Inhibition of Lipid Synthesis in Rat Skin by Tetrolyl-Pantetheine (35182)

VINCENT A. ZIBOH AND ROSCOE O. BRADY

Department of Dermatology, University of Miami School of Medicine, Miami, Florida 33136; and Laboratory of Neurochemistry, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20014

Recent studies have indicated certain advantages of the use of skin tissue for studying regulatory mechanisms of lipid synthesis (1, 2). Lipid synthesis is active in this organ and this process is influenced by various nutritional and hormonal factors. In our studies of substances which may affect lipid synthesis, it was found that tetrolyl-pantetheine (tetrolyl-Pn) profoundly inhibited the synthesis of lipids from acetate in rat skin tissue.

Previous reports have shown that fatty acid synthesis is impaired in the presence of certain sulfhydryl reagents (3–5), and Robinson *et al.* (6) demonstrated that tetrolyl-CoA, the acetylenic analog of butyryl-CoA, strongly inhibited fatty acid synthesis in subcellular preparations of brain, liver, and adipose tissue. In the present communication we report results obtained in studies of lipogenesis in slices of rat skin tissue and the effect of tetrolyl-Pn on this process.

Materials and Methods. Sodium acetate-1-^{14}C (40 mCi/mmole) was purchased from Nuclear Chicago Corp., Des Plaines, Ill. Sephadex was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Unisil was obtained from Clarkson Chemical Co., Williamsport, Pa. Pantethine was purchased from Sigma Chemical Co., St. Louis, Mo., and tetrolic acid was obtained from Farchan Research Laboratories, Cleveland, Ohio.

Pantethine was reduced to pantetheine by treating 700 mg of pantethine in 2 ml of water with 185 mg of $NaBH_4$ at 0°. The pH was adjusted to 4.0 and the reaction was continued for 1 hr. The solution was then neutralized with dilute KOH and finally made slightly alkaline by the addition of 0.4 ml of 1 *M* $KHCO_3$. Tetrolyl-Pn was prepared from the mixed anhydride obtained from the reaction of tetrolic acid with ethyl chloroformate (6). Tetrolyl-Pn was isolated from the reaction mixture and purified by ascending paper chromatography as described previously for tetrolyl-CoA (6).

Preparation and incubation of skin specimens. Adult male Sprague-Dawley rats weighing 300–400 g were maintained *ad libitum* on Purina Laboratory Chow. They were placed in individual cages for at least 48 hr before the experiments to insure that all were well fed. The rats were held firmly to a table and the hair in the posterior dorsum was carefully shaved with an electric clipper. Small skin specimens (60–80 mg) were removed with scissors and the underlying subcutaneous tissue was carefully scraped off with a scalpel. The specimen was immediately weighed and transferred to an incubation vial containing streptomycin (200 μg), gentamicin (200 μg), penicillin (200 units) and a tracer amount of sodium acetate-1-^{14}C (5 μCi, 0.1 μmole) in 2 ml of Krebs–Ringer phosphate buffer (pH 7.4). Tetrolyl-pantetheine in various concentrations was added in 0.02 ml of water. The mixtures were incubated aerobically at 37° in a Dubnoff shaking incubator for 6 hr.

Determination of ^{14}C in lipids. Two procedures were employed for the determination of ^{14}C incorporated into lipids: (1) For the estimation of the total ^{14}C incorporated, the incubation was terminated by the addition of 2 g of potassium hydroxide; and 15 ml of ethanol were added to each incubation vessel and allowed to stand overnight at 60°. The mixture was acidified with HCl and then extracted four times with 100 ml of dichloromethane. Radioactive acetate in the extract was removed by chromatography on Sephadex (G-25 coarse) according to Siakotos and Rouser (7). The efficiency of the

method was established by percolating a mixture of cholesterol-4-^{14}C (8.9×10^5 dpm), palmitic acid-1-^{14}C (8.4×10^5 dpm) and lipids extracted from 60 mg of skin through the column. The radioactive lipids were recovered quantitatively. Similarly, lipids from 60 mg of skin mixed with acetate-1-^{14}C (4.07×10^6 dpm) were recovered free of radioactivity. The ^{14}C in the column effluent was assayed with a Packard Tri-Carb Model 2002 liquid scintillation counter, and the amounts of ^{14}C incorporated into lipids per milligram of skin (wet weight) were calculated.

(2) In order to determine the ^{14}C incorporated into various lipid classes, the tissue was removed from the medium at the end of the incubation, placed in a test tube containing a mixture of chloroform–methanol 2:1, and homogenized with a Polytron (Model PT 10) homogenizer. Tissue debris was removed by filtration on sintered glass funnels and washed with the solvent mixture until no more radioactivity was detected in the wash. The filtrate was evaporated in a rotary evaporator, and the lipid residue was dissolved in chloroform–methanol–water 19:1:0.1 and passed through a Sephadex (G-25 coarse) column as described above to remove radioactive acetate. After evaporation of the solvents, the recovered lipid mixture was dissolved in chloroform and percolated onto a column containing 5 g of Unisil (100–200 mesh) suspended in chloroform according to Borgström (8). Neutral lipids were eluted with chloroform (200 ml) and polar lipids with methanol (200 ml). Aliquots from each fraction were assayed for radioactivity.

The neutral lipids were dissolved in a small amount of hexane and chromatographed further on 5 g of Florisil hydrated with 7% water and suspended in hexane according to the procedure described by Carroll (9). Lipids were successively eluted from the column by the following solvents: (A) 15 ml of hexane; (B) 50 ml of 5% ether in hexane; (C) 75 ml of 15% ether in hexane; (D) 60 ml of 25% ether in hexane; (E) 40 ml of 50% ether in hexane; (F) 40 ml of 2% methanol in water; and (G) 50 ml of 4% acetic acid in ether. The effluent was collected in 5-ml fractions, and aliquots were assayed for radioactivity. Chromatography of reference compounds on Florisil showed that squalene was eluted by (A), sterol esters by (B), tripalmitin by (C), cholesterol by (D), dipalmitin and diolein by (E), monopalmitin and monolein by (F), and palmitic acid and stearic acid by (G).

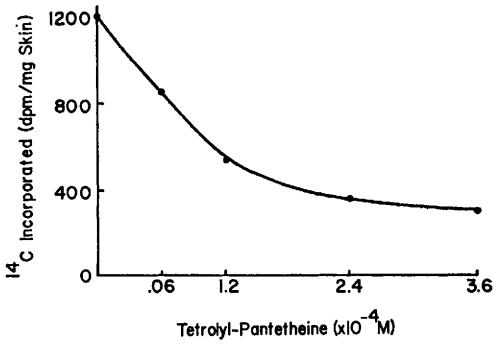

FIG. 1. Inhibition of lipid synthesis by tetrolyl-pantetheine: The amount of ^{14}C incorporated from acetate-1-^{14}C into lipids was determined by Procedure 1. The data are the average of duplicate experiments.

Results. The incorporation of ^{14}C into lipids from acetate-1-^{14}C was approximately 70% inhibited when tetrolyl-Pn was added to the incubation medium at a level of 2.4×10^{-4} *M* (Fig. 1). Little further effect was obtained when the concentration of tetrolyl-Pn was increased to 3.6×10^{-4} *M*. The inhibitory effect of tetrolyl-Pn on the incorporation of ^{14}C into lipids by whole skin preparations was not reversed by the addition of L-cysteine or 2-mercaptoethanol to the incubation medium (Table I). No effect on this process was observed with L-cysteine whereas the addition of 2-mercaptoethanol slightly augmented the inhibitory effect of tetrolyl-Pn [cf. Ref. (4)].

In order to determine wthether the inhibitory effect of tetrolyl-Pn was exerted on the entire lipid synthesizing system or on the synthesis of specific lipid substances, the ^{14}C-labeled lipids obtained from the incubation of skin specimens from 1 rat were separated according to Procedure 2. The addition of 2.4×10^{-4} *M* tetrolyl-Pn to the incubation medium caused a decrease in the amount of ^{14}C incorporated in all of the neutral lipids

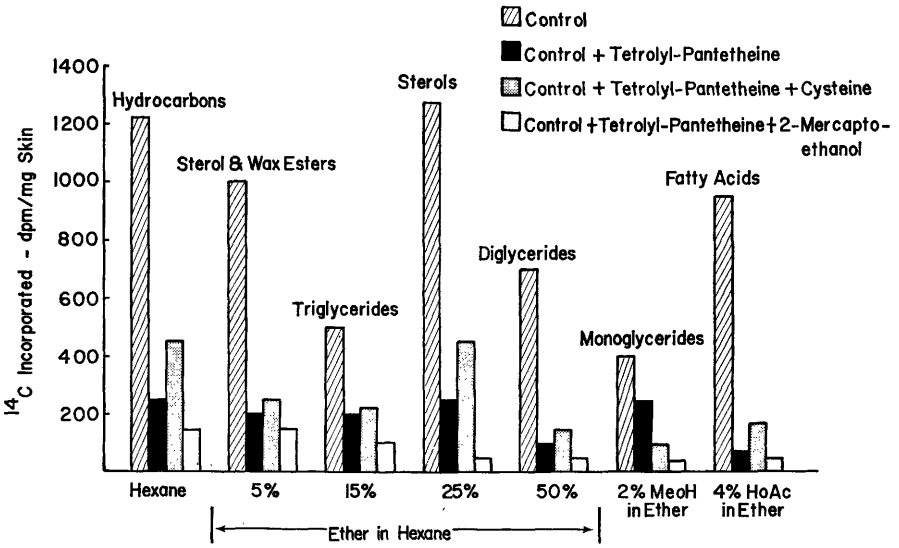

FIG. 2. The effect of tetrolyl-pantetheine on the synthesis of neutral lipids: The neutral lipids were separated on Florisil columns according to Procedure 2 as described in the text. The height of the bars represents ^{14}C incorporated in each lipid fraction.

(Fig. 2). The decreased incorporation in the various lipid fractions was: squalene, 79%; sterol and wax esters, 80%; triglycerides, 60%; sterols, 80%; diglycerides, 86%; monoglycerides, 37%; and fatty acids, 95%. This experiment was repeated with skin from another rat and similar results were obtained.

Discussion. The present experiments conclusively demonstrate an inhibitory effect of tetrolyl-Pn on the biosynthesis of lipid by rat skin tissue. The inhibitory effect was found throughout all of the neutral lipid fractions although there was some minor variation in the extent of this effect in the various lipids. The inhibition of fatty acid synthesis is probably due to the block of the first condensation step in this process, namely the reaction between acetyl-CoA and malonyl-CoA (6, 10).

TABLE I. The Effect of Tetrolyl-Pantetheine, L-Cysteine and 2-Mercaptoethanol on Total Lipid Synthesis from Acetate-1-^{14}C by Rat Skin.[a]

Additions	^{14}C Incorporated into lipids (% of control)
None (control)	100
Tetrolyl-pantetheine (2.4×10^{-4} *M*)	30
Tetrolyl-pantetheine (2.4×10^{-4} *M*) + L-cysteine (5×10^{-4} *M*)	30
Tetrolyl-pantetheine (2.4×10^{-4} *M*) + L-cysteine (2.5×10^{-3} *M*)	34
Tetrolyl-pantetheine (2.4×10^{-4} *M*) + 2-mercaptoethanol (5×10^{-4} *M*)	27
Tetrolyl-pantetheine (2.4×10^{-4} *M*) + 2-mercaptoethanol (2.5×10^{-3} *M*)	22

[a] The procedures for incubation are described in the text. The data are from the average of duplicate experiments.

The inhibition of incorporation of ^{14}C from acetate-1-^{14}C into the sterol fractions is of particular interest since this is the first demonstration of an inhibitory effect of a tetrolic acid thioester on lipid synthesis other than long-chain fatty acids. The present findings suggest that the most likely site of inhibition in the sterol biosynthetic pathway is also the condensation of acetyl-CoA with malonyl-CoA to form a thioester derivative (acetoacetyl-*S*-enzyme). This reaction may therefore be common to fatty acid and sterol synthesis.

Incubation of skin specimens with sulfhydryl compounds such as L-cysteine and 2-mercaptoethanol failed to reverse the inhibition of lipid synthesis by tetrolyl-Pn. The lack of effectiveness of these compounds may

be due to the strong affinity of tetrolic thioesters to the fatty acid synthesizing enzyme complex. The K_i for tetrolyl-CoA in this system is $1 \times 10^{-5} M$ (6).

We conclude that this study has further demonstrated the usefulness of whole skin preparations for the study of regulatory mechanisms of lipid synthesis and that the tetrolyl-Pn employed in the present investigations was sufficiently permeable for it to exert a significant inhibition on lipid synthesis at moderately low concentrations.

Summary. The present study has demonstrated that tetrolyl-pantetheine is a potent inhibitor of lipid synthesis in rat skin. The inhibitory effect was particularly marked with fatty acid and sterol fractions. These findings suggest that tetrolyl-pantetheine may exert its effect by inhibiting reactions common to both fatty acid and sterol synthesis in the skin.

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Received July 17, 1970. P.S.E.B.M., 1971, Vol. 136.