Triglyceride formation and hydrolysis by toad bladder epithelium

A. A. ROSENBLOOM* and P. ELSBACH‡

Department of Medicine, New York University School of Medicine, New York 10016

ABSTRACT Triglycerides of toad bladder epithelium have been labeled in vitro with either palmitate-1-^{14}C or linoleate-1-^{14}C, during incubation of bladders that had been cut in halves. Hydrolysis with pancreatic lipase of triglycerides labeled in this fashion revealed that palmitate-1-^{14}C appeared predominantly in the 1- and 3-position, whereas half of linoleate-1-^{14}C was located in the 2-position.

The hydrolysis of palmitate-1-^{14}C or linoleate-1-^{14}C labeled triglycerides was examined in homogenates of isolated bladder mucosal cells. Lipase activity was evident from pH 3.5 to 8.0, but clearly greatest at pH 4.5. Below pH 6.0 the products of hydrolysis were fatty acid and monoglyceride and the 1- (or 3-) position was preferentially attacked; above pH 6.0 complete deacylation occurred.

Acid-optimum hydrolysis of triglycerides with production of monoglycerides was linear for about 30 min. After 2 hr most of the labeled triglycerides were hydrolyzed. Repeated freezing and thawing of the homogenate enhanced lipase activity. Added Ca^{++}, previously shown to be required for phospholipase A activity in toad bladder, had no effect on hydrolysis of triglycerides.

This lipase activity directed at the considerable store of triglycerides present in toad bladder epithelium may provide fatty acid for energy production or for synthesis of other esters such as in phospholipids.

SUPPLEMENTARY KEY WORDS endogenous triglycerides · fatty acid distribution · hydrolysis · acid lipase

ISOLATED MUCOSAL cells of the urinary bladder of the toad (*Bufo marinus*) convert albumin-bound lysolecithin in the incubation medium to cellular lecithin (1). The pathway of lecithin synthesis is: lysolecithin + fatty acid $\xrightarrow{ATP+CoA}$ lecithin (2, 3). Whereas increasing concentrations of lysolecithin in the medium stimulate lecithin formation by mucosal cells (1), incorporation of fatty acid-1-^{14}C of the medium into lecithin is not enhanced by the addition of lysolecithin to the incubation mixture (see below). This finding suggested the possibility that the fatty acids required for acylation of lysolecithin were derived from endogenous lipid. Observations in this laboratory on granulocytes, cells that are richly endowed with lipases (4, 5), had indicated that cellular triglyceride may serve as a source of fatty acid for synthesis of lecithin (6). We therefore initiated our search for the origin of fatty acid needed for acylation of lysolecithin by seeking evidence for triglyceride-splitting activity in homogenates of isolated mucosal cells of the toad bladder. To this end we studied the hydrolysis of endogenous triglyceride, labeled during preincubation of whole toad bladder with fatty acid-1-^{14}C. Considerable lipase activity directed at endogenous substrate was found. This report concerns a description of several characteristics of this activity.

METHODS

Labeling of Tissue

Urinary bladders of well-hydrated toads were removed and washed in toad Ringer's solution (pH 7.6) containing 112 mM Na^+, 119 mM Cl^-, 2.4 mM HCO_3^-, 3.4 mM K^+, 2.7 mM Ca^{++}, and 5.5 mM glucose. Each bladder was cut in two and incubated in 0.8 ml of this solution, to which had been added 0.2 ml of toad serum and 0.5 μc of linoleate-1-^{14}C or palmitate-1-^{14}C (59.2 and 44.3 mc/mmole, respectively, Nuclear-Chicago Corp., Des Plaines, Ill.). The traces of labeled fatty acid were complexed to the albumin in the toad serum as previously described (7). The mean free fatty acid content of three batches of pooled toad serum was 325 μmoles/liter (8). A stream of water-washed air was blown over the incubation mixture. The pH of the incubation mixture showed little or no change during the course of

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incubation. After this preliminary incubation the bladders were washed twice with Ringer's solution to remove the radioactive medium. The mucosal layers were then removed from the underlying structures by gentle scraping with a glass microscope slide. The isolated cells were collected and washed twice in Ringer's solution by suspension and centrifugation as previously described (1) and incubated for 15 min at 37°C in toad serum–Ringer's solution 1:4 (1 ml for tissue obtained from one bladder) to allow remaining fatty acid-1-^{14}C to become esterified.

Finally, the radiolabeled, isolated mucosal cells were transferred to a glass Potter–Elvehjem tissue grinder, the cells were sedimented by centrifugation in the homogenizing tube, and the medium was removed by pipette. Homogenates were prepared in distilled water by means of a motor-driven Teflon pestle. The flask was kept in ice during and after homogenization.

Enzyme Assay

Assays for lipase activity were carried out in a total volume of 0.4 ml that contained the appropriate buffer (see below) and 0.1 ml of homogenate containing approximately 1 mg of protein as determined by the method of Lowry, Rosebrough, Farr, and Randall (9). The buffers used were (all at 10 mM): acetate for pH 3.5–5.5, phosphate for pH 5.5–7.0, and Tris–maleate for pH 7.0–8.0. When these buffers were used at overlapping pH no differences in activity were found.

Incubations for the assay were carried out in glass tubes at 37°C in a water bath with gentle shaking. Reactions were terminated by addition of 10 ml of chloroform–methanol 1:1 (10). Lipid extraction continued overnight at room temperature.

The extent of hydrolysis of triglycerides was determined by separation of the radioactive-extracted lipids by thin-layer chromatography on Silica Gel H (Brinkman Instruments, Inc., Westbury, N. Y.) and liquid scintillation counting of the isolated fractions (3). Thin-layer chromatography was carried out in two solvent mixtures. Petroleum ether–diethyl ether–glacial acetic acid 80:20:1 was used to separate 1,2- and 1,3-diglycerides, fatty acids, triglycerides, and cholesteryl esters. Monoglycerides are not adequately separated from the origin (at which water-soluble products and phospholipids are retained) in this solvent. For determination of monoglyceride radioactivity, therefore, diethyl ether–hexane–chloroform–glacial acetic acid 55:40:5:0.2 (11) was used. In this solvent, monoglycerides are completely separated from the material at the origin, but free fatty acids are less satisfactorily separated from triglycerides.

The triglyceride content of lipid extracts of isolated mucosal cells was determined by the method of Marsh and Weinstein (12).

RESULTS

Table 1 shows that increasing the lysolecithin concentration of the incubation medium, which reproducibly stimulates incorporation of lysolecithin into lecithin by isolated whole mucosal cells (1), does not enhance incorporation of added linoleate-1-^{14}C or palmitate-1-^{14}C.

The effect of time of incubation on the incorporation of linoleate-1-^{14}C into several lipid fractions is shown in Fig. 1. Labeling of phospholipids and triglycerides tended to level off after 4 hr. In most experiments, therefore, the toad bladder was radiolabeled for this period of time.

The distribution of radioactivity among major lipid fractions of isolated mucosal cells after the incubation with linoleate-1-^{14}C is shown in Table 2. About 90% of the radioactivity incorporated appears in phospholipid and triglyceride. More than 80% of the phospholipid radioactivity accumulates in lecithin (not shown).

The distribution of palmitate-1-^{14}C and linoleate-1-^{14}C among the positions on the labeled triglyceride was examined by hydrolysis with pancreatic lipase (13), which is specific for the 1-fatty acid. Table 3 shows that

TABLE 1 LACK OF EFFECT OF INCREASING LYSOLECITHIN CONCENTRATIONS ON INCORPORATION OF LINOLEATE-1-^{14}C OR PALMITATE-1-^{14}C INTO LECITHIN OF ISOLATED TOAD BLADDER MUCOSAL CELLS

Added Lysolecithin	Added Radioactivity	
	Linoleate	Palmitate
mμmoles	*cpm in lecithin/mg protein*	
0	7670	9260
5	5030	7010
25	7250	8540
50	7500	7000

Whole cells (approximately 1 mg of protein) were incubated for 60 min at 37°C in 0.5 ml of toad serum–toad Ringer's medium 1:4 containing 5 mμmoles of linoleate, 5 mμmoles of palmitate, 0.25 μc of either linoleate-1-^{14}C or palmitate-1-^{14}C, and increasing amounts of lysolecithin.

TABLE 2 DISTRIBUTION OF RADIOACTIVITY AMONG LIPID FRACTIONS OF ISOLATED MUSCOSAL CELLS AFTER INCUBATION WITH LINOLEATE-1-^{14}C

	% of Total Recovered Radioactivity
Phospholipids	52.7 ± 1.9 (5)*
Monoglycerides	0.7 ± 0.1 (4)
1,2- and 1,3-Diglycerides	3.7 ± 0.6 (5)
Free fatty acids	2.1 ± 0.2 (5)
Triglycerides	38.5 ± 2.5 (5)
Cholesteryl esters	2.6 ± 0.2 (5)

Whole bladders were incubated for 4 hr, the mucosal cells were scraped off, and the extracted lipids were separated by thin-layer chromatography.

* Mean ± SEM (no. of determinations).

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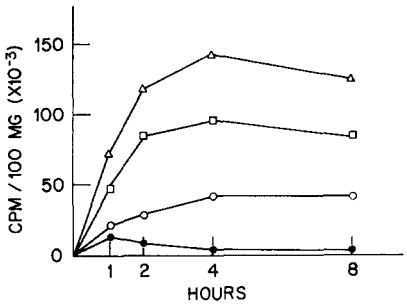

FIG. 1. Effect of time on incorporation of linoleate-1-^{14}C into lipids of toad bladder mucosal cells.

Cellular lipids were radiolabeled during incubation with linoleate-1-^{14}C complexed with toad serum. The mucosal cells were scraped off and the extracted lipids were separated by thin-layer chromatography in petroleum ether–diethyl ether–glacial acetic acid 80:20:1. Water washes of lipid extracts contained negligible radioactivity. Values are expressed per 100 mg wet wt of packed mucosal cells. ○, triglycerides; ●, free fatty acids; □, phospholipids; △, total radioactivity.

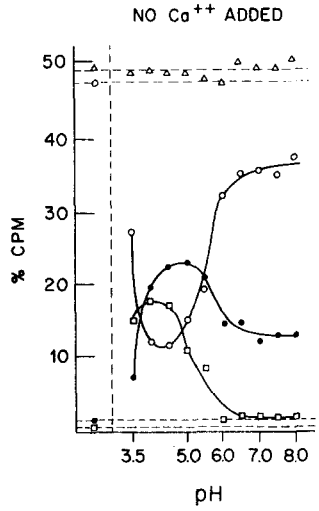

FIG. 2. Effect of pH on triglyceride hydrolysis in homogenates of isolated toad bladder mucosal cells in the absence of Ca^{++}.

Homogenates of mucosal cells previously labeled with linoleate-1-^{14}C were incubated for 60 min. Radioactivity in triglycerides (○), free fatty acids (●), and monoglycerides (□), and total radioactivity in these three fractions (△) are expressed as percentages of total radioactivity in the mucosal lipids. The symbols to the left of the vertical dashed line indicate the values at zero time.

hydrolysis of triglyceride labeled with palmitate-1-^{14}C results in accumulation of radioactivity in the free fatty acid fraction while the remaining monoglycerides contain little radioactivity. Thus, palmitate is preferentially incorporated into the 1-position. By contrast, after hydrolysis of triglyceride labeled with linoleate-1-^{14}C the monoglycerides contain about the same proportion of radioactivity as the fatty acids, so that almost half of the incorporated linoleic acid occupies the 2-position. The drop in monoglyceride radioactivity and the increase in free fatty acid radioactivity in the last 15 min of hydrolysis of linoleate-labeled triglyceride seems plausibly explained by acyl migration of the monoglyceride fatty acid in the 2-position to the 1- or 3-position.

When homogenates of linoleate-1-^{14}C-labeled cells are incubated at 37°C, the radioactive triglyceride undergoes hydrolysis, the course of which depends on the pH (Fig. 2). Lipolytic activity is greatest at pH 4.5. It is evident, however, that hydrolysis takes place across the entire range of pH. Radioactivity accumulates in the monolglyceride only below pH 6.0. The small amount of radioactivity in the 1,2- plus 1,3-diglyceride fraction (Table 2) does not increase at any pH during incubation for 2 hr. The total amount of radioactivity (Δ) in triglycerides, fatty acids, and monoglycerides, expressed as a percentage of total lipid radioactivity, is very much the same (close to 48%) at all pH levels studied. This implies that the radioactive fatty acid released does not stem from other fatty esters such as phospholipids. This observation required clarification since toad bladder mucosal cells contain both phospholipase A and lysolecithinase activities (1, 3). The experiments were therefore repeated in the presence of Ca^{++}, previously shown (3) to be required for this phospholipase A activity. In the presence of Ca^{++} (Fig. 3), the drop of triglyceride radio-

TABLE 3 HYDROLYSIS OF LINOLEATE-1-^{14}C- OR PALMITATE-1-^{14}C-LABELED TOAD BLADDER TRIGLYCERIDE BY PANCREATIC LIPASE

Triglyceride Labeled With	Incubation Time	TG	FFA	DG	MG	FFA/MG*
	min		*% total cpm*			
Linoleate	0†	88.6	3.1	4.1	4.2	
	2	46.0	24.1	8.8	20.1	1.3
	8	25.6	38.1	6.6	29.7	1.4
	15	9.8	45.4	4.2	40.6	1.2
	30	7.5	56.4	3.9	32.2	1.9
Palmitate	0†	85.7	4.0	7.2	3.1	
	2	62.2	22.5	7.9	7.4	4.3
	8	38.0	41.3	7.7	13.0	3.8
	15	25.0	55.9	5.5	13.6	4.9
	30	12.3	68.8	4.2	14.7	5.6

TG = triglyceride; FFA = free fatty acid; DG = 1,2- + 1,3-diglycerides; MG = monoglycerides.

Triglycerides-^{14}C were separated from other labeled mucosal cell lipids by thin-layer chromatography in petroleum ether–diethyl ether–glacial acetic acid 80:20:1 and eluted from the silica gel with chloroform. After removal of chloroform, hydrolysis of triglyceride by pancreatic lipase was examined in 0.5 ml of a reaction mixture that contained 10 mg of sodium chenodeoxycholate (kindly provided by Dr. Scott M. Grundy of The Rockefeller University), 0.08 M Tris buffer (pH 8.5), 0.09 M $CaCl_2$, and 13 mg of pancreatin (Eli Lilly & Co., Indianapolis, Ind.). The triglyceride was dispersed by means of a Vortex mixer.

* Ratio of radioactivities in free fatty acid and monoglyceride, corrected for radioactivity found in these fractions at zero time.

† Analysis of the labeled triglyceride material after incubation for 30 min without pancreatin showed the same distribution of radioactivity.

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activity as a function of pH is comparable to that shown in Fig. 2. However, total radioactivity in triglycerides, monoglycerides, and fatty acids shows a rise over the pH range 6.0–7.5, with a peak at pH 7.0, where phospholipase A activity is optimal (3). In this pH range, the radioactivity in the fatty acid fraction exceeds the loss of triglyceride radioactivity by a value that closely matches the increase in total radioactivity in the three fractions.

The effect of time of incubation at pH 4.5 on hydrolysis of triglycerides labeled with either linoleate-1-^{14}C or palmitate-1-^{14}C is shown in Fig. 4. Hydrolysis of both triglycerides is close to linear for about 30 min. An appreciable amount of radioactivity accumulates in monoglyceride when triglycerides are labeled with linoleate-1-^{14}C (in 1- and 2-positions, see above), but not with palmitate-1-^{14}C (chiefly in 1-position); compare the action of pancreatic lipase, Table 3. The slight increase in total radioactivity is contributed by a small amount of radioactive fatty acid from the slow hydrolysis of labeled diglycerides and cholesteryl esters during the course of incubation.

Water washes of all lipid extracts contained negligible amounts of radioactivity.

A number of chemical agents, some of which are known to inhibit various lipases (14–16), did not affect the lipolytic activity, including *N*-ethyl-maleimide, *p*-chloromercuribenzoate, mercaptoethanol, $HgCl_2$, and sodium iodoacetate, all in final concentrations of 10^{-5} and 10^{-4} M. NaF at a concentration of 10^{-2} M inhibited activity by approximately 40%.

Freezing and thawing of the homogenate, known to enhance lipase activity associated with lysosomes (4, 5), stimulated activity about twofold.

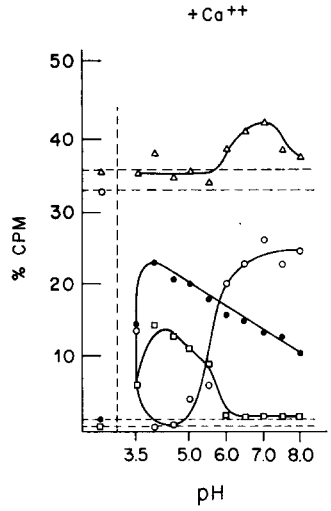

FIG. 3. Effect of pH on triglyceride hydrolysis and the appearance of other labeled fatty acids during 60 min incubation of homogenates of toad bladder mucosal cells in the presence of Ca^{++} (1.4 mM) to stimulate phospholipase activities. For symbols see Fig. 2.

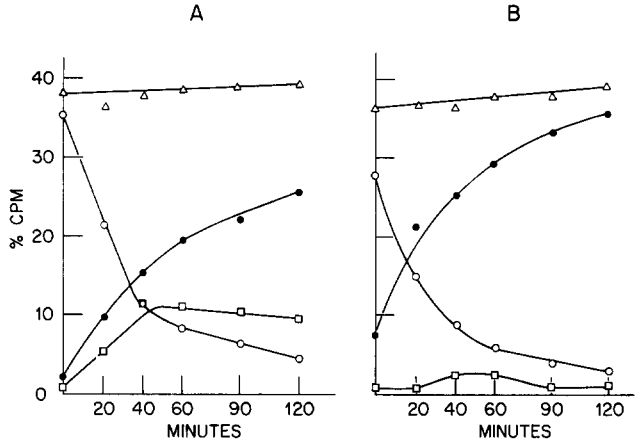

FIG. 4. Effect of time on hydrolysis of triglyceride labeled with linoleate-1-^{14}C (*A*) or palmitate-1-^{14}C (*B*) at pH 4.5. TG (○), FFA (●), MG (□), and total of these (△), all as percentages of total radioactivity in the mucosal lipids.

DISCUSSION

These findings demonstrate that isolated mucosal cells of the toad bladder contain lipase activity directed at endogenous triglycerides. This activity, while evident from pH 3.5 to 8.0, is clearly greatest at acid pH (4.5).

Studies on the hydrolysis of labeled triglycerides with pancreatic lipase (Table 3) revealed that the well-established predominance, in many naturally occurring triglycerides, of unsaturated fatty acids in the 2-position and of saturated fatty acids in the 1- and 3-position (13) also applies to triglycerides of bladder mucosal cells. Hence, the observation (Fig. 4) that monoglyceride radioactivity accumulates when tissue triglycerides were labeled with linoleate-1-^{14}C but not with palmitate-1-^{14}C indicates that the tissue lipase activity at acid pH, like pancreatic lipase, preferentially attacks the 1- and 3-position. Fig. 4 shows, however, that accumulation of radioactive monoglyceride stops after 30 min, even though degradation of triglyceride continues for 2 hr, which indicates that complete deacylation of triglyceride at pH 4.5 does occur. This finding may be explained by acyl migration from the 2- to the 1- or 3-position of monoglyceride, although it is also possible that the mucosal cell homogenates contain several lipid-splitting activities with different pH optima and substrate specificities.

Lipase activity with an acid pH optimum has been found in granulocytes (4, 5). This acid hydrolase activity was shown to be associated, at least in part, with the lysosomes of these cells. The acid pH optimum of the lipase in toad bladder mucosal cell homogenates, as well as an increase in its activity upon freezing and thawing, could mean that this acid lipase also resides

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in the lysosomes of the mucosal cells. While the use of endogenous triglycerides as substrate in the present experiments offers distinct advantages, it suffers from the disadvantage that cell fractionation studies do not allow reliable determination of the intracellular distribution of the activity, since the radiolabeled triglyceride and the lipase activity may be separated by the fractionation. It remains to be determined, therefore, whether the acid lipase activity is of lysosomal origin, and also whether the fatty acid release from triglyceride at higher pH reflects the presence of several lipolytic activities.

It has recently been shown that $^{14}CO_2$ production by toad bladders previously labeled with palmitate-1-^{14}C is stimulated by vasopressin in the presence, but not in the absence, of sodium (17). It was concluded that fatty acid, therefore, provides energy which can be utilized for sodium transport in this tissue. The authors did not identify the lipid species into which palmitate had been incorporated, and which served as a source of fatty acid for oxidation.

We suggest that triglyceride of toad bladder mucosal cells might be this source. This lipid fraction becomes rapidly labeled during incubation with fatty acid-1-^{14}C and the labeled triglycerides undergo almost complete hydrolysis in 2 hr under optimal conditions in vitro. Further, the triglyceride content of six preparations of isolated mucosal cells was 220 ± 38 μg/mg of protein (mean ± SEM). This represents from 600 to 700 mμmoles of fatty acid. Thus, an appreciable store of triglyceride fatty acid appears available for energy metabolism or perhaps for synthesis of other esters such as phospholipids (6).

The fate of triglyceride fatty acid of intact radiolabeled mucosal cells during incubation under various conditions is currently under investigation.

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