Positional Specificity of Lipoprotein Lipase*

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SUMMARY

The stereochemical course of hydrolysis of radioactive triolein was determined using lipoprotein lipase of cow's milk and rat postheparin plasma. [3H]Glycerol trioleate or glycerol [1-14C]trioleate was emulsified with total egg yolk lipids to provide 0.2 to 2.5 μCi of [3H] or 0.03 μCi of [14C] per μM substrate. The hydrolysis products were isolated at 1 to 45 min and free fatty acids and the various positional isomers of mono- and diglycerides were resolved by thin layer chromatography. In a total yield of 0.03 to 0.16 μmole of diglyceride, 9 to 30% was 1,3- and 70 to 91%, 1,2-(2,3-)diglyceride. The 2,3-isomer was 73 to 96% of the latter. The yield of free fatty acids and monoglycerides varied with the length of incubation. In 15 of 17 determinations, the 1-(3-) isomer accounted for 51 to 87% of the monoglyceride. It is suggested that lipoprotein lipase attacks preferentially position 1 in sn-glycerides and follows it by hydrolysis of the positions 2 and 3. An intermediate formation of 2,3-di- and diglycerides during lipoprotein lipase hydrolysis may be important in avoiding stimulation of triglyceride and phospholipid biosynthesis which proceed via 1,2-diglycerides.

Previous investigations (1–3) of the positional specificity of lipoprotein lipase have resulted in conflicting conclusions, which appear to have been due at least in part to a failure to differentiate between the two primary ester groups in the triglyceride molecule. The less extensive hydrolysis of the secondary in relation to the primary positions claimed by Korn (4) and Nilsson-Ehle et al. (3) was based on comparisons of the total rate of hydrolysis of the two primary positions. The preferential hydrolysis of the secondary ester bond demonstrated by Greten et al. (2) involved a relation of the rate of release of the acids of the second and third positions of the glyceride molecule only. Carbon atoms 1 and 3 of the glycerol molecule were pointed out not to be equivalent metabolically, by Karnovsky et al. (5) more than 15 years ago and this difference was re-emphasized recently by the general acceptance of the stereospecific (sn) numbering system (6). In the present study an established method (7) of stereospecific analysis was used for differentiating between diglyceride isomers released as intermediates during hydrolyses with lipoprotein lipase. It is shown that purified preparations of lipoprotein lipase from both cow's milk and rat postheparin plasma release the fatty acids more rapidly from position 1 than from either position 2 or position 3 of the triglyceride molecule. The potential metabolic significance of this observation is briefly discussed.

EXPERIMENTAL PROCEDURE

Materials—All chemicals were of reagent grade and were not purified further. Phospholipase A (Crotalus atrox) was obtained from the Ross Allen Reptile Institute, Silver Springs, Florida; pancreatic lipase (steapsin), from General Biochemicals, Chagrin Falls, Ohio; heparin (1000 i.u. per ml), from the Connaught Medical Research Laboratories, Downsview, Ontario; phenylphosphorodichloridate, from Eastman Organic Chemicals Co., Rochester, N.Y. and Silica gel G and H from Brinkmann Instruments (Canada), Rexdale, Ontario.

The radioactive substrates were tested for purity by thin layer chromatography. [2-3H]Glycerol trioleate, 750 mCi per mm, from Amersham/Searle, had a radioiodin of 99.3%; [carboxyl-14C]glycerol trioleate, 15.2 mCi per mm, from ICN Medical and Radiosotope Division, had 97.8% radiopure with less than 1.5% labeled free fatty acids.

Assay for Lipoprotein Lipase Activity—Lipoprotein lipase was obtained from two sources: fresh cow’s milk prepared by the method of Bier and Havel (8) as a 4% aqueous solution of the lophylized powder; and rat postheparin plasma as described by Fielding (9) as an extract of acetone-ether powder from the plasma in 0.05 M Tris buffer, pH 8.1, containing beef serum albumin and heparin. A preliminary washing of the plasma resulted in a 4-fold purification of the enzyme but a greatly diminished yield. The concentration of the acetone-ether powder of plasma was 1.5% and 5.6% for freshly prepared and frozen-stored, respectively; for washed plasma, the corresponding concentrations were 0.3% and 0.4%.

Radioactive triolein, 0.03 μCi of 14C or 0.2 and 2.5 μCi of 3H per mg of substrate, was emulsified with 14 mg of total egg yolk lipid in 2 ml of 0.09% NaCl solution by sonication for 10 to 15 sec periods. The specific activity of the 14C-labeled emulsion, tested twice by gas chromatography following methylation of fatty acid esters (10) was 109 and 122 dpm per mm triglyceride. Similarly the specific activity of the emulsion more highly labeled with tritium averaged 4318 dpm per mm triglyceride.

Aliquots of the emulsion (0.5 ml) were activated with 0.5 ml of normal rat serum for 30 min at 37° before the addition of 0.1 ml

* This work was supported by the Ontario Heart Foundation, The Medical Research Council of Canada, and the Banting and Best Department of Medical Research.
incubation was started with either 0.5 ml of milk solution or 1 bumin, for a final incubation volume of 5.5 to 6.5 ml and triglyc-
eride content of 3 mg (2.75 pmoles). After equilibration to 37°,
tenths or all of the 1,2-(2,3-)isomer mixture of diglyceride was
borate plates.
free fatty acids, 1, N-diglycerides, 1,2-(2,3-)diglycerides, and or l'ris buffer substituted for the enzyme solution. Two-mil-
litier samples were removed at the stated times during incu-
bation and extracted for total lipids. The labeled triglycerides,
free fatty acids, 1,3-diglycerides, 1,2-(2,3-)diglycerides, and the monoglycerides were isolated by thin layer chromatography
for neutral lipids and prepared for scintillation counting. Nine-
tenths or all of the 1,2-(2,3-)isomer mixture of diglyceride was
reserved for stereochemical analysis, and the monoglyceride for resolution into 1-(3-) and 2-isomers chromatographically
on borate plates.

Thin Layer Chromatography—Thin layer plates (20 cm x
20 cm x 0.3 mm) were prepared from aqueous slurries of Silica
Gel H or from Silica Gel G in 0.4 m borate buffer (11). For
neutral lipids, development was accomplished in petroleum ether
(b.p. 30°-60°)-diethyl ether-acetic acid (70:30:1, v/v/v). The plates
were exposed briefly to ammonium fumes before spraying with
0.05% 2,7-dichlorofluorescein in 50% meanol and were visualized
under ultraviolet light. The system for resolving monoglyceride isomers on borate plates (11) was chloroform-
acetone-methanol (96:4:0.5, v/v/v), and for phenyl phosphatides
(7), chloroform-methanol-3% aqueous NH₄OH (63:30:7,
v/v/v). These plates were sprayed as above but without prior
exposure to ammonium fumes.

Appropriate standard markers were used in all chro-
nomatographic separations. These were obtained by thin layer
chromatography from commercial monodol and triolein, from
pancreatic lipase (12) digestion products of corn oil or as inter-
mediates in stereochemical analysis (7).

Preparation, Recovery, and Radioactive Measurement of Lipid
Samples—Lipid samples were extracted from buffer solutions or
from gel scrapings with chloroform-methanol (2:1, v/v) by
partitioning with water at one-fifth the volume including that
of the sample. The chloroform layer was filtered through an-
hydrous Na₂SO₄ and a cotton plug into a vial to be evaporated at
40° under nitrogen. The residue was taken up immediately
in small amounts of chloroform-methanol for plating and other
procedures. During the final elution of gel scrapings for the
measurement of radioactivity, fluorescein was removed by adding
4 m NH₄OH as one-tenth of the water volume. The chloroform
layer was filtered as before into a scintillation vial and dried at
40°. To the dried residue was added 10 ml of scintillation fluid,
0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis[5-(phenyl-
oxazole)]benzene (Amersham-Searle) in toluene. Tritium was
counted in a Mark I, 6894 series and 4C in an Auto/Subtract
III, 720 series, Nuclear Chicago liquid scintillation system with
quenching monitored by external standard devices.

Stereo specific Analysis of 1,2, and 2,3 Diglycerides—The
stereo specific analysis of 1,2- and 2,3-diglycerides was carried
out according to the method of Brockerhoff (7) with the exception
that the relative concentration of phenyl dichlorophosphate was
doubled and the formation of triethylammonium salt was
omitted. To ensure good detection of diglycerides following
chromatography, 10 mg of carrier 1,2-(2,3-)diglyceride, purified
from the products of pancreatic lipase digestion of corn oil (12)
were added to the extract of total lipid sample. During the
experimental series two other modifications were made which
improved efficiency by reducing the need for rechromatography
and the associated extractions. The first was the substitution
of a wash containing ammonia (13) for one of the two purifica-
tions by thin layer chromatography of the eluted phosphatidyl
phenols. The second, after phospholipase A digestion, con-
cerned the introduction of a preliminary development of the
total lipid extract in a neutral lipid solvent, following which
the same thin layer plate could be redeveloped in a polar lipid
solvent and the residual and lysophosphatidylphenols separated
without interference from free fatty acids. The bands for the
lysophosphatides and the residual phosphatides, representing, respec-
tively, the original 1,2- and 2,3-isomers of the diglyceride frac-
tion, were eluted and the radioactivity measured.

Two tests were made of the efficiency of the stereochemical
method. In one, 500 mg of corn oil mixed with 100 μl of [2-3H]
glycerol trioleate (0.05 μCi) was digested with pancreatic lipase
(12) and the mixture of 1,2-(2,3-) diglycerides isolated by neu-
tral lipid chromatography. After stereo specific analysis (7) the
percentage of distribution of 1,2- to 2,3-isomer, determined
from the radioactivity of the corresponding lysophosphatides,
was 55:45, approximately the 1:1 ratio expected. Any 1,3-diglyceride formed by isomerization would have been
converted to the lys compound (14). In the other test, phos-
thatidylphenol formation and phospholipase A digestion (7)
was performed on the diglycerides derived from egg yolk lecithin
by degradation with phospholipase C (15). Only lysophos-
phatides, indicating the presence of 1,2-diglycerides, were pro-
duced as checked by gas chromatography (10) after methylation
of the corresponding fatty acid esters.

RESULTS

In agreement with the observations of Korn (4) and Bier and
Havel (8), cow's milk provided an excellent source of lipoprotein
lipase. Table I shows that in the presence of 2.75 μmoles of
triglyceride substrate, 0.5 ml of 4% milk solution hydrolyzed
an average of 80% of the tracer triolein in 30 min. This is
compatible to the activity reported for milk lipoprotein lipase
by Bier and Havel (8) and Korn (1). Calculations showed that
free glycerol accounted for 20% in 15 min and 30 to 50% in 30
min of the hydrolyzed triolein. 130th at 15 and 30 min, diglyc-
erides were present in the reaction mixture in small amounts,
representing less than 3% of the total radioactivity. A tran-
sient accumulation of the monoglycerides, also observed by
others (3, 16), amounted to about 40% of the radioactivity at
15 min.

Rat postheparin plasma, in the amounts used, showed less
activity than milk. Nevertheless, the appearance of 62% of
the radioactivity in free fatty acids in 30 min with an extract
from 30 mg of freshly prepared acetone-ether powder, equivalent
to 0.5 ml of plasma, is in agreement with the rate of release of
fatty acids quoted by Fielding (9) for an extract of rat plasma
similarly prepared. Lipoprotein lipase from postheparin plasma,
as from milk, hydrolyzed a large part of the triglyceride to gly-
cerol and free fatty acids within 30 min with a small percentage
of diglyceride and larger amounts of monoglyceride present during
the reaction, whether or not the plasma had been purified by
washing.

It is well known (1-3) that the activity of lipoprotein lipase
is contingent upon activation of the triglyceride substrate by
some component of serum lipoproteins. Omission of serum in
the assay (Table I) resulted in 85% inhibition of triglyceride
hydrolysis in 30 min. The fact that hydrolysis was not pre-
vented completely was due possibly to a small amount of en-
dogenous lipoprotein in the milk, which could have been released
with the enzyme in the rupture of mammary cells during milk
Cow's milk solution or extract of rat postheparin plasma was assayed as described under "Experimental Procedure." In each tube the emulsified and activated substrate provided 2.75 \mu M of triglyceride with specific activity of 0.02 \mu Ci per mg from \[^{14}C\]carboxyl-trioleate or 0.2 \mu Ci per mg from \[^{14}C\]glycerol-trioleate in a total volume of 6.5 ml. The radioactivity of the lipid products was determined after resolution by thin layer chromatography for neutral lipids, in 2-ml samples at the reaction times specified. The results are given in moles % for each class. Blank tubes with no enzyme were run concurrently, and no evidence of hydrolysis was obtained. Concentrations of the powdered enzyme sources and of the inhibitors are for the final medium.

<table>
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<tr>
<th>Experiment</th>
<th>Triglyceride</th>
<th>Diglyceride</th>
<th>Monoglyceride</th>
<th>Fatty acid</th>
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<td>15 min</td>
<td>30 min</td>
<td>45 min</td>
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<tr>
<td>Milk, 4% [^{14}C]H (20 mg)[^{14}C]H</td>
<td>48.3</td>
<td>36.2</td>
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<td>60.2</td>
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<td>3H</td>
<td>84.2</td>
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<td>Milk, 2% (10 mg)[^{14}C]H Plasma</td>
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<td>90.3</td>
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<tr>
<td>7.5 mg</td>
<td>90.4</td>
<td>65.4</td>
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<td>Inhibitors with 4% milk</td>
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<td>Protamine</td>
<td>58.0</td>
<td>46.2</td>
<td></td>
<td>3.8</td>
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\(^{*}\) All experiments were conducted with \[^{14}C\] except where indicated.

secretion (17). Blank tubes showed no hydrolysis during the same period.

Although some investigators (4, 18, 19) have considered that inhibition by sodium chloride and protamine sulfate is characteristic of lipoprotein lipase, Datta and Wiggins (20) demonstrated that either inhibition or stimulation could occur according to the concentration and state of the substrate and the presence or absence of heparin. In Table I 0.3 M NaCl inhibited by 40% the hydrolysis of triglyceride and the production of fatty acids but had little effect on the levels of diglyceride and monoglyceride in the medium. No inhibition was observed for protamine sulfate at 15 or 150 \mu g per ml of incubation medium.

Since the level of diglyceride was only 0.1 \mu mole or less between 15 to 45 min of incubation, an attempt was made to increase the concentration for the proposed stereochromatic analysis by slowing the hydrolytic activity by the substitution of 2% for 4% milk solution. However, the consequent 60% reduction in hydrolysis of the substrate resulted in reduced monoglyceride content without improvement in diglyceride level. As seen in Table II, a 5-min incubation with 4% milk provided a satisfactory level of diglyceride for investigation of the distribution of isomers, especially since the detection of chromatographic fractions was enhanced by addition of carrier 1,2-(2,3)-diglyceride. This period of time resulted in an average triglyceride hydrolysis of 12% for plasma and 24% for milk lipoprotein lipase.

The percentage of the total diglyceride found as the 1,3-isomer is shown in Table III to fall between 10 and 28% for milk lipoprotein lipase. This is considerably higher and more variable than the 3 to 4% reported by Nilsson-Ehle et al. (3). With the plasma enzyme, the proportion of the 1,3-isomer was as much as 50% on three occasions. This apparently resulted from inadequate separation of the 1,3- and 1,2-(2,3)-diglycerides in the presence of too high a proportion of carrier 1,2-(2,3)-diglyceride to labeled 1,3-diglyceride. By scraping the lower part of the band to recover pure 1,2-(2,3)-diglycerides for immediate further processing, we apparently left some labeled 1,2-(2,3)-diglycerides with the 1,3-diglyceride band. Furthermore, since we concentrated on the isolation of pure diglycerides, we may have partially isomerized the monoglycerides. Fifteen of the 17 values for the 1-(3-) mixture of monoglyceride isomers exceed the 2-isomer, ranging from 51 to 87% of the total monoglyceride. The other two values are about equal to those of the 2-isomer. The results for the monoglyceride fractions are at variance with those of Nilsson-Ehle et al. (3), who, however, stressed the effect of isomerism on the levels of both the 1- and 2-monoglycerides.

The results of the stereochemical analyses carried out on the 1,2-(2,3)-fraction of diglyceride are found in Table III. In all 14 determinations the radioactivity of the phosphatidyl phenols, derived from the 2,3-isomers, greatly exceeded that for the lysophosphatides, representing the original 1,2-diglycerides. These results lead to the conclusion that lipoprotein lipase attacks position 1 of the sn-glyceride preferentially, with the consequent appearance of 2,3-diglyceride as intermediate in the reaction mixture. The other positions account for less hydrolysis, and isomerism to 1,3-diglyceride may obscure the pathways.

**DISCUSSION**

There appears to be general agreement that the primary and secondary esters of the triglyceride molecule are hydrolyzed at unequal rates by lipoprotein lipase, whether the source of the enzyme is tissue, plasma, or milk. The contradictory statements as to the preferred site of hydrolysis have arisen through disregard for the stereochromaticity of the substrate. The present investigation which has taken into account the dissimilarity in the three positions of the glyceride may help to resolve some of
higher free fatty acid-formaldehyde ratios were found for substrate and adipose tissue as the source of the enzyme. When based on the almost complete hydrolysis with chylomicrons as the source of the enzyme, may depend upon the nature of the substrate. Thus, his conclusion as to the lack of positional specificity was not supported in these experiments. The conflict. For instance, since 1,2- and 2,3-diglycerides cannot be separated on borate plates, then Nilsson-Ehle and associates must be referring to a mixture of 1,2-(2,3-) isomers when they report that 1,2-diglycerides exceed the 1,3-isomer (3).

The work of Greten et al. (2, 21) has been criticized previously (3) on the grounds that diether monoesters have been assumed unjustifiably to have similar physicochemical properties to triglyceride during hydrolysis. Further emphasis on the importance of a well defined substrate can be made by pointing out that the diether compound used, according to the sn notation, cannot be separated on borate plates, then Nilsson-Ehle and associates must be referring to a mixture of 1,2-(2,3-) isomers (3).

Our results for monoglyceride isomers differ from those of Nilsson-Ehle et al. (3) and cannot be explained without further analyses, which await the development of satisfactory stereospecific analysis. The distribution is reported as percentage of the total class for 1,3-diglyceride and 1,3- and 2-monoglyceride isomers and as percentage of the 1,2-(2,3-) mixture for 1,2- and 2,0-diglycerides. Estimated total diglyceride is indicated as in Table II (*).

The diglycerides and monoglycerides were obtained in the assays described in Table II for 5-min incubations of % milk and plasma, and for % milk in a tube from the same % milk assay in Table I. Radioactivity was measured for 1,3-diglyceride isolated by thin layer chromatography for neutral lipids, for 1,3- and 2-monoglycerides after separation on borate plates and for the lyso- and residual phosphatide derivatives of 1,2- and 2,3-diglycerides after stereospecific analysis. The distribution is reported as percentage of the total class for 1,3-diglyceride and 1,3- and 2-monoglyceride isomers and as percentage of the 1,2-(2,3-) mixture for 1,2- and 2,0-diglycerides. Estimated total diglyceride is indicated as in Table II (*).

### Table II

<table>
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<th>Triglyceride</th>
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<th>Monoglyceride</th>
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<tr>
<td>20 mg</td>
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<tr>
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<td>2359</td>
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<td>520</td>
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<td>1-3-</td>
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### Table III

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<td>4a</td>
<td>9.2</td>
<td>69.5</td>
<td>30.5</td>
</tr>
<tr>
<td>4b</td>
<td>9.8</td>
<td>54.0</td>
<td>46.0</td>
</tr>
</tbody>
</table>

Although the nature of association of lipoprotein lipase with the plasma membrane is not well defined, it is possible that during hydrolysis some diglyceride and monoglyceride could enter the cell interior along with the free fatty acids. An intermediate
formation of 2,3-diglyceride during the hydrolysis of triglycerides by lipoprotein lipase might then be of physiological importance in avoiding stimulation of triglyceride and phosphoglyceride biosynthesis, which proceeds exclusively via the 1,2-diglycerides (23, 24). Likewise, l-(3-)-monoglycerides are not directly utilized by mammary gland (25) or adipose tissue and liver (26), all of which are capable of some direct reacylation of the 2-monoglycerides. Apparently then lipoprotein lipase insures a complete destruction of the original structure of the chylomicron triglyceride by promoting both extensive hydrolysis and the formation of intermediates that cannot be reutilized readily for either triglyceride or phosphoglyceride biosynthesis.

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