*58,* 403.
Green, J. R., Dunn, M. J., Spooner, R. L., and Maddy, A. H. (1974), *Biochim. Biophys. Acta 373,* 51.
Guidotti, G. (1972), *Annu. Rev. Biochem. 41,* 731.
Guthrow, C. E., Jr., Allen, J. E., and Rasmussen, H. (1972), *J. Biol. Chem. 247,* 8145.
Gwynne, J. T., and Tanford, C. (1969), *J. Biol. Chem. 244,* 4406.
Holtzer, A., and Lowey, S. (1959), *J. Am. Chem. Soc. 81,* 1370.
Kato, K., and Bishop, J. S. (1972), *J. Biol. Chem. 247,* 7420.
Knüfermann, H., Bhakdi, S., Schmidt-Ullrich, R., and Wallach, D. F. H. (1973), *Biochim. Biophys. Acta 330,* 356.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Makino, S., Reynolds, J. A., and Tanford, C. (1973), *J. Biol. Chem. 248,* 4926.
Marchesi, S. L., Steers, E., Marchesi, V. T., and Tillack, T. W. (1970), *Biochemistry 9,* 50.
Marchesi, V. T., and Palade, G. E. (1967), *J. Cell. Biol. 35,* 385.
Marchesi, V. T., and Steers, E. (1968), *Science 159,* 203.
Mazia, D., and Ruby, A. (1968), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1445.
Nicolson, G. L., and Painter, R. G. (1973), *J. Cell. Biol. 51,* 265.
Oikawa, K., Kay, C. M., and McCubbin, W. D. (1968), *Biochim. Biophys. Acta 168,* 164.
Painter, R. G., Sheetz, M., and Singer, S. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1359.
Pinto da Silva, P., and Nicolson, G. L. (1974), *Biochim. Biophys. Acta 363,* 311.
Reynolds, J. A. (1972), *Ann. N.Y. Acad. Sci. 195,* 75.
Reynolds, J. A., and Trayer, H. (1971), *J. Biol. Chem. 246,* 7337.
Rosenthal, A. S., Kregenow, F. M., and Moses, H. L. (1970), *Biochim. Biophys. Acta 196,* 254.
Roses, A. D., and Appel, S. H. (1973), *J. Biol. Chem. 248,* 1403.
Singer, S. J. (1974), *Annu. Rev. Biochem. 43,* 805.
Steck, T. L. (1974), *J. Cell. Biol. 62,* 1.
Steck, T. L., and Yu, J. (1973), *J. Supramol. Struct. 1,* 220.
Stevens, C. L. (1975), *FEBS Lett. 56,* 12.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University Press.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N.Y., Wiley.
Tanford, C., Nozaki, Y., and Reynolds, J. A. (1971), *J. Biol. Chem. 246,* 4485.
Tanford, C., Nozaki, Y., Reynolds, J. A., and Makino, S. (1974), *Biochemistry 13,* 2369.
Tilney, L. G., and Detmers, P. (1975), *J. Cell. Biol. 66,* 508.
Trayer, H. R., Nozaki, Y., Reynolds, J. A., Tanford, C. (1971), *J. Biol. Chem. 246,* 4485.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Wyman, J. (1964), *Adv. Protein Chem. 19,* 223.

# Hydrolysis of Tri- and Monoacylglycerol by Lipoprotein Lipase: Evidence for a Common Active Site†

Jer-Shung Twu, Peter Nilsson-Ehle, and Michael C. Schotz*

ABSTRACT: The relationship between triacylglycerol and monoacylglycerol hydrolyzing activities of purified rat heart lipoprotein lipase was studied using emulsified trioleoylglycerol and micellar or albumin-bound monooleoylglycerol as substrates. The maximal reaction rates obtained with the two substrates were similar (650 and 550 nmol of fatty acid released per min per mg of protein, respectively). Addition of apolipoprotein C-II or serum increased the maximal reaction rate for the trioleoylglycerol hydrolyzing activity about fourfold, but had no effect on the monooleoylglycerol hydrolyzing activity. Hydrolysis of the two substrates apparently takes place at the same active site of the enzyme since (1) mutual competitive inhibition between the substrates could be demonstrated; (2) the rate of inactivation of enzymatic activity with the two substrates in 1.2 M NaCl was the same; (3) similar losses of hydrolytic activity with tri- and monooleoylglycerol were observed in the presence of low concentrations of *n*-butyl(*p*-nitrophenyl)carbamide; (4) inhibition of both hydrolytic activities by this compound could be prevented by prior exposure of lipoprotein lipase to either substrate.

Lipoprotein lipases (EC 3.1.1.34) have a comparatively low substrate specificity, both with regard to the chemical structure and the physicochemical nature of the substrate. In contrast to pancreatic lipase (Desnuelle and Savary, 1963; Morgan et al., 1968), lipoprotein lipases readily act upon emulsified tri- and diacylglycerol as well as micellar or albumin-bound monoacylglycerol substrates (Egelrud and Olivecrona, 1973; Nilsson-Ehle et al., 1973), and also catalyze the hydrolysis of truly water-soluble esters (Egelrud and Olivecrona, 1973). The reaction of lipoprotein lipase (LPL[1]) with these substrates has been shown to have certain different properties. Most notably,

† From Research Service, Veterans Administration, Wadsworth Hospital Center, Los Angeles, California 90073, and Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024. *Received November 21, 1975.* This work was supported in part by grants from United States Public Health Service AM 4706, American Heart Association, Greater Los Angeles Affiliate 492-IG2, and the VA Project 7770-01.

[1] Abbreviations used are LPL, lipoprotein lipase; $Ol_3Gro$, trioleoylglycerol; OlGro, monooleoylglycerol; $V_{max}$, maximal reaction rate; $K_m$, Michaelis constant.

activity of LPL with emulsified triacylglycerol, but not with micellar monoacylglycerol or water-soluble substrates, is greatly enhanced by the presence of apolipoprotein C-II (Fielding, 1970; Egelrud and Olivecrona, 1973). Also, enzymatic activity with micellar substrates has a higher pH optimum (Egelrud and Olivecrona, 1973).

These differences may be related to the different physicochemical nature of the substrates, but may also indicate that LPL is a multifunctional complex with separate sites catalyzing the hydrolysis of triacylglycerol and monoacylglycerol (Fielding, 1970). In the present study we have investigated the relationship between the "lipase" and "esterase" activities (Desnuelle and Savary, 1963) of purified LPL, using emulsified trioleoylglycerol ($Ol_3Gro$) and micellar or albumin-bound monooleoylglycerol (OlGro). The data indicate that hydrolysis of these acylglycerol substrates, despite the differences in chemical structure as well as physicochemical properties, is catalyzed at the same active site.

## Materials and Methods

*Materials.* Tri[9,10-$^3$H]oleoylglycerol (>99.2% radiopurity as determined by thin-layer chromatography) was obtained from Amersham-Searle. Monooleoyl[2-$^3$H]glycerol (ICN Pharmaceuticals) was purified by extraction of contaminating fatty acid and glycerol (Belfrage and Vaughan, 1969). The product (99.7% radiopurity) contained about 90% 1(3)-isomer and 10% 2-isomer (Nilsson-Ehle and Belfrage, 1972). Unlabeled $Ol_3Gro$ (NuChek Prep.) and OlGro (Hormel Products) were pure, as determined by thin-layer chromatography. Lysolecithin and crystalline bovine serum albumin were purchased from Sigma Chem. Co. and sodium deoxycholate was from K & K Laboratories. The apolipoprotein C-II, isolated from human VLDL (Brown et al., 1969), was the gift of Dr. Virgil Brown, School of Medicine, University of California, San Diego. *N*-Butyl(*p*-nitrophenyl)carbamide was the gift of Drs. F. Wold and R. E. Scofield, Department of Biochemistry, University of Minnesota.

*Lipolytic Assays.* LPL activity was determined with [$^3$H]fatty acid labeled $Ol_3Gro$ emulsion stabilized by lysolecithin as described previously (Nilsson-Ehle, 1974). After sonication, the $Ol_3Gro$ emulsion was activated by addition of rat serum, which had previously been heated to 60–62 °C for 10 min to eliminate endogenous lipolytic activity (Henson and Schotz, 1975). In experiments using different substrate concentrations, the amounts of $Ol_3Gro$ and lysolecithin were varied to keep the proportions of $Ol_3Gro$ and lysolecithin the same (molar ratio $Ol_3Gro$–lysolecithin was 18). Concentrations of other components in the incubation mixture were kept constant, i.e., crystalline bovine serum albumin (0.85 mg/ml) and rat serum (0.085 ml/ml).

The OlGro hydrolyzing activity of the enzyme preparations was determined in two different systems, one employing OlGro in mixed micellar form (Nilsson-Ehle and Belfrage, 1972) and the other Ol-Gro in albumin-bound form (Nilsson-Ehle et al., 1973). Both OlGro substrate preparations were optically clear after sonication. When OlGro concentration was varied, concentrations of other components of the incubation mixture were kept constant, i.e., sodium deoxycholate (5 mM) or rat serum (0.13 ml/ml). The enzymatic activity recorded with saturating concentrations of the two OlGro substrates was essentially the same, and in experiments monitoring only OlGro hydrolyzing activity the two assays yielded similar results. The presence of bile salt, however, was found to inhibit the $Ol_3Gro$ hydrolyzing activity of LPL (cf. Fielding, 1970), and all experiments performed with mixtures of $Ol_3Gro$ and OlGro substrates were therefore carried out with albumin-bound OlGro.

All assays were performed in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl with enzyme activities of about 5 mU/ml (assayed with $Ol_3Gro$ substrate in the presence of serum). One milliunit of enzymatic activity represents the release of 1 nmol of fatty acid per min. Incubations were carried out for 30 min in a total volume of 0.2 ml. Released fatty acid and glycerol were extracted (Belfrage and Vaughan, 1969) and radioactivity was measured in a Beckman LS 250 scintillation spectrometer. Protein was determined by a standard procedure (Lowry et al., 1951).

*LPL Preparation.* Male Sprague-Dawley rats, weighing 150–200 g, were fasted for 18 h. Acetone powders were prepared from the excised trimmed hearts (Garfinkel and Schotz, 1972), and LPL was purified 1500–2000-fold from these powders by heparin–Sepharose chromatography (Twu et al., 1975). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated that the enzyme preparation was at least 80% pure and showed an apparent molecular weight of 60 000, which was consistent with that determined by Sephadex G-200 chromatography.

The enzyme preparations had a specific enzymatic activity of about 650 mU per mg protein when assayed with a $Ol_3Gro$ emulsion in the absence of serum. Addition of apolipoprotein C-II (10 μg/ml) or serum (8.5%, v/v) increased the initial reaction rate about fourfold, resulting in a specific enzymatic activity of about 2500 mU per mg protein. Specific enzymatic activity assayed with micellar OlGro or albumin-bound OlGro was about 550 mU per mg protein. Addition of serum or apolipoprotein C-II to incubations with OlGro had no effect on the enzymatic activity.

The purified enzyme (obtained in 5 mM barbital buffer (pH 7.2) containing 1.5 M NaCl) was stabilized by addition of crystalline bovine serum albumin to a final concentration of 1 mg per ml. Under these conditions about 70–80% of initial enzyme activity was preserved during storage for a week at −20 °C.

*Titration of LPL with n-Butyl(p-nitrophenyl)carbamide.* Increasing amounts (0–15 nmol) of carbamide, dissolved in acetone, were added to a series of tubes with 25 μg of purified LPL in 1 ml of 5 mM barbital buffer (pH 7.2) containing 1.5 M NaCl. Acetone concentration was adjusted to 1% (v/v). The tubes were incubated for 20 min. at +4 °C under gentle stirring. Aliquots of the reaction mixture were then assayed for hydrolytic activities.

## Results

*Heparin–Sepharose Chromatography of Lipolytic Activities of Rat Heart.* Monoacylglycerol hydrolase activities have been studied in a variety of sources including rat heart, and their properties are different from those of LPL (Björntorp and Furman, 1962; Yamamoto and Drummond, 1967). In extracts of acetone powders of rat heart, the OlGro hydrolyzing activity is about ten times greater than the LPL activity (Table I). When such extracts were fractionated on heparin–Sepharose columns (Table I), more than 80% of the OlGro hydrolyzing activity was not bound to the column. Most of the remaining OlGro hydrolase activity was eluted by 0.75 M NaCl; this fraction showed no hydrolytic activity with $Ol_3Gro$. In contrast, about 40% of the total LPL activity was initially bound to the matrix and eluted with 1.5 M NaCl. This fraction, which will be referred to as "purified LPL", also exhibited OlGro hydrolyzing activity, corresponding to about 20% of its $Ol_3Gro$ hydrolyzing activity.

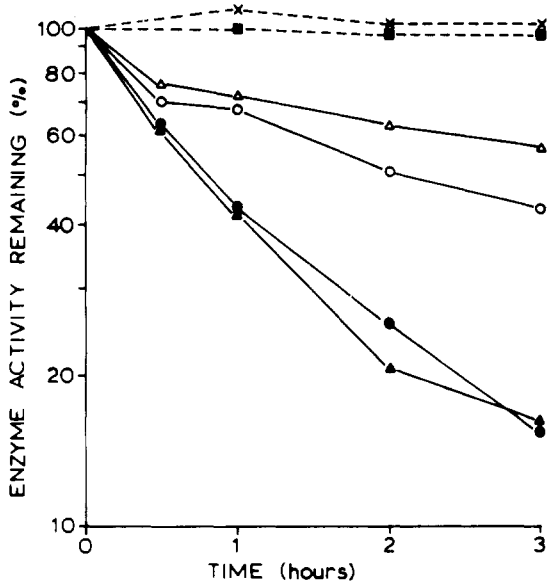


FIGURE 1: Stability of rat heart lipoprotein lipase and monoacylglycerol hydrolase activities in 1.2 M NaCl at +4 °C. (●) Purified LPL, trioleoylglycerol substrate; (▲) purified LPL, monooleoylglycerol substrate; (○) purified LPL with 20% glycerol (v/v), trioleoylglycerol substrate; (△) purified LPL with 20% glycerol, monooleoylglycerol substrate; (X) 0.75 M NaCl eluate from heparin–Sepharose chromatography, monooleoylglycerol substrate; (■) crude extract of acetone powder, monooleoylglycerol substrate. NaCl concentration in the assay was 0.15 M; substrate concentrations were 1.5 mM (trioleoylglycerol) and 3 mM (micellar monooleoylglycerol).

*Stability of Purified Rat Heart LPL.* The activities of purified LPL with $Ol_3Gro$ and OlGro were monitored in 1.2 M NaCl at 4 °C as a function of time in the absence and presence of 20% glycerol (Figure 1). In the absence of glycerol, about 60% of the enzyme activity with both substrates was lost during the first hour and about 85% during 3 h. Both hydrolytic activities could be stabilized by the addition of glycerol to a final concentration of 20%. The similar rates of inactivation of both hydrolytic activities suggest that a single enzyme is involved in the catalysis of the two substrates.

In contrast to the lability of the OlGro hydrolyzing activity of the purified LPL preparation, the OlGro hydrolyzing activity of crude extracts of acetone powders and of the column fractions eluted with 0.75 M NaCl was essentially stable under the same conditions (Figure 1). Although comparisons between enzyme preparations of different purity should be made with care, these data indicate that the OlGro hydrolyzing activity of the purified LPL does not represent contamination by a monoacylglycerol hydrolase.

*Comparison of $Ol_3Gro$ and OlGro Hydrolyzing Activities of Purified LPL.* LPL activity monitored as a function of substrate concentration with the three substrates (emulsified $Ol_3Gro$, micellar OlGro, and albumin-bound OlGro) yielded hyperbolic curves to which Michaelis kinetics could be applied. The $V_{max}$ values for LPL with emulsified $Ol_3Gro$ and micellar OlGro, as calculated from double-reciprocal plots, were 2500 and 550 mU per mg protein, respectively; the corresponding apparent $K_m$ values were 0.3 and 0.33 mM. The $V_{max}$ value obtained with albumin-bound OlGro was similar to that noted with micellar OlGro (550 mU per mg protein); the apparent $K_m$ for LPL with albumin-bound OlGro was found to be about 0.63 mM.

*Competitive Inhibition.* As discussed in detail by Dixon and Webb (Dixon and Webb, 1964), the mixed substrate method can be used to determine whether two reactions are catalyzed at the same active site. Addition of emulsified $Ol_3Gro$ to incubations of purified LPL and OlGro substrate (micellar or albumin-bound) resulted in inhibition of the OlGro hydrolyzing activity. Plot of the reciprocal of initial reaction velocity vs. reciprocal of OlGro concentration (Figure 2A) demonstrated that the inhibition was competitive in nature. Thus, addition of $Ol_3Gro$ to final concentrations of 0.57 and 1.14 mM to assays with albumin-bound OlGro resulted in a shift in apparent $K_m$ from 0.63 to 1.08 and 1.64 mM, respectively, without any change in $V_{max}$. When the hydrolytic activity of LPL with $Ol_3Gro$ was monitored in the presence of albumin-bound OlGro, a competitive inhibition could also be demonstrated (Figure 2B). Comparison of Figures 2A and B shows that the inhibitory effect of OlGro in the $Ol_3Gro$ assay was substantially stronger than that exerted by $Ol_3Gro$ in the OlGro assay. Thus, addition of 0.26 mM OlGro to the $Ol_3Gro$ assay resulted in an increase in apparent $K_m$ from 0.3 to about 9 mM. Addition of micellar OlGro to incubations of LPL with $Ol_3Gro$ substrate yielded inconclusive results, probably due to the inhibition of $Ol_3Gro$ hydrolyzing activity by bile salt (cf. Fielding, 1970). However, the mutual competitive inhibition exerted by emulsified $Ol_3Gro$ and albumin-bound OlGro indicates that the two substrates are hydrolyzed at the same active site.

Experiments were also carried out with the two acylglycerol substrates mixed at equal concentrations (Figure 3). In incubations using labeled $Ol_3Gro$ and labeled OlGro, the fatty acid release was substantially smaller than that noted with $Ol_3Gro$ alone, and only slightly larger than that recorded with OlGro alone. The fact that the hydrolytic activities with the two substrates assayed together did not add up to the calculated sum of the two activities assayed separately is consistent with the view that hydrolysis of the two substrates takes place at the same active site. Addition of unlabeled OlGro in the $Ol_3Gro$ assay resulted in essentially total inhibition of the enzyme activity measured, whereas the presence of $Ol_3Gro$ (labeled or unlabeled) in the OlGro assay did not significantly affect the enzymatic activity recorded. These data show that LPL preferentially catalyzes the hydrolysis of OlGro when incubated with mixtures of the two acylglycerols.

*Inhibition of LPL by n-Butyl(p-nitrophenyl)carbamide.*

Table I: Fractionation of Rat Heart Monoacylglycerol Hydrolase and Lipoprotein Lipase Activities by Heparin–Sepharose Chromatography.[a]

| Fraction | LPL Act.[b] (mU) | Monoacylglycerol Hydrolase Act.[c] (mU) | Ratio LPL/ Monoacylglycerol Hydrolase |
|---|---|---|---|
| Extract | 228 | 2279 | 0.10 |
| Unadsorbed protein | 140 | 1848 | 0.08 |
| 0.75 M NaCl | 0 | 249 | 0 |
| 1.50 M NaCl | 80 | 12 | 6.7 |

[a] One gram of rat heart acetone powder was extracted with 40 ml of 5 mM sodium barbital buffer (pH 7.2) and centrifuged at 30 000*g* for 20 min. The supernatant, designated extract, was applied to a heparin–Sepharose column (1 × 10 cm). Following passage of the sample, the column was washed with 10 ml of buffer. All fractions collected to this point were combined (unadsorbed protein). The column was further eluted sequentially with 30 ml of 0.75 M NaCl and 1.5 M NaCl in 5 mM barbital buffer (pH 7.2). [b] Assayed with trioleoylglycerol (1.5 mM) in presence of serum. [c] Assayed with micellar monooleolyglycerol (3 mM).

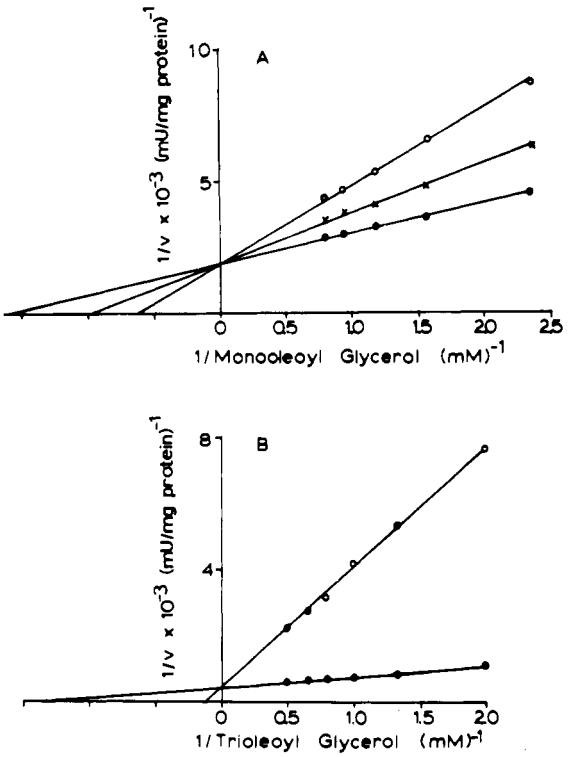


FIGURE 2: Double-reciprocal plot of rate of LPL-catalyzed hydrolysis of trioleoylglycerol in the presence of monooleoylglycerol and vice versa. (A) The incubations contained the indicated amounts of albumin-bound monooleoylglycerol in the absence (●) and presence of 0.57 mM (X) or 1.4 mM (O) emulsified trioleoylglycerol. (B) The incubations contained the indicated amounts of emulsified trioleoylglycerol in the absence (●) and presence (O) of 0.25 mM albumin-bound monooleoylglycerol.

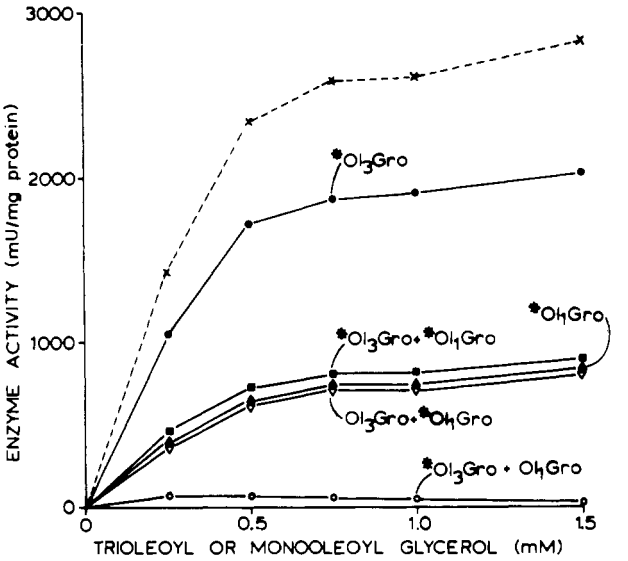


FIGURE 3: Hydrolysis by LPL of emulsified trioleoylglycerol and albumin-bound monooleoylglycerol assayed separately and mixed at equal molar concentrations. The mixed substrates were prepared with one component labeled (*) and the other unlabeled, as well as with both substrate components labeled (*). The dotted line represents the calculated sum of tri- and monooleoylglycerol hydrolytic activities assayed separately.

Preincubation of LPL with increasing concentrations of carbamide resulted in a progressive loss of enzyme activity assayed with both $Ol_3Gro$ substrate and OlGro substrate (Table II). The concentration of carbamide causing about 50% inhibition was comparatively low ($1 \times 10^{-5}$ M). Higher concentrations ($1.4 \times 10^{-5}$ M) resulted in about 85% inhibition of LPL activity. This inhibition, however, could be largely prevented by the presence of $Ol_3Gro$ or OlGro at 1.5 mM concentration (about five times the $K_m$ value). This substrate protection indicates that the carbamide acts at the active site of the enzyme. The fact that preincubation with $Ol_3Gro$ also prevented the inhibition of OlGro hydrolyzing activity by the carbamide supports the view of a common active site catalyzing the hydrolysis of the two acylglycerols. Whether the presence of OlGro would also protect the $Ol_3Gro$ hydrolyzing activity in a similar manner is difficult to determine due to the preferential hydrolysis of OlGro when both substrates are present (Figure 3).

Table II: Inhibition of Lipoprotein Lipase Activity by *n*-Butyl(*p*-nitrophenyl)carbamide in the Absence and Presence of Acylglycerol Substrates.[a]

| | Enzyme Act. (% of Control) | |
|---|---|---|
| | $Ol_3Gro$ Substrate[b] | OlGro Substrate[c] |
| None | 100 | 100 |
| Carbamide ($0.5 \times 10^{-5}$ M) | 82 | 70 |
| Carbamide ($1.0 \times 10^{-5}$ M) | 60 | 48 |
| Carbamide ($1.4 \times 10^{-5}$ M) | 15 | 20 |
| $Ol_3Gro$ + carbamide ($1.4 \times 10^{-5}$ M) | 99 | 78 |
| OlGro + carbamide ($1.4 \times 10^{-5}$ M) | *d* | 89 |

[a] Purified LPL (25 μg per ml) was incubated at +4 °C with and without unlabeled trioleoylglycerol ($Ol_3Gro$; 1.5 mM, emulsified with lysolecithin in the absence of albumin or serum) or unlabeled monooleoylglycerol (OlGro; 1.5 mM, sonicated with deoxycholate). After 2 min, the incubation mixture was titrated with carbamide for 20 min. Aliquots (0.1 ml) of the reaction mixtures were assayed. [b] Emulsified trioleoylglycerol (1.5 mM) in the presence of serum. [c] Micellar monooleoylglycerol (3 mM). [d] Not determined.

## Discussion

The substrate specificity of glycerol ester hydrolases (EC 3.1.1.3) seems to be defined by the physicochemical nature rather than by the chemical structure of the substrate (Desnuelle and Savary, 1963; Morgan et al., 1968). For example, pancreatic lipase acts almost exclusively on insoluble, emulsified substrates, whereas another lipolytic enzyme from the pancreas (pancreatic esterase) reacts preferentially with micellar or water-soluble substrates (Morgan et al., 1968; Erlanson, 1970). Similarly, lipolytic enzymes with predominantly "lipase" or "esterase" activities have been described in preparations from adipose tissue (Vaughan et al., 1964), liver (Belfrage, 1965; Ledford and Alaupovic, 1975), and postheparin plasma (Fielding, 1969; LaRosa et al., 1972; Nilsson-Ehle and Belfrage, 1972).

Lipoprotein lipases, on the other hand, show "lipase" as well as "esterase" activity; also, these enzymes readily act upon esters of different chemical structure, including triacylglycerols and partial acylglycerols of long- and short-chain fatty acids (Egelrud and Olivecrona, 1973). The different characteristics of the LPL reaction with, for example, emulsified triacylglycerol and micellar monoacylglycerol (activation by serum apolipoproteins, pH optimum) might suggest the presence in LPL of different active sites catalyzing the hydrolysis of emulsified and micellar substrates, respectively; the specificity of these hypothetical sites may also be related to the chemical

structure of the substrate (triacylglycerol vs. monoacylglycerol). However, it is also possible that hydrolysis of these different substrates occurs at the same active site, and that the different properties of the hydrolytic activities of LPL reflect differences in the composition of the substrates. In the present study we have attempted to distinguish between these possibilities by investigating the relationship between the LPL-catalyzed hydrolysis of two physiological substrates, triacylglycerol and monoacylglycerol, the former in emulsified form and the latter in either mixed micellar or albumin-bound form. It has been shown that Michaelis kinetics can be applied to enzyme reactions involving emulsified as well as micellar substrates (Sarda and Desnuelle, 1958). However, due to the different physicochemical nature of these substrate preparations, comparison of kinetic parameters involving substrate concentration, i.e., apparent $K_m$, is of little value.

The enzyme employed in this study is a highly purified preparation of LPL from rat heart (Twu et al., 1975). It is interesting to note that the maximal reaction rates with micellar or albumin-bound OlGro and emulsified $Ol_3Gro$ in the absence of serum were similar (550 and 650 mU per mg protein, respectively). Addition of apolipoprotein C-II or serum selectively enhanced the $Ol_3Gro$ hydrolyzing activity; under optimal conditions for each substrate, the OlGro hydrolyzing activity was therefore 20–25% of the $Ol_3Gro$ hydrolyzing activity. Similar relative reaction rates with these substrates have been reported in studies using LPL purified from postheparin plasma and bovine milk, respectively (Fielding, 1972; Egelrud and Olivecrona, 1973). As stated in the Results section, it is unlikely that the OlGro hydrolyzing activity of the purified enzyme preparation would represent contamination by other esterolytic activities.

The major finding in the present study was the demonstration of a mutual competitive inhibition between emulsified $Ol_3Gro$ and albumin-bound OlGro. Although this phenomenon might be caused by effects on the substrate rather than the enzyme (e.g., by the association of one substrate with the other, resulting in an alteration of the substrate characteristics), this mutual competitive inhibition strongly suggests that hydrolysis of the two substrates is catalyzed at the same active site. This view is reinforced by the "mixed substrate" experiments, showing that the enzyme reaction rate using equimolar mixtures of $Ol_3Gro$ and OlGro substrates does not add up to the calculated sum of the reaction rates determined with $Ol_3Gro$ and OlGro separately. Further evidence for a common active site for the two substrates lies in the substrate protection against inhibition by *n*-butyl(*p*-nitrophenyl)carbamide (see below). The different characteristics of LPL-catalyzed hydrolysis of emulsified $Ol_3Gro$ and micellar or albumin-bound OlGro, e.g., stimulation by apolipoprotein C-II, therefore probably are related to the different physicochemical properties of these substrates, and to the interaction of the substrate particles with the enzyme.

It has been demonstrated (Yasuoka and Fujii, 1971) that short-chain triacylglycerol (tributyrin) competitively inhibits hydrolysis of a mixed long-chain triacylglycerol emulsion (Ediol) catalyzed by LPL purified from postheparin plasma. These data, however, are difficult to interpret since the acylglycerols used are heterogenous both with regard to their chemical structure and physicochemical state. Ediol, for example, contains significant amounts of monoacylglycerol (Biale and Shafrir, 1969), and tributyrin is readily water soluble at low concentrations but forms an emulsion at concentrations over about 1 mM (Dixon and Webb, 1964). The results, however, are consistent with the view that hydrolysis of different acylglycerols takes place at the same active site of LPL.

When incubations were performed with both substrates at equimolar concentrations, a preferential hydrolysis of OlGro was noted. This observation is consistent with the earlier demonstration that essentially no 1(3)-monooleoylglycerol accumulates during the sequential degradation of $Ol_3Gro$ to glycerol and fatty acid catalyzed by LPL (Nilsson-Ehle et al., 1973).

An interesting finding was the high affinity of *n*-butyl(*p*-nitrophenyl)carbamide for LPL. If we assume the enzyme were pure, it can be calculated that 50% inhibition would be obtained at concentrations corresponding to a molar ratio of carbamide to enzyme of 25. With protamine sulfate, which is frequently used as a "specific" inhibitor of LPL, similar degrees of inhibition are seen with molar ratios of protamine to enzyme of about 1000 (Twu et al., 1975). The demonstration of substrate protection against inhibition by the carbamide indicates that this compound acts specifically at the active site; we can not, however, rule out the possibility that this phenomenon would represent an impaired accessibility of the inhibitor to its site of action in a less direct way (e.g., by a change in the enzyme's conformation on interaction with the substrates). The presence of $Ol_3Gro$ could also protect the OlGro hydrolyzing activity, again indicating that the two enzymatic activities reside in the same active site. The mechanism of carbamide inhibition is not fully known; however, the chemical structure of this carbamide derivative, containing an alkyl chain and a hydrophilic group, has certain similarities to that of acylglycerols, and it seems possible that the carbamide molecule would behave as a substrate analogue and bind specifically to the active site. It is also possible, however, that the inhibition of LPL activity by the carbamide results from the formation of a covalent linkage between the butylcarbamoyl moiety of the carbamide and functional groups of amino acid residues at the active site of the enzyme, such as serine hydroxy, lysine amino, and cysteine sulfhydryl groups (Twu and Wold, 1973). It is of interest to note that involvement of serine has been demonstrated at the active site of pancreatic lipase (Maylie et al., 1972) as well as a bacterial lipase (Fulton et al., 1974). With regard to LPL, there is strong evidence that histidine is involved in the catalytic site (Fielding, 1973); in addition, reactions of ionizable groups with p$K$ values of 8.0 and 9.8 were indicated. It is possible that these latter groups may represent cysteine and lysine, respectively.

Although hydrolysis of tri- and monoacylglycerol seems to occur at the same active site of LPL, the specific requirements for hydrolysis of these two substrates are different. Elucidation of the nature of the active site may lead to clarification of these differences and to an understanding of the mechanism of interaction of enzyme, substrate, and apolipoprotein activator.

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## References

Arvidson, E. O., and Belfrage, P. (1969), *Acta Chem. Scand. 23,* 232.

Belfrage, P. (1965), *Biochim. Biophys. Acta 98,* 660.

Belfrage, P., and Vaughan, M. (1969), *J. Lipid Res. 10,* 341.

Biale, Y., and Shafrir, E. (1969), *Clin. Chim. Acta 23,* 413.

Björntorp, P., and Furman, R. H. (1962), *Am. J. Physiol. 203,* 323.

Brown, W. V., Levy, R. I., and Fredrickson, D. S. (1969), *J. Biol. Chem. 244,* 5687.
Desnuelle, P., and Savary, P. (1963), *J. Lipid Res. 4,* 369.
Dixon, M., and Webb, E. C. (1964), in Enzymes, 2d ed, New York, N.Y., Academic Press, p 63.
Egelrud, T., and Olivecrona, T. (1973), *Biochim. Biophys. Acta 306,* 115.
Erlanson, C. (1970), *Scand. J. Gastroenterol. 5,* 333.
Fielding, C. J. (1969), *Biochim. Biophys. Acta 178,* 499.
Fielding, C. J. (1970), *Biochim. Biophys. Acta 206,* 109.
Fielding, C. J. (1972), *Biochim. Biophys. Acta 280,* 569.
Fielding, C. J. (1973), *Biochim. Biophys. Acta 316,* 66.
Fulton, J. E., Noble, N. L., Bradley, S., and Awad, W. M. (1974), *Biochemistry 13,* 2320.
Garfinkel, A. S., and Schotz, M. C. (1972), *J. Lipid Res. 13,* 63.
Henson, L., and Schotz, M. C. (1975), *Biochim. Biophys. Acta 409,* 360.
LaRosa, J. C., Levy, R. I., Windmueller, H. G., and Fredrickson, D. S. (1972), *J. Lipid Res. 13,* 356.
Ledford, J. H., and Alaupovic, P. (1975), *Biochim. Biophys. Acta 398,* 132.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Maylie, M. F., Charles, M., Sarda, L., and Desnuelle, P. (1972), *Biochim. Biophys. Acta 276,* 162.
Morgan, R. G. H., Barrowman, J., Filipek-Wender, H., and Borgström, B. (1968), *Biochim. Biophys. Acta 167,* 335.
Nilsson-Ehle, P. (1974), *Clin. Chim.Acta 54,* 283.
Nilsson-Ehle, P., and Belfrage, P. (1972), *Biochim. Biophys. Acta 270,* 60.
Nilsson-Ehle, P., Egelrud, T., Belfrage, P., Olivecrona, T., and Borgström, B. (1973), *J. Biol. Chem. 248,* 6734.
Sarda, L., and Desnuelle, P. (1958), *Biochim. Biophys. Acta 30,* 513.
Twu, J. S., Garfinkel, A. S., and Schotz, M. C. (1975), *Atherosclerosis 22,* 463.
Twu, J. S., and Wold, F. (1973), *Biochemistry 12,* 381.
Vaughan, M., Berger, J. E., and Steinberg, D. (1964), *J. Biol. Chem. 239,* 401.
Yamamoto, M., and Drummond, G. I. (1967), *Am. J. Physiol. 213,* 1365.
Yasuoka, S., and Fujii, S. (1971), *J. Biochem. (Tokyo) 70,* 937.

# Hydrogen Ion Titration of Horse Heart Ferricytochrome *c*†

Robert W. Shaw and Charles R. Hartzell*

ABSTRACT: Continuous hydrogen ion titration curves of deionized solutions of horse heart ferricytochrome *c* have been obtained at 25 °C at a constant ionic strength of 0.10 from pH 3.0 to 11.0. Titration of the oxidized protein in KCl required 28.4 equiv over that pH range, and a small hysteresis between the forward and reverse limbs was displayed. The Linderstrom-Lang approximation, which takes into account electrostatic interactions between charged groups on the protein surface, was used in a computer simulation program to analyze the forward and reverse limbs of the titration curve separately. The results indicated 1 $\alpha$-, 12 $\beta$- and $\gamma$-, and 1 heme propionic carboxylic, 1 imidazole, 1 phenolic, and 18 $\epsilon$-amino residues appear to titrate normally. Variations in the electrostatic interaction factor $\omega$ suggest conformational changes in the protein at the extremes of pH, although the relationship of the variations in $\omega$ to the magnitude of the conformational changes does not appear to be strictly quantitative for cytochrome *c*. These results show the acid–base behavior of cytochrome *c* to be complex in nature, and suggest that the Lindenstrom-Lang model may not be adequate for cytochrome *c*.

In mammalian species, cytochrome *c* is composed of 104 amino acid residues having a molecular weight of 12 400 and an iron porphyrin covalently attached via the porphyrin ring vinyl groups to two cysteine sulfhydryls of the protein. Cytochrome *c*, receiving electrons from cytochrome $c_1$ and in turn reducing cytochrome oxidase, is a necessary link in the mitochondrial electron transfer between NADH or succinate to molecular oxygen (Margoliash and Schejter, 1966). A reversible Michaelis complex between cytochrome *c* and cytochrome oxidase was postulated (Stotz et al., 1938) and some of the properties of the cytochrome *c*–cytochrome oxidase complex were recently reviewed by Nichols (1974). In order to elucidate even the most elementary points of the cytochrome *c*–cytochrome oxidase reaction mechanism and the roles of their interaction in mitochondrial electron transfer and energy coupling, further physical chemical characterization is required. To obtain a better understanding of the thermodynamic factors affecting cytochrome *c* interaction with cytochrome oxidase, we have undertaken the study of hydrogen ion titrations of both oxidized and reduced cytochrome *c* at constant ionic strength.

Acid–base titration curves for this protein have been reported by several authors including Theorell and Åkesson (1941a,b), Paul (1951), Paléus (1954), Bull and Breese (1966a), Greenwood and Wilson (1971), and Marti and Marini (1972). These curves were determined under a variety of experimental conditions including in many instances vari-

† From the Department of Biochemistry and Biophysics, Paul M. Althouse Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received July 22, 1975.* This paper represents the first in a series of studies dealing with the interactions between mammalian cytochrome *c* and cytochrome *c* oxidase. This work was done during the tenure of C. R. Hartzell as an Established Investigator of the American Heart Association and was supported in part with grants from the Central Fund for Research, The Pennsylvania State University and the U.S. Public Health Service, National Heart and Lung Institute (HL 17559). A preliminary report on a part of this work has been presented (Shaw and Hartzell, 1975).