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The *in Vitro* Effects of Triton WR-1339 on Lipid Synthesis by Bone Cells[1] (38766)

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The hyperlipemic action of Triton WR-1339 in whole animals has been accepted for several years (1) although there is some disagreement regarding its mechanism of action. The effect of Triton WR-1339 on individual organ systems has been limited primarily to the liver (2–15), however the brain has been reported to increase cholesterol (6) and phospholipid (16) synthesis in *in vivo* experiments. The effect of Triton on tissue culture systems has been mainly concerned with its inhibition of malignant cell growth (17–20) or its influence on virus propagation (21–24).

In this study, Triton WR-1339 was found to stimulate lipid synthesis in bone, another organ system, when the cells were maintained either as intact newborn rat calvaria or when isolated by collagenase digestion and grown in cell culture. This is the first report demonstrating a stimulation of lipid biosynthesis by Triton in bone and also in a tissue culture system. This stimulation of synthesis of all major lipid classes was observed when Triton was administered *in vitro*. No *in vivo* effect of Triton has been observed in brain slices (16), liver homogenates (3, 25), or rat thymus cells (26). The only other investigation on the *in vitro* effect of Triton on lipid synthesis utilized an isolated perfusion system of liver, and both cholesterol and fatty acid synthesis were increased (4, 13).

Methods. Calvaria. Newborn Charles River CD strain rats were decapitated and the calvaria dissected free of cartilage, dura and periosteum under cold saline. Groups of 10–15 calvaria were preincubated at 37° in Erlenmeyer flasks containing 4 ml of Minimum Essential Medium (MEM) (27) with or without 5% fetal bovine serum (FBS) (4 flasks) or 4 ml of identical MEM containing Triton WR-1339, 250 μg/ml, (4 flasks) under 95% air/5% CO_2. After a preincubation period of 1 or 20 hr, the media were removed and replaced with the respective media containing 1.25 μCi/ml of acetate-1-^{14}C. After a labeling period of 4 hr in all experiments, the calvaria were removed, rinsed three times with cold saline and demineralized with 15% EDTA at pH 7.2, 4° for 24 hr. The lipids were then extracted by homogenizing the demineralized calvaria in 75 ml of chloroform–methanol (2:1) in glass homogenizers (28). The lipid extracts were filtered through Whatman No. 41 filter paper and washed with 15 ml of 0.9% NaCl. After removal of the aqueous phase, the chloroform was washed twice with 2 ml sodium chloride and the solvents evaporated.

The incubation media after removal of the calvaria and centrifugation was retained for lipid extraction as described below.

Tissue Culture: Preparation of cell cultures (29): Cleaned newborn rat calvaria were finely minced, suspended in Hank's balanced salt solution containing 2 mg/ml collagenase and the suspension stirred at room temperature for 60 min. At the end of this time the cells were decanted and centrifuged at 750 rpm for 10 min in an International Centrifuge, Size 1. They were then washed with 0.3% Tris buffered saline, pH 7.3, centrifuged, and the cell pellet resuspended in MEM supplemented with 10% FBS. The cells were grown to a monolayer in glass prescription bottles.

Lipid Synthesis Procedure: For experimental purposes the cells grown in the glass prescription bottles were trypsinized in 0.1% trypsinizing citrate (30), counted, plated in 50 mm Falcon Petri dishes at 7.5×10^5 cells per dish and allowed to attach and grow in MEM supplemented with 5% FBS at 37° in an atmosphere of 95% air/5% CO_2. After 24 hr of growth, the medium was removed and three sets of six dishes were refed the same

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medium with 250 μg/ml Triton WR-1339 and three sets of six dishes were refed with medium lacking the Triton (controls). After a preincubation period of 1 or 20 hr, the media were removed and replaced with the same media containing 1.25 μCi/ml acetate-1-^{14}C and incubated for 4 hr. At the end of the labeling period, the media were removed and saved for lipid extraction as described below. The cells were quickly rinsed with saline, scraped from the dishes with Teflon scrapers and the cells from each set pooled before centrifugation. The pellets of cells were suspended in 24 ml of chloroform–methanol (2:1) and homogenized in glass homogenizers (28). The extracts were filtered and collected in graduated cylinders. The remaining cell debris was washed with 12 ml of the same solvent and combined with the first extract. The combined extracts were washed with 7.2 ml saline and the aqueous phase removed. The chloroform layer was washed twice with 7.2 ml saline. The solvent was evaporated and the lipids analyzed as described below.

Analysis of Incubation Media: The media from both the calvaria and cell incubations were treated similarly. To an aliquot of the media, which had been centrifuged and filtered through Whatman No. 1 filter paper, was added 2.5 vol of methanol. Five volumes of chloroform were added slowly with swirling. After shaking, the phases were allowed to separate and the upper aqueous methanol phase removed. The chloroform layer was washed twice with 0.1 vol of saline and the solvent evaporated.

Analysis of Extracted Lipids: The lipid extracts from the calvaria, tissue culture cells and incubation media were treated identically. The lipid residue from each sample was redissolved in redistilled chloroform and filtered through sintered glass funnels into separate 10 ml volumetric flasks and diluted to 10 ml with chloroform. Duplicate 0.5 ml or 0.1 ml aliquots were taken for phospholipid phosphorus determination according to the micro-Barlett procedure (31). A 7 ml aliquot was evaporated for chromatographic analysis of major lipid classes.

Chromatographic Analysis of Lipids: The lipid extracts (the 7 ml aliquot above) were dissolved in 120 λ of chloroform-methanol (1:1) and 50 λ aliquots were applied to silicic acid paper according to the procedures of Marinetti *et al.* (32) and Wuthier (33). Radioactive spots were located by autoradiography, cut from the papers, and counted in a Packard Tri-Carb Scintillation Spectrometer, Model 3375, using Bray's solution (34). Lipids were identified on the chromatograms by various chemical and enzymatic tests (31, 35–37). Results were compared on the basis of the phospholipid phosphorus (36). Statistical analysis were performed in our computer center.

Results. The effect of Triton WR-1339 on lipid synthesis of all major classes of lipid bone cells is shown in Table I (newborn calvaria) and Table II (cell tissue culture). There appears to be a stimulation of synthesis of most lipid classes by Triton in both systems. Table I shows the results in detail of one of three experiments where calvaria were incubated with and without Triton WR-1339, 250 μg/ml, as the only variable. While synthesis of all major lipid classes was significantly stimulated by Triton ($P < 0.001$), with exception of spingomyelin and lysolecithin, the T values on which these are based varied from 7.3 to 18.2. Synthesis of triglycerides, free fatty acids and phosphatidylethanolamine showed the greatest effect. The other lipid classes, except for sphingomyelin which was not significant, had lower T values. In other experiments where calvaria were preincubated 20 hr prior to the 4 hr labeling period, the cpm differed but the stimulatory effect of Triton on lipid synthesis was similar. The results of these experiments are summarized, i.e., P values given, in the last two columns of Table I. Preincubation of the calvaria for 1 hr or 20 hr did not seem to alter the extent of labeling of the lipid classes. Further, deletion of fetal bovine serum from the incubation medium did not effect lipid synthesis. Triton does not seem to change the rate of synthesis of one lipid class more than another class as shown by calculating the percentage of counts in a specific lipid class. For example triglyceride containing 27% of the total counts (108.8/406.2 × 100) in control calvaria and 24% of the total counts (229.2/966.5 × 100) in Triton treated calvaria. All other lipid classes are similar to each other in the percentage of the total

TABLE I. LIPID SYNTHESIS IN NEWBORN RAT CALVARIA.

Lipid Class	5-Hr incubation with 5% FBS: Control[a] cpm × 10^3	5-Hr incubation with 5% FBS: Triton, 250 μg/ml[a] cpm × 10^3	5-Hr incubation with 5% FBS: *P* Value	24-hr incubation with 5% FBS *P* Value	24-hr incubation no FBS *P* Value
Triglycerides	108.8 ± 5.1	229.2 ± 10.3	<0.001	<0.001	<0.001
Phosphatidylcholine	108.4 ± 8.2	213.8 ± 12.1	<0.001	<0.001	<0.001
Mono- and diglycerides	18.6 ± 3.0	44.0 ± 2.0	<0.001	<0.001	<0.001
Cholesterol	95.0 ± 9.2	155.4 ± 11.0	<0.001	<0.001	<0.001
Free fatty acids	16.4 ± 7.3	190.1 ± 12.0	<0.001	<0.001	<0.001
Cholesterol esters	10.1 ± 0.6	15.4 ± 0.7	<0.001	<0.001	<0.001
Phosphatidylethanolamine	15.8 ± 2.5	51.5 ± 2.4	<0.001	<0.001	<0.001
Phosphatidylinisitol	16.7 ± 1.5	36.9 ± 2.0	<0.001	<0.001	<0.001
Sphingomyelin	7.5 ± 1.1	10.7 ± 5.7	NS	NS	<0.01
Lyso-lecithin	2.4 ± 0.7	4.5 ± 1.1	<0.05	NS	<0.01
Phosphatidylserine	6.5 ± 0.8	15.0 ± 1.0	<0.001	<0.01	<0.001

[a] Mean cpm and standard deviation of four groups of 12 Calvaria.

TABLE II. LIPID SYNTHESIS IN CULTURES OF BONE CELLS.

Lipid class	5-Hr incubation: Control[a] cpm × 10^3	5-Hr incubation: Triton, 250 μg/ml[a] cpm × 10^3	5-Hr incubation *P* Value	5-hr incubation *P* Value	24-hr incubation *P* Value
Triglycerides	112.9 ± 6.2	379.0 ± 8.9	<0.01	<0.01	<0.01
Phosphatidylcholine	312.0 ± 24.2	637.0 ± 54.8	<0.05	NS	NS
Mono- and diglycerides	29.0 ± 0.8	93.0 ± 17.6	<0.05	<0.05	NS
Cholesterol	66.5 ± 5.4	145.6 ± 2.1	<0.01	<0.01	NS
Free fatty acids	32.4 ± 5.4	70.0 ± 6.3	<0.05	NS	<0.02
Cholesterol esters	17.5 ± 1.5	16.8 ± 0.0	NS	NS	<0.02
Phosphatidylethanolamine	39.6 ± 1.8	207.6 ± 5.4	<0.01	<0.01	<0.05
Phosphatidylinisitol	21.1 ± 1.9	67.5 ± 1.8	<0.01	<0.01	<0.05
Sphingomyelin	2.7 ± 0.2[b]	2.7 ± 0.4[b]	NS	NS	<0.05
Lysolecithin				NS	NS
Phosphatidylserine	20.8 ± 1.8	58.2 ± 0.4	<0.02	<0.05	NS

[a] Mean cpm and standard deviation of three sets (six dishes pooled per set) of bone cells.

[b] Spingomyelin and lysolecithin spots overlapped in this experiment and were counted together.

counts when the control and Triton-treated calvaria are compared.

Table II shows the results of three experiments measuring lipid synthesis in bone cell cultures. Again the cpm in one experiment is shown as well as the *P* values of two other experiments. In this 5 hr experiment, the results in the tissue culture system are similar to those in the calvaria system, i.e., synthesis of all major classes of lipids was stimulated by inclusion of Triton in the medium. Only sphingomyelin, lysolecithin and cholesterol esters were not affected. The *P* values are larger than were the *P* values in Table I. This reflects, we believe, the smaller number of degrees of freedom in these calculations since the incubations were done in triplicate compared with the calvaria with quadruplicate incubations. This also may explain why there are more lipid classes with insignificant differences in the tissue culture systems. In the experiments where a 24 hr incubation period was employed, fewer lipid classes in the Triton treated cells were significantly different when compared to the control values. In the culture dishes incubated for 24 hr with Triton, the cell monolayer was observed to thin out and more floating cells

were observed. This probably represents a toxic effect of Triton on cells in tissue culture although previous reports suggested that normal cells grown in tissue culture with this concentration of Triton show no toxic effects (17–19). Cytological studies of these bone cells were not done since our primary objective was to study lipid synthesis.

The incubation media from both the calvaria and the cell cultures were centrifuged to remove any floating cells and the cell free media subjected to a Folch extraction (28). The radioactivity of extracted lipids from both the control and Triton-containing media was insignificant. There was no difference in the radioactivity of lipid from the media of control or Triton-treated cells. This suggests that while bone cells have the ability to synthesize lipids from precursors such as acetate, they are unable to secrete significant amounts of the newly synthesized lipid into the incubation fluid.

Discussion. In *in vitro* experiments, Triton WR-1339 has been found to have no effect upon lipid synthesis in liver homogenates (3, 25) and brain slices (16). The only *in vitro* effect recorded to date was in liver where Triton added to the perfusion fluid is reported to have resulted in an increased intracellular cholesterol and fatty acid synthesis with no labeled lipids in the perfusion fluid (4) and in another study, an increased perfusion fluid cholesterol content without an increased liver cholesterol content (13). If, however, the Triton was administered *in vivo*, then Triton stimulated lipid synthesis in liver slices (2, 5–10, 12, 14), brain slices (6, 16), and liver homogenates (and some subcellular liver systems) (3, 11, 15) obtained from the treated animals. In the present study, a stimulation of lipid synthesis of most lipid classes (but not the same extent in all classes) in cells grown in tissue culture and in intact calvaria was observed, thus demonstrating an *in vitro* effect of Triton. Since there was no appreciable difference in lipid labeling between preincubation of the bone cells with Triton for 1 hr or 20 hr prior to the labeling period of 4 hr, it seems reasonable to assume that Triton has a direct and immediate effect upon the enzyme systems involved in lipid synthesis. Because the radioactivity of all lipid classes containing fatty acids was elevated and the percent of each class of total incorporated counts did not markedly change between control and Triton-treated cells, it is probable that Triton's action is on the incorporation of acetate into fatty acids.

Cholesterol and cholesterol ester synthesis from acetate was also stimulated by Triton. Should Triton affect only a single enzymatic step during synthesis of both cholesterol and fatty acids, then this action must occur in the early phases during conversion of acetate to lipids, i.e., beginning with cell uptake of acetate but before the routes of acetate to cholesterol or fatty acid separate to form the respective end products. It seems more likely that Triton affects more than one enzymatic site, i.e., acts on fatty acid biosynthesis and cholesterol biosynthesis at different steps in their respective synthetic pathways. Some evidence has been reported supporting this suggestion. Triton increases acetate activation (15) and increases β-hydroxy-β-methylglutaryl Coenzyme A reductase (14, 15).

We believe the results of our study indicates something about the mechanism of action of Triton in experimental hyperlipemia. The mechanism of the hyperlipemic effect of Triton has been suggested as (I) coating or otherwise altering blood lipoproteins thus retarding their removal from the vascular system, (II) inhibiting lipoprotein lipase, and (III) increasing the rate of lipid synthesis especially in liver. While the inhibition of lipoprotein lipase hydrolysis has been well documented (38–44), there are no reported effects of Triton on the enzyme itself (42), concluding that the only effect of Triton is to alter the substrates (38, 41, 42, 44) (mechanism I). Inhibition of lipoprotein lipase does not explain the hypercholesterolemia associated with Triton treatment. It is possible that the alteration of circulating lipoprotein (mechanism I) could result in increased lipid synthesis (mechanism III) by removing the feedback inhibition of lipoproteins on the tissue's endogenous rate of lipid synthesis. Such a mechanism has previously been suggested (2, 6, 16) and it has been shown that the inclusion of rat lipoproteins in the incubation mixture inhibits lipid synthesis by isolated rat hepatocytes (45). As shown in the present study, a feedback mechanism from incubation medium

lipids cannot explain the effect of Triton since the incubation media did not contain significant levels of radioactive lipid. The fetal bovine serum in the media could possibly serve as an acceptor for any secreted lipid, but inclusion of serum did not appreciably change the extent of lipid labeling nor was there a difference in cell-free media radioactivity whether FBS was included or not. The mechanism of action of Triton seems likely then to be a direct effect on lipid synthesis, probably that of fatty acids and cholesterol. This is compatible with our previous study (16) in which phospholipid synthesis in brain from Triton-treated rats was greater than in normal brain. Thus, with increased fatty acid synthesis in Triton-treated animals, more fatty acids would be available for phospholipid synthesis, thereby incorporating more ^{32}Pi or ^{14}C-choline into into phospholipids as previously observed (16).

We have demonstrated in this report that Triton stimulates lipid synthesis in bone in addition to the previously reported organs brain and liver. Triton exhibits an *in vitro* effect on lipid synthesis of most classes of lipids. The only previous *in vitro* effect was limited to cholesterol and fatty acid synthesis in perfused liver (4, 13). We have also shown that Triton exhibits an *in vitro* effect on lipid synthesis in a tissue culture system which has not been previously observed to our knowledge.

Summary. Triton WR-1339, administered parenterally, has long been known to be a potent hyperlipemic agent. *In vitro* lipid biosynthesis is stimulated in liver and brain preparations from animals injected with Triton. Only in a perfused isolated liver system has an *in vitro* effect of Triton on lipid synthesis been demonstrated. In the present study, lipid biosynthesis has been shown to increase in bone, a third organ system, under the influence of *in vitro* Triton WR-1339. This stimulation affects most major lipid classes. Triton similarly stimulates lipid synthesis in tissue cultures of bone cells. This is the first report of an effect of Triton on lipid synthesis (1) in bone and (2) in any tissue culture system.

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