JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Generation of Monochloropropanediols (MCPDs) in Model Dough Systems. 2. Unleavened Doughs

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The contribution to monochloropropanediol (MCPD) levels in cooked wheat flour dough from glycero lipid precursors present in white flour and flour improver agents has been investigated. The results showed that monoacylglycerols, lysophospholipids, and phosphatidylglycerols present in white flour together with diacetyl tartaric acid esters of monoacylglycerols used in flour improvers were precursors of MCPDs. Diacyl- and triacylglycerols and phosphatidylchloline did not form measurable levels of MCPDs. Precursor compounds with −OH adjacent to phosphoryl or acyl groups on the glycerol skeleton were able to form MCPDs suggesting that the formation reaction involved a neighboring group mechanism. The relative rates and regioselectivity could be explained by differences in leaving group abilities and the steric and electronic effects of the acyl and phosphoryl groups, respectively. Kinetic data obtained from the addition of high purity reference lipids were used to quantify the potential contribution to MCPDs in wheat dough.

KEYWORDS: DATEM; dough; glycero lipids; 3-MCPD; monochloropropanediol; phospholipids; wheat

INTRODUCTION

Monochloropropanediols (MCPDs) are contaminants that can be found in a wide range of processed foods and ingredients (*1*). The toxicological effects of these compounds are well-known, and a provisional maximum tolerable daily intake of 2 μg kg^{-1} body weight has been proposed (*2*, *3*) for 3-monochloropropane-1,2-diol (3-MCPD). It has been known for some time that MCPDs can be generated during the manufacture of certain soy sauces and seasonings using acid hydrolysis (*4*). In this instance, it is the reaction of concentrated hydrochloric acid with residual lipids present in the raw materials that can lead to the levels formed (5, *6*). However, the occurrence of MCPDs in heat-treated and predominantly cereal-based foods and ingredients (*7*, *8*) has prompted more recent investigation (*9*).

In a preceding paper (*10*), we established that glycerol was a major precursor of MCPDs in leavened wheat dough. Glycerol, generated primarily by the yeast during proving, can react with chloride ion (Cl^-) from added cooking salt to generate MCPDs in the dough when baked. It was found that glycerol could account for up to 68% of the contribution to MCPDs generated in proved dough. The remaining 32% contribution was attributed to precursors present in commercial white flour (21%) and components of the flour improver (11%) that have not yet been identified. Furthermore, it was shown that the relatively abundant and structurally related triacylglycerols did not contribute to MCPD formation under the conditions employed.

Although the contribution to MCPD levels from unidentified precursors in flour may be relatively small in leavened doughs, this cannot be the case for many nonyeasted cereal products where these compounds are likely to be the major source of MCPDs. For example, MCPDs have been found in unleavened biscuit products such as matzos (flour + water) and short dough biscuits (*11*). In this paper, we describe an investigation of the precursors of MCPDs in commercial white flour and flour improver agents.

MATERIALS AND METHODS

Bakery Ingredients. Ascorbic acid, diacetyl tartaric acid esters of mono- and diglycerides (DATEM), enzyme active soya flour, and white bread flour were obtained from commercial baking suppliers.

Chemicals and Reagents. Diphosphatidylglycerol (linoleic acid, 98%), glycerol (>99%), dl-α-glycerophosphate (disodium salt, hexahydrate, >85%), β-glycerophosphate (water 4.7 mol/mol, >99.9%), 1-monopalmitoyl-*rac*-glycerol (1-MPG; >99%), 2-monopalmitoylglycerol (>99%), 1-monostearoyl-*rac*-glycerol (1-MSG; >99%), 1,2-dipalmitoly-*rac*-glycerol (>99%), l-α-phosphatidyl-DL-glycerol (palmitoyl and stearoyl, >99%), L-α-lysophosphatidylcholine (palmitoyl and stearoyl, >99%), and DL-α-phosphatidylcholine (dipalmitoyl, >99%) were obtained from Sigma (Poole, U.K.). Hexamethlydisilazane, β-phenylglucopyranoside, and trifluoroacetic acid were obtained from Aldrich Chemical Co. (Poole, U.K.). Chloroform, super purity grade, was from Romil (Cambridge, U.K.). Ultrapure deionized water (resistivity > 18.0 MΩcm) was prepared in-house (NANOpure DIamond, Thame, U.K.).

Preparation of Dough Samples. *Lipid Addition Experiments*. Finely powdered or crystalline high purity reference lipids were dispersed in white flour (10.000 ± 0.005 g) contained in a 100 mL beaker. Salt

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10.1021/jf035078o CCC: $27.50
Published on Web 03/06/2004

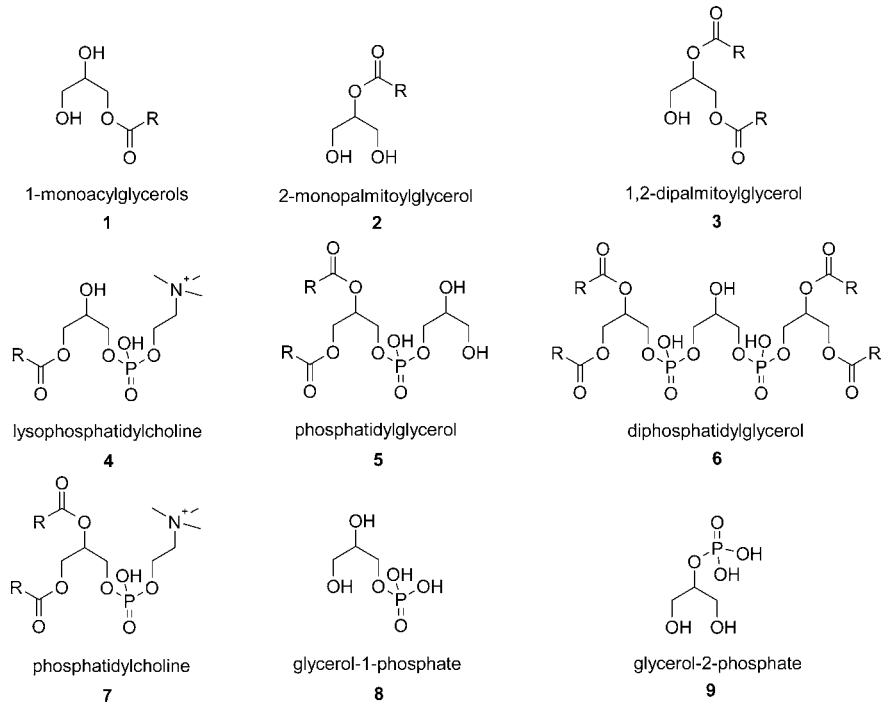

Figure 1. Acylglycerols and phospholipids representative of potential precursor compounds in white flour [R = $(CH_2)_{14}CH_3$ and/or $(CH_2)_{16}CH_3$ or $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$]. Each of the compounds **1**–**9** was added to model dough to determine which would promote MCPD formation.

(0.200 ± 0.002 g) and water (6.00 ± 0.01 mL) were added and mixed by spatula until a dough was formed. The dough piece was kneaded and repeatedly folded by hand (powder free latex gloves) for 2 min. Lipids were added in the concentration range of 1−4% (on flour weight), and prepared dough samples were cooked within 15 min of mixing.

Flour Improver Agents. Model bread dough (white flour + salt + water, 100:2:60 w/w) was vacuum mixed to a work input of 36 kJ kg^{-1} on a 1 kg (flour) scale by the Chorleywood bread process (*12*, *13*). DATEM and enzyme active soya flour were blended with the flour prior to the addition of the dough water. Ascorbic acid was dispersed in the dough water immediately before mixing.

Generation of MCPDs. Dough samples were cooked using a custom built pressure-cooking apparatus used previously to simulate the conditions of baking (*9*, *10*). Briefly, samples were contained in stainless steel HPLC tubes of 75 mm length with an internal diameter (i.d.) of 7.75 mm (Hichrom Ltd., Reading, U.K.) and secured with stainless steel end caps modified to accept 1.6 mm o.d. calibrated type K thermocouple probes (Labfacility, Bognor Regis, U.K.). MCPDs were generated by cooking the samples in a Carlo Erba Mega series (Milan, Italy) gas chromatograph (GC) oven at 180 °C for 20 min.

Analytical Methods. *Measurement of pH*. Replicate pH measurements were made using a Gelplas calibrated double junction flat tip probe, model 309/1070/09 (BDH/Merck, Lutterworth, U.K.) to an accuracy of ±0.02 pH unit. The pH of uncooked samples was measured directly at the dough surface; cooked samples were determined as a slurry with deionized water (1:1 w/w).

Chloride Analysis. Samples (1−3 g) were dispersed in freshly boiled deionized water (80 mL, ≥90 °C) prior to making to volume with deionized water (100 mL). Chloride was measured in cooled and filtered samples by electrochemical titration with silver ions using a 926 Chloride Analyzer (Corning Ltd., Halstead, U.K.).

Analysis of Glycerol. Glycerol was determined as the GC volatile derivative, triacetin, using a procedure described previously (*10*). GC-MS was carried out using a Saturn 3 GC-MS system (Varian Inc., Walnut Creek, CA) fitted with a 30 m × 0.25 mm i.d. Rtx-50 (Restek, Bellefonte, PA) column with a 0.25 μm film thickness.

Analysis of Monoacylglycerols. Stock solutions of 1-MSG and 1-MPG were prepared at 2 mg mL^{-1} in chloroform. A stock solution of β-phenylglucopyranoside (PGP, internal standard) was prepared at 2 mg mL^{-1} in pyridine.

Table 1. Apparent First-Order Rate Constants (k) for the Formation of 3-MCPD from Glycerol and Glycerol Esters Added to Model (Flour + Salt + Water) Dough[a]

precursor	rate constant $10^8 \times k$ (s^{-1})	relative rate (to glycerol)	isomer ratio 3-MCPD: 2-MCPD
phosphatidylglycerol (**5**)	6.069	4.70	6.6:1
diphosphatidyl glycerol (**6**)	3.616	2.80	9.8:1
glycerol-1-phosphate (**8**)	1.582	1.23	5.3:1
glycerol-2-phosphate (**9**)	1.413	1.09	5.7:1
glycerol[b]	1.290	1.00	3.3:1
lysophosphatidylcholine (**4**)	0.328	0.25	7.9:1
1-monopalmitoylglycerol (**1**)	0.254	0.20	6.5:1
1-monostearoylglycerol (**1**)	0.244	0.19	6.4:1
2-monopalmitoylglycerol (**2**)	0.208	0.16	7.4:1
phosphatidylcholine (**7**)	0.043	0.03	6.7:1
1,2-diapalmitoylglycerol (**3**)	0.000		7.7:1

[a] Samples were cooked at 180 °C for 20 min. [b] Data from ref *10*.

Samples (10−100 mg equivalents in chloroform) or 1-MPG reference standard (2 mL) were transferred into a 25 mL McCartney bottle (slender neck), and the solvent was removed by nitrogen stream. PGP (1 mL of 2 mg mL^{-1}) was added, followed by 0.9 mL of hexamethlydisilazane. Trifluoroacetic acid (0.1 mL) was added slowly with care (exothermic), and the bottle was capped, shaken, and allowed to stand at room temperature for ≥30 min. Completion of the reaction was indicated by the disappearance upon shaking of the two phases present initially. Aliquots of the 1-MSG stock standard (0.5, 1.0, 2.0, and 5.0 mL) together with PGP (1 mL) were treated in the same way to give a series of calibration standards.

All determinations were made using a 3900 GC (Varian Inc.) equipped with a split/splitless injector and FID. Samples were separated on a 30 m × 0.22 mm i.d. Ultra II (Hewlett-Packard) column with a 0.33 μm film thickness under the following conditions: injector temperature, 275 °C (split ratio, 100:1); injection volume, 1 μL; hydrogen carrier gas, flow rate, 0.7 mL min^{-1}; temperature program, 1 min at 220 °C then 2 °C min^{-1} to 260 °C followed by 10 °C min^{-1} to 300 °C, hold for 5 min. Monoacylglycerols were quantified in samples from the 1-MSG/1-PGP responses, and the slope of a least squares line was fitted to a calibration data set.

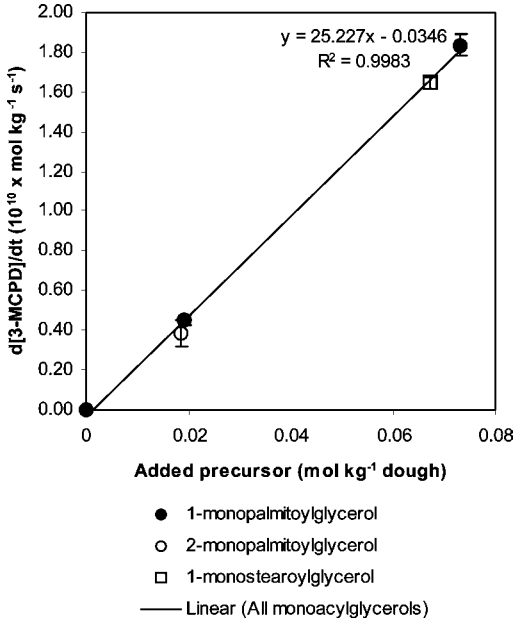

Figure 2. Rate of formation of 3-MCPD from the addition of monoacylglycerols to model dough. Data points are means of duplicate experiments, and vertical bars represent the standard errors of the means (SE).

Analysis of MCPDs. The isomers, 2-MCPD and 3-MCPD, were determined as the heptafluorobutyryl esters by a procedure reported previously (*9*). GC-MS was carried out using a Saturn 2000 ion trap (Varian) equipped with a BPX5 (SGE, Milton Keynes, U.K.) capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness).

RESULTS AND DISCUSSION

Precursors in White Flour. Previous investigations had shown that white flour could account for approximately 30% of the contribution to MPCDs in commercial proved dough (*10*). Of this contribution, 33% was attributed to glycerol present naturally in the flour. To determine the remaining contribution to MCPDs from flour, mono- and diacylglycerols and phospholipids representative of those present in wheat starchy endosperm were investigated as potential precursors (**Figure 1**). Triacylglycerols were not included in this investigation since they are not believed to be precursors in dough (ibid). The glycerol phosphates (**8**, **9**) are not natural components of wheat flour, and these compounds were included for mechanistic studies. All rates were compared to the known precursor, glycerol.

Table 1 shows the results obtained from the addition of compounds **1**−**9** to model dough, and **Figure 2** shows the data obtained from the addition of monoacylglycerols. The apparent first-order rate constants (k) were obtained from the gradient of the least squares fit to the data obtained from the addition of each compound over the concentration range of 0−0.1 mol kg^{-1}. These results were also shown not to be attributable to pH changes in the dough since, on the basis of previous studies (*9*), the measured variation in pH was insufficient to affect the stability of 3-MCPD and 2-MCPD.

Mono- and Diacylglycerols. The addition of monoacylglycerols (**1**, **2**) to model dough produced a first-order response in MCPD formation (**Figure 2**). The relative rates of formation (**Table 1**) suggest that mole for mole **1** and **2** were approximately five times less effective in generating 3-MPCD as compared to glycerol. The ratio of 3-MPCD and 2-MPCD formed from **1** and **2** was close to that obtained for model (flour + salt + water) dough without added lipid, i.e., 7.7:1, and was therefore consistent with these compounds being present in the flour. Dipalmitoylglycerol (**3**) did not react rapidly enough to contribute significantly to MPCD production in dough.

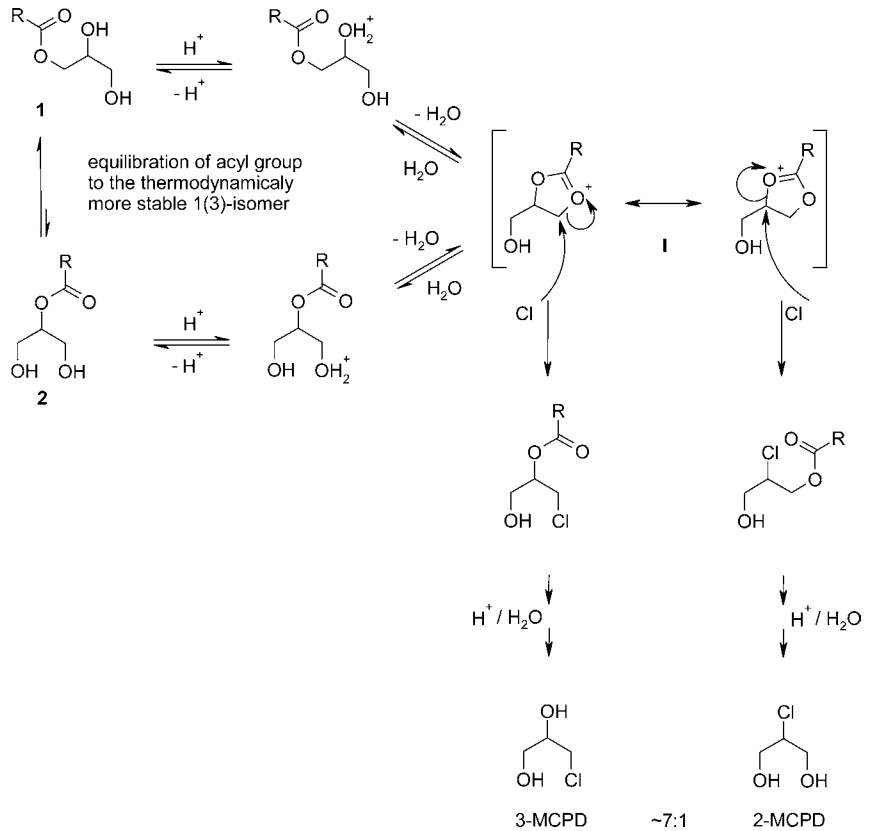

Figure 3. Possible mechanisms of formation of MCPDs from 1- and 2-monoacylglycerols. The incorporation of Cl$^-$ is facilitated by a neighboring group mechanism involving a cyclic acyloxonium ion intermediate (**I**).

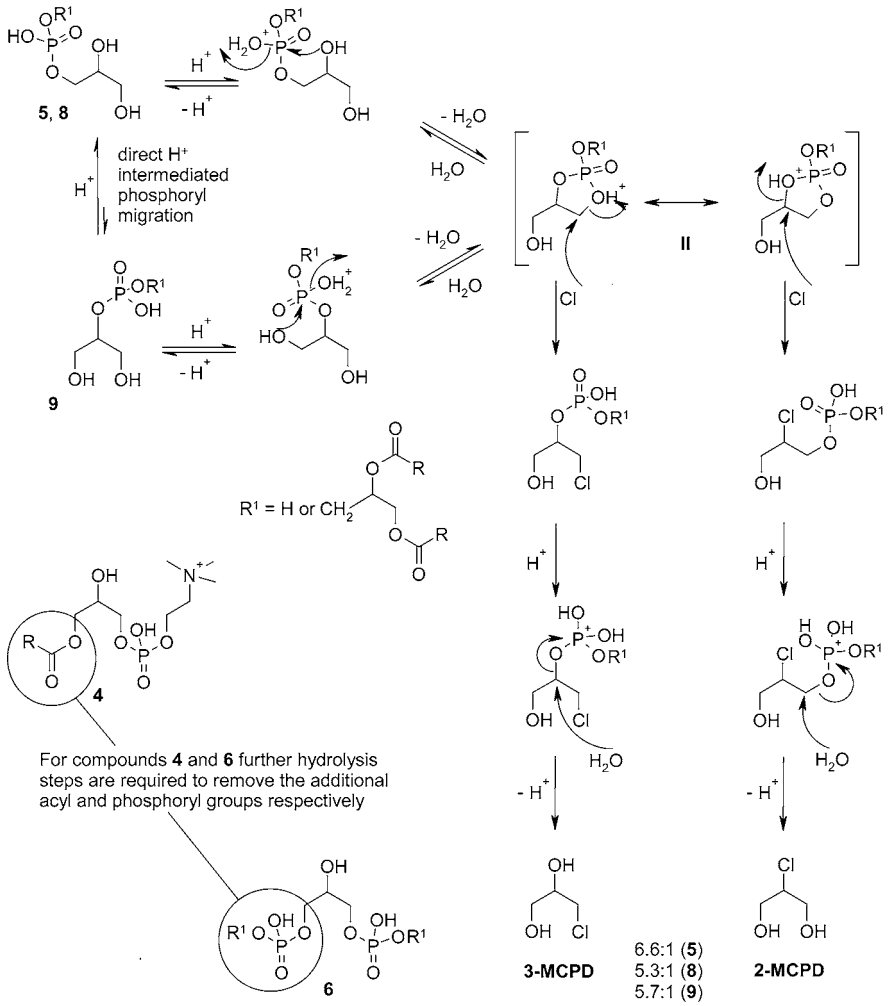

Figure 4. Proposed MCPD formation mechanisms from glycerolphosphates. MCPD isomer ratios are controlled by steric and electronic effects arising from nucleophilic substitution at a cyclic phosphate diester (**II**).

Direct substitution of the acyl group by Cl^- is unfavorable due to the poor leaving group ability of the former. The equivalent reactivity exhibited by 1- and 2-monoacylglycerols (**1**, **2**) suggests that MCPDs are formed via a common mechanism. A factor in this mechanism is very probably the rapid equilibration of the acyl groups to the thermodynamically favored 1(3)-isomer (*14*) via a neighboring group mechanism involving a common cyclic acyloxonium ion intermediate (*15*, *16*). The cyclic intermediate (**I**) can be opened by Cl^- at one of two positions determined by steric and electronic effects. Hence, the proposed mechanism shown in **Figure 3** can account for the equivalent reactivity and selectivity exhibited by **1** and **2**. It is likely that the final ester hydrolysis steps are rate determining.

Phospholipids. The addition of lysophosphatidylcholine (**4**), phosphatidylglycerol (**5**), and diphosphatidylglycerol (**6**) produced a first-order response in MCPD formation. The addition of phosphatidylcholine (**7**) at a level of up to 0.03 mol kg^{-1} had no impact on MCPD generation. On a molal (mol kg^{-1}) basis, **5** and **6** were, respectively, five and three times more reactive and **4** was four times less reactive than glycerol. In keeping with the results from the addition of the monoacylglycerols (**1**, **2**), the measured isomer ratio (3-MCPD:2-MCPD) from the addition of each phospholipid was approximately 7:1.

For the phospholipids, there exist two plausible mechanistic routes by which Cl^- may be introduced into the glycerol skeleton: (i) direct acid-catalyzed nucleophilic substitution of the phosphoryl group by Cl^- and (ii) acid-catalyzed opening of a favored five-membered cyclic phosphate diester (can only form when there is an adjacent $-OH$ group on the glycerol skeleton) by Cl^-. Like the monoacylglycerols, a significant factor in the MCPD formation mechanisms is likely to be the acid-catalyzed migration of the phosphoryl group to the thermodynamically more stable 1(3)-isomer. It is well-known that 1,2-phosphoryl migrations can occur by two pathways: via a cyclic intermediate or through a direct H^+-intermediated transfer (*17*, *18*). In the case of (i), the relative amounts of 3- and 2-MCPD will be determined by the equilibrium ratio of phosphoryl isomers, and this should favor almost exclusively 3-MCPD formation whereas in (ii) the MCPD isomer ratio will be controlled by steric and electronic effects arising from nucleophilic substitution at a cyclic phosphate diester.

To confirm this hypothesis, 1- and 2-glycerolphosphates (**8**, **9**) were added to model dough since migration equilibrium constants and rate data were known for these compounds (ibid). The equivalent reactivity and lower than predicted 3-MCPD:2-MCPD isomer ratios (predicted value $\gg$ 10:1) obtained from each compound (**Table 1**) suggest that MCPDs are more likely to be formed via the cyclic intermediate **II**. The rate-determining step probably involves nucleophilic substitution of phosphate by OH_2. Leaving group ability is related to basicity, and because alkyl phosphates are weaker bases than phosphates, this is consistent with the observed reactivity of **5** and **6** > **8** and **9**. The reduced reactivity of **6** as compared to **5** may be a

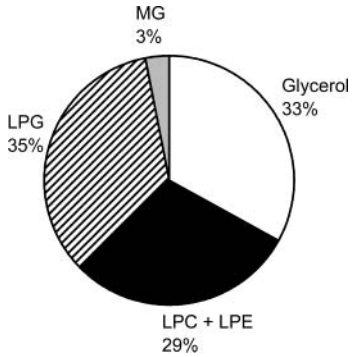

Figure 5. Calculated contributions to 3-MCPD levels in wheat dough from precursors in flour. The contributions were derived from the rate constants given in **Table 1** and the known levels of lipids in flour (*19*). The glycerol contribution was derived from ref *10* (MG = monoacylglycerols; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; and LPG = lysophosphatidylglycerol).

consequence of an additional hydrolysis step to remove the second phosphate group. Although the diphosphate-substituted glycerol (**6**) is a minor component of wheat starchy endosperm, it is interesting to compare the superior reactivity of this compound, and hence the phosphate groups, as compared to the diacyl-substituted glycerol (**3**), which did not generate MCPDs.

The similar reactivity exhibited by **4** and the monoacylglycerols (**1**, **2**) despite the more reactive leaving group in the former (phosphate is a better leaving group than $-OH_2^+$) suggests that these compounds may share a common rate-determining step, possibly involving hydrolysis of the acyl group. Possible mechanisms of formation of MCPDs from glycerolphosphates are given in **Figure 4**.

Contributions to 3-MCPD Levels in Wheat Doughs. The contributions to 3-MCPD levels in wheat dough from flour lipid precursors (**Figure 5**) were calculated using the data given in **Table 1** and known lipid distributions in flour (*19*). Because phosphatidylglycerol (**5**) is a minor component in wheat starchy endosperm (the lyso equivalent was not commercially available), the contribution to 3-MCPD levels was derived for the more abundant lysophosphatidylglycerol for which a similar reactivity was assumed. Similar reactivities may also be expected for lysophosphatidylcholine and other structurally similar wheat lyso lipids, i.e., lysophosphatidylethanolamine, lysophosphatidylinisitol, and lysophosphatidylserine.

Precursors in Flour Improvers. Commercial improvers used in bakery products typically contain ascorbic acid, soya flours, and a source of monoacylglycerols, e.g., glycerolmonostearate or modified monoacylglycerols, e.g., DATEM. Because the contribution from monoacylglycerols had been determined from the lipid addition experiments, the remaining ingredients were selected for investigation.

Neither soya flour nor the ascorbic acid produced any change in the rate of formation of MCPD. The addition of DATEM to model dough over the concentration range of 0−2.4% (dough basis) was found to promote MCPD formation. When chloride was omitted from the recipe, generation did not occur at measurable levels confirming that DATEM and chloride were required to form MCPDs. On a molal basis, the reactivity of DATEM was found to be 0.7 relative to that of glycerol in model dough (average molecular weight of major DATEM components assumed). Furthermore, the isomer ratio obtained from each addition of DATEM (6.4:1) was similar to that obtained from the addition of the lysophospholipids and monoacylglycerols

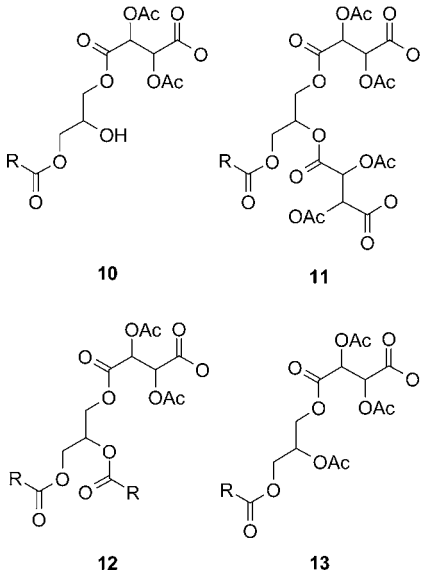

Figure 6. DATEM: the major reaction product is **10** together with varying amounts of **11**–**13** (*21*).

suggesting that similar mechanisms of formation might apply. These results were shown not to be attributable to residual levels of glycerol (927 ± 54 mg kg^{-1}) and monoacylglycerols (2.0%) in DATEM, which were too low to promote MCPD formation at the dilutions employed in dough.

Although DATEM is a complex mixture of many components (*20*), the structure of the major active ingredients (**Figure 6**) suggests that compound **10** could be a MCPD precursor. There remains, however, a difficulty in explaining the relative reactivity of this compound, which is structurally similar to the nonreactive diacylglycerols. A factor in this may be the much lower pK_a of the diacetyl tartaric acid moiety and hence better leaving group ability as compared to the diacylglycerol fatty acid group.

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Received for review September 22, 2003. Revised manuscript received January 27, 2004. Accepted January 28, 2004.

JF035078O