In Vitro Model for Lipase-Catalyzed Lipophile Release from Fats

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A simple method was developed to study the release and dispersion of lipophilic substances into the aqueous phase from an edible oil emulsion and to estimate their potential availability for transport across the intestinal lumen. The rates of cholesterol release and dispersion from butter oil–cholesterol and from butter oil fatty acid based sucrose polyester–cholesterol emulsions were compared when both systems were exposed to lipoprotein lipase or bile–pancreatic fluid, under conditions that simulated in vivo hydrolysis. The cholesterol lipophile remained associated with the oil phase in the butter oil emulsion until appreciable hydrolysis had taken place. Results clearly indicate that cholesterol release is intimately linked to the extent of lipolysis likely via cholesterol entrapment in the micelles as they are formed. No detectable lipolysis took place in experiments with sucrose polyester–cholesterol emulsions and no cholesterol was released. The method provides a facile technique for in vitro measurements of lipophile release, and the results obtained conform to in vivo hypotheses of lipid transfer and absorption.

INTRODUCTION

A variety of lipophiles are associated with triglycerides which dominate food lipids, with some of them being of concern from a nutritional or a toxicological standpoint. Classic examples include cholesterol in butter, fat-soluble vitamins, or lipophylic pesticides. Although in vivo studies provide definitive data on the extent of transfer and uptake of lipophiles, their initial release from the triglyceride matrix is obviously an important factor related to their subsequent absorption.

Although gastric lipases may also contribute to the lipolysis of dietary triglycerides (Gargouri et al., 1989), the action of pancreatic lipases in the intestinal lumen is generally accepted as a key step in fat absorption. As it enters the intestine, lipophile dispersed in a dietary triglyceride carrier is emulsified by bile salts to form droplets of large surface area. Pancreatic lipase is known to be active only at an oil–water interface (Sarda and Desnuelle, 1958) and to hydrolyze esters of primary but not secondary alcohols (Mattson and Volpenhein, 1966, 1968). As early as 1964, Mattson and Volpenhein reported that hydrolysis of oleoyl glycerides in the rat lumen proceeded by way of α,β-diglycerides to yield predominantly β-monoglycerides and glycerol and that approximately 75% of the fatty acid and glycerol of this "dietary" triglyceride was absorbed into the intestinal wall as free acids or β-monoglycerides, respectively, through the intermediacy of micelles. The bulkier triglycerides are excluded from these micelles, and both glycerol and monoglycerides undergo new acylations to re-form triglycerides in the intestinal wall. This resynthesized lipid is then transported to the lymphatic or blood system. The means by which lipophiles, dissolved in an edible fat, are transferred into the aqueous phase have not been clearly elucidated, although micelles as a suitable carrier have been implicated (Surpuriya and Higuchi, 1972). In vivo, lipophiles such as cholesterol are considered to be transferred from the lipid droplets to the micellar phase, whence they can be transferred to and absorbed by intestinal mucosal cells and ultimately transported to the bloodstream. The extent of partitioning of the lipophile between the emulsified triglyceride and preformed micelles is governed by the relative solubility of the lipophile in the two phases (Jandacek, 1982).

In the course of studies of potential substitutes for dietary glycerides which would, inter alia, reduce caloric intake, considerable attention has been paid to sucrose polyester (SPE, olestra). This is a fatlike synthetic compound, prepared from sucrose and the methyl esters of fatty acids (Rizzi and Taylor, 1978), having physical and interfacial properties similar to those of mixtures of triglycerides with the same fatty acid composition (Fallat et al., 1976; Crouse and Grundy, 1979; Adams et al., 1981) but which is neither digested by pancreatic lipases (Mattson and Volpenhein, 1972) nor absorbed by the intestinal tract (Mattson and Nolen, 1972; Glueck et al., 1980). SPE thus remains as an oil phase in the intestinal lumen and is secreted almost unchanged together with any dissolved lipophiles (Glueck et al., 1980). These properties have led to SPE being considered as a potential substitute for dietary triglycerides, which would help reduce the absorption of cholesterol (and other lipophiles) and, because of the equilibria between lipophiles in tissue, in serum, and in bile fluids, would reduce both total and low-density lipoprotein cholesterol (Glueck et al., 1979). In clinical trials, olestra (SPE) has been demonstrated to reduce (but not eliminate) the intestinal absorption of cholesterol and to inhibit the reabsorption of endogenous biliary cholesterol (Mellies et al., 1985; Mattson and Jandacek, 1985). By contrast, no statistically significant differences were evident in the extent of absorption of four relatively lipophilic drugs by human subjects that had ingested the drug orally together with olestra, partially hydrogenated soybean oil, or water (Roberts and Leff, 1989). Similarly, in vivo intestinal absorption experiments with rats demonstrated that the presence of SPE (at 10 g/L of perfusate) decreased the net absorption rate of vitamin A by only 8.7% (Sletten et al., 1985). The intestinal lumen thus contains sufficient emulsifying agents and micelles so that lipophilic solutes can be partially extracted from an oil emulsion without the necessity of hydrolyzing the carrier oil.

The work described in this paper illustrates how a simple, in vitro model system can be used to measure the effects

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of lipolysis on cholesterol release from butter triglycerides under conditions comparable to those in vivo. The method can be readily extended to measure the effects of lipolysis and the release of lipophiles under a variety of conditions.

MATERIALS AND METHODS

Preparation of Butter-Based Sucrose Polyesters (BSPE). Molten summer butter (Agropur, Coopérative Agro-Alimentaire, Granby, Québec) was dehydrated at 60 °C over anhydrous sodium sulfate and filtered. Butter fatty acid methyl esters were prepared by mixing equivalent amounts of sodium methoxide in methanol with anhydrous molten butter. After a 12-h reaction at room temperature, the methanol layer was separated, washed with distilled water until negative to phenolphthalein, and dried over anhydrous sodium sulfate and the methanol removed under vacuum. A portion of the product was then converted to its respective fatty acid salts by the addition of a 50% (w/v) aqueous solution of potassium hydroxide. An additional 5% of butter fatty acid methyl esters was added to the crude reaction mixture to ensure that all of the base had reacted. After reaction, volatiles were removed under reduced pressure, and the powdered residues were washed with cold methanol, followed by *n*-hexane, and dried under vacuum.

BSPEs were prepared according to the interesterification method of Rizzi and Taylor (1978). Fifteen grams (0.03 mol) of anhydrous sucrose powder was added to 50 mL of the butter methyl esters (0.09 mol) and 5 g of the anhydrous potassium fatty acid salts. The mixture was reacted at 95 °C for 1.3 h under a partial vacuum (10 mmHg) and then cooled to 50 °C, and sodium hydride (0.1 g of 60% dispersion in mineral oil, Aldrich Chemical Co., Milwaukee) was added. The mixture was reheated to 150 °C to initiate the reaction and then was allowed to proceed for 2 h without further heating during which time the sucrose dissolved. A second 0.1-g aliquot of sodium hydride was added, and the reaction was continued for a further 1.5 h. A second addition of methyl esters (150 mL) was made and the mixture again heated to 150 °C for and additional hour, followed by a third aliquot of sodium hydride. The reaction mixture was reheated and held at 150 °C for 3 h and then allowed to cool, with 1 mL of acetic acid then added to quench the reaction. The residue was washed nine times with methanol (300 mL) to remove excess methyl esters. Cooling in an ice bath facilitated phase separation. The solids were redissolved in *n*-hexane, decolorized by warming in the presence of activated charcoal, and filtered, and the solvent was removed under vacuum. The resulting pale yellow viscous butter fatty acid based SPE residue solidified at room temperature.

Lipases. The bile pancreatic ducts of eight male rats (250 g, CD Charles River, Longueil, Québec) were cannulated at a point near the entrance into the duodenum and the bile–pancreatic fluid was collected over a 24-h period into a test tube maintained at 4 °C. Lipoprotein lipase (EC 3.1.1.3, activity 50 units/mg of protein) was purchased from Sigma Chemical Co., St. Louis.

Lipid–Cholesterol Preparation. Cholesterol (99% pure, Sigma) was taken up in 1 mL of chloroform and appropriate aliquots mixed thoroughly with 10 g of either dry summer butter or the synthesized BSPE, and the chloroform was removed under vacuum. The final concentration was 8 mg of cholesterol/g of lipids. Emulsions were prepared as needed for enzyme assays by using a Polytron (Brinkman Instruments Ltd.) to mix 1 g of the lipid–cholesterol mixture with 100 mL of 0.1 M Tris buffer (0.1 M, pH 7.2, containing 5% bovine serum albumin and 0.1 g of $MgCl_2 \cdot 6H_2O$) at maximum speed for 5 min.

Lipolysis. Two enzyme solutions were prepared either by dissolving lipoprotein lipase (550 units) in Tris buffer (0.1 M, 50 mL, pH 7.2) or diluting the bile–pancreatic fluid (4 mL) with the same buffer to give a final volume of 50 mL. The lipid–cholesterol substrate and enzyme solutions were separately thermostated at 37 °C for 1 h prior to mixing 25 mL of each to initiate the reaction. At 3-min intervals, 5-mL aliquots of the incubation mixture were withdrawn and immediately immersed in an ice bath to arrest lipolysis and solidify the lipid droplets. The solidified fat was quantitatively recovered on Whatman No. 1 filter paper by filtration and the clear filtrate collected. The filtrate was lyophilized, and the residue, resuspended in 0.2 mL of distilled water, was subjected to cholesterol analysis using a Boehringer-Mannheim (Mannheim, Germany) cholesterol assay kit. A control trial containing identical concentrations of butter oil–cholesterol emulsion and the Tris buffer (containing 5% BSA and $MgCl_2$) was conducted for 8 h for comparison.

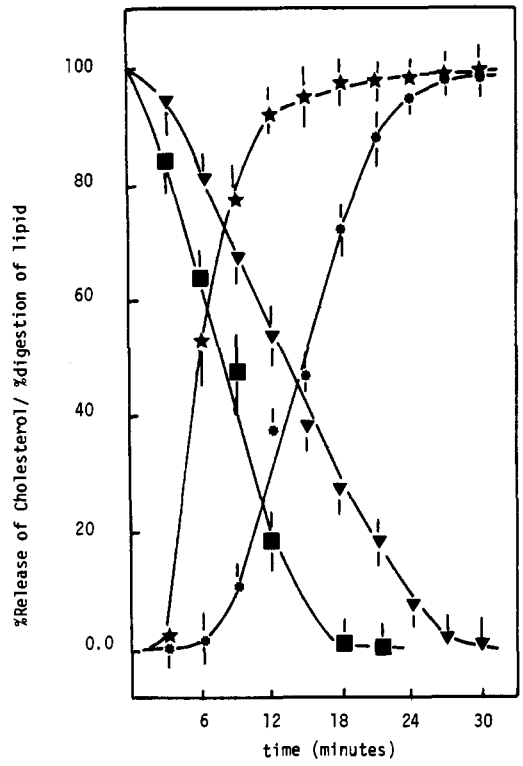

Figure 1. Variation, with time, of the percentage of lipolysis of a butter oil triglyceride emulsion in the presence of bile–pancreatic fluid (*) or lipoprotein lipase (★) and the percentage cholesterol release (▲ and ■, respectively). Error bars, representing ±1 relative standard deviation from the mean (n = 6), have been added to individual data points.

Lipid Recovery/Analysis. The solid lipid material on the Whatman filter and any adhering to the test tube were extracted by using six 5-mL rinses of warm *n*-hexane. The rinses were combined and evaporated, and the residue was mixed with tetrahydrofuran (0.2 mL) for total lipid analysis. Gel permeation chromatography (GPC) of the solid lipid material was carried out by using a Waters Associates (Milford, MA) system composed of a Model 510 pump, a U6K universal injector, an R401 differential refractometer (8 × 8 attenuation), and three serially connected 7.8 mm i.d. × 30 cm Ultrastyragel columns having exclusion limits of 10^3, 500, and 100 Å, respectively. The permeation and void volumes of the columns were determined to be 36 and 18 mL, respectively. Tetrahydrofuran was used as the elution solvent at a rate of 1 mL min^{-1} and the injection volume was 10 μL. Peak detection and integration were performed by using a Spectra-Physics Model SP-4270 integrator. Experiments were performed in triplicate, and measurements were carried out in duplicate.

RESULTS AND DISCUSSION

Under the specified experimental conditions, the final 0.5% emulsions of butter oil triglycerides were completely hydrolyzed after 30 min by the bile–pancreatic fluid. The appearance of cholesterol in the aqueous phase was measured enzymatically, and the disappearance of triglycerides was measured by decreases in the peak area of triglycerides as determined by GPC. The rate of hydrolysis of the butter oil triglycerides, as measured by their disappearance, was linear and numerically equal to the rate of release of cholesterol into the aqueous buffer solution (Figure 1). Under in vitro conditions, cholesterol

is apparently released into the aqueous phase as micelles (of fatty acids, biles salts, and mono- and possibly diglycerides) are formed, much in the same way as in vivo prior to transport into intestinal mucosal cells.

It is notable that the release of cholesterol shows an induction period of ca. 20% of the total hydrolysis time in the bile–pancreatic fluid experiments. One possible explanation for the lag is that initial lipolysis yields mostly diglycerides at the oil–water interface of the emulsion droplets which are not readily released from the lipid matrix. Upon formation of monoglycerides, these are likely released into the aqueous medium as individual molecules until they reach the critical micelle concentration. From this point on, it is likely that monoglycerides can only be released as micelles from the fat droplet surface. As a consequence, the hydrophobic core of the micelle can act as a vehicle for cholesterol release from the lipid droplet. Inspection of the Figure 1, however, indicates clearly that this explanation is tenable only if the rate of hydrolysis of diglycerides is equal to, or greater than, that of triglycerides under the experimental conditions, as the rate of cholesterol release after the induction period is equal to that of triglyceride disappearance. Another mechanism of cholesterol release is simply through interfacial collisions of preformed micelles with the oil droplet, leading to transfer of cholesterol to the micelle as the lipid droplet grows smaller. The rate of cholesterol release by this mechanism has been shown to be much slower (Surpuriya and Higuchi, 1972). It is likely that this mode of cholesterol release is secondary in comparison to the lipolytic mechanism.

For lipoprotein hydrolyses, the activity of added enzyme (225 units per hydrolytic trial) was chosen to approximate the activity of the bile–pancreatic fluid trials. When lipoprotein lipase was used for lipolysis, the rates of hydrolysis of butter oil triglycerides and of cholesterol release increased by factors of 1.46 and 1.86, respectively. Presumably, the differences in rates for the hydrolysis using lipase from different sources reflect differences in enzyme activities. Accompanying these faster rates of reaction was an approximate 4-fold decrease in the induction period for cholesterol release (Figure 1). The control trial, in the absence of lipolytic enzyme, resulted in less than 5% transfer of cholesterol to the aqueous phase over 8 h corroborating that transfer to the bulk medium is unimportant (Surpuriya and Higuchi, 1972).

Replacement of the butter oil triglycerides by BSPE in either of these lipolysis reactions resulted, as anticipated, in neither detectable lipolysis (Mattson and Volpenhein, 1972) nor release of cholesterol over the reaction time. This result is in qualitative accord with in vivo studies of the hypocholesterolemic effect of SPE which showed that fatty droplets containing cholesterol or other lipophiles such as vitamins A and E (Glueck et al., 1979) or DDT (Volpenhein et al., 1980) in SPE were removed from the intestine almost chemically unchanged (St. Clair et al., 1981). Clearly, the process of lipolysis and the concomitant formation of micelles are important factors in the rapid release of cholesterol from the fat droplets. While this property of SPE has obvious advantages, the regular intake and excretion of undigested SPE may cause some depletion of the mucinous glycoprotein layer of the gastrointestinal tract.

CONCLUSION

This in vitro model offers a simple system for the study of the kinetics, catalyzed by lipolysis, of the release of lipophiles from lipid-in-water emulsions. The study with the nonhydrolyzable, butter oil based sucrose polyester (synthetic fat) indicates that, in the absence of lipolysis and/or micelles capable of stripping the cholesterol from the fat like carrier, it is not released. Thus, the BPSE is a useful reference material against which to measure the release and dispersion of cholesterol relative to butter oil. This model carrier, which can be customized for any edible lipid system, can be used to obtain release data for other lipophiles (i.e., vitamins A, D, and E or pesticides such as DDT) and provides an in vitro indication of the availability of the lipophile in question for transport across the mucosal membrane. With the inclusion of an appropriate concentration of a micellar phase, the BPSE model system should permit an estimate of the relative importance of transfer between the emulsion and preformed micelles and the transfer directly into micelles as they are formed (and concomitantly released) from the emulsified oil.

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