INTERMOLECULAR SPECIFICITY OF PANCREATIC LIPASE AND
THE STRUCTURAL ANALYSIS OF MILK TRIGLYCERIDES

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SUMMARY
Pancreatic lipase released butyrate and palmitate in equimolar quantities
from glyceryl 1-palmitate 2,3-dibutyrate (PBB), an absence of intramolecular
specificity. However, when glyceryl 1-oleate 2,3-dioleate (MOO) was mixed
with PBB, the latter was hydrolyzed more rapidly, indicating intermolecular
specificity. Primary position specificity was maintained during 5-min diges-
tions when glyceryl 2-butyrate 1,3-dipalmitate was the substrate. Data from
the pancreatic lipase digestions of milk fat are presented. It is concluded that
pancreatic lipase digests some classes of milk triglycerides more rapidly than
others.

Pancreatic lipase, which is highly specific
for the primary esters of glycerides, has been
used to study the structure of milk triglycer-ides (TGs) (2, 5, 12, 18, 19). However, Des-
nuelle and coworkers (10, 11) found that this
enzyme caused the preferential release of short-
chain acids from glycerides containing both
long- and short-chain acids. We have desig-
nated this type of specificity as intramolecular
and define it as a major difference in the rate
of release of one primary ester as compared
to the other primary ester (13). Clement et al.
(4) observed migration of butyrate from the
secondary to the primary position during long
digestions (2 hr) and more rapid digestion of
short-chain acids where long-chain TGs were
combined with TGs containing both long- and short-chain acids. We have
confirmed this type of specificity as intramolecular
and define it as a major difference in the rate
of release of one primary ester as compared
to the other primary ester (13). Clement et al.
(4) observed migration of butyrate from the
secondary to the primary position during long
digestions (2 hr) and more rapid digestion of
short-chain acids where long-chain TGs were
combined with TGs containing both long- and short-chain acids. It is apparent that
if pancreatic lipase differentiates between long-
and short-chain acids attached to the primary
positions of the same triglyceride (intramo-
lecular specificity), or if the enzyme differen-
tiates between classes of TGs (intermolecular
specificity), then results obtained on milk tri-
glycerides must be viewed with reservation. In
support of this, Coleman (6) has recently ob-
tained evidence that pancreatic lipase prefer-
entially attacked unsaturated TGs when lard
was the substrate.

Stimulated by our finding that milk lipase
did not exhibit intramolecular specificity when
glyceryl 1-palmitate 2,3-dibutyrate (PBB) was
the substrate (14), we have carried out similar
studies with PBB and pancreatic lipase. PBB
was combined with glyceryl 1-oleate 2,3-
dioleate (MOO) and the equimolar mixture
digested to investigate intermolecular speci-
ficity. Glyceryl 2-butyrate 1,3-dipalmitate
(PBP) was employed as a substrate to check
migration of butyrate. Milk fat was digested
for varying periods of time to determine if
either type of specificity existed with this sub-
strate. Results are reported and discussed
herein.

EXPERIMENTAL PROCEDURE
The synthetic triglycerides PBB and MOO
were prepared as previously described (14)
and purified by column chromatography. PBP
was synthesized from 1,3-dipalmitin (17) and
butyryl chloride and purified by crystallization
from acetone. All operations were monitored
by thin-layer chromatography (TLC). Milk
fat was obtained by churning fresh cream,
melting the butter at 55°C, and filtering the
oil. Two digestions of PBB and MOO, three
of PBB, five of equimolar mixtures of PBB
and MOO, and three of milk fat were completed
as described (15), except that NH₄Cl-NH₃OH
buffer replaced Tris buffer and 400 mg of
substrate used. In preliminary work, 25
digestions of PBB were carried out. Synthetic
substrates were incubated at 38°C for 5 min
and milk fat for 5, 10, and 15 min with enzyme-
less controls. In addition, one portion of the
milk fat was digested for 1 hr with 120 mg of
lipase. Samples were extracted using 250 ml
of CHCl₃:MeOH (9:1) (15). The solvent ex-
tract was divided into two equal portions and


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one portion titrated immediately. The solvent was removed from the other portion and the long-chain FFAs estimated by use of the Keeney column (1) and further analyzed by gas-liquid chromatography as methyl esters. The short-chain FFAs were determined by difference. Solvent was removed from the total titration and the material applied to two TLC plates (20 by 20 cm) coated with a 1-mm layer of silica gel G. The plates were developed with petroleum ether (30-60 C):ethyl ether:acetic acid (90:30:1) (3), the bands visualized with iodine vapor, and removed by scraping. The fats were extracted with ethyl ether and the fatty acid composition of each fraction; TGs, FFAs, DGs, and MGs determined (14). Only the MG fraction of the 60-min digest was analyzed, to ascertain if the composition of the MGs varied with time. Glycerol content, since the MGs contained largely 4:0, there was little loss of positional specificity. When PBP was the substrate, results were very close to those calculated from theory. There was no loss of positional specificity if a short reaction time was employed. This is in contrast to the findings of Clement et al. (4), who noted a loss of positional specificity when PBP was incubated for 2 hr. MOO as a substrate yielded FFAs and glycerides of the composition expected.

To study intermolecular specificity, PBB and MOO were mixed in approximately equimolar quantities. Fatty acid compositions of the FFAs and glycerides produced by the action of pancreatic lipase on the mixture are presented in Table 2. To clarify intra- and intermolecular specificity, calculations labeled intra- and intermolecular are included which represent, respectively, the relative M% of acids within and between the two TGs. There was no apparent intramolecular specificity, since approximately equimolar quantities of 4:0 and 16:0 and 14:0 and 18:1 were released from PBB and MOO, respectively. The compositions of the DGs and MGs also agreed closely with theoretical values (see Table 1). As evidenced by comparisons of the intermolecular figures, preference was for the TG containing 4:0, PBB. Intermolecular specificity is most clearly illustrated by the composition of the FFAs; the more rapid release of 4:0 and 16:0 as compared to 14:0 and 18:1. The other composition figures also mirror intermolecular specificity; the more rapid lipolysis of PBB. Finally, positional specificity was maintained.

The concept of intermolecular specificity described above and illustrated in Table 2 is in contrast to the generally accepted theory of

TABLE 1

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<tr>
<th>Substrate</th>
<th>Control TG</th>
<th>Residual TG</th>
<th>DG</th>
<th>MG</th>
<th>FFA</th>
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RESULTS AND DISCUSSION

In Table 1 are presented the fatty acid compositions of PBB, MOO, and PBP and of the glycerides and FFA derived from the individual digestion of these compounds. Included are theoretical values assuming positional but no intramolecular specificity. Palmitate and butyrate were released from PBB in approximately equimolar quantities. The slightly higher concentration of 16:0 in the FFA was reflected by a higher than theoretical value for 4:0 in the DGs. Under the digestion conditions employed with the 5-min incubation period, pancreatic lipase did not differentiate between 4:0 and 16:0 attached to the 1- and 3-positions of the same triglyceride. Thus, there was little or no intramolecular specificity. On the other
intramolecular specificity for short-chain acids (10). This assumption has been based almost completely on the following evidence: (a) Preferential release of 4:0 from the diglyceride, glyceryl 1-butyrate 3-palmitate (PB) (11). While several attempts to prepare the glyceride have been made by us, these were unsuccessful with respect to obtaining a product which contained a marker acid in the 2-position after acylation and redigestion. It is entirely possible that 4:0 may be released preferentially from PB, but results in Table 2 do not support the contention that 1,2 PB preferentially yields 4:0. The short period of digestion used would reduce the comparatively rapid acyl migration of 2-position short-chain acids (10). (b) Relatively rapid hydrolysis of tributyrin as compared to longer-chain simple TGs (10). Because of the great difficulty in obtaining similar emulsions and, therefore, digestion conditions, these comparisons should not be made. (c) Lipolysis of a random mixture of 4:0 and 18:1 chains prepared by interesterification yielded 76 and 24 M% of the two acids (11). At first glance, intramolecular specificity can explain these results, but intermolecular specificity can also. A random mixture prepared from equimolar quantities of 4:0 (B) and 18:1 (O) would contain, based upon expansion of the binomial equation, 12.5% tributyrin (BBB), 12.5% BOB, 25% BBO, 25% OOB, 12.5% OBO, and 12.5% triolein (OOO). Since tributyrin is digested about three times faster than triolein (10), BBB and probably BOB would be attacked at this rate. If intermediate rates are assigned to BBO and OOB, and lower rates for OBO and OOO, FFAs with compositions of about 70% B and 30% O can be obtained. These are not greatly different from the figures above and are based on intermolecular specificity. In this regard, Coleman and Fulton (9) have noted what appears to be intramolecular specificity with preferential release of oleate when glyceryl-1 oleate 2-palmitate 3-stearate was the substrate.

The results of Clement et al. (4) can be interpreted as a preferential lipolysis of TGs containing butyrate, but it is not possible to decide between intra- and intermolecular specificity from their data. This also applies to the findings of Coleman (7, 8). To differentiate between the two types of specificities, it is necessary to use a mixture of two unsymmetrical TGs, as we have done.

Apparent intramolecular specificity and loss of positional specificity were the objections to the use of pancreatic lipase for the study of milk TG structure. Data in Tables 1 and 2 and accompanying discussion emphasize the absence of intramolecular specificity and the retention of positional specificity during short digestions of synthetic TGs containing butyrate. However, another factor, intermolecular specificity, was discovered. Heretofore, the concept of intermolecular specificity has not been applied to the results obtained by the lipolysis of milk fat. Accordingly, milk fat was lipolyzed for 5, 10, 15, and 60 min with a fourfold quantity of pancreatic lipase added to the 60-min trial and the fatty acid composition of the intact and residual TGs, DGs, MGs, and FFA reported in Table 3. Extent of digestion, as per

### TABLE 2

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<th>Substrate and products of lipolysis</th>
<th>Fatty acids M%</th>
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*Intramolecular refers to the relative M % of fatty acids within each TG, whereas intermolecular refers to the relative M % of acids between TGs.*
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Table 3: Fatty acid composition of the intact triglycerides and the products of pancreatic lipolysis of milk fat.

*M %

*Minutes of digestion.

b Estimated by column chromatography.
cent of available esters was 25.8, 33.6, 37.8, and 90.8, respectively. The last figure was calculated on the basis of all three TG positions, because of the extensive hydrolysis. About 0.5 μM of glycerol was present after each of the 5-, 10-, and 15-min trials. Presence of substantial amounts of glycerol would have implied that considerable acyl migration could have occurred.

The specificity of pancreatic lipase for some of the milk TGs containing butyrate was apparent from the data in Table 3. Apparently, because of intermolecular specificity, these fast TGs were hydrolyzed with greater velocity than the residual or slow TGs. Residual TGs should always be examined. The difference in 4:0 content between intact and residual TGs was appreciable and this is the most rigorous evidence that the fast TGs contained larger than random amounts of butyrate. Interestingly, intermolecular specificity did not appear to mediate the lipolysis of TGs containing 6:0 and 8:0, which may be due to the relatively small quantities of these acids in the intact fat.

It is impossible to decide between intra- and intermolecular specificity from the data in Table 3. However, if the concept of intermolecular specificity gained from the data in Tables 1 and 2 applies to milk fat, then pancreatic lipase attacks certain classes of TGs more readily than others and the utility of this enzyme for the study of milk TG structure is limited. The changing composition of the FFAs with time precludes using these data to calculate the distribution of fatty acids in milk fat, at least on an over-all basis, although distribution of the fast TGs can be calculated (2).

Because of changing composition, MGs cannot be used as proposed by Coleman (6, 7, 9). Furthermore, the quantity of MGs, obtained colorimetrically (15), at 5, 10, and 15 min represented only 8, 10, and 15% of the 2-position esters present. If the length of digestion is extended, or a more concentrated enzyme used, then appreciable glycerol has been reported (7, 12) to be produced which, in turn, indicated acyl migration and apparent loss of positional specificity. The MGs after 60 min consisted largely of saturated acids, a marked change in composition. If the composition had not changed, then the MG data could have been used to calculate distribution.

Therefore, the data in Table 3, except for the 60-min MGs, represents those acids associated with the fast TGs. In this class of TGs, the butyrate is essentially located in Position 1 (or 3), whereas 14:0 and 16:0 are apparently occupying Position 2 to a greater extent. Distribution of individual acids was nonrandom as calculated by Coleman's method (6). Distribution of the acids in Positions 1 and 3 or 2 as saturates (S) or unsaturates (U) was random (2, 6-8). Use of U and S in these calculations obscures the nonrandom distribution of individual acids. These statements apply only to the fast TGs and are invalid for the intact milk fat.

The residual TGs were not devoid of 4:0, containing 6:9 M% after 15 min. This agrees with Jack et al. (12), who noted a similar 4:0 content after two-thirds hydrolysis. Some of this butyrate could be associated with disaturated TGs which would be digested relatively slowly and some may well be in Position 2. Kumar et al. (16) presented evidence that butyrate was located exclusively at the primary position of milk TGs. Clement et al. (5) recently concluded that at least 75% of the butyrate in milk fat was located in the 1-position, with some of the remainder possibly in the 2-position. Preferential attack of TGs containing butyrate was also noted.

Investigators studying the structure of milk fat TGs with pancreatic lipase should realize that short periods of digestion yield information essentially about the fast TGs. Longer periods are accompanied by acyl migration, which obscures positional specificity (10). The problem can possibly be approached by previous fractionation of milk fat, either by column chromatography as was done by Clement et al. (5) or by fractional crystallization. It might even be more profitable to use an esterase which preferentially releases butyrate from milk fat and then analyze the products of digestion. Pregastric esterase will accomplish this (20), and thus studies are in progress on the specificity of this enzyme toward synthetic and milk TGs.

REFERENCES

(4) CLEMENT, G., CLEMENT, J., AND BEZARD, J. Action of Human Pancreatic Lipase on Synthetic Mixed Symmetrical Triglycerides