Hydrolysis of primary and secondary esters of glycerol by pancreatic juice

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ABSTRACT The relative rates of hydrolysis of the secondary ester in glycerol 1,3-benzylidene 2-oleate and in glycerol 1,3-dihexadecyl ether 2-oleate, and of the primary and secondary esters in triolein were determined. Both unaltered and selectively inactivated rat pancreatic juice were used as sources of enzyme. It was found that rat pancreatic juice contains an enzyme that can hydrolyze fatty acids esterified at the 2-position of a glyceride. This enzyme is not pancreatic lipase. It may be sterol ester hydrolase. Partial glycerides, as well as complete glycerides, can serve as substrates. Pancreatic lipase, if it can hydrolyze the 2-positioned fatty acids of a triglyceride, does so at a very slow rate.

KEY WORDS enzyme specificity · digestion · absorption · pancreatic lipase · cholesterol esterase · structure of triglycerides

Several years ago we reported that pancreatic lipase, glycerol ester hydrolase (EC 3.1.1.3), specifically hydrolyzes the esters of the primary hydroxyl groups in a triglyceride (1). On the basis of this observation, the use of this enzyme as a tool for determining the structure of triglycerides was proposed (2, 3). However, in our original report and in subsequent work, particularly from this laboratory (4) and that of Borgström (5) and Desnuelle and Savary (6), there remained a question as to the absolute specificity of this enzyme.

Obtaining a satisfactory answer to this question has been complicated by the instability of the hydrolysis products (7, 8). Thus isomerization of 1,2-diglyceride to 1,3-diglyceride or 2-monoglyceride to 1-monoglyceride would result in glycerides that would be completely hydrolyzed by an enzyme that was specific for primary esters. For example, Hofmann and Borgström (9) observed hydrolysis of 2-monoolein by rat pancreatic juice, but they believed this was preceded by an isomerization step.

A further complication was introduced by our demonstration of an enzyme in pancreatic juice and tissue that can split esters of secondary, as well as primary, alcohols (10). As a consequence of these observations, the finding of a free fatty acid that was originally esterified at the 2-position of a triglyceride in the digestion products did not necessarily mean that it had been released by pancreatic lipase.

In the experiments described here we have overcome the problem of isomerization by using some compounds of glycerol in which the 1- and 3-positions are occupied by unhydrolyzable groups. This same approach has been used by others (5, 11). The digestions were carried out with selectively inactivated pancreatic juice, which contains only pancreatic lipase, and with untreated pancreatic juice, which contains nonspecific lipase[1] as well as lipase.

MATERIALS AND METHODS

Oleic acid was isolated from olive oil. Glycerol-1,3-^{14}C and oleic-1-^{14}C acid were purchased. These materials were processed to a chemical and radiochemical purity of 99% as determined by R_f, retention time, and radioassay after thin-layer and gas–liquid chromatography.

Abbreviations: TG, triglyceride; DG, diglyceride; MG, monoglyceride; FFA, free fatty acid.

[1] We suggest the trivial name *nonspecific lipase* for this enzyme. As we pointed out earlier (10), there is the likelihood that this enzyme is sterol ester hydrolase (EC 3.1.1.13). However, the existing systematic and trivial names imply a specificity which the enzyme does not possess. Rather it appears to hydrolyze equally well esters of all alcohols. The trivial name lipase is already established for glycerol ester hydrolase (EC 3.1.1.3). However, this enzyme is specific for esters of primary alcohols. To distinguish it from the nonspecific lipase, an appropriate systematic name would be carboxylic primary ester hydrolase.

The methods for preparing triolein and 1,3-benzylidene glycerol have been described (7). The 1,3-benzylidene glycerol was dissolved in chloroform containing a 20 moles % excess of pyridine and of oleoyl chloride. Acylation was allowed to take place at room temperature for 24 hr. After washing the reaction mixture three times with 1% of aqueous HCl, we removed the chloroform by evaporation, and the benzylidene glycerol ester was recrystallized three times from acetone at −35°C. Thin-layer chromatography (ether–hexane 15:85) of the product revealed the presence of two components. A large-scale separation of these was made by silica gel column chromatography. The first component was eluted with benzene–hexane 1:1 and the second component with benzene. Nuclear magnetic resonance spectra of these two materials were obtained on a Varian Aerograph HA-100 spectrometer, operating at a frequency of 100 MHz. Tetramethyl silane was used as an internal reference. The spectra established the two components as the *cis* and *trans* isomers of glycerol 1,3-benzylidene 2-oleate. Configurational assignment was based on a comparison of the chemical shift of the methine protons adjacent to the phenyl ring and the chemical shift of the methylene protons adjacent to the carbonyl group of the ester chain. Baggett, Dobinson, Foster, Homer, and Thomas (12) have reported that both of these chemical shifts are more shielded for the *trans* isomer than for the *cis* isomer. Our spectra are very similar to those of Serdarevich and Carroll (13) who published 60-MHz spectra of the palmitoyl esters of these two isomers. Any differences between the spectra obtained in their laboratories and those obtained in ours could be attributed to our use of the higher field strength and the presence of an oleate rather than a palmitate ester. Since the *cis* isomer was hydrolyzed twice as rapidly as the *trans* isomer,[2] the *cis* isomer was used in the experiments described here. The synthesis of glycerol 1,3-dihexadecyl ether and the acylation of this with oleoyl chloride have been described (14). Thin-layer chromatography of all of the final products showed them to have chemical and radiochemical purities of about 99%.

In these experiments two enzyme solutions were used. One of these, untreated, was reconstituted pancreatic juice and contained both lipase and the nonspecific lipase. The other, treated, was a solution of pancreatic juice which had been maintained at pH 9 and 40° for 2 hr. This exposure inactivates the nonspecific lipase but causes little alteration in the activity of lipase. The details of the methods for obtaining, storing, and treating the pancreatic juice have been described (10).

[2] Unpublished observations.

Two different techniques were used in studying the rates of hydrolysis of these esters. In the longer time studies, the digestion mixture consisted of 330 μmoles of $CaCl_2$, 0.73 mg/ml (1.35 mM) sodium taurocholate[3] (if present), 0.1 M Tris, 1 M NaCl, and 0.3 mg of lyophilized pancreatic juice (1.0 mg if taurocholate was added) in a total volume of 55 ml at a pH of 8.0 and at 25°C. If the digest contained taurocholate, a flask containing all of these components, except the enzyme, was placed on a wrist action shaker (330 cycles/min, amplitude 25°) for 90 min. The enzyme was then added and the shaking was continued. Those digests that did not contain taurocholate were treated in a similar fashion, but the preliminary 90 min of shaking was omitted. At the end of the digestion period, the mixture was acidified to pH 2, the lipids were extracted, and the free fatty acids were determined by titration (10). The titrated solution was reacidified, and the lipids were recovered. These were separated into free fatty acids and esters by an ion-exchange resin. The ester component was fractionated into its constituent glycerides by silica gel column chromatography (15). The amount of each glyceride was determined gravimetrically. Measurements of radioactivity were made on the free glycerol and on the free fatty acids that were obtained from the glycerides by saponification, acidification, and separation into ether-soluble and water-soluble components. The amount of free glycerol that was formed during enzymatic digestion was determined by counting the aqueous phase of the digest after extraction of the lipids.

The *initial* rates of hydrolysis were determined with the aid of a pH-stat. The digestion mixture consisted of 225 μmoles of substrates, 330 μmoles of $CaCl_2$, 0.73 mg/ml (1.35 mM) sodium taurocholate (if present), 0.002 M histidine, 1 M NaCl, and 6 mg of lyophilized rat pancreatic juice (0.3 mg when the substrate was triolein) in a total volume of 55 ml at a pH of 9.0 and at 25°C. If taurocholate was added, the digestion mixture, minus the enzyme, was shaken for 90 min as described above. The flask was then transferred to the pH-stat, and the enzyme was introduced. The course of hydrolysis of the esters was followed with 0.02 N KOH. The rate of stirring during digestion was such that a further increase in the amount of agitation did not cause an increase in the rate of enzymatic hydrolysis. The gas space in the flask was continuously flushed with purified, water-saturated nitrogen. When the digestion is carried out at pH 9, all of the resulting free fatty acids are titrated (16). This pH is within the broad pH optimum

[3] Nutritional Biochemicals Corporation, Chagrin Falls, Ohio. The possible nature of the contaminants in this material have been discussed (10).

of lipase, but the nonspecific lipase is restricted to 90% of its maximal activity (10).

Besides the selective inactivation of pancreatic juice, the important variable in both of these techniques is the presence or absence of taurocholate. The nonspecific lipase has an absolute requirement for bile salts, whereas, under these experimental conditions, bile salts inhibit the activity of lipase (10).

RESULTS

The initial rates of hydrolysis of the three substrates as determined with the aid of the pH-stat are shown in Table 1. Triolein, because it contains primary ester groups, is hydrolyzed more rapidly than the other two substrates. The inhibition of lipase hydrolysis of primary ester groups by bile salts under these conditions has been reported earlier (10). In the digest that contained bile salts, the untreated pancreatic juice hydrolyzed the triolein twice as rapidly as did the treated solution, whereas the two enzyme solutions showed similar activities towards this substrate in the absence of bile salts.

The two compounds that contained only a secondary ester group, glycerol-*cis*-1,3-benzylidene 2-oleate and glycerol 1,3-diether 2-oleate, were hydrolyzed at measurable rates only by the untreated pancreatic juice in the presence of bile salts. With the treated enzyme preparation, or if bile salts were absent, the hydrolysis was so slow that a measurable initial rate could not be obtained on the pH-stat. In an attempt to obtain measurable rates under the three conditions that allowed only a very slow hydrolysis, the amount of enzyme was increased to 12 mg. Digestions were carried out on the wristaction shaker and digestion times of up to 180 min were used. Regardless of whether the flasks contained the treated pancreatic juice with or without added taurocholate or the untreated pancreatic juice without added bile salts, a small and approximately equal amount of fatty acid was released from both substrates. The amount produced increased linearly with time at a rate of approximately 0.002 μeq/min per mg enzyme preparation. These results demonstrate the presence in pancreatic juice of an enzyme that can hydrolyze a fatty acid esterified at the 2-position of glycerol. The enzyme is more unstable than lipase and to be active requires the presence of bile salts. After inactivation of this enzyme, pancreatic juice can still split the secondary ester but at an extremely slow rate.

In the next series of experiments, the substrate was triolein in which the fatty acid in the 2-position was labeled. The digestions were carried out on the wrist-action shaker in the presence and absence of bile salts using both the treated and untreated enzyme preparations.

TABLE 1 INITIAL RATE OF HYDROLYSIS*

Substrate	Pancreatic Juice, Untreated, Taurocholate Added (+)	Pancreatic Juice, Untreated, Taurocholate Added (−)	Pancreatic Juice, Treated, Taurocholate Added (+)	Pancreatic Juice, Treated, Taurocholate Added (−)
	μeq/min/mg enzyme preparation			
Glycerol 1,3-benzylidene 2-oleate	1.1	<0.05	<0.05	<0.05
Glycerol 1,3-diether 2-oleate	0.65	<0.05	<0.05	<0.05
Triolein	4.8	36.0†	2.3	38.1†

* Digestion conditions were those described under Methods for the pH-stat.

† Only 0.3 mg (dry weight) of enzyme preparation used.

Figs. 1 and 2 illustrate the similar pattern of digestion products that were obtained under two of these conditions, namely when hydrolysis was due to lipase (treated enzyme preparation) in the absence of taurocholate (Fig. 1) and when hydrolysis was due to both enzymes (untreated enzyme preparation) in the presence of taurocholate (Fig. 2). The other two conditions of digestion yielded similar patterns of products.

The important measurement in these experiments was the presence or absence of labeled free fatty acid after digestion. Digestion with the treated enzyme preparation and in the absence of taurocholate resulted in the release of less than 1 μmole of labeled fatty acid during the initial 45 min. The 60 min sample contained 2 μmoles of labeled free fatty acid. When the triolein was digested for 60 min with untreated enzyme in the absence of taurocholate, labeled acid constituted less than 1% of the free fatty acids. Thus in the absence of taurocholate only very small amounts of the 2-positioned fatty acid were released by either enzyme preparation.

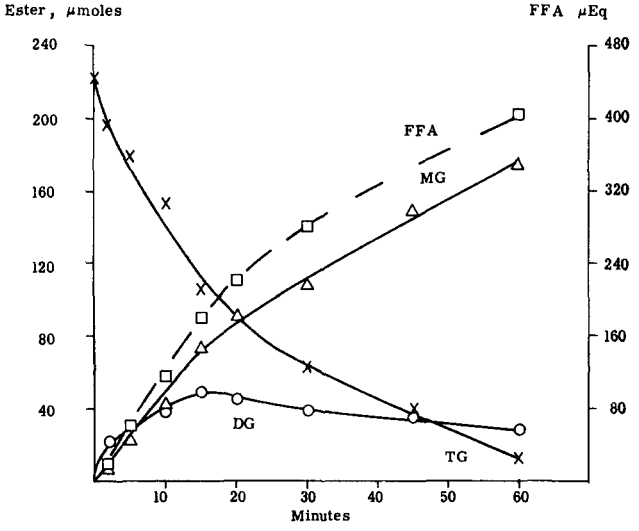

FIG. 1. Products of hydrolysis of triolein by 0.3 mg (dry weight) of treated pancreatic juice in the absence of taurocholate.

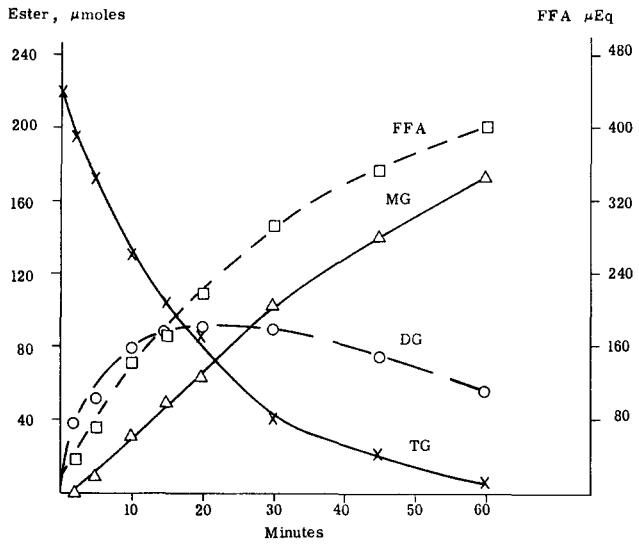

FIG. 2. Products of hydrolysis of triolein by 1 mg (dry weight) of untreated pancreatic juice in the presence of taurocholate.

As shown in Table 2, there was a similar paucity of labeled free fatty acid when taurocholate was added to the digest containing the treated pancreatic juice. However, when digestion was by the untreated enzyme preparation in the presence of bile salt, an appreciable amount of labeled free fatty acid was found, and the amount increased with the time of digestion. At each time interval, labeled acid constituted about 5% of the total free fatty acids. Thus the relative rate of hydrolysis of primary to secondary ester in this system was about 20 to 1.

The previous experiments have demonstrated that untreated pancreatic juice in the presence of bile salts can hydrolyze the fatty acid from the 2-position of glycerol. The substrate for this hydrolysis in the case of the glycerides could be triglyceride or the digestion products, 1,2-diglyceride and 2-monoglyceride. To investigate which it was, we used triolein in which both the glycerol and the fatty acid in the 2-position were labeled as the substrate. After digestion for 60 min with the treated enzyme preparation in the absence of taurocholate only trace amounts of labeled free glycerol and labeled free fatty acid could be found. The amounts of labeled free fatty acid and labeled free glycerol formed on digesting the doubly-labeled triolein with the untreated enzyme preparation in the presence of taurocholate are shown in Table 3. The presence of free glycerol when untreated pancreatic juice was used, but not when treated pancreatic juice was used, shows that the nonspecific lipase is capable of splitting monoglycerides. Except for the 5 min digestion period, the amount of labeled free fatty acid at all times exceeded that of the labeled free glycerol. This was particularly marked during the initial 30 min of digestion before

TABLE 2 FREE FATTY ACIDS FORMED DURING HYDROLYSIS* OF TRIOLEIN CONTAINING LABELED FATTY ACID IN THE 2-POSITION

	Enzyme Preparation				
	Treated		Untreated		
Time	Total FFA	Labeled FFA	Total FFA	Labeled FFA	Labeled/ Total
min	*μmoles*		*μmoles*		%
2	7	0.3	29	1.2	4.7
5	15	1.0	62	2.3	3.7
10	32	0.3	116	4.7	4.0
15	55	0.7	181	9.3	5.1
20	74	0.9	223	10.7	4.8
30	121	1.0	281	15.4	5.5
45	180	1.5	—	20.1	—
60	261	2.6	406	23.5	5.8

* Digestion conditions were those described under Methods and included the use of a wrist-action shaker. Taurocholate was present and 1 mg (dry weight) of pancreatic juice was used.

TABLE 3 PRODUCTS FORMED DURING HYDROLYSIS* OF GLYCEROL AND 2-POSITIONED FATTY ACID LABELED TRIOLEIN

Time	Labeled FFA	Labeled Free Glycerol	Labeled FFA/ Labeled Free Glycerol
min	*μmoles*		
0	0.4	0	—
5	2.5	3.2	0.8
10	5.4	2.0	2.7
15	9.2	2.3	4.0
20	12.1	2.5	4.8
30	15.1	4.2	3.6
45	20.1	7.5	2.7
60	25.3	13.7	1.8

* Digestion conditions were those described under Methods and included the use of a wrist-action shaker. Taurocholate was present and untreated pancreatic juice (1 mg dry weight) was used.

monoglyceride became the dominant glyceride species (see Fig. 2). The excess of labeled free fatty acid over labeled free glycerol must mean that the 2-positioned fatty acid in the triglyceride or 1,2-diglyceride was hydrolyzed.

The amounts of labeled glycerol and labeled fatty acid in the mono- and diglycerides were determined. In both compounds, the ratio of the two labeled moieties still was approximately 1. Thus it was not possible to determine whether fatty acid was being split from the 2-position of the triglyceride, the 1,2-diglyceride, or from both glycerides. The small difference in ratio that would be expected was probably beyond the sensitivity of the method.

DISCUSSION

The presence in pancreatic juice of an enzyme that can hydrolyze the ester bond at the 2-position of a triglyceride

helps to explain some of the conflicting reports on the mechanism of fat digestion and absorption. In an earlier study (17), we concluded that about 20% of the dietary triglyceride was completely hydrolyzed. As a mechanism, we proposed that the digestion product, 2-monoglyceride, isomerized to 1-monoglyceride and that this could then be hydrolyzed by lipase. However, Hofmann (18) has shown that in the presence of bile salts 2-monoglycerides isomerize at a very slow rate. The presence of an enzyme that can directly hydrolyze this secondary ester removes the necessity for an isomerization step when a triglyceride undergoes complete hydrolysis.

Nevertheless, the possibility remains that isomerization of 2-monoglycerides prior to hydrolysis, as well as direct hydrolysis are operating. It is now generally agreed that the main hydrolysis product of dietary triglyceride is 2-monoglyceride. One step in the absorptive process is the formation of a monoglyceride-free fatty acid–bile salt micelle. However, this is not a fixed structure. There will be a continuous exchange among the monoglycerides in the micelles, in the monomeric form in the aqueous phase, and in the oil phase. As a consequence, some 2-monoglyceride molecules can have a considerable residence time in the lumen of the intestine with a corresponding increase in the possibility of isomerization.

Complete hydrolysis could occur also if lipase can split the ester group at the 2-position of a triglyceride. If one assumes that the treatment of pancreatic juice that we employed completely inactivates the nonspecific lipase, then the results reported here show that there is only a very small amount of splitting of the secondary ester group by lipase. Thus, based on the rates of hydrolysis with the treated enzyme preparation when glycerol 1,3-diether 2-oleate and glycerol 1,3-benzylidene 2-oleate were the substrates, these secondary esters are split by the primary ester lipase at a rate that is insignificant relative to that of the primary esters in triolein, i.e., 0.002 vs. 38.

Recent papers by Borgström (5) and by Entressangles, Sari, and Desnuelle (19) have been directed at the question of the specificity of pancreatic lipase. Borgström in a portion of his experiments used human pancreatic juice. He concluded that lipase cannot hydrolyze the ester linkage at the 2-position of a triglyceride or 1,2-diglyceride. However, he proposed that this enzyme can hydrolyze a 2-monoglyceride. Entressangles, et al. used partially purified pig pancreatic tissue lipase. It is their opinion that the positional specificity of pancreatic lipase is almost absolute, i.e., the enzyme cannot hydrolyze a fatty acid from the 2-position of a glyceride. The differences among the results from the laboratories at Lund, Marseille, and Cincinnati could of course be due to differences in experimental procedures. However, we believe that there is substantial agreement, if the results from the three laboratories are interpreted on the basis of the presence of two enzymes in pancreatic juice and tissue. One of these is a primary ester hydrolase, pancreatic lipase (EC 3.1.1.3), that hydrolyzes fatty acids only from the 1- and 3-positions of a glyceride. The resulting 2-monoglyceride can isomerize to 1-monoglyceride. The extent of this isomerization is dependent on the environment of the digest, such as pH (7), free fatty acids (20), and bile salts (18), and of course the time of exposure. The resulting 1-monoglyceride can be hydrolyzed by pancreatic lipase with the amount of hydrolysis being markedly increased by bile salts (9).

In addition to lipase, there is another enzyme, which we refer to here as nonspecific lipase, in pancreatic tissue and juice. This enzyme can hydrolyze a fatty acid from the 2-position of a glyceride; it is not limited to 2-monoglycerides. Although the absolute activity of these two enzymes cannot be determined until both have been isolated, an approximation of their relative activity can be made from our experiments. From Table 1 it can be estimated that lipase (treated enzyme, taurocholate absent) hydrolyzes the 1- or 3-positioned fatty acids in triolein at 35 and 58 times the rate at which the nonspecific lipase (untreated enzyme, taurocholate present) hydrolyzes the 2-positioned fatty acid in glycerol 1,3-benzylidene 2-oleate and in glycerol 1,3-diether 2-oleate, respectively. In the experiment with labeled triolein and the untreated enzyme preparation (Table 2), the fatty acids from the primary esters appeared as free fatty acid approximately 20 times more rapidly than did the fatty acids from the secondary esters. Since a triglyceride contains twice as many primary as secondary esters, the relative rate would be 10 to 1. Comparisons of the rates for the two enzymes is complicated by their having different optimal conditions, e.g., the effect of taurocholate. Nevertheless, lipase is indicated to have an activity 10–60 times greater than that of nonspecific lipase. The presence of this nonspecific lipase of this activity in the enzyme preparation used by Borgström (5) would explain the complete hydrolysis of triolein that he observed. At the same time, the activity of this enzyme is sufficiently low that the rates of hydrolysis of the synthetic substrates that he observed would be considered as insignificant.

The method for determining the distribution of fatty acids in a triglyceride that was developed in this laboratory (4), and the subsequent extensions of this method by Brockerhoff (21) and Lands, Pieringer, Slakey, and Zschocke (22), all depend on pancreatic lipase's hydrolyzing only those fatty acids that are esterified at the 1- and 3-positions of the triglyceride. Most laboratories use one of the commercial pancreatin preparations, usually without purification, as a source of the enzyme.

The presence in pancreatic tissue of an enzyme that can hydrolyze the ester linkage at the 2-position would vitiate these methods. Although we have made no systematic investigation of commercial materials, we have examined a sufficient number of samples to know that some contain an appreciable amount of nonspecific lipase. Coleman (23) has reported that partially purified pancreatic lipase has greater positional specificity than the several commercial preparations that he tested. This specificity can also be gained by subjecting the commercial materials to the same treatment that we used for selective inactivation of pancreatic juice. Since nonspecific lipase is either identical with cholesteryl ester hydrolase or has very similar properties, a convenient way to check for the presence of this enzyme in a tissue preparation is to test its ability to hydrolyze cholesteryl ester.

In the past we have recommended that the monoglycerides formed during digestion, rather than the free fatty acids, be used in studying the structure of triglycerides. In this way, erroneous results due to isomerization of the 2-monoglyceride and subsequent hydrolysis can be avoided. This recommendation also will help to minimize, but will not overcome, the effect of the presence of nonspecific lipase.

The isomeric structure of the 2-oleoyl-1,3-benzylidene glycerol was established by T. J. Flautt of these laboratories.

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