Participation of Phosphorylated Intermediates in *in vivo* Synthesis of Triglycerides from α-Monoglycerides in Rat Intestinal Mucosa. (30246)

ROGER PARIS AND GUY CLÉMENT (Introduced by J. F. Mead)
Laboratory of Animal Physiology, School of Science, University of Dijon, France

In a previous study (Clement and Paris, 1), we demonstrated the rapidity of absorption of doubly labeled (glycerol-C^{14} and palmitic acid-H^3) α-monopalmitin and studied the biosynthesis of triglycerides by the intestinal mucosa *in vivo*.

Our technique involves a 30-minute incubation of the lipid constituents in a segment of intestine *in situ* in rats in which the pancreatic and biliary secretions have been directed exteriorly for 24 hours previously. The lipids (20 mg of doubly labeled α-monoglycerides and equimolecular amounts of oleic and palmitic acids), 1.5 ml of Ringer solution and 25 mg of bile salts are introduced into the intestinal segment *in situ* with intact nervous connections. For analysis, the mucosa of 2 rats are combined. Our first results, using α-monopalmitin (glycerol-C^{14}, fatty acid-H^3), demonstrated that in the mucosa the istotopic ratio H^3/C^{14} increased from monoglycerides to diglycerides to triglycerides; there was, therefore, acylation of the monopalmitin by labeled palmitic acid. Since this palmitic acid could arise only by hydrolysis of part of the labeled monopalmitin our experiments furnished new proof of the existence of a monoglyceride lipase in the intestinal mucosa and demonstrated that this enzyme is active *in vivo* on α-monopalmitin.

The biosynthesis of triglycerides from α-monoglycerides could be explained satisfactorily by direct acylation according to Clark and Hubscher(2). However, since we had observed at the same time in this study that the phosphatidic acids were highly radioactive and had a H^3/C^{14} ratio intermediate between those of the monoglycerides and diglycerides, the existence of a phosphorylated intermediate in the synthesis of triglycerides from monoglycerides seemed reasonable.

To clarify this point, we therefore repeated the experiment with α-monopalmitin (glycerol-1-C^{14}, 1 μc, and palmitic acid 9,10-H^3, 3.6 μc) but with separation of the phospholipids by methods particularly designed to obtain the phosphatidic acids and lysophosphatidic acids in as pure a state as possible. These methods included both column and thin-layer chromatography as described by Di Costanzo and Clement(3). By this method, the lysophosphatidic acids are eluted with the phosphatidylethanolamines. To separate these we developed a 2-dimensional thin-layer chromatography (first eluant: $CHCl_3$:MeOH:H_2O-65:25:4 v/v; second eluant as described by Abramson and Blecher (4). The efficacy of the method was tested with use of a lysophosphatidic acid synthesized by the method of Pieringer and Hokin (5).

Results and discussion. The very high radioactivity (H^3) of the monoglycerides is immediately apparent (Table I), a result of the fact that it is the labeled material initially added to the intestinal loop; it was evidently well absorbed (5% of the initial activity).

Aside from the monoglycerides, the most active fractions are, in increasing order: free fatty acids, phosphatidic acids, diglycerides and triglycerides. The other phospholipids, although some types are present in significant amounts in the mucosa, are only weakly labeled.

Considering the H^3/C^{14} rations, it can be seen, as previously, that the monoglycerides of the mucosa have a ratio somewhat higher than that of the monoglycerides originally placed in the loop. This increase may be explained by the simultaneous presence of α-monopalmitin absorbed without hydrolysis and of monoglycerides formed from fatty acids only part of which is the labeled palmitic acid and of glycerol-C^{14} for which the dilution by endogenous glycerol is greater than the dilution of the fatty acids. The latter are diluted by the oleic and palmitic

TABLE I. Radioactivity of the Lipid Fractions of the Mucosa During Absorption of Doubly Labeled α-Monopalmitin.

	α MG substrate	MG	DG	TG	FFA	PA	LPA	PE	PS	PC	LL, LC
% of H^3 activity in lipid constituents of mucosa		48	10	27	5	9			1		
% of H^3 activity of the different PL in comparison to total PL						95.6	.9	1.7	.2	1.3	.3
$\frac{H^3}{C^{14}}$	3.6	3.9	4.6	6.0		4.5	3.2	.3	1.2	1.9	.9

MG = monoglyceride; DG = diglyceride; TG = triglyceride; FFA = free fatty acids; PA = phosphatidic acids; LPA = lysophosphatidic acids; PE = phosphatidylethanolamine; PS = phosphatidylserine; PC = phosphatidylcholine; LL = lysolecithin; LC = lysocephalin; SG = sphingomyelin.

acids placed in the loop with the monopalmitin.

The H^3/C^{14} ratio increases in the following order: monoglycerides, diglycerides-phosphatidic acids, triglycerides (3.9, 4.5-4.6, 6.0 respectively). It is apparent, moreover, that the lysophosphatidic acids have a H^3/C^{14} ratio slightly lower than that of the monoglycerides. It is interesting to note that the value of this ratio increases by the same amount (1.4) with each acylation:

$$\text{lysophosphatidic acids } (3.2) \rightarrow \text{ or } \rightarrow \begin{matrix} \text{phosphatidic acids} \\ (4.5) \\ \text{or} \\ \text{diglycerides} \quad (4.6) \end{matrix} \rightarrow \text{ triglycerides } (6.0)$$

Since the glycerol cannot be diluted after it is esterified, this figure of 1.4 gives the measure of activity of the fatty acids in the mucosal pool.

In addition, since the specific activity of the phosphatidic acids (50,000 dpm/mg) is higher than that of the diglycerides (40,000 dpm/mg), the latter are not likely to be the precursors of the phosphatidic acids. We therefore consider that the lysophosphatidic acids might be the intermediates in the reactions leading to the synthesis of the triglycerides from the α-monoglycerides by way of the phosphatidic acids and diglycerides. In this experiment we did not determine the specific activity of the lysophosphatidic acids; however, it must have been very high from the significant activity of the traces of these compounds on the thin-layer chromatograms. A control experiment confirmed the fact that there was no degradation of phosphatidic acids during the various manipulations. If a labeled phosphatidic acid is added to non-radioactive mucosal phospholipids and all the manipulations are repeated, it is found that the activity in the region of the lysophosphatidic acids is less than 0.2% of that of the phosphatidic acids.

Two hypotheses may explain the formation of lysophosphatidic acids from α-monoglycerides. Either 1) the α-monoglycerides are completely hydrolyzed in the intestinal mucosa as suggested by Mattson and Volpenhein(6), and the glycerol is reutilized by the classical route (Weiss and Kennedy, 7) or 2) the α-monoglycerides are directly phosphorylated to lysophosphatidic acids as has been demonstrated in liver and brain (Pieringer and Hokin, 5, 8).

It is probable that the direct acylation of the α-monoglycerides is not of great importance: on the one hand, the experiments of Ailhaud and his collaborators(9), and of Mattson and Volpenhein(6) do not favor this mechanism in the case of the α-monoglycerides and, on the other hand, according to our results, from a consideration of the H^3/C^{14} ratio of the monoglycerides of the mucosa (3.9) and the activity of the fatty acid pool, there should be a ratio of 3.9 + 1.4 = 5.3 for the diglycerides formed by this pathway. In fact, the diglycerides of the mucosa have a distinctly lower ratio.

In conclusion, if, according to the current concept the β-monoglycerides produced by digestive hydrolysis of the triglycerides are directly acylated, our results indicate that the α-monoglycerides yield triglycerides with

phosphorylated compounds as intermediates whether they are hydrolyzed and the glycerol reutilized after phosphorylation or whether they are directly phosphorylated to lysophosphatidic acids.

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Tissue Culture of Tonsillar Lymphocytes.* (30247)

A. R. GOLDFARB AND I. ULLAL (Introduced by P. P. Foà)
Division of Research, Sinai Hospital of Detroit, Mich.

It is well established that lymphoid tissue is involved in the immunologic reactions of experimental animals. In cases of severe allergic systemic reactions in man, the regional lymph nodes frequently become enlarged. As part of a study of the allergic reaction in human subjects, we used tonsils from patients in apparent good health. These tonsils were considered a prototype of "normal" lymphatic tissue. Cultures of these tonsils yielded a variety of cells, consisting predominantly of small lymphocytes with smaller numbers of plasmacytes, large lymphocytes and other cells which were similar to those described in histologic sections of tonsils(1). We have found that the growth of the tonsillar lymphocytes in tissue culture was considerably improved by the presence of human gamma globulin.

Materials and methods. The basic medium used was Eagle's minimum essential medium (MEM, Microbiological Associates) containing fetal calf serum at a concentration of 15%. Penicillin-G (100 units/ml) and streptomycin sulfate (50 μg/ml) were added. The medium was sterilized by passage through a 0.54 μ Millipore® filter. Trypsin (1:250, Difco Laboratories) was used at a concentration of 0.5% (w/v) in Hanks' balanced salt solution (BSS, Microbiological Associates). The proteins (Pentex Corp.) were crystalline preparations of human serum albumin (HSA), bovine serum albumin (BSA), human gamma globulin (HGG) and egg albumin (OA).

Tonsils were obtained from male and female donors between the ages of 7½ months and 14 years, most being between 2 and 6 years. A portion of tonsil was finely minced with sterile scissors, washed 3 times with BSS, centrifuged and resuspended in 0.05% trypsin solution. A fairly uniform suspension was obtained after trypsinization at room temperature (23°-26°C), using a magnetic stirrer for about 15 to 20 minutes. The suspension was allowed to settle for about one minute and the supernatant was withdrawn and centrifuged at 1500 rpm in a refrigerated centrifuge. The cells were washed twice with basic medium by resuspension and centrifugation and finally dispersed in 5 to 10 ml of basic medium. The precise volume was determined by the number of culture bottles to be set up. Each bottle, containing 9.5 ml of medium and 0.5 ml of the cell suspension, was incubated at 37°C in an atmosphere of air and 5% CO_2. The control samples were cultured in MEM with 15% fetal calf serum

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