Regioselective Lipase-Catalyzed Transesterification of Tributyrin. Influence of Salt Hydrates on Acyl Migration

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ABSTRACT: The influence of the nature of solid salt hydrates on the rate of hexanoic acid catalyzed acyl migration in 1,2-dibutyrin has been examined in hexane. The results show that the rate of acyl migration is faster when hydrogen phosphate salts are included compared to sulfate salts. The rate is essentially the same in the presence of sulfate salts and without salts. In regioselective lipase-catalyzed acidolysis of tributyrin with hexanoic acid, the use of hydrogen phosphate salts to control water activity leads to a higher rate of migration than the use of sulfate salts. Minor differences are observed in interesterification with ethyl hexanoate.
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KEY WORDS: Acyl migration, 1,2-diglyceride, salt hydrates, transesterification.

Lipase-catalyzed acidolysis with fatty acid and interesterification with fatty acid ester are commonly used to modify readily available, low-value fats and oils into high-value products. Exchange of all or only the primary ester group can, in principle, be achieved by using a non- or a 1,3-selective lipase, respectively. However, changes in the 2-position may occur also when a 1,3-selective lipase is used due to acyl migration in mono- and diglycerides, which are present as reaction intermediates (1). In addition, water will influence the level of partial glycerides through hydrolysis. Re-esterification of migrated 1,2-diglycerides may yield triglycerides in which the fatty acid in the 2-position has been exchanged. Migration in both mono- (2,3) and diglycerides (4) is important in the formation of isomeric diglycerides.

The effects of acyl migration can be exemplified by regioselective lipase-catalyzed acidolysis and interesterification of palm oil mid-fraction to produce cocoa butter substitutes. The main triglyceride in palm oil mid-fraction is 1,3-dipalmitoyl-2-oleoyl-glycerol. Acidolysis and interesterification with stearic acid or ester should ideally only result in production of StOP and StOSt (where St = stearic acid; O = oleic acid; P = palmitic acid). Due to acyl migration, trisaturated StStSt may also be formed (5,6). This leads to a lower commercial value of the product.

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In a previous study of water activity (a_w) and uncatalyzed isomerization of 1,2-dibutyrin in hexane, it was observed that solid salt hydrates, used to control the a_w, influenced the rate of acyl migration (7). When pairs of different hydrate forms of hydrogen phosphate ($Na_2HPO_4 \cdot XH_2O$) were included, the rate of acyl migration decreased compared to reactions that had been pre-equilibrated to similar a_ws without salt hydrates. The only sulfate salts used (a mixture of $Na_2SO_4 \cdot 10H_2O$ and anhydrous Na_2SO_4) did not influence the rate. No obvious explanation for the effect of hydrogen phosphate salts was found. However, the results showed that acyl migration is not directly catalyzed by the salt hydrates. When 1,2-dibutyrin was dissolved in hexane in the presence of hexanoic acid, the rate of migration increased when a mixture of $Na_2HPO_4 \cdot 12H_2O$ and $\cdot 7H_2O$ was included. This was partly attributed to the increased a_w, which may have facilitated acid catalysis. However, we suspected that the nature of the salt hydrate also had an effect. As a consequence, the influence of the nature of salt hydrates (hydrogen phosphates and sulfates) on the rate of fatty acid- catalyzed isomerization of 1,2-dibutyrin in hexane was examined and is reported here.

The effect of the two types of salt hydrates (hydrogen phosphates and sulfates) was further examined in regioselective lipase-catalyzed transesterification. As a model reaction, acidolysis and interesterification of tributyrin with hexanoic acid and ethyl hexanoate, catalyzed by the 1,3-selective lipase Lipozyme (Novo Nordisk, Bagsvaerd, Denmark), was used. The production of 1,3-dibutyrin and tricaproin was examined as a function of the type of salt hydrate used.

EXPERIMENTAL PROCEDURES

Chemicals. Tributyrin, hexanoic acid, and ethyl hexanoate were purchased from Fluka Chemika AG (Buchs, Switzerland). Tricaproin and dicaproin for calibration curves were from Sigma Chemical Co. (St. Louis, MO).

Synthesis of 1,2-dibutyrin (Ref. 8). The primary hydroxy group of 3-chloro-1,2-propanediol was tritylated with triphenylchloromethane. The secondary hydroxy group and the chloro group were acylated successively using butanoyl chloride and sodium butanoate, respectively. The trityl group was substituted by trifluoroacetyl with trifluoroanhydride in

toluene. The triphenyl methanol formed was partly removed by treating the product with light petroleum ether before distillation, b.p. 86°C (0.075 mmHg). The isomeric ratio by ^{1}H nuclear magnetic resonance (NMR) was 1,2:1,3 = 97:3, ^{1}H NMR: 1,2-isomer (ABNMX-system) 5.32 (quintet), 4.59, 4.45, 4.32, and 4.18, 1,3-isomer (AA′BB′X-system) 5.44 (quintet), 4.41, and 4.21. Finally, the trifluoroacetyl group was removed by pyridine and methanol. The resulting diglyceride was >99% pure. The isomeric ratio was as for the trifluoroacetyl ester. ^{1}H NMR: 1,2-dibutyrin (ABNMX-system) 5.09 (quintet) (H-2), 4.31, and 4.25, both *dd* (2H-1), J_{gem} = 11.95 Hz, J_{vic} = 4.52 and 5.67 Hz, 3.75 and 3.73, both *dd* (2H-3), 1,3-dibutyrin: (AA′BB′X-system) 4.09 (quintet) (H-2), 4.18, and 4.13, both *dd* (2H-1 and 2H-3).

Enzyme. Lipozyme IM20, 1,3-selective lipase from *Rhizomucor miehei* was kindly donated by Novo Nordisk AS.

Analysis. Gas–liquid chromatography (GLC) was performed in a 3400 Varian (Palo Alto, CA) instrument equipped with an 8100 Varian auto-sampler. Column, DB-1701 (J&W Scientific (Folsom, CA), 30 m x 0.25 mm i.d., film thickness 0.25 μm; carrier gas, H_2 at 10 psi; injector, 270°C, split/splitless, split ratio 70:1; detector, 270°C.

1,2/1,3-Diglycerides. Samples were silylated with *bis*(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane before analysis. The temperature was programmed at 60–190°C (15°C/min), 190–260°C (5°C/min) with a 2-min hold time at 260°C. The results obtained by GLC were compared with those obtained by ^{1}H NMR (Bruker AM 500; Bruker, Rheinstetten, Germany). The results were virtually identical.

Composition (transesterification). The temperature was programmed at 60–90°C (5°C/min), 90–220°C (15°C/min), and 220–260°C (5°C/min) with a 4-min hold time at 260°C.

a_w. Pairs of solid salt hydrates (50:50, w/w) (see following paragraph) were added directly to the reaction mixture. For pre-equilibration, the organic phase and the diglyceride or enzyme were equilibrated separately with the same salt hydrate with direct contact and through the vapor phase, respectively, for three days before mixing. No salt was present during the reaction.

Pairs of salt hydrates. The numbers in parentheses refer to the hydration state of the pairs of salt. The a_w refers to 20°C (9). The salt hydrates used were: Na_2SO_4 (10:0) a_w = 0.76, Na_2HPO_4 (12:7) a_w = 0.74, $ZnSO_4$ (7) a_w = 0.58, Na_2HPO_4 (7:2) a_w = 0.57, $CaSO_4$ (2:0.5) a_w = 0.35, and Na_2HPO_4 (2:0) a_w = 0.15. For pre-equilibration, Na_2SO_4 (10:0) a_w = 0.76, Na_2HPO_4 (7:2) a_w = 0.57, and (2:0) a_w = 0.15 were used.

Reactions. Isomerization of dibutyrin (28.7 μmol/mL, 98% 1,2-isomer) was studied in pure hexane (1 mL) and with hexanoic acid or ethyl hexanoate (262.5 μmol/mL) present. The reactions were carried out at 22°C. The model reaction for regioselective lipase-catalyzed transesterification consisted of tributyrin (75.3 μmol/mL), hexanoic acid, or ethyl hexanoate (262.5 μmol/mL), hexane (8 mL), decane (internal standard, 60 μL), and Lipozyme (20 mg). The reactions were carried out in sealed vessels (10 mL) at 22°C with shaking (100 strokes/min).

RESULTS

The rate of isomerization of 1,2-di- to 1,3-dibutyrin in hexane was measured with and without hexanoic acid present. A detailed description of the conditions is as follows: (i) with hexanoic acid: in the presence of hexanoic acid and various salt hydrates [Na_2HPO_4 (12:7) a_w = 0.74, (7:2) a_w = 0.57, (2:0) a_w = 0.15, Na_2SO_4 (10:0) a_w = 0.76, $ZnSO_4$ (7) a_w = 0.58, and $CaSO_4$ (2:0.5) a_w = 0.35] or pre-equilibrated to a_w = 0.76, 0.57, and 0.15 with the appropriate salt hydrates; (ii) without hexanoic acid: in the presence of ethyl hexanoate and various salt hydrates or pre-equilibrated as above and with no water added (i.e., without pre-equilibration or salt hydrates added); in pure hexane pre-equilibrated to a_w = 0.76, 0.57, 0.15, and with no water added (i.e., without pre-equilibration or salt hydrates added).

The influence of the nature of the salt hydrates was evaluated by measuring initial rates of acyl migration when hydrogen phosphate salts, sulfate salts, or no salt were present in the reaction mixture. The reactions without salt hydrates were pre-equilibrated to different a_ws to correct for a possible dependence on the a_w.

The results for reactions without hexanoic acid (data not shown) confirmed earlier observations (7). The initial rate of acyl migration was higher in pure hexane than in reactions with ethyl hexanoate. Similarly, the rate was higher in reactions with no water added compared to reactions with a_w = 0.15 and above. In both cases the initial rate of uncatalyzed migration increased with decreasing polarity of the reaction mixture. Hydrogen phosphate salts led to decreased migration, whereas sulfate salts had no influence.

The initial rates of migration in the presence of hexanoic acid are given in Figure 1. Compared to the uncatalyzed migration, the initial rate was five times higher at a_w = 0.76 (0.02 μmol/mL h and 0.1 μmol/mL h) in the pre-equilibrated reaction in the presence of hexanoic acid.

The initial rate of hexanoic acid-catalyzed acyl migration in reactions at a_w pre-equilibrated to a_w = 0.15–0.76 were approximately constant (Fig. 1). A reasonably valid comparison with reactions with $CaSO_4$ (2:0.5) and $ZnSO_4$ (7), for which the a_w achieved may be somewhat uncertain (10), could therefore be made.

Similar initial rates were obtained for hexanoic acid-catalyzed acyl migration with sulfate salts and with a_w adjusted by pre-equilibration (Fig. 1). This suggests that the presence of the sulfate salts does not significantly influence the rate of acyl migration. In contrast, the presence of hydrogen phosphate salts strongly influenced the rate of hexanoic acid-catalyzed acyl migration. The initial rate of migration increased with decreasing a_w, i.e., 0.7, 1.1, and 1.9 μmol/mL h for Na_2HPO_4 (12:7), (7:2), and (2:0), respectively.

Increased rates of acyl migration for reactions with hydrogen phosphate salts included were only observed in the presence of the acid. As a conclusion, it appears to be the combination of fatty acid and hydrogen phosphate salt that leads to a higher rate of migration. It is possible that the salt can af-

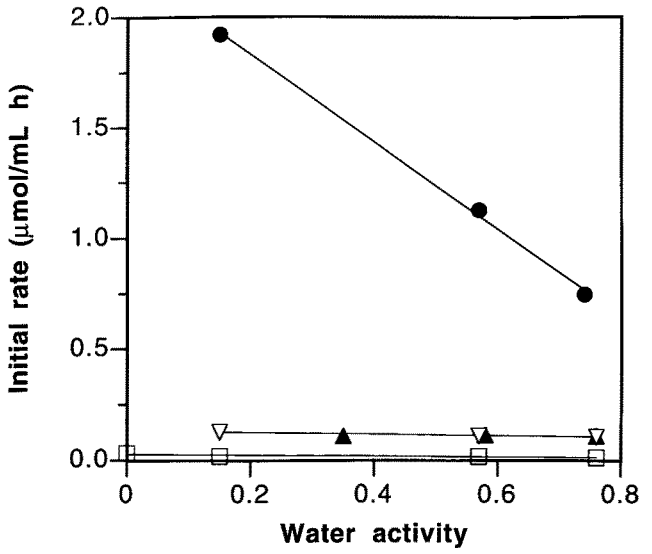

FIG. 1 Initial rate (μmol/mL h) for isomerization of 1,2-di- to 1,3-dibutyrin. In the presence of hexanoic acid and various salt hydrates: hydrogen phosphates (●), and sulfates (▲), and pre-equilibrated to water activity (a_w) = 0.15–0.76 (▽) together with, uncatalyzed acyl migration (□) in hexane.

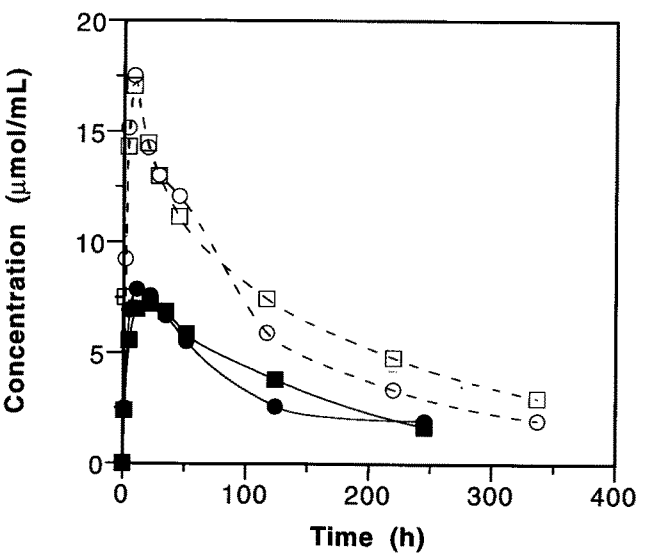

FIG. 2. Progress curves for total dibutyrin. Acidolysis of tributyrin in the presence of Na_2SO_4 (10:0) water activity (a_w) = 0.76 (■) and Na_2HPO_4 (12:7) a_w = 0.74 (●). Interesterification of tributyrin in the presence of Na_2SO_4 (10:0) a_w = 0.76 (□) and Na_2HPO_4 (12:7) a_w = 0.74 (○).

fect the ionization state of the acid, and thereby the rate of migration. Hydrogen phosphate is more alkaline than sulfate.

The effects observed for separate components do not necessarily give the same effect in a reaction system. The salt hydrates were therefore used in regioselective lipase-catalyzed acidolysis and interesterification of tributyrin with hexanoic acid and ethyl hexanoate.

The rate of acyl migration was examined by comparing the production of 1,3-dibutyrin for reactions with hydrogen phosphate salts and sulfate salts with similar a_ws. The first step in the transesterification is regioselective hydrolysis of tributyrin. This will yield 1,2-dibutyrin, and 1,3-dibutyrin will be formed by migration. The production of 1,3-dibutyrin will depend on both the degree of hydrolysis and the rate of acyl migration. If the production of the total dibutyrin is the same, as could be assumed at similar a_ws, then differences in the production of 1,3-dibutyrin can be attributed to different rates of acyl migration.

In this work, the production of 1,3-dibutyrin has been evaluated. This avoids any indication about the pathway of isomerization in diglycerides (mentioned in the Introduction section).

Production of 1,3-dibutyrin. A comparison was made between the reaction profiles of 1,3-dibutyrin formed in acidolysis and in interesterification of tributyrin. These profiles were obtained from reactions that were performed in the presence of the following salts: Na_2SO_4 (10:0) a_w = 0.76 and Na_2HPO_4 (12:7) a_w = 0.74, $ZnSO_4$ (7) a_w = 0.58 and Na_2HPO_4 (7:2) a_w = 0.57.

The reaction profiles of total dibutyrin in both acidolysis and interesterification were similar in reactions with either Na_2HPO_4 (12:7) a_w = 0.74 or Na_2SO_4 (10:0) a_w = 0.76 (Fig. 2). In contrast, the production of 1,3-dibutyrin was higher in reactions with Na_2HPO_4 (12:7) a_w = 0.74 than in reactions with Na_2SO_4 (10:0) a_w = 0.76 in acidolysis (Fig. 3). The differences were considerably less in interesterification (Fig. 3). Similar results were obtained for reactions with $ZnSO_4$ (7) a_w = 0.58 and Na_2HPO_4 (7:2) a_w = 0.57 (Figs. 4 and 5).

Because the reaction profiles of total dibutyrin were similar for the reactions compared, the higher production of 1,3-dibutyrin in acidolysis when hydrogen phosphate salts were included may be attributed to a higher rate of acyl migration. Differences were also observed in interesterification, but the initial production of 1,3-dibutyrin was the same. Free fatty acids produced by hydrolysis may be the reason for this effect.

The initial production of 1,3-dibutyrin in acidolysis and interesterification with salt hydrates was compared with reactions which were pre-equilibrated to the same a_w (data not shown). The results for the reactions pre-equilibrated to a_w = 0.76 and 0.57 corresponded to the results obtained for reactions with Na_2SO_4 (10:0) a_w = 0.76 and $ZnSO_4$ (7) a_w = 0.58 included. This indicates that Na_2SO_4 (10:0) and $ZnSO_4$ (7) do not significantly influence the rate of acyl migration.

Production of tricaproin. Migration can also be examined on the basis of triglycerides produced by this reaction. In the reactions conducted here, tricaproin is a product of migration. The content of tricaproin in acidolysis and in interesterification (same reaction conditions as before) was measured after 14 d. The relative amounts are shown in Figure 6.

The content of tricaproin was higher in acidolysis with Na_2HPO_4 (7:2) a_w = 0.57 than with $ZnSO_4$ (7) a_w = 0.58. The content of tricaproin was also high in acidolysis with Na_2HPO_4 (2:0) a_w = 0.15. There were no differences in acidolysis between reactions with Na_2HPO_4 (12:7) a_w = 0.74 and

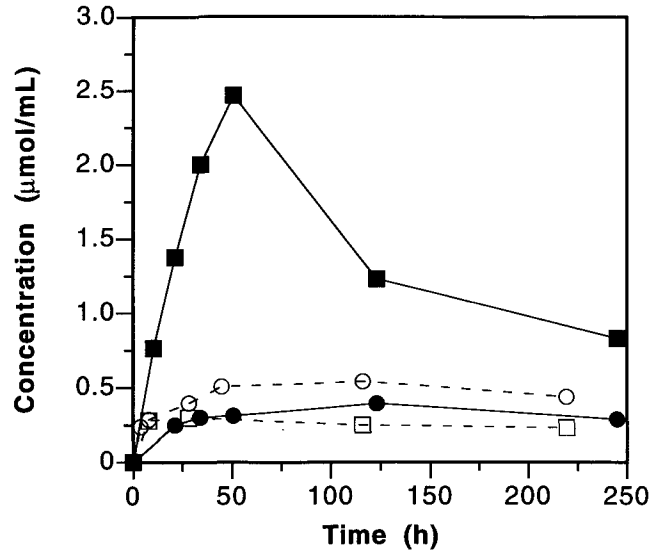

FIG. 3. Progress curves for 1,3-dibutyrin. Acidolysis of tributyrin in the presence of Na_2SO_4 (10:0) $a_w = 0.76$ (●) and Na_2HPO_4 (12:7) water activity (a_w) = 0.74 (■); interesterification of tributyrin in the presence of Na_2SO_4 (10:0) $a_w = 0.76$ (□) and Na_2HPO_4 (12:7) $a_w = 0.74$ (○).

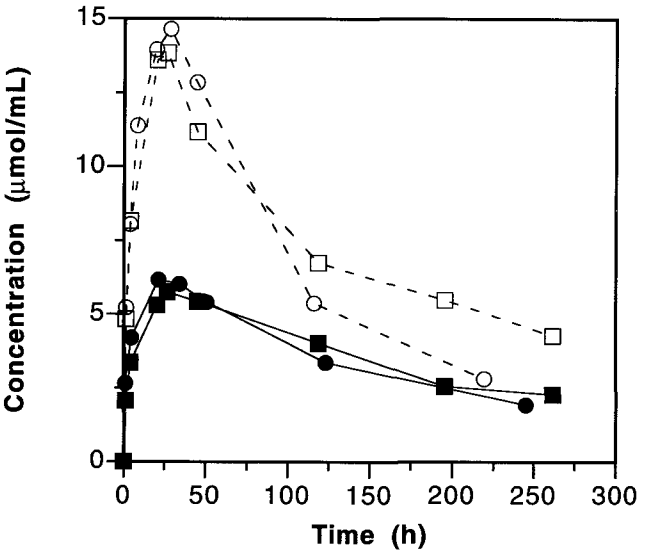

FIG. 5. Progress curves for 1,3-dibutyrin. Acidolysis of tributyrin in the presence of $ZnSO_4$ (7) water activity (a_w) = 0.58 (■) and Na_2HPO_4 (7:2) $a_w = 0.57$ (●). Interesterification of tributyrin in the presence of $ZnSO_4$ (7) $a_w = 0.58$ (□) and Na_2HPO_4 (7:2) $a_w = 0.57$ (○).

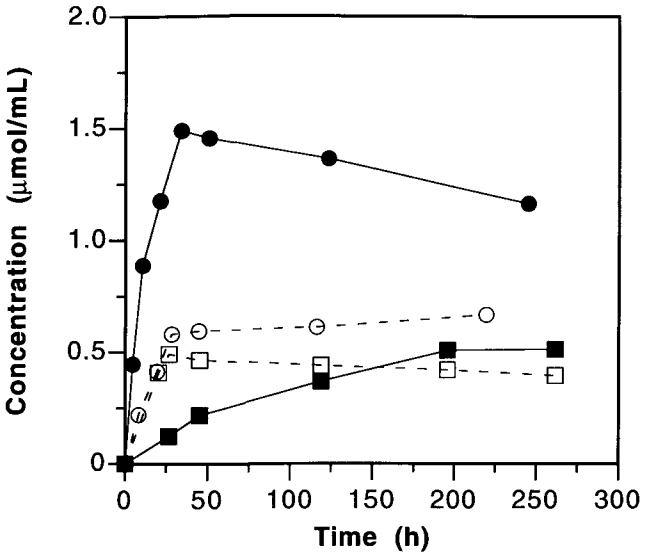

FIG. 4. Progress curves for total dibutyrin. Acidolysis of tributyrin in the presence of $ZnSO_4$ (7) water activity (a_w) = 0.58 (■) and Na_2HPO_4 (7:2) $a_w = 0.57$ (●). Interesterification of tributyrin in the presence of $ZnSO_4$ (7) $a_w = 0.58$ (□) and Na_2HPO_4 (7:2) $a_w = 0.57$.

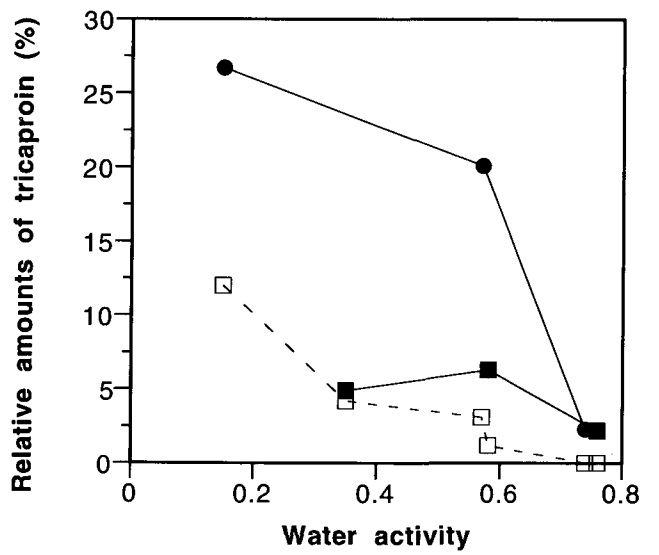

FIG. 6. Relative amounts of tricaproin after 14 d given as percentage of the total amount of triglycerides. Acidolysis with various salt hydrates, hydrogen phosphates (●) and sulfates (■) included, and interesterification with both types of salt hydrates included (□).

Na_2SO_4 (10:0) $a_w = 0.76$; the high degree of hydrolysis at the time of measurement may be the reason why only small effects were observed with respect to the production of tricaproin at the higher a_ws. Only minor differences in the content of tricaproin were observed in interesterification when Na_2HPO_4 (7:2) $a_w = 0.57$ and $ZnSO_4$ (7) $a_w = 0.58$ were compared. The relative amounts of tricaproin ranged from 7 to 15% in both acidolysis and interesterification reactions preequilibrated to $a_w = 0.76$, 0.57, and 0.15 (data not shown).

DISCUSSION

The rate of fatty acid-catalyzed acyl migration in 1,2-dibutyrin was higher when hydrogen phosphate salts were present compared to those with sulfate salts or with no salts. Because migration is not directly catalyzed by hydrogen phosphate salts, it can be concluded that it is the combination of fatty acid and hydrogen phosphate salts that leads to an increase in the rate of acyl migration.

The same effect of the nature of the salt hydrate on the rate of acid-catalyzed acyl migration was found when these salts were used in lipase-catalyzed transesterification. The use of solid salt hydrates is a convenient and efficient method to control a_w during a reaction and may be used in lipase-catalyzed reactions with glycerides. In view of the results obtained here, hydrogen phosphate salts are not recommended in reactions where free fatty acids are present if acyl migration is to be minimized. Their use in reactions with fatty acid esters should also be avoided because free fatty acids will be produced by hydrolysis. Phosphate salt deposited on the enzyme preparation after immobilization has also been observed to affect the rate of acyl migration (11).

Acyl migration is commonly connected with hydrolysis and the presence of water in the reaction system. The influence of water on the level of partial glycerides through the hydrolytic equilibrium is well known. However, the influence on the rate of acyl migration is less clear.

An increase was obtained for hexanoic acid-catalyzed acyl migration when Na_2HPO_4 (12:7) was present compared to when hexanoic acid alone was present and when no water was added (7). In view of the present results, this is attributed to the nature of the salt hydrate rather than to the a_w. The rate was strongly dependent on the a_w in acid-catalyzed acyl migration in 1,2-dibutyrin in the presence of hydrogen phosphate salts (Fig. 1). Whether this is an effect of a_w, or is influenced by the salt hydrate at different a_ws, has to be examined. Further research is necessary to reveal the precise dependence of the rate of acyl migration on the a_w.

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