Digestion of Butyrate Glycerides by Pancreatic Lipase[1,2]

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ABSTRACT

The racemic triglycerides, glyceryl-1-palmitate-2,3-dibutyrate (PBB), glyceryl-1-butyrate-2,3-dipalmitate (PPB), glyceryl-2-butyrate-1,3-dipalmitate (PBP), and the diglyceride, racemic glyceryl-1-palmitate-3-butyrate (P-B) were synthesized and digested with pancreatic lipase. Each triglyceride was mixed with equimolar amounts of triolein (OOO) prior to incubation.

The following order of digestion rates was observed: PBB > PPB > PBP > P-B. There was no evidence for short-chain fatty acid specificity; however the triglycerides containing butyric acid were hydrolyzed more rapidly than OOO. Based upon the fatty acid composition of partial glycerides, digestion of butyrate glycerides was not a simple phenomenon. For example, in the digestion of PBB, butyric acid accumulated faster than palmitic acid in the diglycerides, and monobutyrin was found to accumulate when the diglyceride, P-B, was digested. As evidenced by the fatty acid composition of the monoglycerides, positional specificity of pancreatic lipase was always maintained.

INTRODUCTION

MILK LIPASE was found to digest glycerides in a manner almost identical with pancreatic lipase (1). Nevertheless one major difference appeared to exist between these otherwise similar lipolytic enzymes. Entressangles and co-workers (2) concluded that pancreatic lipase possessed a short-chain fatty acid specificity, but this laboratory found that milk lipase did not differentiate between a short- and long-chain fatty acid attached to the primary positions of the same triglyceride (3).

This difference was confusing since it had been observed that both of these lipases yielded similar fatty acid patterns in the products of lipolysis from the digestion of milk fat, namely, large quantities of butyric acid (4:0) in the free fatty acids and lesser quantities of 4:0 in the residual triglyceride as compared with the original triglyceride. Thus, contrary to expectations, the milk enzyme gave results which could be interpreted as short-chain fatty acid specificity.

At that time it was not understood how milk lipase could preferentially release butyric acid from the mixed natural fat and not from a synthetic triglyceride, such as racemic glyceryl-1-palmitate-2,3-dibutyrate (PBB). These paradoxical results were reconciled when the milk enzyme was incubated with an equimolar mixture of the synthetic triglycerides, PBB and triolein (OOO), and greater quantities of both palmitic acid (16:0) and 4:0 compared with oleic acid (18:1) were released but not more 4:0 relative to 16:0 (4). In considering lipolysis of the butyrate triglyceride, there was not a preferential digestion of the 4:0 linkage but instead a more rapid hydrolysis of the entire triglyceride as compared with OOO. This differential rate of lipolysis between glycerides was termed intermolecular specificity, as contrasted to fatty acid specificity or intramolecular specificity, but is more correctly defined as nonrandom hydrolysis of glyceride classes.

The results of the lipolysis of milk fat by the milk enzyme could now be attributed to intermolecular specificity for the butyrate triglycerides, not necessarily to a simple preferential digestion of the butyrate ester bonds. It was a necessary, logical step to ask whether the data reported for the pancreatic lipolysis of short-chain fatty acid triglycerides were in actuality the result of intermolecular specificity and not short-chain fatty acid specificity. Indeed, the authors are convinced that this is the case and have already presented some preliminary data which support this hypothesis (5). Nevertheless data in the literature (2) can only be interpreted as short-chain fatty acid specificity, suggesting that these results with PBB may have been a special case. Therefore the research reported herein was initiated to explore more fully the behavior of pancreatic lipase in the hydrolysis of butyrate glycerides.

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MATERIALS AND METHODS

Substrates

Triolein (OOO) was purchased from the Hormel Institute (Austin, Minn.) and purified by elution through alumina (6). Racemic glyceryl-1-palmitate-2,3-dibutyrate (PBB), racemic glyceryl-1-palmitate-3-butyrate (P-B), and glyceryl-2-butyrate-1,3-dipalmitate (PBP) were prepared by reacting 1-monopalmitin with the appropriate quantities of acid chloride. The synthesis of PBP involved the intermediate, 1,3-dipalmitin. All intermediates and final products, including the 1-monopalmitin, were synthesized according to established procedures (7,8).

Racemic glyceryl-1-butyrate-2,3-dipalmitate (PPB) was prepared by reacting the diglyceride, P-B, with palmitoyl cholride or by reacting glyceryl-1,2-dipalmitin with butyryl chloride (7); the dipalmitin was synthesized from palmitoyl chloride and 1-tetrahydropyranyl glyceryl ether essentially as described by Krabisch and Borgstrom (9) and was purified by crystallization from petroleum ether (30-45C) at room temperature.

PBB, PBP, and PPB were purified by elution through alumina (6). P-B was purified by procedures outlined by Mattson and Volpenhein for 1,3-diglycerides (7) except that the temperature of crystallization was –25C. As determined by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) (8,10), the purity of all intermediates and final glycerides, including the OOO, was estimated to approach 99%.

Digestion Conditions

Each of the butyrate triglycerides (1.05 millimoles) was mixed with an equimolar quantity of OOO, 56 ml of 0.25 molar Tris buffer (pH 8.0) containing 10% w/v gum arabic, 3.5 ml of 4 molar $CaCl_2$, and 1.4 ml of 1% w/v bile salts. The mixtures were emulsified with the aid of a Waring Blendor and distributed so that each digestion flask contained approximately 300 micromoles of substrate, 8 ml of Tris buffer, 0.5 ml of $CaCl_2$, and 0.2 ml of bile salts. Samples were incubated in the presence of 25 mg of pancreatic lipase for 2.5, 5, and 10 min at 37C. The diglyceride substrate, P-B, was treated in essentially the same manner except that no OOO was used; 2.5- and 15-minute incubation periods were employed, and each sample contained 600 micromoles of substrate and 50 mg of pancreatic lipase. Duplicate samples of each substrate was analyzed at each time-interval. In addition, one control containing no enzyme was incubated for each substrate at the longest time-interval employed. The enzyme preparation and the general enzymatic procedure have been described (11).

Extraction and Analysis

Samples were extracted with chloroform-methanol (9:1) by a procedure adapted from Harper et al (12). Digestion mixtures were acidified with 0.5 ml of 20% H_2SO_4 and carefully rinsed with water and ethyl ether into a casserole containing activated silicic acid. The sample was triturated and mixed with 75 ml of extracting solvent; the resulting slurry was transferred to a column fitted with a sintered glass disc. The sample was eluted from the silicic acid with the aid of a vacuum from a water aspirator. Before the column was dry, the casserole was rinsed with 175 ml of solvent, and this was used to elute the sample completely. After extraction the samples were divided into two equal portions. One portion was separated by preparative TLC (5) into residual triglyceride, diglyceride, monoglyceride, and free fatty acids; the other portion was used to determine 4:0 in the free fatty acids as described below. Fractions separated by TLC were converted to butyl esters and analyzed by temperature-programmed GLC (10). Free fatty acids were esterified by refluxing in butanol with H_2SO_4 as the catalyst (13); neutral lipids were esterified with sodium butoxide in the presence of dibutyl carbonate (10).

Estimation of Butyric Acid in the Free Fatty Acids

One-half of the extracted sample was first titrated to the thymol blue end-point to determine total free fatty acids. Then the solvent was removed with the aid of a rotary flash evaporator, ca 10 mg of 4:0 were added to facilitate discrimination during chromatography, and the contents were acidified and separated by conventional column chromatography according to the AOAC procedure for the estimation of 4:0 (14). The long-chain fraction was collected and titrated; 4:0 was calculated by the difference between the total titration and the long-chain titration. The ratio of 16:0 to 18:1 in the latter fraction was obtained from GLC data. Several "digestion mixtures" containing known amounts of 4:0 and 16:0 were prepared and conducted through the extraction and free fatty acid estimation steps to determine the recovery of 4:0 by this method. Prior to extraction, each of these samples was mixed with 0.5 ml of $CaCl_2$, 0.2

TABLE I

Recovery of Standard Mixtures[a] of Butyric and Palmitic Acid

Samples analyzed	Fatty Acid Added 16:0	Fatty Acid Added 4:0	Fatty Acid Recovered[b] Total[c]	16:0[d]	4:0[e]	Mole Percentage 4:0 Added	Found	Recovery
	Microequivalents			Percentage				
8[f]	74.6	77.7	99.3	93.4	105.3	51.0	54.1	106
3	287.9	58.0	90.9	89.8	95.5	16.8	17.6	105
3	375.4	48.9	97.3	95.0	115.1	11.5	13.6	118
2	198.4	73.9	90.0	91.3	86.6	27.1	26.1	96
2	151.0	198.0	91.6	99.3	86.0	56.7	53.2	94
2	149.8	152.2	100.1	98.9	101.2	50.4	51.0	101
1	223.0	325.0	97.9	101.2	95.1	59.3	57.8	98
1	263.3	146.6	92.7	91.3	95.4	35.8	36.8	103
Average % recovery			95.0	95.0	97.5	....	....	103

[a] These were mixed with 0.5 ml of $CaCl_2$, 0.2 ml of 1% bile salts, and 10 ml of 10% gum arabic in pH 8.0 Tris buffer.

[b] These are averages of number of samples analyzed.

[c] Recovery after extraction step is shown.

[d] Recovery includes extraction and column chromatography.

[e] The 4:0 contents were calculated by difference [total free fatty acids-long-chain fatty acids].

[f] These samples also contained 125 mg of purified olive oil triglycerides.

ml of 1% bile salts, and 10 ml of pH 8.4 Tris buffer containing 10% gum arabic, then was allowed to incubate with shaking at 37C for 5 min. Eight of these samples also contained 125 mg of purified olive oil triglycerides (6). A total of 22 samples was analyzed. Data concerning the ratios and microequivalents of fatty acid added as well as the recovery values are recorded in Table I.

RESULTS AND DISCUSSION

As can be seen in Table I, the recoveries for 4:0, on an absolute basis, varied from 86-115% but, on the average, were slightly higher than those for 16:0. This can be attributed to the extra step of column chromatography required for the determination of 16:0. Butyric acid, in this method, can be lost only before the total titration step, thus the recovery of 4:0 relative to 16:0 is generally high (last column, Table I) and averages 103%. The addition of olive oil triglycerides did not appreciably alter the recoveries.

The recovery of total fatty acids in this silica gel extraction method was 95% and was comparable with those previously reported (12). No attempt was made during this study to ascertain the recoveries of each individual digestion product; however, based on previous work (15,16), the average recoveries of all products were judged to be 90% or greater. An estimate of the relative recovery of the triglycerides can be made by comparing the compositions of these fats from the intact and enzymeless controls (Table II). The values for the intact fat were from GLC analyses of butyl esters of an aliquot of the digestion mixture prior to extraction whereas those for the enzymeless control were based on similar analyses after extraction and TLC. Some loss of oleate triglyceride did occur (average recovery = 97.7%) and may have been caused by oxidation during TLC as noted by Nichaman et al. (17). From data in Tables I and II it was concluded that the method was suitable for the analyses under investigation.

The data from the pancreatic lipase digestions are summarized in Tables III-VI as mole percentage of each product in each fraction from TLC. The results from the triglyceride mixtures are compared with theoretical values calculated from control samples by assuming the absence of both fatty acid and intermolecular specificity. In the runs involving PBB and OOO (Table III) it was apparent that PBB had been preferentially hydrolyzed as, after 10 minutes of digestion, only 16.9 M% remained compared to an initial value of 52.9 M%.

TABLE II

Fatty Acid Composition of Synthetic Triglycerides Before[a] and After[b] Extraction and Thin-Layer Chromatography

Sample		4:0	16:0	18:1
		Mole percentage		
PBB + OOO[c]	Before	33.1	16.9	50.0
	After	35.3	17.5	47.2
PPB + OOO[c]	Before	16.2	32.4	51.4
	After	16.5	32.9	50.6
PBP + OOO[c]	Before	17.4	34.7	47.9
	After	16.6	35.2	48.2

[a] Before = intact TG sampled immediately after mixing triolein with the butyrate triglyceride.

[b] After = enzymeless control sample.

[c] P = palmitate, B = butyrate, O = oleate.

TABLE III

Some Lipolytic Products from Equimolar Mixture of PBB + OOO[a]

Minutes	Residual Triglyceride		Diglyceride Mole percentage		
	PBB	OOO	PB–	–BB	–OO
2.5	45.2	54.8	20.8	52.1	27.1
5.0	29.2	70.8	21.6	48.7	29.7
10.0	16.9	83.1	13.6	46.6	39.8
Theory	52.8	47.2	26.4	26.4	47.2
Minutes	Monoglyceride		Free fatty acid		
	–B–	–O–	P	B	O
2.5	50.0	50.0	35.9	31.3	32.8
5.0	61.6	38.4	33.5	28.1	38.4
10.0	58.4	41.6	28.2	28.8	43.0
Theory	52.8	47.2	26.4	26.4	47.2

[a]These were incubated with 25 mg of pancreatic lipase at 37C; P = palmitate, B = butyrate, O = oleate.

TABLE V

Some Lipolytic Products from Equimolar Mixture of PBP + OOO[a]

Minutes	Residual Triglyceride		Diglyceride Mole percentage	
	PBP	OOO	PB–	–OO
2.5	49.8	50.2	53.6	46.4
5.0	49.8	50.2	47.1	52.9
10.0	44.5	55.5	49.6	50.4
Theory	51.8	48.2	51.8	48.2
Minutes	Monoglyceride		Free fatty acid	
	–B–	–O–	P	O
2.5	73.7	26.3	61.9	38.1
5.0	58.7	41.3	58.9	41.1
10.0	56.2	43.8	55.7	44.3
Theory	51.8	48.2	51.8	48.2

[a]These were incubated with 25 mg of pancreatic lipase at 37C; P = palmitate, B = butyrate, O = oleate.

This decrease in butyrate triglyceride could have resulted from specificity for either triglyceride or fatty acid. Examination of the free fatty acid data however eliminates the possibility of a simple specificity for butyric acid which would require a greater than theoretical content of 4:0. Instead, both 16:0 and 4:0 were released in greater than theoretical amounts. These results are inconsistent with a specificity for butyric acid but can be explained by a more rapid hydrolysis of the butyrate triglyceride with equal lipolysis rates of either primary ester linkage.

The pattern in the partial glycerides is more difficult to interpret. The accumulation of monobutyrin was expected since the butyrate triglyceride was being preferentially digested, and monobutyrin is hydrolyzed relatively slowly compared with longer-chain monoglycerides (18). The diglyceride values however were unexpected. The high values of dibutyrin may be attributable to the partial solubility of this diglyceride in water, which resulted in a preferential digestion of the PB diglyceride. This is partially substantiated by the higher amounts of 16:0 relative to 4:0 in the 2.5- and 5.0-minute free fatty acids.

In Table IV are summarized the results for the digestion mixtures of PPB-OOO. Again, butyrate triglyceride has been preferentially digested, and the free fatty acid data do not support a simple concept of short-chain fatty acid specificity. As for PBB, the diglyceride values are difficult to interpret. The BP diglyceride relative to the dipalmitin should be present in equal amounts; however, at all times sampled, there was relatively more BP present. Also in the free fatty acid data, relatively more 16:0 than 4:0 was released. These results are consistent with a faster digestion rate for the PP diglyceride. As for PBB, it was the diglyceride containing the least butyric acid which appeared to be digested most rapidly and is contrary to what would have been predicted. Similar results would be expected if the natural substrate for pancreatic lipase was the 1,2-diglyceride as compared with the 1,3-diglyceride and if preferential acyl migration occurred so that the diglycerides which contained more butyrate isomerized to the 1,3-diglycerides the fastest. However numerous alternative hypotheses could be formulated, all of which would be equally speculative.

TABLE IV

Some Lipolytic Products from Equimolar Mixture of PPB + OOO[a]

Minutes	Residual Triglyceride		Diglyceride Mole percentage		
	PPB	OOO	BP–	–PP	–OO
2.5	48.7	51.3	37.9	24.5	37.6
5.0	45.1	54.9	44.8	16.1	39.1
10.0	35.2	64.8	34.5	20.1	45.4
Theory	49.4	50.6	24.7	24.7	50.6
Minutes	Monoglyceride		Free fatty acid		
	–P–	–O–	B	P	O
2.5	47.6	52.4	28.5	31.6	39.9
5.0	50.0	50.0	23.4	36.8	39.8
10.0	48.5	51.5	23.6	28.2	48.2
Theory	49.4	50.6	24.7	24.7	50.6

[a]These were incubated with 25 mg of pancreatic lipase at 37C; P = palmitate, B = butyrate, O = oleate.

TABLE VI

Some Lipolytic Products from Glyceryl-1-Palmitate-3-Butyrate[a]

Minutes	Monoglycerides[b]		Free fatty acids[b]	
	Mole percentage			
	—B—	—P—	B	P
2.5	58.0	42.0	39.3	60.7
15.0	66.1	33.9	39.1	60.9

[a]The 240 mg per sample were incubated at 37C with 50 mg of pancreatic lipase.

[b]B=butyrate, P=palmitate.

The results from the PBP-OOO mixtures are displayed in Table V. Evidence for a preferential attack of the butyrate triglyceride was most pronounced in the 10-minute residual triglyceride, the monoglyceride, and the free fatty acids. In the digestions of PBP-OOO it was possible to detect no 4:0 in the free fatty acids and only traces (less than 1 M%) of monopalmitin. These results with PBP contrast those of Clement et al. (19), who found 25 M% 4:0 in the free fatty acids when this glyceride was digested with human pancreatic lipase. However the latter digestions were conducted for two hours as compared with the 10-minute digestions in this study. Undoubtedly acyl migration is a serious problem in lengthy digestions since Clement et al. reported only 2 M% 4:0 in the free fatty acids when the digestions were limited to 20 minutes.

The absence of 4:0 in the free fatty acids and the virtual absence of 16:0 in the monoglyceride derived from PBP in this study are good evidence that the positional specificity of pancreatic lipase can be maintained during digestions involving butyrate glycerides. Also the absence of any 4:0 in the free fatty acids excludes the possibility that the preferential hydrolysis of this butyrate triglyceride is attributable to a short-chain fatty acid specificity.

Some of the results from the digestion of the diglyceride, P-B, are presented in Table VI. In the free fatty acid more 16:0 rather than 4:0 is present, a finding which is in contrast to what has been reported (2). From these results it would be predicted that the pattern in the monoglyceride should be the opposite of the free fatty acid values, that is, if more 16:0 was liberated, then more 4:0 should remain behind in the monoglyceride. This was indeed observed as monobutyrin accumulated.

These results would be expected if the diglyceride isomerized during the digestion process and if this isomerization involved the preferential migration of the 4:0 from the primary to the secondary hydroxyl. The 4:0 present as the secondary position ester would be less available to the pancreatic lipase. However, when these digestion mixtures were checked by boric acid TLC (20) for the presence of 1- and 2-monoglycerides, only the 1-isomer was observed. Furthermore, in experience with synthetic glycerides, the 1,3-diglyceride has been found to be much more stable than the 1,2-diglyceride.

A more plausible explanation for the results in Table VI assumes the preferential digestion of 1-monopalmitin. This is understandable when it is recognized that monobutyrin is water-soluble and that pancreatic lipase is known to act at a water-oil interface (21). Therefore monobutyrin might be relatively unavailable to this enzyme. This is supported by the report (18) that monobutyrin was hydrolyzed more slowly than monolaurin.

In the digestion of P-B it should be pointed out that, compared with the triglyceride substrates, the diglyceride was not hydrolyzed readily. A comparison of the 2.5-minute values, based on the ueq of fatty acid released per minute per 25 mg of enzyme gave: PBB-OOO, 63.4; PPB-OOO, 52.7; PBP-OOO, 40.0; and P-B, 17.5. Similar differences have been noted when other triglycerides and diglycerides were compared (18,22). Nevertheless, when digestion rates of diglycerides were determined in a triglyceride $\rightarrow$ diglyceride $\rightarrow$ monoglyceride reaction (16,23), the diglycerides and triglycerides appeared to be digested at comparable rates. This may simply reflect differences in ease of emulsification, but it is relevant that the natural substrate for pancreatic lipase is the 1,2-diglyceride formed at the interface by lipolysis. Therefore comparisons drawn from separate incubations of 1,2- or 1,3-diglycerides may not be valid.

ACKNOWLEDGMENT

The work was supported in part by Public Health Service Research Grant AM-02605-09 from the Institute of Arthritis and Metabolic Diseases.

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[Received Dec. 13, 1966]