Benzoolysis of diacylglycerophosphocholines: dephosphorylation and sequential formation of isomeric reaction products

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Abstract Benzoolysis experiments are reported in which diacylglycerophosphocholine is heated at 100°C with benzoic anhydride for variable periods of time. It is shown that more than 90% of the phospholipid is dephosphorylated after 5 h of heating. Lipid extracts of the reaction mixture contained 1,2- and 1,3-diacylglycerobenzoate and 1,2- and 1,3-diacylglycerol in nearly constant isomer ratios of about 3:1 and 1:2, respectively, independent of the heating and extraction time. The total amount of isomeric diacylglycerobenzoates increased more slowly with increasing heating time than corresponded with the dephosphorylation rate, complete benzoylation being attained only after a 15 h heating period. The total amount of isomeric diacylglycerols went through a maximum after about 4 h and vanished after 15 h of heating. Addition of 4-dimethylaminopyridine subsequent to the heating period resulted in rapid and complete benzoylation of dephosphorylated phospholipid. However, the ratio of 1,2- to 1,3-diacylglycerobenzoate then found in the lipid extract depended on heating time, changing from less than 1:1 to about 3:1 upon an increase of heating time from 1 to 15 h. The results are interpreted in terms of two consecutive reactions. In a relatively fast first step, a dephosphorylated intermediate is formed, which, in the molten benzoic anhydride, is slowly benzoylated. The intermediate yields diacylglycerols upon extraction in the absence of 4-dimethylaminopyridine and diacylglycerobenzoates upon extraction in the presence of 4-dimethylaminopyridine.–**Gelsema, W. J., O. F. van den Brink, and H. Van den Bosch.** Benzoolysis of diacylglycerophosphocholines: dephosphorylation and sequential formation of isomeric reaction products. *J. Lipid Res.* 1996. **37:** 1224–1233.

Supplementary key words dipalmitoyl GPC • dioctanoyl GPC • dephosphorylation • benzoylation • isomeric diacylglycerols • isomeric diacylglycerobenzoates • high performance liquid chromatography • thin-layer chromatography

Recently (1) we demonstrated that heating of diacylglycerophosphocholines (GPC) with benzoic anhydride in the presence of boric acid for 5 h at 100°C, followed by treatment with 4-dimethylaminopyridine (DMAP) at room temperature, converted these lipids quantitatively into the corresponding diacylglycerobenzoates. Addition of boric acid was shown to be necessary for reproducible and complete dephosphorylation within the 5-h heating period. However, as complete benzoylation was not attained during this period, a subsequent treatment with DMAP was required.

Moreover, it was found that substantial acyl migration occurred as a result of the procedure. Addition of ammonia to the reaction mixture after the heating period, hexane extraction of the lipid products formed, and TLC analysis showed the presence of both 1,2- and 1,3-diacylglycerol in addition to diacylglycerobenzoates. Using the same extraction procedure after the DMAP treatment, GCMS analysis of the lipid products revealed that both 1,2- and 1,3-diacylglycerobenzoates were formed (1).

In this report the phenomenon of acyl migration is studied in more detail. Benzoolysis experiments are described that permitted the determination of the isomer ratio of the products formed, both after the heating procedure and after DMAP treatment. To this end, we used 1,2-diacyl GPCs with rather short acyl groups, viz., caproyl to octanoyl. This allowed the two isomeric 1,2- and 1,3-diacylglycerobenzoates to be easily separated by HPLC.

Abbreviations: DMAP, 4-dimethylaminopyridine; GCMS, gas chromatography–mass spectrometry; GPC, glycerophosphocholine; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography.

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MATERIALS AND METHODS

Chemicals

1,2-Dicaproyl-, 1,2-dioctanoyl-, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, dicaproin (mixed 1,2- and 1,3-dicaproyl-*rac*-glycerol isomers), 1,2-dicaproyl-*rac*-glycerol, 1,2-dioctanoyl-*rac*-glycerol, 1,2-dipalmitoyl-*rac*-glycerol, 1,3-dipalmitoyl-*rac*-glycerol, 1-octanoyl-*rac*-glycerol, 1-palmitoyl-*rac*-glycerol, 2-palmitoyl-*rac*-glycerol, and glycerol were obtained from Sigma, St. Louis, MO. 1-Octanoyl-2-heptanoyl[1-^{14}C]-*sn*-glycero-3-phosphocholine (sp act 2.1 GBq • mmol^{-1}) was purchased from NEN-DuPont, Dordrecht, The Netherlands. Benzoic anhydride (98%, Merck, Darmstadt, Germany) was purified by repeated precipitation by hexane from a benzene solution. 4-Dimethylaminopyridine (99%, Aldrich, Bornem, Belgium) was recrystallized in chloroform/diethylether. All other chemicals and solvents were of analytical grade.

Apparatus

A ChromSpher-5 Si analytical column (250 × 4.6 mm) with guard column (Chrompack, Middelburg, The Netherlands) was used for HPLC separations. The equipment further consisted of a LKB 2150 pump, a Rheodyne 7125 injector with a 20-µl sample loop, a Perkin-Elmer LC-55 variable wave length spectrophotometer with an HPLC flow cell, and a Spectra Physics SP-4270 integrator. Eluents were filtered through Schleicher and Schuell RC-55 (0.45 µm) membrane filters and ultrasonically degassed. TLC separations were performed on silica 60 F-254 plates (Merck). For phosphorus determinations, a Milton Roy Spectronic 601 variable wavelength spectrophotometer was used. Liquid scintillation counting was performed on a Packard Tri-Carb 1500 spectrometer, using Packard Emulsifier-Safe™ liquid scintillation cocktail. TLC plates were scanned for radioactivity on a Multi-TraceMaster-20 linear scanner (Berthold, Bad Wildbad, Germany).

Preparation of reference compounds

The diacylglycerols, monoacylglycerols, and glycerol were benzoylated using the method of Blank, Cress, and Snyder (2). HPLC analysis of the reaction products demonstrated the absence of isomerization for the diacylglycerobenzoates and less than 5% isomerization for the acylglycerodibenzoates. The products were used to identify HPLC peaks (see Fig. 1 and 3).

Preparation of stock solutions

Appropriate amounts of 1,2-dicaproyl-, 1,2-dioctanoyl-, and 1,2-dipalmitoyl GPC were dissolved in chloroform–methanol 2:1 (by volume) to concentrations of about 0.75 µmol • ml^{-1}. The exact concentrations were determined by phosphorus analysis. An aliquot of about 0.37 MBq of the ^{14}C-labeled GPC was dissolved in 100 ml of the 1,2-dioctanoyl GPC stock solution (concentration 0.74 µmol • ml^{-1}). This labeled stock solution was used for experiments described in the next sections. The corresponding unlabeled stock solution of the same concentration was used to determine the extent of dephosphorylation upon benzoolysis.

Benzoolysis

Aliquots of the stock solutions generally containing 0.75 µmol of diacyl GPC were evaporated to dryness in 10-ml tubes fitted with Teflon-lined screw caps. After adding 10 mg of boric acid and 250 mg of benzoic anhydride, the tubes were heated in an oven at 100°C for periods of time ranging from 1 to 15 h.

Determination of the extent of dephosphorylation

After benzoolysis, 4 ml of chloroform and 4 ml of water were successively added to the tubes. The mixtures were vigorously stirred and left overnight. Appropriate aliquots of the water layer were evaporated to dryness and treated with perchloric acid for 30 min at 190°C, whereafter the amount of water-soluble phosphorus was determined using a slightly modified (1) method of Rouser, Fleischer, and Yamamoto (3).

Extraction of lipid products

After benzoolysis, 600 µl of benzene or 600 µl of a 2.5% (w/v) solution of DMAP in benzene was added to the tubes. After 30 min at room temperature, 4 ml of *n*-hexane and 5 ml of 25% (w/v) ammonia were successively added, and the mixtures were vigorously shaken and left overnight.

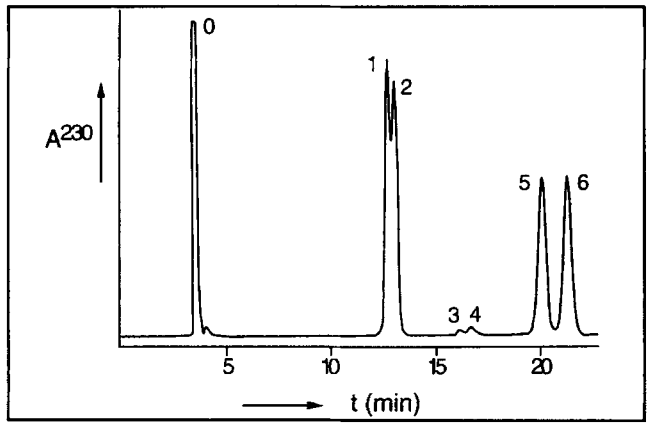

Fig. 1. HPLC chromatogram of lipid products formed in benzoolysis and DMAP-treatment of a mixture of dicaproyl GPC (0.63 µmol) and dipalmitoyl GPC (0.71 µmol). Benzoolysis (5 h, 100°C), DMAP-treatment, extraction of lipid products, and HPLC analysis were performed as described in Materials and Methods. Components: 0, benzene; 1, 1,2-dipalmitoylglycerobenzoate; 2, 1,3-dipalmitoylglycerobenzoate; 3, 2-palmitoylglycerodibenzoate; 4, 1-palmitoylglycerodibenzoate; 5, 1,2-dicaproylglycerobenzoate; 6, 1,3-dicaproylglycerobenzoate.

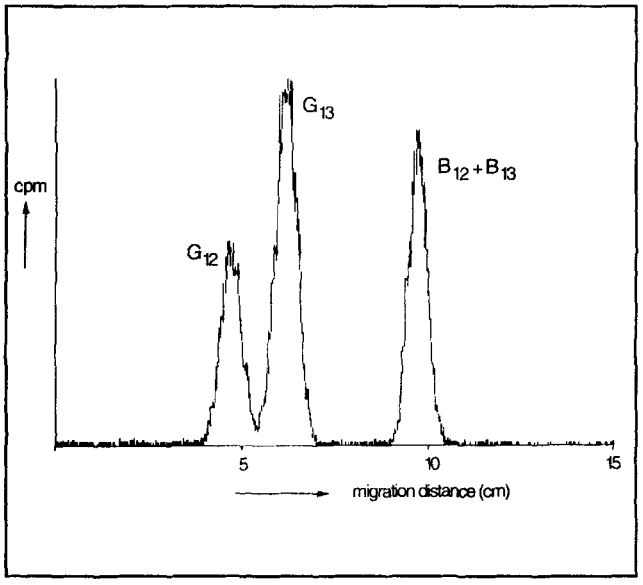

Fig. 2. TLC scan of lipid products upon extraction (without DMAP) in hexane/benzene after benzoolysis (5 h, 100°C) of dioctanoyl GPC spiked with a trace of 1-octanoyl-2-[1-^{14}C]heptanoyl GPC. G_{12} = 1,2-dioctanoylglycerol; G_{13} = 1,3-dioctanoylglycerol; B_{12} = 1,2-dioctanoylglycerobenzoate; B_{13} = 1,3-dioctanoylglycerobenzoate.

Analysis

Aliquots (20 µl) of the top layers were analyzed for benzoylated products using the indicated HPLC column, an eluent consisting of *n*-hexane–diethyl ether–ethanol 485:15:0.4 (by volume) at a rate of 1 ml • min^{-1} and UV detection at 230 nm. Integrated peak areas were recorded. Samples (100 µl) from both top and bottom layers were taken to counting vials, 4.5 ml of liquid scintillation cocktail was added, and the radioactivity was measured for 10 min. The remaining top layers (about 3.5 ml) were transferred to pear-shaped flasks, evaporated to dryness in a nitrogen stream, and stored overnight on phosphorus pentoxide in vacuo. The residues were dissolved in 50 µl of *n*-hexane and the solutions were spotted on a TLC plate. After development with petroleum ether–ethylacetate 2:1 (by volume) and drying, the plates were scanned (5 min counting time per lane). In preliminary experiments, dicaproin was used for the optimization of the eluent composition.

RESULTS AND DISCUSSION

Reliability of the extraction procedure

Experiments with dicaproyl GPC were used to check whether the relatively long period of contact with hexane/benzene and ammonia after benzoolysis induces any change of the lipid composition of the organic layer, both in the absence and presence of DMAP. To that end, the benzoolysis experiment was performed with 10 times larger amounts (relative to the procedures described in Materials and Methods) of dicaproyl GPC (7.5 µmol), benzoic anhydride (2.5 g), and boric acid (100 mg). After heating for 2 h at 100°C the melt was divided into two about equal parts, to which 3 ml of benzene and 3 ml of DMAP solution in benzene, respectively, were added. After 30 min at room temperature, 20 ml

TABLE 1. *Benzoolysis of radioactive dioctanoyl GPC*

Benzoolysis Time (h)	Phosphorus Determination[a] (% water-soluble phosphorus)	Scintillation Counting (% of total activity in top layer)	HPLC of Top Layer	
			S_{tot}	X_{B12}
Without DMAP				
1	14.6 ± 0.5	12.1 ± 0.1	546 ± 110	0.85 ± 0.01
2	27.0 ± 1.8	39.1 ± 1.2	5092 ± 366	0.79 ± 0.01
3	83.2 ± 1.5	82.9 ± 1.2	12711 ± 583	0.75 ± 0.01
5	92.7 ± 1.1	87.1 ± 1.2	20723 ± 913	0.74 ± 0.01
10	98.0 ± 0.3	88.9 ± 1.2	50694 ± 1044	0.72 ± 0.01
15	98.8 ± 0.8	94.8 ± 0.3	77140 ± 955	0.73 ± 0.01
With DMAP				
1	14.6 ± 0.5	11.5 ± 0.9	16227 ± 1148	0.45 ± 0.01
2	27.0 ± 1.8	35.5 ± 1.0	59240 ± 2103	0.43 ± 0.01
3	83.2 ± 1.5	76.7 ± 0.2	67963 ± 881	0.45 ± 0.01
5	92.7 ± 1.1	90.3 ± 0.3	75618 ± 497	0.48 ± 0.01
10	98.0 ± 0.3	91.4 ± 0.3	76513 ± 934	0.67 ± 0.02
15	98.8 ± 0.8	94.4 ± 0.1	77906 ± 260	0.73 ± 0.01

Values represent mean of triplicate determinations ± SD. The meaning of S_{tot} and x_{B12} is explained in the text.
[a]As the extent of dephosphorylation is determined by a different procedure, the data are identical in the presence and absence of DMAP.

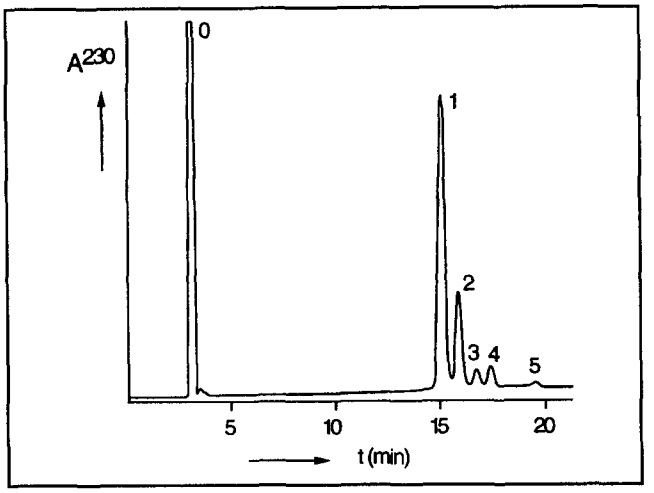

Fig. 3. HPLC chromatogram of lipid products upon extraction (with DMAP) in hexane/benzene after benzoolysis (15 h, 100°C) of 1-octanoyl-2-[1-^{14}C]heptanoyl GPC-labeled dioctanoyl GPC. Components: 0, benzene; 1, 1,2-dioctanoylglycerobenzoate; 2, 1,3-dioctanoylglycerobenzoate; 3, 2-octanoylglycerodibenzoate; 4, 1-octanoylglycerodibenzoate; 5, glycerotribenzoate. From the integrated peak areas S_1 to S_5, mole percentages of dioctanoylglycerobenzoates, octanoylglycerodibenzoates, and glycerotribenzoate can be calculated as $100\ (S_1 + S_2)/S_{tot}$, $100(S_3 + S_4)/2S_{tot}$, and $100S_5/3S_{tot}$, respectively, where S_{tot} equals $S_1 + S_2 + {}^1/_2\ (S_3 + S_4) + {}^1/_3\ S_5$. At t = 15 h, both in the presence and absence of DMAP, the results are 94.2 ± 0.2; 5.4 ± 0.2, and 0.4 ± 0.1%, respectively. (At t ≤ 10 h, no octanoylglycerodibenzoates and glycerotribenzoate were found in extracts without DMAP; in extracts with DMAP the content of octanoylglycerodibenzoates increases gradually with benzoolysis time.)

of *n*-hexane and 25 ml of ammonia were successively added to both samples. After thorough shaking, the systems were left at room temperature to separate into two layers that cleared within about 1 h. Aliquots (3 ml) were taken from both top layers at 5 min and at 1, 3, 5, 7, and 24 h after the addition of ammonia, evaporated to dryness in a stream of nitrogen, and redissolved in 1 ml of *n*-hexane. Samples (20 µl) of these solutions were analyzed by HPLC as described in Materials and Methods. Except for the result after a 5-min contact period, it was found for both DMAP-treated and non-treated samples that the total area of the 1,2- and 1,3-dicaproylglycerobenzoate peaks and the ratio of these peak areas do not depend on contact time (data not shown). Thus, beyond a 1-h contact period (when the layers are definitively settled), there is no measurable change in the glycerobenzoate recovery and isomeric composition in the organic layer. In all further experiments, the two-phase systems were allowed to settle overnight and (HPLC) analyses were performed 15–20 h later.

Absence of intermolecular reactions

Experiments with mixtures of dicaproyl GPC and dipalmitoyl GPC at two 5-fold different concentration levels were used to check whether intermolecular reactions leading to mixed caproylpalmitoylglycerobenzoates occur. **Figure 1** shows the HPLC chromatogram obtained at the highest concentration level. At the lowest concentration level only dipalmitoyl- and dicaproylglycerobenzoate peaks are found. The small peaks 3 and 4 in Fig. 1 are, however, not due to mixed caproylpalmitoylglycerobenzoates, but to 2-palmitoylglycerodibenzoate (peak 3) and 1-palmitoylglycerodibenzoate (peak 4). Indeed, we found earlier (1) that in the benzoolysis procedure of dipalmitoyl GPC some (~1%) palmitoylglycerodibenzoates are formed. (Probably about the same amount of caproylglycerodibenzoates are formed. These are, however, not separated from peak 6 (Fig. 1)). Their elution positions and relative peak heights correspond exactly to those of peaks 3 and 4 (mixed caproylpalmitoylglycerobenzoates are expected to elute slightly earlier). It can be concluded, therefore, that no intermolecular reactions take place at a total GPC concentration of 1.3 µmol/250 mg of molten benzoic anhydride. This agrees with the observation of Renkonen (4) who found no evidence for intermolecular reactions during acetolysis at comparable diacyl GPC concentration levels. In our experiments with dioctanoyl GPC (next sections) the GPC concentration is 0.74 µmol/250 mg; consequently, intermolecular reactions can be ignored.

Recovery and stability of lipid products during extraction

To determine the efficiency of the extraction and the stability of the possible lipid products obtained in benzoolysis of dioctanoyl GPC, the products were first prepared in radioactive form. To that end dioctanoyl GPC was spiked with a trace amount of 1-octanoyl-2[1-^{14}C]heptanoyl GPC, assuming the former behaves identically to the radioactive tracer. For simplicity, this mixture will be termed radioactive dioctanoyl GPC. Benzoolysis for 5 h at 100°C, followed by extraction (without DMAP treatment) and TLC of the hexane/benzene extract, yielded the compounds as depicted in **Fig. 2.** The silica containing the respective products was scraped and the compounds were desorbed using *n*-hexane–diethyl ether–ethanol 80:15:10 (by volume). The extracts were evaporated to dryness and the ^{14}C-labeled reference compounds prepared in this way were again carried through the extraction procedure by treatment with 250 mg of benzoic anhydride, 10 mg of boric acid, 600 µl of benzene, 4 ml of *n*-hexane, and 5 ml of ammonia. After thorough mixing and overnight equilibration, 400-µl samples of top and bottom layers were assayed for radioactivity by liquid scintillation counting and the remaining top layers were analyzed again by TLC as described under Materials and Methods. The results of the radioactivity measurements indicated that

the total radioactivity associated to all three isolated lipid products is recovered in the hexane/benzene layer in a nearly quantitative yield of 98.6%. The 1.4% of radioactivity in the ammonia layer can be explained either by assuming a distribution ratio of about 98.6:1.4 of both dioctanoylglycerols and dioctanoylglycerobenzoates over the top and bottom layers, or, more probably, by an about 1.4% hydrolysis of the lipid products during the extraction and separation procedure, liberating free fatty carboxylate ions to the bottom phase. The (repeated) TLC showed a distribution of radioactivity over the G_{12}, G_{13} and B_{12} + B_{13} spots (see Fig. 2) of 32.2:67.0:0.8, 32.3:67.0:0.7 and <0.5:1.1:>98.4 for the three isolated products 1,2-dioctanoylglycerol, 1,3-dioctanoylglycerol, and 1,2-/1,3-dioctanoylglycerobenzoate, respectively. Thus, the extraction procedure induces (in the absence of DMAP) less than 1% benzoylation and a constant isomer ratio of the dioctanoylglycerols irrespective of the starting isomer. This means that the extraction medium induces acyl migration to give a 1,2- to 1,3-isomer equilibrium amounting to approximately 1:2. In addition, the separation procedure appears to induce minimal hydrolysis of dioctanoylglycerobenzoates, yielding slightly less than 1.6% of dioctanoylglycerols in agreement with the 1.4% hydrolysis mentioned before.

Benzoolysis of radioactive dioctanoyl GPC

An extensive series of experiments, covering benzoolysis times ranging from 1 to 15 h and complete analysis of reaction products by scintillation counting, TLC and HPLC as described in Materials and Methods was performed in triplicate with 0.74 µmol amounts of radioactive dioctanoyl GPC. Moreover, the extent of dephosphorylation was determined in triplicate with 0.74 µmol amounts of unlabeled dioctanoyl GPC. A summary of these results is presented in **Table 1.**

First, we comment on the results of radioactivity measurements, both by scintillation counting and thin-layer scanning. From the total scintillation count rates of top plus bottom layers obtained in all experiments, the recovery of radioactivity was calculated as 99.6 ± 2.0 and 103.1 ± 2.6%, for DMAP-treated and non-treated samples, respectively. No systematic trend of recovery as function of benzoolysis time was observed. Thus, no losses of lipid products occur during the benzoolysis and extraction procedures.

The increase with increasing benzoolysis time of the percentage of the radioactivity recovered in the top layers for both DMAP-treated and non-treated samples largely parallels the increasing degree of dephosphorylation (see Table 1). However, TLC analysis of these top layers reveals that the lipid products obtained from DMAP-treated and non-treated samples are totally different. In the former no dioctanoylglycerols are found and dioctanoylglycerobenzoates constitute the main products. In the latter the radioactivity is distributed over 1,2-dioctanoylglycerol, 1,3-dioctanoylglycerol, and dioctanoylglycerobenzoates in proportions depending on benzoolysis time. The dioctanoylglycerols account for about 93, 90, 80, 68, 30, and zero % of the radioactivity at benzoolysis times of 1, 2, 3, 5, 10, and 15 h, respectively. The relative amount of dioctanoylglycerobenzoates increases slowly from 5% at 1 h to 100% at 15 h. The isomeric 1,2- and 1,3-dioctanoylglycerols are found in a molar ratio of about 1:2, virtually independent of benzoolysis time. This is expected, as preliminary experiments (see previous section) demon-

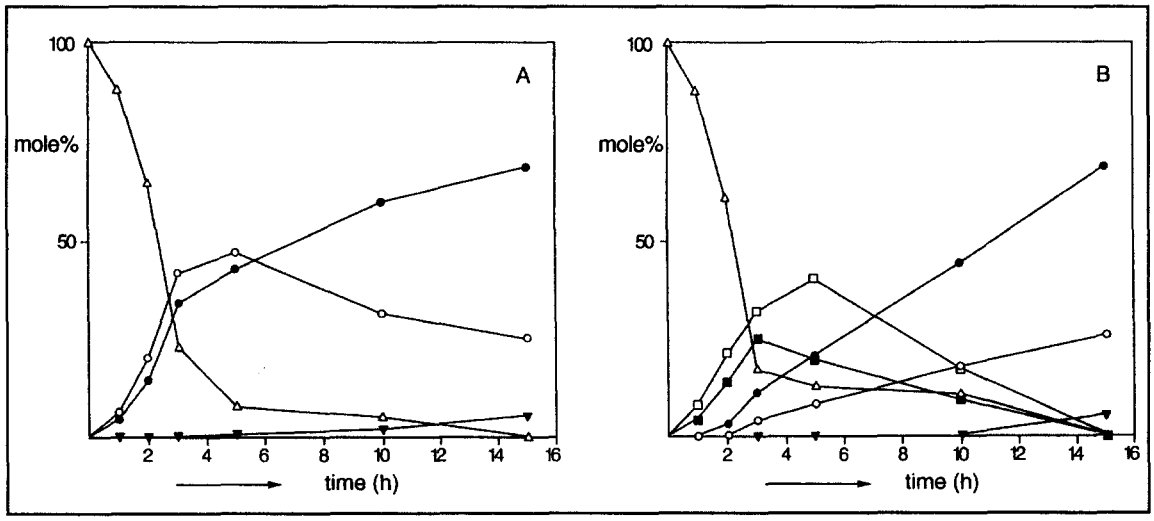

Fig. 4. Relative amounts of species found after extraction with DMAP (panel A) and without DMAP (panel B). Species: 1,2-dioctanoyl GPC (△); 1,2-dioctanoylglycerobenzoate (●); 1,3-dioctanoylglycerobenzoate (○); 1,2-dioctanoylglycerol (■); 1,3-dioctanoylglycerol (□); octanoylglycerodibenzoates plus glycerotribenzoates (▼).

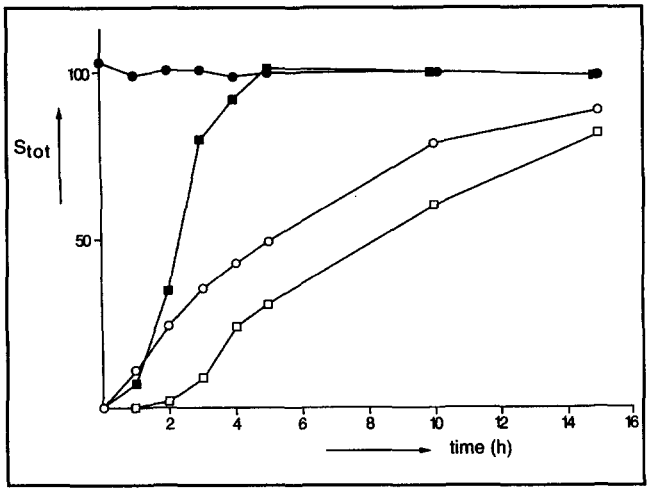

Fig. 5. Total area of HPLC peaks of lipid products from extracts with (filled symbols) and without DMAP (open symbols) after benzoolysis (100°C) of 1,2-dipalmitoyl GPC (■, □) and 1,2-dipalmitoylglycerol (●, ○).

strated that the extraction procedure induces a constant mole fraction of $x_{G12} \approx 0.32$. At t = 15 h, about 5% of the total radioactivity still remains in the bottom layers, while the corresponding top layers contain only dioctanoylglycerobenzoates. This is substantially more than the proportion (~1.5%) found by distributing dioctanoylglycerobenzoates over both phases (compare previous section) and must arise, therefore, as a result of the benzoolysis process. This conclusion is confirmed by the results of HPLC analysis of the top layers, which show, at long benzoolysis times, the presence of octanoylglycerodibenzoates and, at t = 15 h, even some glycerotribenzoate (see Fig. 3; note that in TLC these di- and tribenzoates co-chromatograph with dioctanoylglycerobenzoates). This means that an equivalent amount of fatty acid is liberated, which, of course, dissolves as carboxylate ion in the bottom phase. Therefore, if we assume that in the formation of di- and tribenzoates the *sn*-1 and *sn*-2 acyl of the original 1,2-diacyl GPC have an equal chance to be substituted by benzoyl, the presence of x and y mole % of octanoylglycerodibenzoates and glycerotribenzoate, respectively, in the top layer (as exemplified by HPLC analysis) would result in a contribution of (0.5x + y) % of the total radioactivity in the bottom layer. This is approximately born out by the data at t = 15 h.

Next, the results of HPLC analysis of the top layers are discussed. Here, as a result of the lack of a chromophore, dioctanoylglycerols are not detected; only dioctanoylglycerobenzoates, octanoylglycerodibenzoates, and glyceroltribenzoate are found. As an example, **Fig. 3** shows the separation of these compounds present in the top layer obtained in an experiment with DMAP at t = 15 h. Total area entries in Table 1 are calculated from the integrated areas of peaks 1 to 5 as: $S_{tot} = S_1 + S_2 + {}^1/_2 (S_3 + S_4) + {}^1/_3 S_5$ and the mole fraction of the 1,2-dioctanoylglycerobenzoate isomer as $x_{B12} = S_1/(S_1 + S_2)$. The total area data in Table 1 measured from top layers obtained from DMAP-treated samples increase rapidly with increasing benzoolysis time and reach a plateau value for benzoolysis times exceeding 5 h, i.e., these data parallel the increase of the dephosphorylation percentage (see Table 1, 1st column). Those derived from samples without DMAP treatment increase at a much lower rate, i.e., the benzoylation rate is much lower than the dephosphorylation rate. These trends confirm the TLC results, which, in the absence of DMAP treatment, also indicate the initial accumulation of dephosphorylated dioctanoylglycerols ($G_{12} + G_{13}$) in large excess of the benzoylated dioctanoylglycerobenzoates ($B_{12} + B_{13}$).

The combined scintillation counting, TLC, and HPLC results enable the calculation of the composition of the top and bottom layers. Thus, the fate of dioctanoyl GPC upon benzoolysis and extraction can be traced down in terms of the amounts of the various species formed as a function of benzoolysis time. **Figure 4** shows the results of these calculations (expressed as the mole percentages of the dioctanoyl GPC originally present) for both DMAP-treated and non-treated samples.

It should be stressed that the validity of these results relies on the virtually complete recovery of the radioactivity (as exemplified by scintillation counting) and

TABLE 2. Isomer ratios in lipid extracts with and without DMAP after benzoolysis

	Without DMAP		With DMAP	
Lipid Extract	x_{G12}[a,b]	X_{B12}[a]	X_{B12} (2 h)[c]	x_{B12} (15 h)[c]
Dicaproyl GPC	0.32 ± 0.01	0.74 ± 0.01	0.38 ± 0.01	0.74 ± 0.0±
Dicaproylglycerol	0.33 ± 0.01	0.71 ± 0.01	0.37 ± 0.01	0.67 ± 0.02

Benzoolysis was performed (at 100°C, variable time) with 4.4 μmol amounts of 1,2-dicaproyl GPC and 1,2-dicaproylglycerol. Abbreviations: x_{G12} = mole fraction of 1,2-isomer in dicaproylglycerols; x_{B12} = mole fraction of 1,2-dicaproylglycerobenzoate isomer in dicaproylglycerobenzoates.

[a]Independent of benzoolysis time, mean ± SD (n = 7).

[b]Determined by HPLC analysis using the silica column indicated in Materials and Methods, *n*-hexane–2-propanol 96:4 (by volume) as the eluent and differential refractive index detection.

[c]Mean ± SD (n = 2).

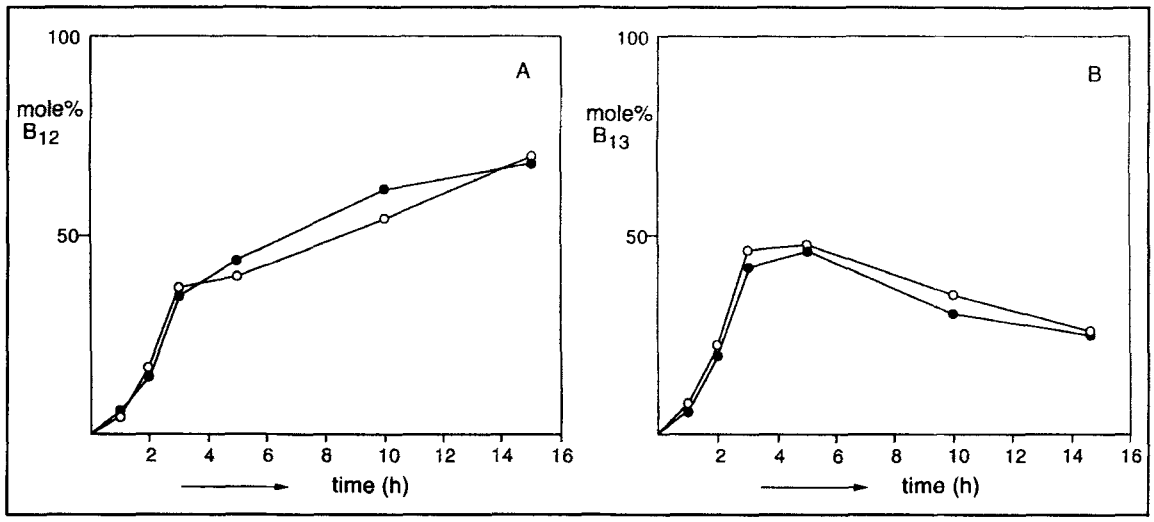

Fig. 6. Experimental (●) and calculated (○) mole percentages of 1,2-dioctanoylglycerobenzoate (panel A) and 1,3-dioctanoylglycerobenzoate (panel B) upon extraction in the presence of DMAP.

nearly 100% extraction efficiency of dephosphorylated benzoolysis products (see previous section). In fact, the only assumptions made are a 1:2:3 ratio of the molar absorbancies of dioctanoylglycerobenzoate, octanoylglycerodibenzoate, and glycerotribenzoate, respectively, and an equal release of ^{14}C-labeled and unlabeled acyl groups in the formation of dibenzoates and tribenzoate. Of these assumptions, the former is quite realistic, the latter is arbitrary. However, the ultimate alternative assumption, i.e., selective release of the ^{14}C-labeled acyl group, yields results that are only slightly different (maximally about 3%) as the total amount of dibenzoates and tribenzoate is at maximum about 6% (see Fig. 3).

The most conspicuous result is the appearance of dioctanoylglycerols in the extracts (without DMAP), the concentration of which goes through a maximum at about 3–5 h benzoolysis time. These glycerols must be either already present in the melt at the end of the heating period or produced upon extraction from some intermediate dephosphorylated species. Apparently, either these glycerols or such an intermediate are formed rather rapidly by dephosphorylation of dioctanoyl GPC, but are benzoylated at a much slower rate during the heating period, giving rise to the observed concentration maximum.

The behavior of diacylglycerols in our benzoolysis procedure was therefore also studied. To that end, equal molar amounts (0.68 μmol) of 1,2-dipalmitoylglycerol and 1,2-dipalmitoyl GPC were separately submitted to the benzoolysis and extraction procedure (with and without DMAP) and the extracts were analyzed by HPLC. The results are presented in **Fig. 5** (the total areas S_{tot}, calculated as indicated before from the areas of dipalmitoylglycerobenzoate, palmitoylglycerodibenzoate, and glycerotribenzoate peaks, are normalized to the mean result from DMPA-treated samples obtained in the benzoolysis of 1,2-dipalmitoylglycerol). It is clearly seen in Fig. 5 that the formation rate of benzoylated products (without DMAP) from dipalmitoyl GPC and dipalmitoylglycerol is nearly equal for benzoolysis times beyond 4 h. This might indicate that diacylglycerols are also present in the benzoolysis mixture of diacyl GPC: in Fig. 4 it is seen that at t = 4 h the total diacylglycerol concentration in the non-DMAP-treated extracts has risen to about 58% of the original diacyl GPC concentration; from Fig. 5 it can be concluded that at t = 4 h the total diacylglycerol concentration in the non-DMAP-treated extracts has decreased to 58% of the original diacylglycerol concentration. Thus, beyond t = 4 h the total diacylglycerol concentration and thereby the benzoylation rate would be roughly the same. Analogous results were obtained by submitting equal molar amounts of 1,2-dicaproyl GPC and 1,2-dicaproylglycerol to the benzoolysis procedure. Moreover, in those experiments, the isomer ratios of dicaproylglycerols and dicaproylglycerobenzoates in extracts with and without DMAP were determined and proved to be nearly identical in both experiments (**Table 2**). This also seems to indicate that diacylglycerols are produced during the heating period. However, the results do not rule out the possibility that not diacylglycerols, but some intermediate species, identical in both experiments, is present in the benzoolysis mixtures, yielding diacylglycerols only upon extraction.

Another, though related, remarkable phenomenon exhibited by the data in Fig. 4A is the change with increasing benzoolysis time of the relative amounts of the isomeric glycerobenzoates formed upon benzoolysis and subsequent extraction (with DMAP). At short ben-

zoolysis times, more 1,3- than 1,2-dioctanoylglycerobenzoate is formed from 1,2-dioctanoyl GPC, a rather unexpected result, but the isomer ratio is reversed after a 5-h heating period. This is in contrast with the almost constant ratio in which the two isomers are formed from the same benzoolysis mixtures upon extraction without DMAP (see Fig. 4B). DMAP is known to catalyze the benzoylation of free hydroxyl groups of glycerides. The mechanism of its action, which involves a highly reactive N-benzoyldimethylpyridinium cation, has been amply discussed (5). When performed at room temperature, the reaction appears not to induce any acyl migration (compare the preparation of 1,2- and 1,3-diacylglycerobenzoates, described in Materials and Methods). Therefore, dioctanoylglycerols (whether already present in the melt in relative amounts given in Fig. 4B or produced in these amounts by the extraction procedure without DMAP) can be expected, upon DMAP-treatment, to yield the corresponding dioctanoylglycerobenzoates without acyl migration. If so, the relative amounts of 1,2- and 1,3-dioctanoylglycerobenzoates, given in Fig. 4A, could be calculated from those in Fig. 4B by simply adding the amounts of the corresponding dioctanoylglycerols and dioctanoylglycerobenzoates given in Fig. 4B. It is demonstrated in **Fig. 6** that, indeed, values calculated in this way are in good accordance with experimental values.

Thus, dioctanoylglycerobenzoates occurring in the DMAP-treated extracts (see Fig. 4A) appear to be formed via two different routes: *a)* relatively slowly at 100°C in molten benzoic anhydride by benzoylation of a rapidly formed dephosphorylated intermediate (or a mixture of isomeric dioctanoylglycerols), and *b)* rapidly at room temperature in a benzene solution of benzoic anhydride by a DMAP-catalyzed benzoylation of this intermediate (or the mixture of isomeric dioctanoylglycerols; compare Fig. 7). Route *a)* yields both dioctanoylglycerobenzoate isomers with $x_{B12} \approx 0.75$ (Table 1), whereas route *b)*, by virtue of the extraction medium-induced constant isomer ratio of the diacylglycerols, leads to $x_{B12} \approx 0.33$. When DMAP is not added, only route *a)* is left and the extracts contain besides dioctanoylglycerobenzoates both dioctanoylglycerol isomers (with $x_{G12} \approx 0.33$). The concentration of the latter, whether already present in the melt or produced from a dephosphorylated intermediate only upon extraction, initially increases rapidly with benzoolysis time, goes through a maximum at about 4 h, and decreases slowly at prolonged heating. Accordingly, the isomer ratio of 1,2- to 1,3-dioctanoylglycerobenzoates in DMAP-containing extracts changes gradually with benzoolysis time from less than 1:1 to about 3:1, as at prolonged benzoolysis times more of the dioctanoylglycerobenzoates are produced via route (a).

The results of comparative benzoolysis experiments of 1,2-diacyl GPC and 1,2-diacylylycerol (see Fig. 5 and Table 2) suggest that both compounds become benzoylated in molten benzoic anhydride via a common intermediate. Previously (1), we tentatively suggested a cyclic carbocation as a possible intermediate in the benzoolysis of 1,2-diacyl GPC. A similar proposal was made by Renkonen (4) to explain acyl migration occurring during acetolysis of diacyl GPC. This intermediate is supposed to be formed by protonation of the substrate and subsequent nucleophilic attack by the carbonyl group at the glycerol *sn*-2 position, liberating choline phosphate.

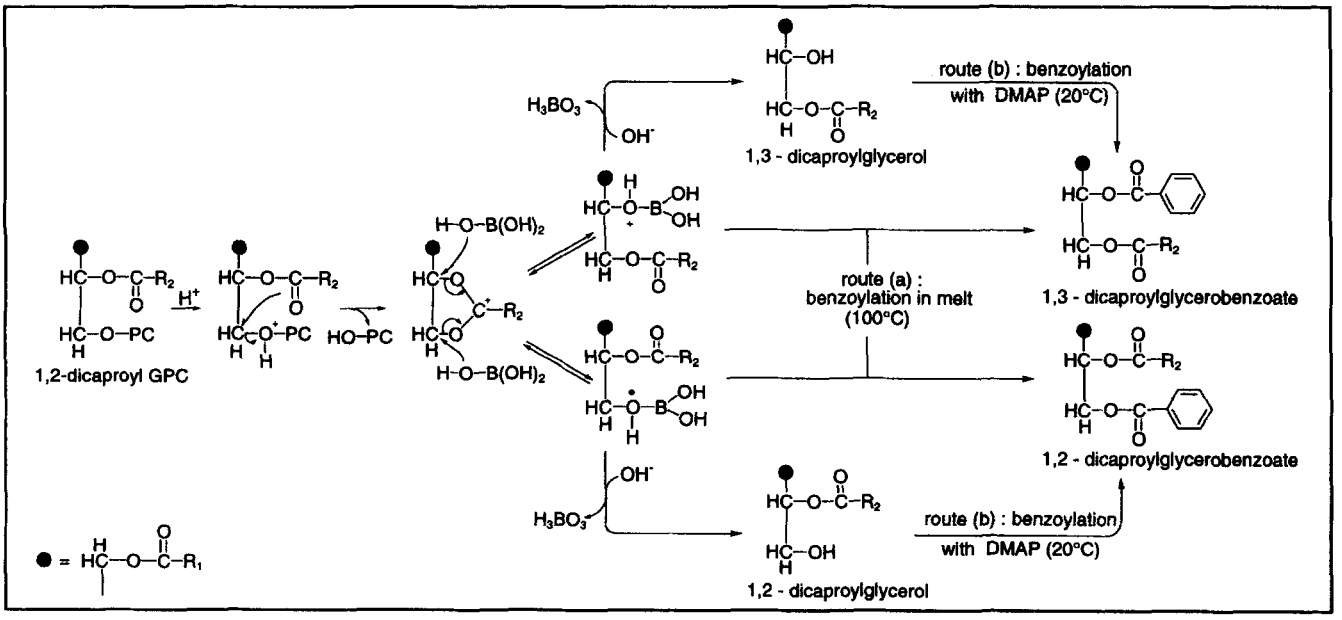

Fig. 7. Proposed mechanism and sequence of reactions involved in the dephosphorylation and benzoylation of diacyl GPC by benzoolysis.

It is interesting to note that this cyclic intermediate can also be imagined to be formed by an analogous reaction of 1,2-diacylglycerol, i.e., protonation and subsequent nucleophilic attack by the *sn*-2 carbonyl group, liberating water, which is withdrawn in the dehydrating benzoic anhydride medium, producing benzoic acid.

The role of boric acid in the benzoolysis process remains incompletely understood. However, some remarks can be made. First, it is recalled that with dipalmitoyl GPC we found earlier (1) that omission of boric acid leads to a strongly reduced dephosphorylation rate. The same effect was noticed with (4.4 μmol amounts of) dicaproyl GPC: without boric acid the extent of dephosphorylation upon benzoolysis for 4 h at 100°C is only 4.6% compared to 82.3% in the presence of 10 mg of boric acid (results not shown). It should be stressed that during the heating period the added crystals of boric acid do not visibly dissolve in the melt. As was found with dipalmitoyl GPC (1), the amount of boric acid added (varied from 2.5 to 50 mg) appeared to exert no significant influence on the dephosphorylation rate of dicaproyl GPC. It is therefore improbable that heterogeneous catalysis is involved. Instead, some boron-containing species, soluble in molten benzoic anhydride, must be responsible for the acceleration of the dephosphorylation reaction. Therefore, a melt, obtained by heating 10 mg of boric acid with 250 mg of benzoic anhydride for 4 h at 100°C followed by filtration at 100°C to remove undissolved boric acid, was prepared. A weighed aliquot of the filtrate was dissolved in chloroform, extracted with water, and analyzed by inductively coupled plasma spectrometry. Indeed, this indicated that the melt contained about 8 μmol of boron, i.e., a 2-fold excess with respect to the amount of dicaproyl GPC used in the experiments described above and a 10-fold excess with respect to the amount of dioctanoyl GPC generally used.

Second, it is known from literature (6–9) that boric acid reacts with acid anhydrides under the experimental conditions of our benzoolysis procedure to give mixed anhydrides according to:

$$H_3BO_3 + 3(RCO)_2O \rightarrow B(O_2CR)_3 + 3RCOOH$$

and

$$2H_3BO_3 + 5(RCO)_2O \rightarrow [(RCO_2)_2B]_2O + 6RCOOH$$

Indeed, infrared spectroscopy of a chloroform solution of the above-described filtrate revealed the presence of benzoic acid. Thus, it is conceivable that the protons needed in the first step of the dephosphorylation reaction (see Fig. 7) are provided by the partial ionization of benzoic acid.

Finally, it may seem curious that ionic reactions take place in molten benzoic anhydride. However, this is not unlikely. According to Charlot and Trémillon (10), acid anhydrides $(RCO)_2O$ have a tendency to dissociate according to $(RCO)_2O \rightleftarrows RCO^+ + RCOO^-$. In such solvents quaternary ammonium carboxylates NR'_4OOCR are known to behave as strong carboxylate ion donors. We checked, therefore, the effect of the addition of (0.5 mmol of) tetramethylammoniumbenzoate on the dephosphorylation rate of (0.75 μmol of) 1,2-dipalmitoyl GPC in our benzoolysis procedure. The result (no dephosphorylation, no benzoylation after 4 h at 100°C) is in line with the expected effect of excess benzoate ion on the dissociation of benzoic acid. Thus, the effect of boric acid seems to be related to the (indirect) delivery of protons needed in the dephosphorylation step. However, the use of benzoic acid alone instead of boric acid in the benzoolysis procedure does not lead to any appreciable dephosphorylation of diacyl GPC (1). Therefore, boric acid must fulfill yet another role, probably connected with stabilization of the intermediate carbocation. In **Fig. 7** a tentative picture is given of the dephosphorylation reaction and the benzoylation reactions via the routes *a)* and *b)* referred to above. In this scheme it is understood that the cyclic carbocation is at equilibrium with protonated 1,2-diacylglycero-3-borate and protonated 1,3-diacylglycero-2-borate, probably in a molar ratio of about 1:2 as a result of the higher stability of the latter, i.e., the molar ratio of 1,2- and 1,3-dioctanoylglycerols that is experimentally found in extracts without DMAP. In view of this reaction scheme it is not surprising that the molar ratio (about 3:1) of 1,2- to 1,3-dioctanoylglycerobenzoate produced during the heating period (i.e., via route *a)* is totally different, as the more stable protonated dioctanoylglycero-2-borate can be expected to be the least susceptible to attack by the benzoylating agent.

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