Glyceride Isomerizations in Lipid Chemistry

B. SERDAREVICH, Collip Medical Research Laboratory, University of Western Ontario, London, Canada

Abstract

Isomerization of isopropylidene glycerol ketals and benzylidene glycerol acetals was studied, and isomerization equilibria were established. Reaction of benzaldehyde with glycerol gave four benzylidene glycerol isomers, which were separated by column chromatography and characterized by NMR spectroscopy and other methods.

Isomerization of 1- and 2-monoglycerides and of 1,2- and 1,3-diglycerides, and their separation by column chromatography, are described. Mechanisms of isomerization in mono- and diglycerides and factors which affect them are discussed.

Isomerization of 1- and 2-glycerophosphates and of cyclic glycerophosphates by acid and base was also studied. Hydrolysis products of L-3-glycerylphosphorylcholine and 2-glycerylphosphorylcholine were separated by column chromatography and characterized by periodic acid oxidation, optical rotation, and NMR spectroscopy. No isomerization of unhydrolyzed L-3-glycerylphosphorylcholine and 2-glycerylphosphorylcholine was observed. Evidence indicated that acid-catalyzed hydrolyses of phosphoglycerides are under thermodynamic control whereas most base-catalyzed hydrolyses are under kinetic control.

Introduction

ALTHOUGH DIFFERENT TYPES OF ISOMERIZATION are possible in lipid chemistry, this paper deals only with those kinds which pose major difficulties in the preparation, isolation, and characterization of lipids. These include acetal and ketal isomerizations in glycerides with isopropylidene and benzylidene blocking groups, and acyl migration (intramolecular transesterification) in esters of carboxylic acids and phosphoric acid. Acyl migration has been a problem from the very beginning of lipid chemistry, and this phenomenon has been studied many times in the past. Extensive experimental data and conclusions are available in the literature, and an excellent summary is given by Hanahan (1).

The recent development of conformational analysis and reaction kinetics has shown that most rearrangements in organic compounds involve electronic, steric, stereoelectronic, and entropy control. Improvements in methods for isolation and characterization of isomerization products in lipid chemistry have made it possible to study mechanisms of isomerization of lipids by these methods. An attempt is made to review isomerizations in lipid chemistry in the light of these more recent developments and to give up-to-date interpretations of the reaction mechanisms which are involved. The paper also contains new experimental data which were required to support the proposed reaction mechanisms.

Isopropylidene Glycerol

The most commonly used intermediate for synthesis of 1-monoglycerides and 1-monoglyceryl ethers is isopropylidene glycerol, in which the vicinal hydroxyls of glycerol are blocked by formation of a ketal with acetone (2). The free hydroxyl group in position 1 of glycerol is reacted with a fatty acid or fatty alcohol derivative, and subsequent removal of the blocking group by boric acid or hydrochloric acid leaves 1-monoglyceride or 1-monoglyceryl ether (3,4). The purity of the 1-isomer depends primarily on how much 1,3-isomer is present in the 1,2-isopropylidene glycerol. Although it is known that isopropylidene glycerol exists almost entirely in the 1,2-form (2), experience with the synthesis of normal- and branched-chain 1-monoglycerides and 1-monoglyceryl ethers has shown that a small amount of 1,3-isomer is always present in 1,2-isopropylidene glycerol. Similar isomerizations have been observed in the preparation of cyclic acetals and ketals of carbohydrates and other compounds, but these occur to only a limited extent in ketals (5–7).

Experimental Procedure and Results

The 1-monoglycerides and 1-monoglyceryl ethers were prepared from isopropylidene glycerol by previously described methods (3,4). When freshly prepared isopropylidene glycerol was used, the products contained only a trace of 2-isomer whereas about 5% of 2-isomer was obtained when commercial isopropylidene glycerol was used. If freshly prepared isopropylidene glycerol was treated with dry hydrochloride at room temperature for five days, then washed with sodium hydroxide solution, and purified by chromatography on Florisil, the product gave monoglycerides and monoglyceryl ethers which contained about 5% of the 2-isomer. When freshly prepared isopropylidene glycerol was treated with methanolic sodium hydroxide (0.5 N) for 2 hr under reflux, purified, and converted to derivatives, the results indicated that only a trace of 2-isomer was present. The monoglycerides and monoglyceryl ethers were purified under conditions that prevent isomerization of monoglycerides (3,4,8). Purification by recrystallization was avoided because the small amount of 2-isomer was readily removed after one or two recrystallizations. The amount of isomer was determined by oxidation of monoglyceryl products with periodic acid (9) and by thin-layer chromatography on Silica Gel G, impregnated with boric acid (3,4,8). Quantitation was achieved on thin-layer chromatograms by measuring the intensity of charred spots by densitometry (Photovolt Densitometer-Photometer, Models 52C and 520A) (10).

Discussion

These results confirm that cyclic ketals can be isomerized under acidic but not under basic conditions when a free hydroxyl group is located at a convenient distance and in suitable steric orientation with respect to the ketal.

From Figure 1 it is obvious that isomerization of the 5-membered ring 1,2-isopropylidene glycerol (I) to the two possible 6-membered ring 1,3-isopropylidene glycerols (II and III) is not favorable. The strong steric interactions in the 6-membered rings between the axial methyl group at position 2 and the axial hydrogens at positions 4 and 6 push the

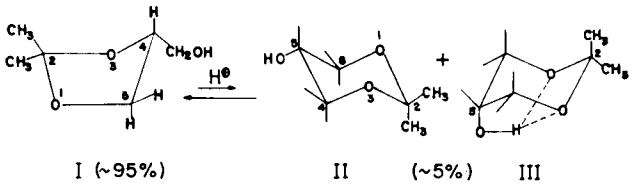

FIG. 1. Isomerization of isopropylidene glycerol.

isomerization equilibrium toward the 1,2-isomer (I).

The experimental findings and this analysis indicate that isopropylidene glycerol exists at equilibrium as a mixture of 1,2- and 1,3-isomers in a ratio of about 95:5, and this is the main reason for the presence of small amounts of 2-isomer in the synthetic monoglycerides and monoglyceryl ethers. This would also explain the observation of Kates (11) that optically active isopropylidene glycerol gradually loses its optical activity on standing. Since isomerization of the cyclic ketal occurs only under acidic conditions, storage over pellets of sodium hydroxide should prevent its occurrence.

Benzylidene Glycerol

The 1,3-benzylidene glycerol, the most commonly used intermediate for preparation of 2-monoglycerides and 2-monoglyceryl ethers, is prepared by the reaction of glycerol with benzaldehyde (12–14). This reaction has been extensively investigated by previous workers because of low yields of 1,3-benzylidene glycerol and because of the variable melting-points of the isolated product. Hibbert and Carter (15) detected 1,2- and 1,3-benzylidene glycerols in the reaction mixture but were able to isolate only crystalline 1,3-isomers. Verkade and van Roon (13) reported isolation of two crystalline 1,3-benzylidene glycerols, which they assigned as *cis* and *trans* with regard to the position of phenyl group and hydroxyl group in the 6-membered ring. Baggett et al. (16) separated acetyl and benzoyl derivatives of these two forms on alumina and determined the structure of their hydrolysis products by infrared and other methods. Further proof of the *cis* and *trans*-1,3-benzylidene glycerol conformations was obtained by Baggett et al. (17) by NMR spectroscopy of their acetyl derivatives.

Since these studies only partially solved the problem of isomerization of benzylidene glycerols, the products of synthesis by separation of the isomers on a Florisil column were reinvestigated and their structures determined by preparation of monoglyceryl derivatives and by NMR spectroscopy (4). Two oily isomers were identified as 1,2-benzylidene glycerols and two crystalline isomers as 1,3-benzylidene glycerols. The 1,2-isomers made up 80%–85% of the reaction product and the 1,3-isomers, the remainder. Further studies on equilibria between these isomers, as determined by NMR spectroscopy, are now reported.

Experimental Procedure and Results

NMR Spectra of Benzylidene Glycerols. All spectra were obtained with a Varian A-60 spectrometer by using 10%–16% solutions (w/v) in $CDCl_3$ or CCl_4. Band positions are given relative to internal tetramethylsilane (TMS) reference in parts per million (ppm). Spectra of the separated benzylidene glycerols and of the whole reaction mixture are shown in Figure 2, together with a spectrum of isopropylidene glycerol for purposes of comparison.

The spectrum of the oily 1,2-benzylidene glycerol (B) shows a pattern similar to that of 1,2-isopropylidene glycerol (A) in the region of 3.5–4.5 ppm. The five protons attached to glycerol resonate in this region but, since they are all non-equivalent, they appear as a group of bands which are not easy to interpret in a simple way. The band at 2.83 ppm is attributable to the hydroxyl proton, and bands at 7.2–7.6 ppm represent protons on the phenyl group.

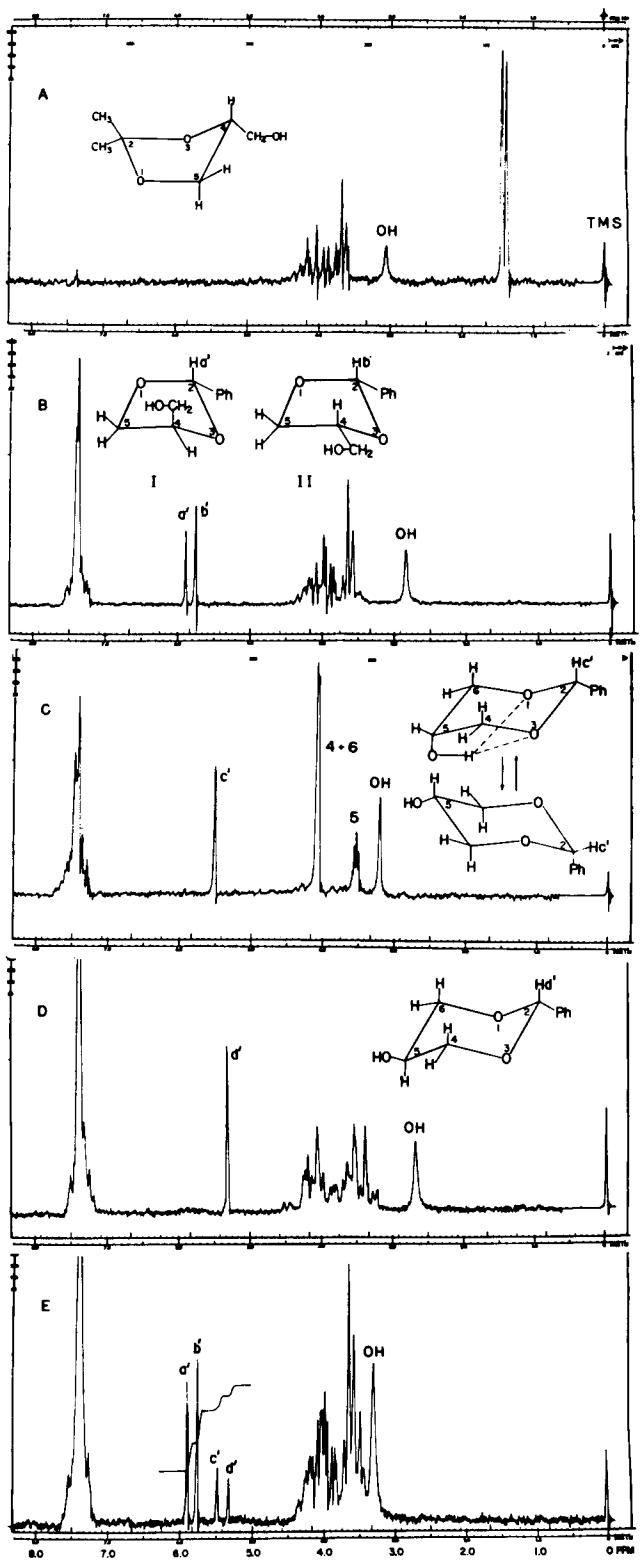

FIG. 2. NMR spectra of 1,2-isopropylidene glycerol (A), 1,2-benzylidene glycerols (B), *cis*-1,3-benzylidene glycerol (C), *trans*-1,3-benzylidene glycerol (D), and reaction mixture (E). Ph = phenyl group.

The two bands at 5.75 ppm (b′) and 5.88 ppm (a′) are from the proton at position 2 of the 5-membered ring. This proton resonates at the lower field in the presence of a *cis* orientated hydroxymethyl group at position 4, and at the higher field in the presence of *trans* orientated substituent at this position, as shown by Baggett et al. (18–20) on similar compounds. The presence of both bands in spectrum B (Figure 2) indicates that the 1,2-benzylidene glycerol is a mixture of two conformational isomers corresponding to the two structural formulae shown (I and II). Integration of bands (a′) and (b′) indicated that the isomers were present in the ratio of 44:56 of isomers I and II.

Spectrum C represents the crystalline 1,3-benzylidene glycerol (mp 82–83C) which can be assigned the *cis* conformation by inspection of the spectrum bands and by comparison with the spectra of its *cis* glyceryl derivative (Fig. 3,C) and with spectra of the *cis* acetyl derivative reported by Baggett et al. (17). The band at 3.2 ppm is from the hydroxyl proton at position 5 in the ring, the band at 3.5–3.6 ppm from the other proton at position 5, and the band at 7.2–7.6 ppm from the protons of the phenyl group at position 2. The four protons at positions 4 and 6 appear as one band at 4.00–4.15 ppm, split in a weak doublet by the proton at position 5. This indicates that these protons are nearly equivalent and that the compound exists as an equilibrium mixture of two conformational forms as shown in spectrum C. The band at 5.52 ppm (c′) is from the proton at position 2 in the ring. This proton has less interaction in the ring than the corresponding proton in the 5-membered ring of 1,2-benzylidene glycerols (Fig. 2,B) and therefore appears at a higher field.

Spectrum D is from the minor crystalline product (mp 34C–35C) and represents *trans*-1,3-benzylidene glycerol with stable chair conformation, where axial and equatorial protons are not equivalent and differ in chemical shifts and splitting (21–22). The band at 2.7 ppm is from the hydroxyl proton in position 5, and the band at 7.2–7.6 ppm from the protons of the phenyl group. Bands from axial protons at positions 4, 5, and 6 appear at approximately 3.2–3.9 ppm whereas the bands from equatorial protons at positions 4 and 6 are, as usual, at a lower field (3.9–4.3 ppm). The band at 5.31 ppm (d′) is from the axial proton at position 2. This proton has less interaction in the ring than corresponding protons in the other benzylidene glycerols (described above) and therefore appears at the highest field.

Spectrum E represents the product of an acid-catalyzed reaction of benzaldehyde and glycerol containing all four types of benzylidene glycerol. The relative proportions of both of the 1,2- and *cis*-1,3- and the *trans*-1,3-benzylidene glycerol can be determined directly from this spectrum by integrating the bands in the region of 5.3–5.9 ppm. Bands (a′) and (b′) are derived from the two 1,2-benzylidene glycerols, band (c′) from *cis*-1,3- and band (d′) from *trans*-1,3-benzylidene glycerol (*see* spectra B, C, and D). Since all four bands correspond to the same proton in different isomers, the ratio of their integrals gives directly the percentage of each isomer present in the mixture. This method is useful for following the isomerization process since the sharp bands give clear-cut integrals which permit good quantitative estimation without reference to other parts of the spectrum.

NMR Spectra of Benzylidene Glycerol Derivatives. The NMR spectra of palmitoyl derivatives of benzylidene glycerol (Fig. 3) provided further proof of the existence of two 1,2- and two 1,3-benzylidene glycerol isomers in the products of reaction of benzaldehyde and glycerol.

Spectrum B of 1-palmitoyl-2,3-benzylidene glycerols (mp 15–17C) is again similar to spectrum A of 1-palmitoyl-2,3-isopropylidene glycerol (an oil) in the region of 3.5–4.5 ppm. The peaks from 2.5 ppm up-field in all spectra represent protons from the fatty acid chain, and the peaks at 7.2–7.6 ppm represent protons of the phenyl group. Two bands at 5.88 ppm (a′) and 5.75 ppm (b′) again show the presence of two conformational forms of 1,2-benzylidene glycerol derivative, as noted previously (Figure 2, B). Partial separation of the two forms was achieved by chromatography of a 300-mg mixture on a column of Florisil (1.5 × 42 cm), eluting with

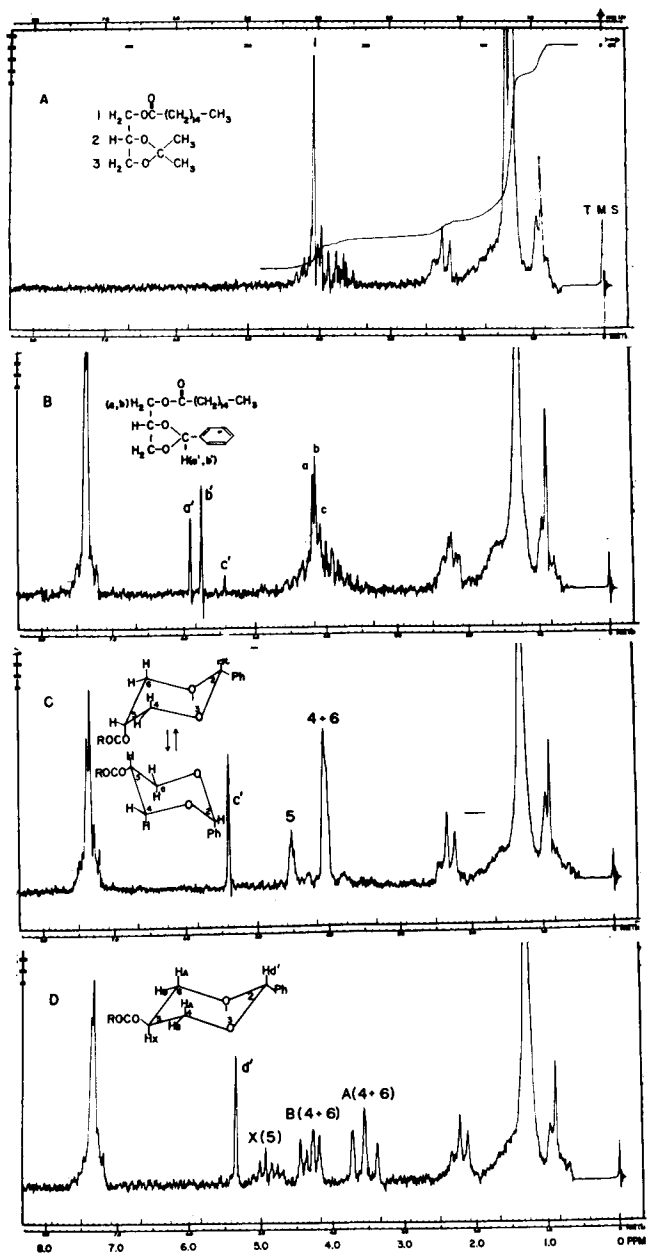

FIG. 3. NMR spectra of 1-palmitoyl-2,3-isopropylidene glycerol (A), 1-palmitoyl-2,3-benzylidene glycerols (B), *cis*-2-palmitoyl-1,3-benzylidene glycerol (C), *trans*-2-palmitoyl-1,3-benzylidene glycerol (D). R = palmitoyl chain; Ph = phenyl group.

Skellysolve B-ether 100:0 (100 ml), 90:10 (200 ml), and 85:15 (300 ml). The separation was evidenced by differences in the proportion of bands (a') and (b') in NMR spectra of column fractions. The first fractions eluted with Skellysolve B-ether (85:15) gave about 75% of product (a') and 25% of product (b'), and in later fractions the proportion of product (a') decreased whereas that of product (b') increased proportionately. Inspection of these spectra showed further that bands (a') and (a) were derived from one isomer while bands (b') and (b) were derived from the other isomer. Bands (c') and (c) were caused by the presence of a small amount of *cis*-2-palmitoyl-1,3-benzylidene glycerol (*see* spectrum C).

The C and D spectra represent *cis*-2-palmitoyl-1,3-benzylidene glycerol (mp 50–51C) and *trans*-2-palmitoyl-1,3-benzylidene glycerol (mp 62–63C) respectively. They are identical to the spectra of Baggett et al. (17) for similar *cis* and *trans* compounds. In spectrum C the unstable *cis* compound exists in two conformations in equilibrium, where axial and equatorial protons in positions 4 and 6 are not distinguished and appear as a singlet at 4.05 ppm, therefore give approximately spectra of type AX_4. Spectrum D represents the *trans* form with the acyl and phenyl groups in equatorial position. In such stable conformation the axial and equatorial protons in positions 4 and 6 are not equivalent and can be distinguished by their chemical shifts and coupling (17,21,22). In spectrum D of the approximate type ABX, the coupling constant (J_{AX}) between the vicinal axial protons at positions 5(X), 4 and 6(A) is about 10.2 cps whereas the coupling constant (J_{AB}) between the geminal protons in positions 4 and 6(A and B) is about 11.3 cps. The bands of axial protons from positions 4 and 6(A) appear therefore as a triplet at 3.3–3.8 ppm and are composed of two doublets whose two inner bands coincide. The coupling between axial protons in position 5(X) and equatorial protons in positions 4 and 6(B) is smaller, $J_{BX} = 4.8$ cps, and the bands of equatorial protons from positions 4 and 6 appear as two doublets at lower field (4.2–4.5 ppm), separated by the J_{AB} coupling constant. The proton in position 5(X) resonates at the lower field (4.7–5.1 ppm) as a multiplet, as in the case of 2-monoglycerides, 1,2-diglycerides and triglycerides (3). The band at 5.35 ppm is from the proton in position 2 and appears at a higher field than in any of the other benzylidene glycerol isomers.

Gas-Liquid Chromatography (GLC) of Benzylidene Glycerols. A partial separation of the mixture of benzylidene glycerols, obtained by reaction of benzaldehyde and glycerol, was also achieved by GLC, using a 6-ft × 1/8-in. column of 3% SE-30 on Chromosorb W at 120C. Similar isomers obtained from reaction of acetaldehyde and glycerol have been separated with GLC by Aksnes et al. (7).

The chromatogram shows only three peaks although there are four different benzylidene glycerol isomers in the synthetic mixture, as shown by NMR spectroscopy. By separation of the benzylidene glycerol mixture on Florosil and by checking the fractions by NMR and GLC, it was found that the peak of retention time, 8.5 min, is a mixture of 1,2-benzylidene glycerol (Fig. 2,B,II) and *cis*-1,3-benzylidene glycerol (Fig. 2,C). The peak of retention time, 9.5 min, corresponds to the other form of 1,2-benzylidene glycerol (Fig. 2,B,I) and the peak of retention time, 13 min, represents the *trans*-1,3-benzylidene glycerol (Fig. 2,D).

Isomerization Equilibria of 1,2- and 1,3-Benzylidene Glycercols. It was noted by earlier workers that treatment of the reaction mixture from benzaldehyde and glycerol with mineral acids increased the yield of 1,3-isomers (13,23). Isomerization equilibria of 1,2- and 1,3-benzylidene glycerols was therefore studied by treating the reaction product, or the separated 1,2-isomers, with dry hydrochloride in dry benzene at room temperature for different periods of time. After 5 days, 10 days, 15 days, one month, and three months, aliquots were taken, the acid was neutralized by sodium carbonate solution, and the isomerization products were isolated by distillation at Bp = 170–175C (15 mm Hg) or by chromatography on a Florisil column. The NMR spectra of the products were recorded, and the amount of isomers was determined by integration of bands (a'), (b'), (c'), and (d') (Fig. 2, spectrum E). The bands (a') and (b') decreased during isomerization, and bands (c') and (d') increased proportionately. Isomerization equilibrium was achieved in about 30 days and showed a ratio of 44:56 of 1,2- to 1,3-isomers. While the ratio of 1,2-isomers remained unchanged throughout (44:56 = a':b'), the ratio of 1,3-isomers changed during an extended time of isomerization and reached an equilibrium of 29:71 of isomer (d') to (c'). Further exposure to acid had little effect on the equilibrium. When the mixture was treated with 0.5 N NaOH in methanol under reflux for two hours, no isomerization could be detected, in agreement with earlier reports (24).

Further isomerization studies of 1,2-benzylidene glycerols were done by column chromatography. On acid-treated Florisil or silicic acid, isomerization occurred and was proportional to the time of contact with the adsorbent. It was however slower than that observed on hydrochloride treatment. Chromatography on Florisil and alumina columns did not cause isomerization.

Discussion

It is well known that aldehydes and ketones react smoothly with vicinal *cis* hydroxyl groups to form cyclic acetals and ketals whereas reaction proceeds more slowly when the hydroxyls are lying farther apart (25). It therefore seems reasonable to assume that benzaldehyde reacts primarily with vicinal hydroxyls of glycerol to form 1,2-isomers, particularly since the products of reaction contain about 80% of 1,2-benzylidene glycerols. This is further supported by the observed isomerization of 1,2- to 1,3-isomers on treatment with acid.

As shown by NMR spectra (Fig. 2 and 3), the 1,2-benzylidene glycerol is a mixture of two 5-membered ring conformational forms (Figure 4). 5-membered rings are usually not planar because of interactions between eclipsed hydrogens and substituents. Although there are many possible conformations, two simplified projections are commonly used: C_s, with one carbon out of the plane of the ring as drawn in Figure 4 (I and II); or C_2, with two carbons out of the plane (5,26). In these two conformations there is partial staggering of the hydrogens which diminishes nonbonding interactions but to some extent increases angle strain. As a result, the nonplanar 5-membered ring has less strain than a planar ring (27,28).

The most stable 5-membered ring conformation

should have the larger substituents in *cis* and equatorial orientation as depicted for product II (Figure 4), in which eclipsed interactions with neighboring hydrogens are minimized (5,26,29). The 1,2-benzylidene glycerol present in a smaller amount in the reaction mixture may therefore have the conformation depicted in Figure 4 (I) and the proton at position 2 *cis* oriented to the hydroxymethyl group at position 4. This interaction would result in less stability and would push the resonance of the proton (a′) to lower field (spectra B, Fig. 2 and 3). The other 1,2-isomer present in larger amount would then have the conformation shown in Figure 4 (II) and the proton at position 2 *trans* oriented to the group at position 4. This results in less interaction and the resonance of the proton (b′) therefore appears at higher field (spectra B, Fig. 2 and 3).

Although the 5-membered ring usually has more strain than the corresponding 6-membered ring (30–33), formation of the 5-membered ring proceeds faster than the 6-membered ring (26). Faster formation of a less stable compound results from a kinetically controlled reaction. If the steric and inductive conditions of the substituent in the 5-membered ring are optimal, the compound can then readily be isomerized to the more stable 6-membered ring form by a thermodynamically controlled reaction. In the present case, isomerization of 5-membered (I and II) to 6-membered ring isomers (III and V) is possible through nucleophilic attack of the free hydroxymethyl group on the electron deficient carbon at position 2. The isomerization is probably not a simple concerted reaction as shown in Figure 4 (I) but rather a two-step reaction as proposed by Baggett et al. (20). It was shown above by NMR studies that the 1,2-benzylidene glycerol isomers both decrease in amount during isomerization and the 1,3-isomers both increase proportionately. From the isomerization scheme in Figure 4 one can see that 1,2-benzylidene glycerol (II), which is present in larger amount in the reaction mixture (56%), is isomerized to *cis*-1,3-isomers (V + VI), which constitute the larger proportion of the 1,3-isomers. The 1,2-benzylidene glycerol (I), which is present in smaller amount in the reaction mixture (44%), is isomerized to *trans*-1,3-isomers (III + IV), which are present in smaller percentage in the 1,3-isomers.

The 6-membered ring conformation in which the large substituents are in equatorial positions (III) is normally the most stable form and would therefore be expected to predominate in the equilibrium mixture of 1,3-isomers. However Baggett et al. (16,17) showed, by infrared studies of intramolecular hydrogen bonding and by NMR spectroscopy, that the *cis*-1,3-isomer (V) with the axial hydroxyl was the major product. This was explained on the basis that the ring conformation is stabilized by intramolecular hydrogen bonding of the axial hydroxyl at position 5 with the oxygens at positions 1 and 3. The *trans*-1,3-isomers (III and IV) made up most of the remainder and were present in about equal amounts (16).

The *cis*-1,3-benzylidene glycerol had mp 82C–83C, and the *trans*-1,3-benzylidene glycerol had mp of 34–35C. Earlier workers had assigned a mp of 63C to *cis*- and 83C to *trans*-1,3-benzylidene glycerol. The product of the mp, about 63C, believed to be *cis*-1,3-benzylidene glycerol, is actually a mixture of about 90% *cis*-1,3-isomer and 10% of a mixture of 1,2- and *trans*-1,3-benzylidene glycerols. By repeated recrystallization or chromatography on Florisil, pure *cis*-1,3-isomer of mp 82–83C can be obtained from this mixture. The pure samples of *cis*, *trans*, or 1,2-benzylidene glycerols stored at room temperature are not stable and, after a few days, show evidence of some isomerization and of splitting to benzaldehyde and glycerol. They are however stable if stored in cold. Each of the four benzylidene glycerol isomers, when treated individually with acid, was isomerized after some time to give a mixture of all four isomers.

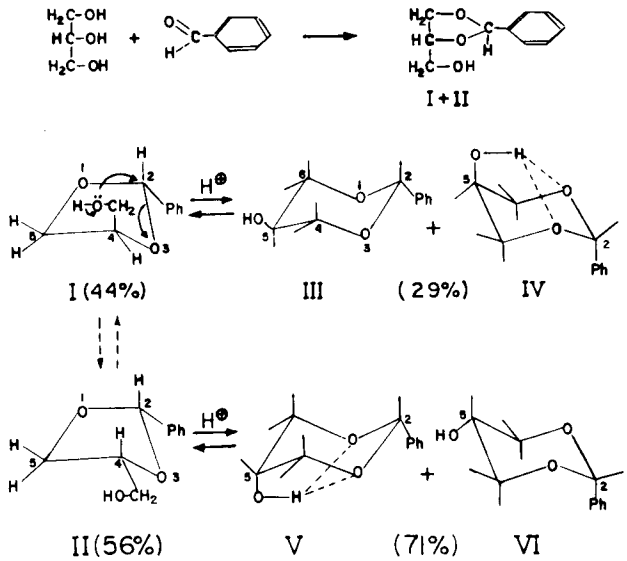

FIG. 4. Synthesis of 1,2-benzylidene glycerols and their isomerization to 1,3-benzylidene glycerols.

The above observations on the separation of the products of reaction by chromatography on Florisil, preparation of their 1- and 2-monoglycerides and monoglycerol ethers, isomerization studies, and analysis by GLC and NMR indicate that two stereoisomeric 1,2-benzylidene glycerols are the first products of the reaction of benzaldehyde and glycerol and that these subsequently isomerize to 1,3-benzylidene glycerols.

Monoglycerides

Monoglycerides occur naturally as intermediates in the biosynthesis and degradation of lipids, and they are also often used as intermediates in the chemical synthesis of mixed diglycerides, triglycerides, and phospholipids (14,34). Isomerization is one of the main problems in any kind of work with monoglycerides. They easily isomerize under acidic, basic, or thermal conditions to an equilibrium of about 90% of 1-monoglyceride and 10% of 2-monoglyceride (12,35–37). The isomerization velocity is strongly dependent on the size and nature of the fatty acids whereas the equilibrium ratio is only slightly influenced (37,38). This type of isomerization is called acyl migration or intramolecular transesterification.

Removal of the isopropylidene and benzylidene blocking groups by acids results in some isomerization of monoglycerides. This is usually avoided by removal of the group with hydrogenolysis (limited to saturated compounds) or more recently by boric acid in trimethylborate (3,14,35,39).

Isomerization of 1- and 2-monoglycerides occurs during chromatography on silicic acid, Florisil, and acid-treated Florisil (3,14,40). This can be prevented however by impregnating the adsorbents with 10% (w/w) of boric acid, thus allowing purification of

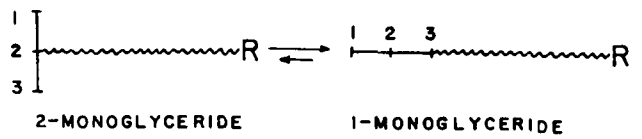

FIG. 5. Isomerization scheme of monoglycerides. R = fatty acid chain; 1,2,3 = glycerol moiety.

monoglycerides by column chromatography without significant isomerization (3).

Isomerization of 1- and 2-monoglycerides was observed also during biosynthesis of diglycerides and triglycerides from monoglycerides and during hydrolysis of triglycerides to monoglycerides by lipases (41,42). The rate of isomerization was dependent on the size of the fatty acid chain and pH of the medium in which the enzymatic reactions were carried out.

Experimental Procedure and Results

Although 1- and 2-monoglycerides were not separated by column chromatography on acid-treated Florisil which had been impregnated with boric acid, nearly complete separation was achieved by using Florisil impregnated with boric acid. A mixture of 1- and 2-monoglycerides (150 mg) was chromatographed on a column (1.5 × 43 cm) of Florisil impregnated with 10% (w/w) boric acid. The 2-monoglyceride was eluted first with 290 ml of ether-methanol (98:2), then the 1-monoglyceride with an additional 380 ml of the same solvent. There was about 20% of the mixture overlapping in the middle fractions. Separation of 1- and 2-monoglycerides on boric acid-impregnated adsorbent by column chromatography, or by TLC (3), is possible because the vicinal hydroxyls of 1-monoglycerides form a complex with boric acid, which migrates more slowly than 2-monoglycerides; the latter have no vicinal hydroxyls. Isomerization was not significant during the average elution time of two to three hours for an impregnated Florisil column, but when 2-monoglycerides wer left for 15 hours on such a column and then eluted, isomerization to the 1-isomer was detected as follows: for C_{15}-anteiso acid, 53% of 1-isomer; for C_{17}-anteiso acid, 49% of 1-isomer; and for palmitic acid, 37% of 1-monopalmitin. However, when 1- and 2-monoglycerides (5 mg each) were incubated separately with boric acid, $B(OH)_3$, in 1 ml of Skellysolve B-ether (50:50) at room temperature for 15 hours, no significant isomerization was observed. Incubation of monoglycerides under the same conditions with $Al(OH)_3$, $Zn(OH)_2$, and $B(OCH_3)_3$ also failed to cause significant isomerization.

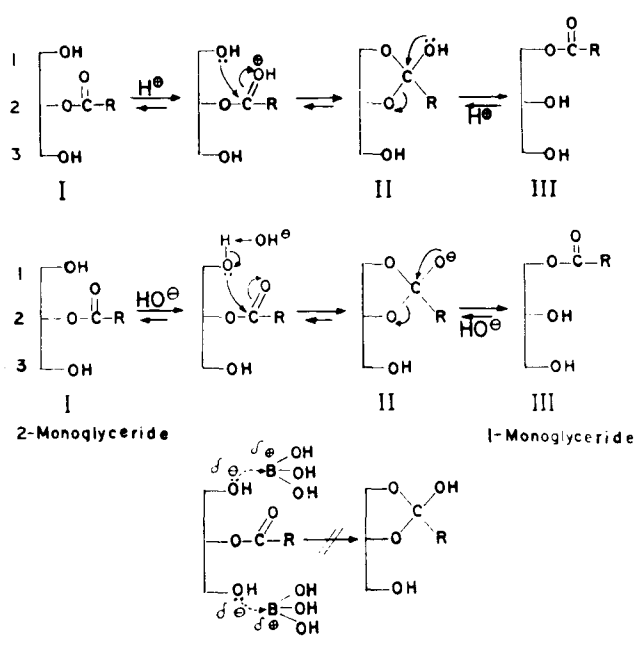

FIG. 6. Isomerization of monoglycerides by acid and base catalysis.

In the above experiments the relative proportions of 1- and 2-isomers were determined by periodate oxidation (9) and by TLC, followed by densitometry (10), but the latter gave more satisfactory results. NMR spectroscopy was also useful in studying isomerization of monoglycerides because the spectra of 1- and 2-monoglycerides can be readily distinguished (3,43).

Comment

This laboratory's experience in the preparation of normal- and branched-chain 1- and 2-monoglycerides showed that branched-chain monoglycerides isomerize faster than monoglycerides with normal-chain fatty acids (3). It is also known that monoglycerides with unsaturated fatty acids isomerize faster than their saturated analogs (14). Further observations have shown that monoglycerides with shorter-chain fatty acids isomerize faster than those with longer-chain acids (3,37,41–43). The melting points of 2-monoglycerides are usually lower than those of corresponding 1-monoglycerides. Branched- and unsaturated-chain monoglycerides also have lower melting-points than monoglycerides with straight-chain and saturated fatty acids. This is in agreement with general observations for organic compounds, that straight-chain compounds usually have higher melting-points than their unsaturated- or branched-chain analogs. As is already known, steric effects are mainly responsible for this. Normal-chain compounds fit better in a crystal lattice than a sterically disturbed packing of branched and unsaturated analogs.

The simplified representation of branched and linear shapes of the monoglycerides in the scheme (Fig. 5) shows that the main driving force for acyl migration in monoglycerides may be the intramolecular steric effect of branching. The 2-monoglycerides, having a branched form, isomerize to the more linear 1-monoglycerides, escaping in that way a great deal of branching interaction. An estimate of the driving force was obtained by measuring the heats of combustion of some 1- and 2-monoglycerides (44). The less stable 2-monoglycerides had higher heats of combustion than the 1-isomer analogs, and from the difference the isomerization force was calculated.

Fischer (45) and later workers (46,47) postulated that acyl migration proceeds by the formation of cyclic intermediates. The neighboring hydroxyl group, possessing optimal steric requirements, reacts with the ester carbonyl through a 5-membered ring intermediate to form the more thermodynamically stable monoglyceride (Fig. 6). The ester group at position 2 in 2-monoglycerides has larger interactions with the adjacent hydroxymethyl groups than after migration to position 1 or 3. The isomerization equilibrium is therefore pushed strongly to the more thermodynamically stable 1-monoglycerides. If an additional steric effect in the fatty acid chain is in-

troduced by branching or unsaturation, the isomerization equilibrium will be reached faster. Similar observations have been made in carbohydrates, where acyl groups on ring structures tend to migrate to the position with the smallest steric interaction and the most thermodynamic stability (5,48). Such migration is usually from a secondary to a primary hydroxyl group, from ring structure to side-chain, or to some other favored position in the ring.

One would expect larger fatty acids to exercise greater steric effects, therefore their monoglycerides should isomerize faster. This might be so if there were no competetion of rate-controlling steps in the transition state during formation of the cyclic intermediate (49,50). From the isomerization mechanism in Figure 6 it can be seen that there are transition state steps in the conversion of one monoglyceride isomer to another, one of which is the rate-controlling step. The first step is formation of the 5-membered ring intermediate (II) from reactant (I), and the second is the conversion of this intermediate to the product (III). According to experience, monoglycerides with smaller fatty acids isomerize faster, and this fact suggests that the first step has to pass a higher transition state free energy (T_1), making it the rate-controlling step as shown in the qualitative free-energy diagram (Figure 7). Once the cyclic intermediate (II) is formed, the conversion to product proceeds faster by lower-transition-state free-energy (T_2). Monoglycerides with smaller fatty acid chains would have a smaller free-energy barrier to the transition state (solid line, Fig. 7) and therefore form the intermediate (II) faster than monoglycerides with larger acyl groups. The larger fatty acids will introduce stronger opposition to the formation of the 5-membered ring intermediate by larger ring deformations, therefore the first rate-controlling step has to proceed by higher activation energy (broken line, Fig. 7), resulting in slowing of the over-all reaction. By introducing different substituents into the fatty acid chain, van Lohuisen and Verkade (37) showed that acyl migration velocity was inversely proportional to the size of the substituents. If three methyl groups in the benzene ring in 2-benzoyl-glycerol or three phenyl groups in 2-acetyl-glycerol were introduced, practically no isomerization occurred. The steric interaction of the large substituents in these cases prevented formation of the transition state intermediate (II), therefore no isomerization was possible. However the steric hindrance in the formation of the cyclic intermediate is largest when the substituents in the fatty acid chain are next to the ester group. When they are farther removed, this effect gradually disappears. Characteristics of the fatty acid chain other than its size and substitution can also have some influence on isomerization. For example, the larger electron density on the carbonyl carbon of an ester group with an aromatic chain can slow the rate of migration (37).

The slower isomerization of 1- and 2-monoglycerides on boric acid-impregnated columns and thin-layer plates may be related to partial charge interactions. Boric acid is a Lewis acid, being two electrons short on the boron atom, and can partially block unshared electron pairs on free hydroxyl groups in monoglycerides and prevent their nucleophilic attack on the ester carbonyl (Fig. 6,IV). The negligible isomerization of monoglycerides exposed for 15 hours to boric acid as well as $B(OCH_3)_3$, $Al(OH)_3$, and $Zn(OH)_2$, which are also Lewis acids, supports the above statement. A similar explanation could be given for lack of isomerization of monoglycerides during removal of isopropylidene and benzylidene blocking groups with boric acid in trimethyl borate. The fact that monoglycerides isomerize during prolonged contact with Florisil impregnated with boric acid, but not when exposed to pure boric acid, might be explained by incomplete coverage of the adsorbent surface by boric acid, but impregnation with a larger percentage of boric acid did not change the situation.

Although acyl migration in monoglycerides could be theoretically promoted by any other acid and base, one has to consider that such isomerization takes time. If monoglycerides are exposed to a weak acid or base, the isomerization will proceed slowly and take more time to reach an equilibrium. If manipulations of monoglycerides under such conditions are performed rapidly, they can be done without significant isomerization.

Diglycerides

Like monoglycerides, 1,2- and 1,3-diglycerides are not stable and are easily isomerized under acidic, basic, or thermal conditions to an equilibrium of about 40% of the 1,2- and 60% of the 1,3-isomer (14, 41,51,52). Crossley et al. studied isomerization of different diglycerides and observed that isomerization velocity depended to some extent on the size and nature of the fatty acids (51). Unlike monoglycerides, 1,2- and 1,3-diglycerides do not isomerize significantly during chromatography on silicic acid (40,53). They do however isomerize when chromatographed on Florisil (14).

Experimental Procedure and Results

Diglycerides obtained from lipids of *L. monocytogenes* (3) were separated into 1,2- and 1,3-isomers by chromatography on acid-treated Florisil as follows. First, 400 mg of mixed diglycerides were applied to a column (1.5 × 43 cm). Then elution with 280 ml of Skellysolve B-ether (80:20) gave 237 mg of 1,3-isomer (mp 30C–32C), and subsequent elution with 180 ml (75:25) gave 136 mg of 1,2-isomer (an oil). Separation of 1,2- and 1,3-isomers of dipalmitin and diolein was achieved in the same way. Rechromatography of the separated isomers showed that 1,3-isomers passed through the column without isomerization but that 1,2-isomers always showed a trace of 1,3-isomer.

Analysis of these isomeric mixtures was carried out primarily by TLC, followed by densitometry (10). The 1,2- and 1,3-isomers can be separated on Silica Gel G alone (10), but Silica Gel G impregnated

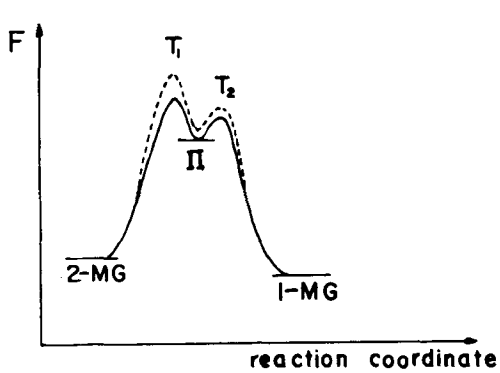

FIG. 7. Free-energy (F) diagram for isomerization of monoglycerides (MG). T = transition state; II = intermediate.

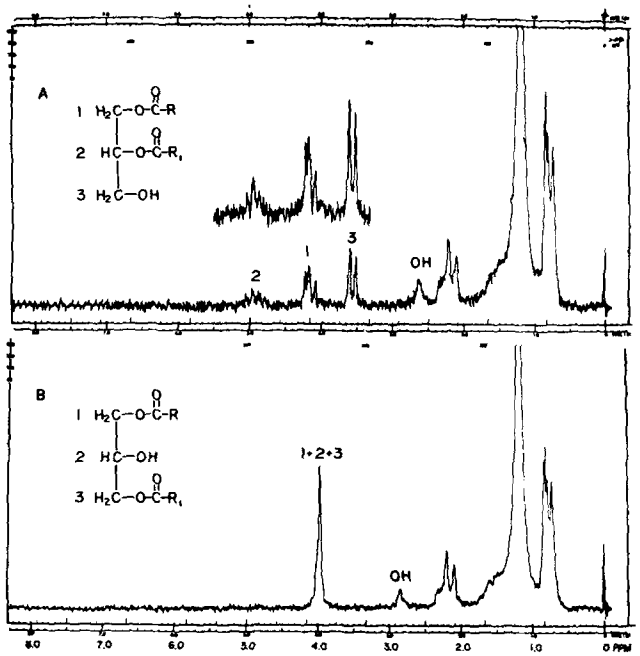

FIG. 8. NMR spectra of 1,2- and 1,3-diglycerides of branched-chain fatty acids.

with 10% boric acid was used in most experiments because 1- and 2-monoglycerides and triglycerides can be separated as well in the one system.

NMR spectroscopy was also useful for following the isomerization process because 1,2- and 1,3-diglycerides give distinctly different spectral patterns (Figure 8). The characteristic proton bands of the glycerol moiety, seen between 3.3 and 5.0 ppm in the above spectra, correspond to those reported by other workers (54,55) and to the proposed scheme for differentiating glyceride isomers (3). Bands of the glycerol moiety of 1,2- and 1,3-diglycerides do not coincide in the spectrum and can therefore be integrated to give a quantitative measure of the proportion of 1,2- and 1,3-isomer in a mixture. The bands at 0.6 to 2.4 ppm are attributable to protons on the fatty acid substituents and may be ignored for purposes of this analysis.

The 1,2- and 1,3-diglycerides of palmitic acid, oleic acid, and branched-chain fatty acids were isomerized separately on a Florisil column, with heating by the method of Crossley et al. (51). After different periods of time the ratio of isomers was determined by separation on an acid-treated Florisil column, by TLC, and NMR spectroscopy. The isomerization equilibrium for diglycerides on Florisil was reached between two and three hours in each case, but isomerization was fastest for branched-chain fatty acid diglycerides and slowest for dipalmitin. Diglycerides left for 15 hours on a Florisil column were split to the extent of about 50% to glycerol, fatty acids, and monoglycerides. After isomerization of diglycerides by heating for different lengths of time, TLC always showed some monoglycerides and triglycerides present. The average analytical results for separated isomers by column chromatography, TLC, and NMR showed the isomerization equilibrium ratio for branched-chain diglycerides to be about 36% of 1,2- and 64% of the 1,3-isomer; for diolein, about 43% of 1,2- and 57% of the 1,3-isomer; and for dipalmitin, 41% of 1,2- and 59% of the 1,3-isomer.

Next, 5 mg of each of these diglycerides were incubated separately with 500 mg $B(OH)_3$, $B(OCH_3)_3$, $Zn(OH)_2$, and $Al(OH)_3$ in 1 ml of Skellysolve B-ether (50:50) for 10 hours at room temperature. The diglyceride solutions were simply decanted after each reaction except that the $B(OCH_3)_3$ first had to be precipitated by adding water and then decanting the solution. Diglyceride solutions checked on TLC did not show significant isomerization.

Comment

In general, the interpretation of isomerization of diglycerides is similar to that of monoglycerides. The main driving force for isomerization may again be a steric effect, which tends to convert the branched form of 1,2-diglycerides to the more thermodynamically stable linear form of 1,3-diglycerides. The ester group in position 2 of 1,2-diglycerides has larger interactions with the adjacent ester group in position 1 and the adjacent hydroxyl in position 3 than is the case in 1,3-diglycerides. In diglycerides at equilibrium the ratio of 1,2- to 1,3-isomers is about 40:60 whereas in monoglycerides the ratio is about 10:90 of 2- to 1-isomers. This may be explained by the attraction between the nonpolar fatty acid chains (R and R_1) in 1,2-diglycerides because of the van der Waals forces (56–58), which would partially counteract the steric effect of branching in 1,2-isomers and therefore decrease their tendency to isomerize to 1,3-diglycerides.

Diglycerides isomerize in the same way as monoglycerides through formation of a 5-membered ring intermediate, but the two fatty acid chains in diglycerides cause larger deformations in the cyclic intermediate and thus act as a greater hindrance to its formation. Therefore the rate-controlling step in the transition state during isomerization of diglycerides has to pass a higher free-energy barrier than in the case of monoglycerides with the same type of fatty acids. This means that the over-all slower acyl migration in diglycerides compared with monoglycerides is attributable to slower formation of the transition state intermediate. Additional steric effects because of chain-length, branching, unsaturation, etc., in fatty acid chains have the same influence on rate of isomerization in diglycerides as in monoglycerides (14,37,38,41,51).

Phosphoglycerides

The most extensive investigations of isomerization in lipid chemistry have been carried out on phosphoglycerides. The main points of interest in these studies were the position of the phosphate group in the glycerol moiety of naturally occurring phosphoglycerides and its position in the inositol of naturally occurring phosphoinositides. It has now been established that the phosphate group is attached to position 1 of glycerol and to position 1 of inositol in natural phosphoglycerides (59–67).

Experimental Procedure and Results

Isomerization of Glycerophosphates and Glycerylphosphorylcholines. DL-1-glycerophosphate (1-GP) was prepared from 1-glycerochlorhydrin according to the method of McMurray et al. (68), and 2-glycerophosphate (2-GP) was obtained commercially (Fisher Scientific Company, Toronto, Ont.). The sodium was removed from these compounds by exchange on Dowex (H^+) resin. The resulting products were analyzed by periodic acid oxidation according to the method of Karnovsky and Brumm (9), except that water was used rather than absolute ethanol be-

cause of the solubility properties of glycerophosphates, and by NMR spectroscopy. The results showed a purity of about 98% for 1-GP and 95% for 2-GP. Attempts to separate a mixture of 1- and 2-GP on columns of acid-treated Florisil or silicic acid with different mixtures of chloroform:methanol were not successful. They were eluted together on an acid-treated Florisil column with chloroform:methanol (90:10). Neither was any separation achieved by TLC on Silica Gel with a variety of solvent systems. Silica Gel impregnated with boric acid could not be used because the boric acid was eluted by solvent systems that contained methanol and water. Some separation of these isomers was reported by Kennedy, using paper chromatography (69).

Cyclic glycerophosphate (CGP) was prepared from 2-GP by dicyclohexylcarbodiimide according to the method of Khorana et al. (70) and was further converted to its barium cyclic phosphate by the method of Ukita et al. (71,72).

The 1-GP and 2-GP were treated with 1 N HCl at 37C, and CGP with 1 N HCl and 1 N NaOH at 25C. During isomerization, aliquots were taken at different time-intervals, neutralized to pH 7, then analyzed by periodic acid oxidation and by NMR spectroscopy. Each aliquot contained 50 mg of glycerophosphates, of which 1 mg was used for periodic acid oxidation and the rest for NMR spectroscopy. To obtain better NMR spectra, the water was evaporated in vacuum at room temperature, and the remaining 49 mg of glycercophosphates were dissolved in 0.3 ml D_2O.

The curves in Figure 9 for acid isomerization of 1-GP and CGP show a small inflection in the earlier stages of isomerization, similar to the results of Baer and Kates (73) for acid hydrolysis of L-3-glycerylphosphorylcholine. It therefore appears that there is a higher percentage of 2-GP in the mixture during the early stages of isomerization than at final equilibrium, and this was confirmed by NMR spectroscopy.

The acid hydrolysis of L-3-glycerylphosphorylcholine (L-3-GPC), $[\alpha]_D^{25} = -2.99°$ ($c = 7.692$, in water) (a gift from K. P. Strickland, University of Western Ontario), was repeated under the conditions used by Baer and Kates (73). Aliquots (100 mg) of the hydrolysis mixture were removed at intervals, the hydrochloric acid was neutralized with an equivalent amount of NaOH, the sample was dried in vacuum over NaOH pellets at room temperature and separated on an acid-treated Florisil column (1 × 24 cm). Elution with chloroform, followed by chloroform-methanol (90:10, 70:30, and 20:80), gave a mixture of 1-GP and 2-GP in the 90:10 fraction, choline in the 70:30 fraction, and unreacted GPC in

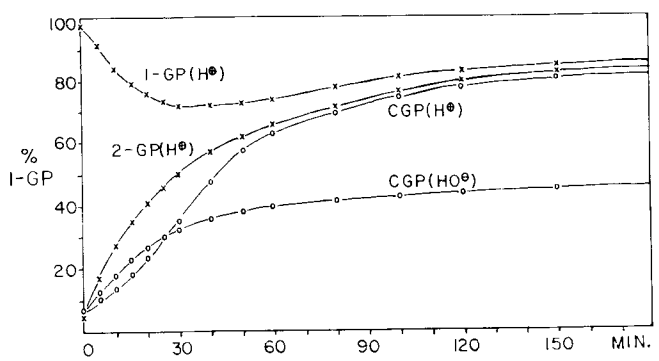

FIG. 9. Isomerization of 1-GP and 2-GP with 1 N HCl at 37C, and hydrolysis of CGP with 1 N HCl and 1 N NaOH at 25C. The curves show the percentage of 1-GP in the reaction mixture as determined by periodic acid oxidation.

the 20:80 fraction. Each of these fractions was analyzed by TLC on Silica Gel G with the solvent system: chloroform:methanol:water (20:70:10). The GP and GPC fractions were also analyzed by periodic acid oxidation, measurement of optical rotation, and NMR spectroscopy. Although the results of periodic acid oxidation and optical rotation of aliquots of the isomerization mixture coincided with those reported earlier (73), the unreacted GPC, separated by column chromatography, showed no difference in NMR spectra from the starting material (L-3-GPC). Optical rotation measurements and periodic acid oxidation of the GPC isolated from aliquots at all stages of the isomerization gave values which indicated that at least 90 to 95% was present in the form of L-3-GPC.

Similar acid hydrolysis studies were carried out also on 2-glycerylphosphorylcholine (2-GPC). This compound was prepared from monophenylphosphoryl dichloride, 1,3-benzylidene glycerol (mp 83C), and choline by using the procedure of Baer and Kates (74) for L-3-GPC with some modifications. The reaction products were not separated as Reinecke salts (74) but were chromatographed on a column of acid-treated Florisil or silicic acid, eluting with chloroform, followed by chloroform-methanol (90:10) and (60:40). The (60:40) fraction was split by hydrogenolysis, with a platinum oxide catalyst in absolute alcohol, and rechromatographed on acid-treated Florisil by elution with chloroform:methanol (60:40) and (20:80). The (20:80) fraction consisted of 2-GPC in about 23% yield. This was identified by NMR spectroscopy (Figure 10,D). This compound was subjected to acid hydrolysis, and the products were separated by column chromatography and analyzed by NMR and by periodic acid oxidation. The results showed that no isomerization to 1- or 3-GPC had occurred.

NMR Spectra of Glycerophosphates and Glycerylphosphorylcholine. The spectra of these compounds were recorded under the same conditions as described for glycerides except that D_2O was used as solvent and sodium trimethylsulfopropylsilane was used as internal reference (Fig. 10).

The 1-GP (spectrum A) and 2-GP (spectrum B) have practically the same band dispositions of the protons on glycerol carbons as the 1- and 2-monoglycerides (3). The two protons on the 3-carbon in 1-GP (A) resonate at 3.55–3.75 ppm whereas the protons on the 1-carbon are more deshielded by the phosphate group and resonate at a lower field (3.9–4.15 ppm). The proton on the 2-carbon appears in between these two bands with partial intersecting, and the strong band at 5.5 ppm is from water protons present in the D_2O. Spectrum B of 2-GP has four protons from the 1- and 3-carbons approximately equivalent, which appear at 3.65–3.90 ppm as a doublet split by the proton from the 2-carbon. The 2-carbon proton deshielded by the phosphate group appears as a multiplet shifted to a lower field (4.1–4.5 ppm). The small doublet at 3.9–4.1 ppm corresponds to 1-carbon protons from 1-GP present as an impurity (about 5%). Since the large bands in the NMR spectra of 1-GP and 2-GP had different chemical shifts, their ratio could be used for following the isomerization process.

In spectrum C (L-3-GPC) there is a strong band at 3.15–3.30 ppm from the protons of the methyl groups of the choline nitrogen. The protons at the 1- and 2-glycerol carbons give a band at 3.55–3.80

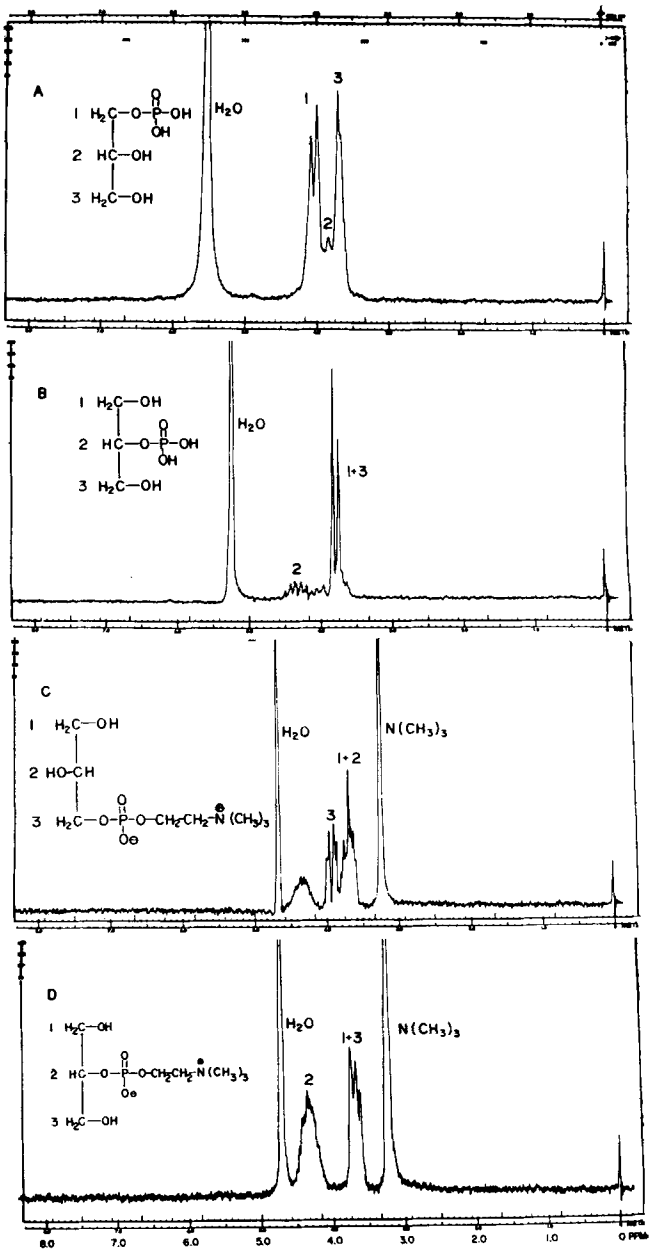

FIG. 10. NMR spectra of 1-GP (A), 2-GP (B), 3-GPC (C), and 2-GPC (D).

ppm, and the protons on the 3-carbon appear as a quartet at 3.8–4.1 ppm. The broad peak at 4.1–4.6 ppm corresponds to one methylene group from the choline moiety; 2-GPC (spectrum D), like 2-GP, has protons from positions 1 and 3 at 3.5–3.8 ppm, but the proton from position 2 appears together with the methylene protons of the choline moiety at 4.1–4.5 ppm. The hydroxyl protons from glycerol and phosphate moiety are exchanged and resonate together with water protons (originating from 93% D_2O) in the band at the lowest field in each spectrum. This band changes its position in the field, depending on temperature and concentration.

Comment

Like acyl migration in monoglycerides and diglycerides, phosphate group migration in phosphoglycerides involves formation of a cyclic intermediate with the vicinal hydroxyl group of glycerol, as proposed by Verkade et al. (75), Baer and Kates (63, 73), and Brown et al. (65,76,77). The transition state involved in phosphate group migration is still not entirely certain, but the stability of pentacovalent phosphorus suggests the possibility that it proceeds by the formation of a cyclic orthophosphate intermediate (63,65,78). The strongly substituted 5-membered ring intermediate (II, Fig. 11) would be extremely unstable because of high ring strain and would be quickly converted to a more stable intermediate (III) with an exo-double bond on the ring. The 5-membered ring with exo-double bond is more stable because of elimination of some nonbonding interactions (79). The cyclic phosphates have been prepared and identified (70,71,80,81). The 5-membered ring cyclic phosphates are unstable to acid or base treatment, but 6- and 7-membered ring phosphates are quite stable (70,71,82).

Acid-catalyzed Hydrolysis of Phosphoglycerides. As in monoglycerides and diglycerides, steric effects seem to be the main reason for the phosphate group to be in position 1 of glycerol. Since isomerization of phosphate monoesters (1- and 2-GP) does not occur in base (59,77), acid-catalyzed isomerization of 1- and 2-GP may proceed as depicted in Figure 11 (lane a). It is reasonable to assume that the phosphate group, with a large van der Waals radius, would interact strongly with adjacent hydroxymethyl groups when it is in position 2 whereas this interaction would be minimized when it is in position 1 or 3. Therefore acid-catalyzed isomerization results in an equilibrium of about 85% 1-GP and 15% 2-GP (59, 60,63,64,73). The free-energy diagram for acid isomerization of GP would be approximately as shown in Figure 12 (H^+). The formation of the unstable orthophosphate intermediate (II) involves the highest free-energy barrier, and this intermediate is quickly transformed to the more stable cyclic phosphate (III). The intermediate (III) is then split by acid and water predominantly in the direction of the lowest energy product, 1-GP (thermodynamically controlled reaction).

Similar steric effects may account for the natural occurrence of glycerophosphate in the form of L-3-GP, which acts as a precursor for the biosynthesis of phosphoglycerides (83). Natural L-3-GP is derived mainly from hexose-1,6-diphosphate through triose phosphate by the glycolytic pathway, and these precursors have their phosphate groups on the ter-

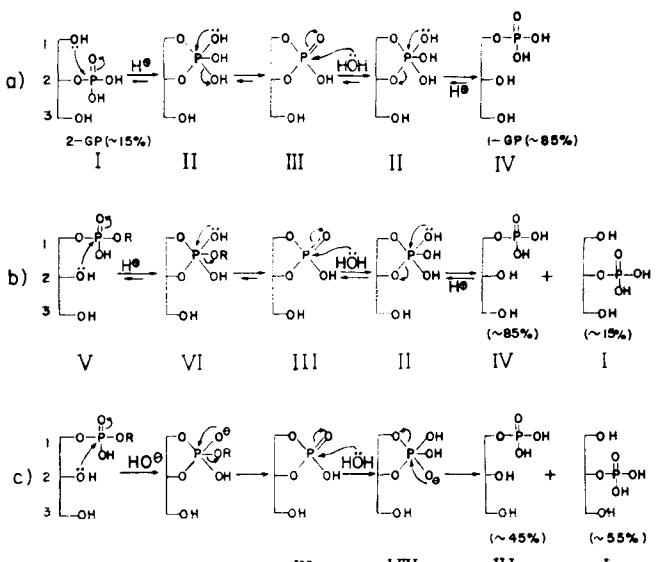

FIG. 11. Acid- and base-catalyzed hydrolysis of phosphoglycerides. R = choline, ethanolamine, etc.

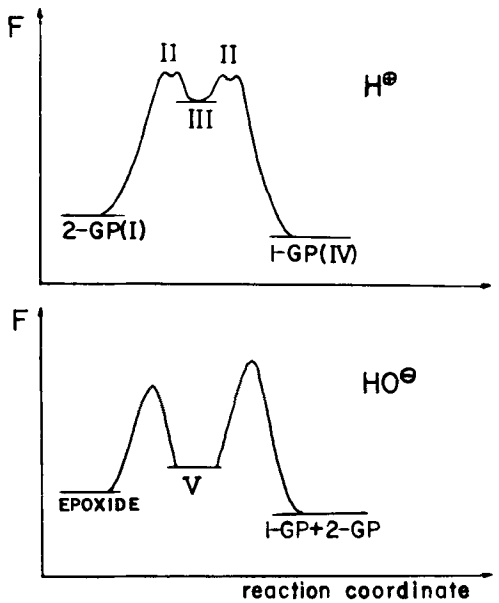

FIG. 12. Free-energy (F) diagrams for acid-(H^+) and base-(HO^-) catalyzed hydrolysis of phosphoglycerides.

minal carbon of the chain in the position of highest thermodynamic stability.

Phosphate diesters (phosphatidyl choline, phosphatidyl ethanolamine, etc.) isomerize in the presence of acid in the same way as GP, losing an alkyl group (choline, ethanolamine, etc.) during the formation of the transition state intermediate (III), as shown in Figure 11 (lane b). This was confirmed by the experiment with acid-catalyzed hydrolysis of L-3-glycerylphosphorylcholine (L-3-GPC), in which unreacted GPC was isolated from the reaction mixture by column chromatography. The NMR spectra of unreacted GPC were found to be identical with that of starting L-3-GPC (Fig. 10,C) and did not show any evidence of 2-GPC bands. Results of periodic acid oxidation and optical rotation were also similar to those obtained for the starting L-3-GPC. This indicates that no significant formation of 2-GPC occurs during hydrolysis and that the alkyl group in phosphoglycerides is split before the formation of a cyclic intermediate (III), as shown in Figure 11 (lane b). Similar experiments on acid hydrolysis of 2-GPC likewise showed no evidence of 1- or 3-GPC in the reaction mixture. Steric considerations also indicate that the cyclic intermediate (III) is more probable than the corresponding intermediate with an alkyl chain containing a bulky amino group, in agreement with the proposed mechanism of Brown and Todd (65,84) and Long Maguire (64). Thus formation of cyclic intermediate (III) during acid hydrolysis of phosphate diesters involves a higher free-energy barrier in the formation of the cyclic orthophosphate intermediate (II) containing an alkyl group (Fig. 12, H^+). The observed mixture of 1- and 2-GP from acid hydrolysis of natural L-3-phosphoglycerides, with partially racemized 1-GP, is therefore the result of isomerization after splitting of the cyclic intermediate (III).

Base-catalyzed Hydrolysis of Phosphoglycerides. Base-catalyzed hydrolysis of phosphoglycerides (Fig. 11, lane c) is similar to acid-catalyzed hydrolysis except that the resulting mixture of glycerophosphates contains about 45% 1-GP and 55% 2-GP. This differs from the results of acid hydrolysis and from what would be expected from thermodynamic stability of these compounds. On inspection of the acid hydrolysis kinetics of L-3-GPC in earlier experiments of Baer and Kates (73) and of acid isomerization of 1-GP and hydrolysis of cyclic glycerophosphate (CGP) in the present studies (Fig. 9), it is seen that the periodic acid oxidation curves show an inflection in the earlier stages of isomerization. This effect was thought by earlier workers to be attributable to the presence of larger amounts of 2-GP in the earlier stages of hydrolysis (63,72,73), and this was confirmed in the present studies by NMR spectroscopy.

These observations suggest that, in both acid and base hydrolysis, splitting of cyclic intermediate (III) proceeds similarly and results in some excess of 2-GP over 1-GP. The excess of the less stable product may be the result of steric or stereoelectronic controlled splitting of the cyclic intermediate (50), which is usually true of cyclic phosphates which possess a free vicinal hydroxyl group (72). Since no isomerization of monoesters (GP) occurs in an alkaline medium, this result is final for base hydrolysis whereas in an acidic medium both GP isomers may isomerize further until an equilibrium of about 85% 1-GP and 15% 2-GP is reached. This explanation is also supported by experiments (63,64) which showed that, after base-catalyzed hydrolysis of optically active phosphoglycerides, the decrease in optical rotation of the resulting 1-GP was less than after acid hydrolysis.

Base-catalyzed hydrolysis of phosphodiesters has been studied further by Brown and Usher (76,85), using model compounds of the type shown in Figure 11, lane c (V), and introducing different R groups and different substituents on the 2-carbon. They found that, when the R group or the substituent on the 2-carbon was small, the hydroxyl on the 2-carbon attacked the phosphorus predominantly and splitting proceeded by release of the R group alcohol and glycerophosphate (Fig. 11, lane c). When larger substituents were introduced as the R group or at the 2-carbon, the adjacent hydroxyl attacked the 1-carbon preferentially, forming an epoxide and an alkyl phosphate. The hydroxyl group probably has less chance to be properly oriented for nucleophilic attack on the phosphate group to form a cyclic intermediate (III) because of the larger steric hindrance, therefore reacts with the 1-carbon to form an epoxide. Although a 3-membered ring epoxide has less stability than glycerophosphate (1-GP and 2-GP), its formation from compound (V) involves a lower free-energy barrier (Fig. 12, HO^-), and it is therefore the main product (26). This is an example of a reaction which is kinetically controlled by the mode of formation and conversion of a transition state intermediate. Most base-catalyzed hydrolyses of natural phosphoglycerides involve this type of control. This finding is valid for aliphatic substituents, but with aromatic R groups the splitting proceeds only by way of glycerophosphate formation (Fig. 11, lane c) and no epoxide is found (85).

Base-catalyzed hydrolysis of phosphoglycerides is also dependent on the polarity of the medium in which the hydrolysis is carried out. The mild alkaline conditions used by Dawson (86) for hydrolysis of fatty acid ester groups of phosphoglycerides also liberate appreciable amounts of the base moiety (choline, ethanolamine, etc.) of the phosphoglyceride. Brockerhoff (87) studied the hydrolysis of different phosphoglycerides by using mild alkali in media of different polarities and found that more base was liberated in chloroform-ethanol medium than in the more polar methanol-water medium. This is ex-

plained by the greater ability of the adjacent hydroxyl on the glycerol moiety of the phosphoglyceride for nucleophilic attack on the phosphate group in less polar media, leading to formation of a cyclic intermediate with elimination of the base moiety (Fig. 11, lane c). In more polar media the adjacent hydroxyl must compete with the high electron density of the medium.

Hydrolysis of Phosphoinositides. In isomerization studies of phosphoglycerides the phosphoinositides have received much attention (66,80,81,88–90). During hydrolysis of phosphoinositides under acidic conditions, the glycerol-phosphate linkage is split to a greater extent than the inositol-phosphate linkage. To simplify hydrolysis studies, Brown and co-workers (88) prepared glycerol-1-(2′-hydroxy)-cyclohexyl phosphates with *cis* and *trans* oriented hydroxyl groups on the cyclohexanediol moiety. Hydrolysis of these products with acid or base gave about 85% cyclohexanediolphosphate with the *cis*-cyclohexanediol derivative and about 75% glycerophosphate with the *trans*-cyclohexanediol derivative. This was explained by the faster formation of the cyclic phosphate intermediate on the cyclohexanediol moiety in the *cis*-1,2-diol, which has a shorter distance between axial-equatorial hydroxyls, than in di-equatorial *trans*-1,2-diol in which the hydroxyls are farther apart. A *cis*-junction between a 5-membered ring and a 6-membered ring is more stable than a *trans*-junction (26,91). Formation of intermediate (II) (Fig. 13) would correspond to *cis*-fusion and therefore proceed faster.

Hanahan and Olley (89) hydrolyzed monophosphoinositides of liver and found that, after acid-catalyzed hydrolysis, the phosphate group was predominantly found in the form of inositol phosphate whereas, after base-catalyzed hydrolysis, it was found mainly as glycerophosphate. They suspected that this difference resulted from the slower splitting of the fatty acid ester groups under acidic conditions. Thus, in acidic conditions, the vicinal hydroxyl of glycerol is blocked and hydrolysis proceeds mainly by formation of a cyclic phosphate intermediate with the inositol moiety (Fig. 13, lane a). In base-catalyzed hydrolysis the fatty acyl groups are easily removed, and the cyclic phosphate intermediate is formed faster with the vicinal hydroxyl of the glycerol than with the inositol hydroxyl (Fig. 13, lane b). Through further studies on natural phosphoinositides, methods have been developed for determining the ester linkage position on the inositol ring (80,81,90). By oxidation with periodic acid and other methods it was found that naturally occurring phosphoinositides have the ester linkage in position 1′ of the inositol moiety. Five hydroxyl groups in myo-inositol have equatorial orientation whereas the sixth in position 2′ is axial (Fig. 13, I). In the same way it was determined that the phosphate is linked to the 1-position of glycerol in phosphoinositides as it is in other phosphoglycerides.

Glycerol-1-myo-inositol-2′-phosphate gave about 60% glycerophosphate and 40% inositol phosphate on base-hydrolysis whereas glycerol-1-myo-inositol-1′-phosphate gave about 66% glycerophosphate and 34% inositol phosphate. Brown et al. (90) considered this difference to be one of the proofs for 1′ and 2′ positioning of the ester linkage in inositol. The small excess of inositol phosphate in the first case is probably caused by slower hydrolysis of axial phosphate esters than of equatorial (Fig. 13, lane a, I),

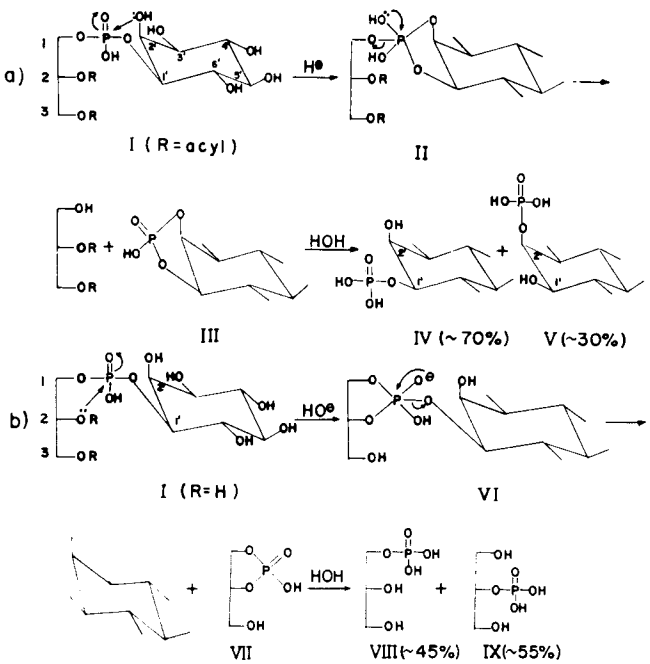

FIG. 13. Acid- and base-catalyzed hydrolysis of phosphoinositides.

related to similar observations for carboxylic acid ester hydrolysis (26). However this statement was later weakened by the possible presence of diastereomers in synthetic glycerol myo-inositol phosphates (81). By acid or base hydrolysis of glycerol-1-myo-inositol-1′-phosphate and glycerol-1-myo-inositol-2′-phosphate they found that the resulting inositol phosphate is a mixture of 70% inositol-1′-phosphate (IV) and 30% inositol-2′-phosphate (V). The cyclic phosphate (III) splits preferably to the more stable 1′-isomer with the phosphate group in equatorial position (IV) rather than to the 2′-isomer, in which the axial phosphate group has two strong 1:3 interactions with axial hydrogens in positions 4′ and 6′ (V). In the same way the inositol-1,2-cyclic phosphate splits with acid or base predominantly to a more thermodynamically stable product, inositol-1-phosphate (80).

Although hydroxyls in the 1′ and 2′ position in myo-inositol are *cis*, the base hydrolysis of naturally occurring phosphoinositides (Fig. 13, lane b) favors the formation of cyclic glycerophosphate VII (about 60%) more than that of inositol phosphate III (about 40%) (1,89). This is in opposition to the results with *cis*- and *trans*-cyclohexanediol-glycerophosphates. Since inositol has four more hydroxyl groups than cyclohexanediol, the larger size of the inositol moiety and the additional ring strain may pose more difficulty in the formation of a cyclic phosphate intermediate on inositol than on glycerol (VI). The resulting cyclic glycerophosphate (VII) is found to be split by base to about 45% 1-GP and 55% 2-GP for the same reason as kinetically controlled hydrolysis of the cyclic intermediate glycerophosphates. Similar product ratios have been obtained by hydrolysis of some ribonucleoside cyclic phosphates. Other examples are known, such as propane-1,2-diol and some carbohydrate cyclic phosphates, which split by acid and base to the more thermodynamically stable products (82).

Isomerization in Other Types of Lipids. The foregoing discussion has been concerned to a large extent with acyl migration in glycerides and phosphoglycerides. Acyl migration could also theoretically occur

in other types of lipids, as in sphingolipids, where there is a possibility for acyl migration between adjacent amino and hydroxyl groups, in the erythro more so than in the threo form.

There are also many possibilities for isomerization in steroids but, since acyl migration and acetal and ketal isomerizations in steroids are of less concern to lipid chemists, this subject is not covered. Excellent summaries of isomerizations in steroids have been given by Fieser and Fieser (92) and by Wendler (93).

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