bands appear to indicate a methylene subcell with perpendicularly packed chains [20] but a

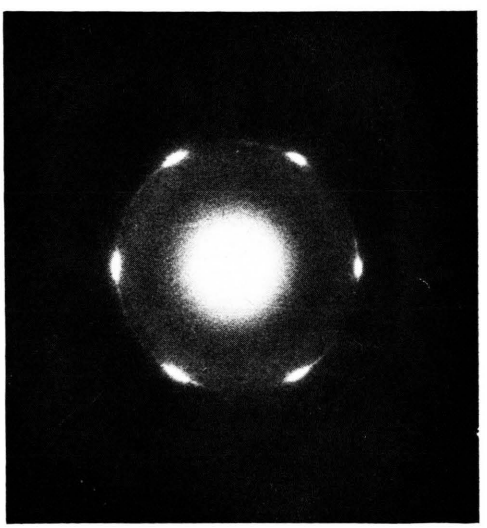

Fig. 7. Electron diffraction pattern (h k 0) from α-form of (1,3/2) pseudotripalmitin. Accelerating voltage: 100 kV.

Fig. 8. Crystal habit of 1,3/2 pseudotripalmitin, α-form.

subcell such as $O_{\perp}$, found for the β' polymorph of triglycerides, does not give a powder diffraction spacing at 4.38 Å. This was, in fact, verified by obtaining a powder pattern of orthorhombic *n*-hexatriacontane, which showed strong bands at 4.22 Å and 3.95 Å only. A *caveat* given by Larsson [24] immediately suggests the presence of mixed polymorphic forms in the solution grown bulk material.

The assumption is borne out in electron diffraction studies. In crystals grown from *n*-hexane, and α-form is most commonly seen giving diffraction patterns nearly identical to Fig. 7. The crystal habit is also similar. From alcohol, two forms are seen. One form, associated with dendritic growths (Fig. 9) gives diffuse diffraction rings with spacings at 4.08 Å and 3.83 Å, consonant with the $O_{\perp}$ subcell (001) projection. Another form, the habit being tiny laths, gives a doublet of diffraction spots representing a spacing of 4.39 Å, in accord with the most intense ring of the X-ray powder diffraction pattern.

Crystals grown from *n*-pentanol are somewhat larger than those obtained from the other two solvents. A well defined (001) single crystal pattern from the untilted $O_{\perp}$ methylene subcell (Fig. 10) can be found for the β'-polymorph. There is also evidence of yet another polymorph, which gives a strong doublet of spots at 3.73 Å. Additional weak spots indicate that this polymorph may be a tilted-chain triclinic form, but repeated efforts to grow larger crystals have failed.

Fig. 9. Dendritic crystals of β'-polymorph, (1,2/3) pseudotripalmitin from ethanol.

Tripalmitate of (1,2,3/0) cyclopentane-1,2,3-triol

Mats of lath-like microcrystals are grown from solvents, the largest coming from *n*-hexane (Fig. 11). Using the large selective-area aperture given above, a typical h k 0 diffraction pattern is obtained as shown in Fig. 12. Its appearance is somewhat similar to the epitaxial overgrowth of two polymorphic forms reported for a phosphatidylethanolamine diether [23]. Occasionally single patterns from the two components can be obtained. One is hexagonal and similar to Fig. 7; the other is a structure packing in the $O_{\perp}$ methylene subcell as shown in

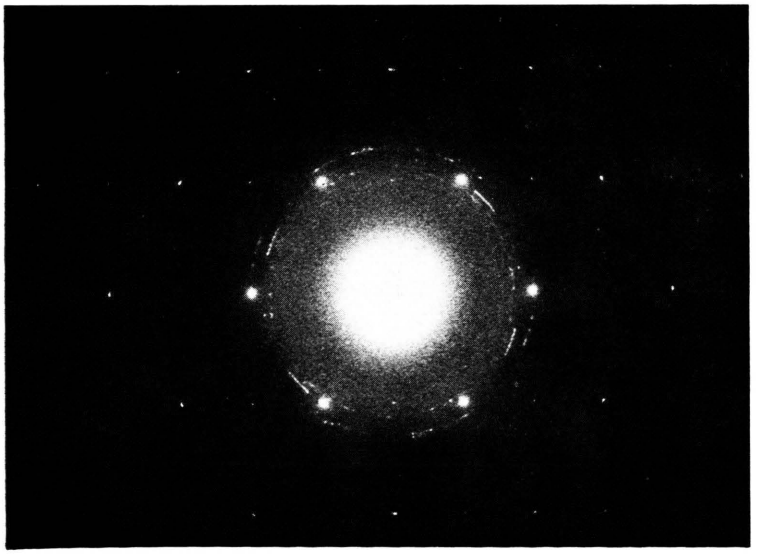

Fig. 10. Electron diffraction pattern (h k 0) from β'-form, tripalmitate of 1,2/3 cyclopentane-1,2,3-triol.

Fig. 11. Microcrystals of (1,2,3/0) analog from *n*-hexane.

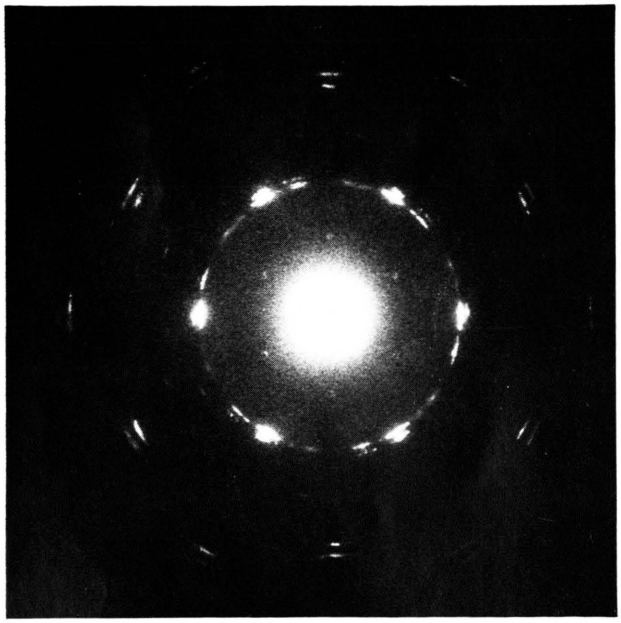

Fig. 12. Compound h k 0 electron diffraction pattern from (1,2,3/0) analog.

Fig. 13. A transition of the latter to the former is indicated by several pseudohexagonal patterns which have slightly different spacings of inner spots – *e. g.* 4.05 Å and 4.08 Å. Careful inspection of the $O_{\perp}$ (001) pattern in Fig. 13 also reveals the presence of a superlattice structure with axes twice the subcell dimensions $a = 7.66$ Å, $b = 4.75$ Å. These superlattice reflections account for the innermost hexagonally disposed doublet of Fig. 12 with spacings at approx. 7.2 Å and 6.9 Å.

Although Greenwald *et al.*[4] obtained infrared spectra with an absorption doublet at 726 cm^{-1} and 717 cm^{-1}, consonant with the $O_{\perp}$ methylene packing of solution-grown microcrystals, the powder X-ray diffraction patterns of the bulk material (giving spacings at 4.35 Å and 4.25 Å in addition to expected spacings at 4.02 Å and 3.75 Å) again indicate a mixture of polymorphs. Our powder X-ray diffraction patterns verify this finding, but the electron diffraction studies do not. Comparison of the work on tripalmitin monolayers on a water surface[25], which apparently must assume the conformation of this analog, anticipates the presence of a tilted chain β-form packing with the $T_{\parallel}$ methylene subcell.

The other polymorph found in this study is inconsistent with the tilted-chain β'-structure, also indicated from monolayer studies on natural triglycerides[25]. A single pattern from a microcrystal of this form grown from alcohol gave a strong doublet of spots with a spacing $d = 2.86$ Å. The C-form of fatty acids[26] and the γ_4-form of fatty alcohols[27] give a strong (020) reflection at 2.50 Å, consistent with an $O_{\perp}$ methylene subcell, a chain

Fig. 13. Single crystal pattern of untilted β'-form of (1,2,3/0) pseudotripalmitin.

tilt of 52°, and also with the β'-form proposed by Bursh *et al.* [25]. However, since the (020) reflection represents a distance of 1/2 the subcell *b*-axis, the 2.86 Å doublet represents too large a spacing to be congruent with normal $O_\perp$ methylene subcells [28]. Its origin thus remains unknown.

Discussion

Based on an analysis of their infrared spectra, it was recently proposed by Fringeli *et al.* [29] that the conformation of solvent-grown tripalmitin microcrystals is an all *cis* arrangement of the chains (comparable to the 1,2,3/0 isomer above) rather than the all *trans* "tuning-fork" arrangement (comparable to the 1,3/2 isomer above) found in single-crystal studies. The basis of their conclusion lay in part on their interpretation of C = O stretching modes in their spectra, requiring a pseudotrigonal arrangement of carbonyl groups in the molecule. The hexagonal symmetry of cited electron diffraction patterns from tristearin was adduced to this argument. Moreover, it was claimed that the molecular conformation in multilayer structures was identical for all layers, including the surface layer. The spectra of multilayers and microcrystals are virtually identical.

The analysis presented here conclusively demonstrates that the postulate of an all *cis* conformation for triglycerides in the solid state is incorrect. Only the all *trans* pseudotriglyceride (1,3/2) gives a diffraction pattern similar to that found in solvent-grown microcrystals of the natural triglyceride, further substantiating the expected uniformity of crystal packing in microcrystals with the tuning-fork conformation found in macrocrystalline samples of homologs [11–16]. The hexagonal symmetry cited by Fringeli *et al.* can be characteristic of diffraction patterns of all three conformers, as is shown here, and merely bespeaks a similar chain packing for these isomers. An assumed identity of conformation for an interfaced monolayer and a monolayer within a multilamellar array has also been shown to be incorrect by Bursh *et al.* [25]. The dangers of structure analysis based solely on infrared data are again revealed. The *sole* use of even the 720 cm^{-1} band to identify the type of subcell packing has also been shown to be unreliable [30].

That the phase behavior is largely determined by the long chains is substantiated by a comparison of melting points of two single crystal polymorphs (tuning-fork) [31] with the analogous critical temperature of a phase for triglyceride monolayers on a water surface (all *cis*) [24]. The similarities in bulk melting points for the conformationally-restricted analogs [1, 4] also support this contention.

Yet, there are some perturbations to the structure imposed by the "polar group" difference. Natural triglycerides, for example, exhibit several β' forms with $O_\perp$ packing which have not yet been observed in the 1,3/2 analog series either by X-ray diffraction or electron diffraction. This also appears to be the case for analogs where the glycerol is replaced by tris(hydroxymethyl) 1,1,1-propane or tris(hydroxymethyl) 1,1,1-*n*-pentane [19]. The X-ray short spacings given for the so-called β'-forms by Huu and Perron [19] do not correspond to Larsson's [24] rules for denoting triglyceride polymorphs. Furthermore, the melting points of their models differ more from the parent triglycerides than do the analogs based on cyclopentane-1,2,3-triol.

Additional evidence for perturbed molecular packing is the presence of superlattice type diffraction patterns in two cases. The full significance of this effect will only be realized after a quantitative X-ray crystal structure analysis has been carried out.

The use of electron diffraction structural analysis of conformationally-fixed analogs of natural lipids, even in the qualitative way presented in this paper, has many exciting ramifications for crystallographic study of microsystems. Since (due to imperfections in crystal packing) electron diffraction patterns often originate mostly from the long chains [32], an *a priori* posited conformation of the polar group is valuable for later analysis of packing of the whole molecule in the crystal. Given that the scattering cross section of matter for electrons is some 10^3 greater than for X-rays, microcrystals can be used to obtain single crystal patterns via electron diffraction, in contrast to the powder patterns obtained by X-ray methods. The use of microcrystals ensures the identification of several polymorphs in a single bulk sample as is shown in this paper, whereas X-ray powder patterns in this case are very difficult to interpret (see also ref. 24).

Synthetic analogs of this kind will also be valuable for assessing the favored conformation of a lipid molecule under physiological involvement in a variety of biological situations. Systematic modi-

fications of lipid molecules have provided valuable insight into the dimensional and electronic demands of lipid-associated proteins at interfaces [33, 34]. However, these synthetic alterations have not directly addressed the question of the *mutual conformational demands* of lipid and protein components of the enzyme or membrane system. The exigencies of the protein conformation at the lipid binding site may well require conformations in both neutral and polar lipids which differ greatly from the thermodynamically preferred conformational state of the unassociated lipid (that is, the lipid in a self-associated state). Such deviations may be tolerable in the natural lipid, but either impossible, or actually desirable, in the conformationally-restricted cyclopentanoid analogs. That this enforced relative orientation of the polar and non-polar aspects of lipid amphipaths is indeed critical is amply shown by preliminary biological experiments with the analogs. Morrisett *et al.* [35] have studied the interaction of apolipoprotein C-III, isolated from human high density lipoprotein, and a variety of phosphorylated cyclopentanoid analogs. The studies clearly showed that the induced conformational change in the protein and the binding affinity both vary with the conformation of the analog. Furthermore, the configuration of the substituents in the cyclopentane ring has recently been found markedly to affect the susceptibility of the cyclopentanoid phosphatidic acid analogs [3] to the dephosphorylation action of a highly specific phosphatase isolated from canine lung [35]. These indications that the lipid conformation in the protein (enzyme)-lipid complex is significant encourages us to examine not olny the biological relecance of the findings, but also the rich field of physical techniques available to study intra and inter-molecular associations in the lipid moieties themselves.

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