Rapid increase in hepatic HMG CoA reductase activity and in vivo cholesterol synthesis after Triton WR 1339 injection[1]

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Abstract Triton WR 1339, injected intravenously into rats, caused a 12% decrease in hepatic cholesterol within 30 minutes and a 34% decrease after 2 hours. An early and progressive increase in plasma cholesterol and triglycerides was also confirmed. Although hepatic HMG CoA reductase activity was unchanged after 30 minutes, it had increased seven-fold after 105 minutes. In vivo cholesterol synthesis measured by determining incorporation of intraperitoneally-injected ^3H_2O into cholesterol also showed an early increase, suggesting that the enzyme was rate controlling for cholesterogenesis under conditions of rapid stimulation. These findings strongly suggest that Triton WR 1339 stimulates hepatic cholesterogenesis by depleting hepatic cholesterol and trapping it in the blood compartment. The rapidity with which the drug acts supports the hypothesis that hepatocellular flux of cholesterol or its derivatized product could mediate the diurnal and hormonally induced fluctuations of cholesterogenesis.

Supplementary key words tritiated water · triglycerides · plasma lipids

Triton WR 1339, a nonionic detergent, causes a marked increase in hepatic cholesterol synthesis (2, 3) and HMG CoA reductase (mevalonate:NADP oxidoreductase (acylating CoA), EC 1.1.1.34) activity (4–6) in rodents within 24 hr of intravenous or intraperitoneal injection. Even more rapid than the reported stimulation of cholesterogenesis is the accumulation of triglycerides and cholesterol in the plasma (7) which is thought to be a consequence of "trapping" in the blood compartment of physically altered lipoproteins (8–10) whose degradation by lipoprotein lipase is impaired (11–13). Since the detergent does not stimulate cholesterogenesis by a direct effect on microsomal membranes (5) or by stimulating membrane biogenesis (14), these findings support the speculation that blockage of the return of serum lipoproteins or their degraded products to the liver somehow stimulates an increase in cholesterol synthesis (2, 3, 15). The detergent's effects offer an interesting model for studying the integration of hepatic cholesterol and lipoprotein synthesis under fasting conditions when the marked diurnal fluctuations of HMG CoA reductase activity (5, 16) and cholesterogenesis (17) are less prominent. Accordingly, earlier studies were repeated and extended by comparing the rate of hepatic cholesterogenesis and HMG CoA reductase activity with the level of hepatic cholesterol very soon after injecting Triton WR 1339. The results suggest that depletion of hepatic cholesterol after Triton WR 1339 injection is an extremely early event that precedes and probably induces the increase in hepatic HMG CoA reductase activity and cholesterogenesis.

MATERIALS AND METHODS

Male Holtzman rats weighing 110–170 g, housed one to a cage, were fed a synthetic diet containing, by weight, 75% dextrose, 20% casein, and no lipids, previously referred to as basal diet (18). Conditions of lighting and feeding were controlled. Food was placed in the cages daily at 9:00 AM when lights were turned off. The food was removed at 5:00 PM and the lights were turned on at 9:00 PM daily. This schedule was continued for at least 7 days prior to the experiments, but all food was withheld after Triton injection. Triton WR 1339 (oxyethylated tertiary octylphenol polymethylene polymer) from Ruger Chemical Co., Irvington, NJ, was injected as a 10% solution in 0.9% saline at a dose of 100 mg/100g body weight into tail veins of rats under light ether anesthesia. When experiments were concluded within 5 hr, rats were injected with Triton WR 1339 at 9:00 AM at the end of their regular 16-hr fasting period.

Abbreviation: HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

[1] A preliminary report of this work was presented at the 1975 Meeting of the American Society of Biological Chemists (1).

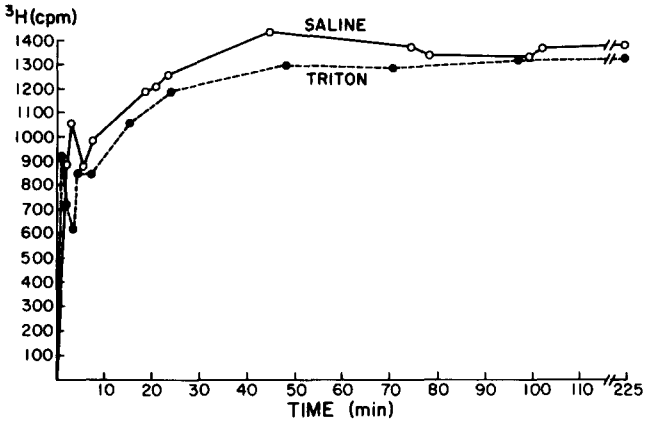

Fig. 1. Plasma radioactivity in 5-μl aliquots taken at frequent intervals from two rats of identical weight after intravenous injection of either isotonic saline or Triton WR 1339 90 min prior to intraperitoneal injection of ^3H_2O. (Injection of ^3H_2O was at 0 time.) See text for details of experiment.

Other rats were injected at 5:00 PM, the end of their regular feeding period, and killed 18 hr later. Rats used as controls for all experiments were injected with 1 ml of saline per 100 g body weight at the time of Triton injection. Groups of 4–7 rats were killed at each time point. Since the experiments were conducted over a period of 2 months, each group of experimental animals was matched with its own group of control rats.

Lipids were extracted from 1-g pieces of liver or 1 ml of plasma by the method of Folch, Lees, and Sloane Stanley (19). Triglycerides were quantitated by the method of Van Handel and Zilversmit (20) on aliquots of the lipid extract. Other aliquots of these extracts were dried under nitrogen, treated with alcoholic KOH, and extracted with petroleum ether. Sterols were precipitated from the nonsaponifiable lipid fraction with digitonin (21), and the digitonides were dissolved in glacial acetic acid. Measured volumes of this solution were taken for cholesterol determination by the method of Zak (22). For quantitation of radioactivity by liquid scintillation counting, 1 ml of the digitonide solution was added to 10 ml of toluene–PPO solution. In some experiments, the amount of nonesterified cholesterol was also determined by precipitation with digitonin without prior saponification of the lipid extract.

HMG CoA reductase activity was determined as described earlier using a double label assay procedure (23) incorporating improved assay conditions (18). Rats, three in each group, were killed at different times after Triton WR 1339 injection. The specific activity of ^{14}C-labeled HMG CoA was 4.7×10^6 dpm/μmol. Microsomal membrane protein was separated by a modification of a technique designed to rupture microsomes and remove intravesicular and adsorbed protein (24). Small aliquots of a suspension of the microsomes in incubating solution were diluted with a 20-fold excess of distilled water adjusted to pH 8.0 with NaOH, incubated for 15 min at 30°C, and then centrifuged in the cold at 100,000 *g* for 2 hr. Protein was determined on a suspension of this pelleted membrane material by the method of Lowry et al. (25).

In vivo cholesterogenesis was measured in two experiments by estimation of the μmol of ^3H_2O incorporated into the 3β-hydroxysterols in 1-g pieces of liver or in 1-ml volumes of plasma from rats injected intravenously with either Triton (100 mg/100 g body weight) or saline either 135 or 150 min prior to being killed. Approximately 10 mCi of ^3H_2O was injected intraperitoneally 60 min before the rats were killed and the specific activity of the plasma water, taken as a measure of intracellular water, was determined at the time of death.

In order to validate the use of this technique, it was necessary to first show that the intraperitoneally injected ^3H_2O equilibrated with body water pools at the same rate in Triton-injected and saline-injected rats. This was confirmed in two experiments in which two pairs of rats of identical weight were injected intravenously with either isotonic saline or Triton WR 1339 (100 mg/100 g body weight) 1.5 hr prior to injection of 50 μCi of ^3H_2O. Ten-μl samples of blood were then taken in capillary pipettes at frequent intervals from a small incision in the rats' tails. The blood was flushed into centrifuge tubes with 1 ml of distilled water. After precipitating the blood proteins with 1 ml of 10% trichloroacetic acid, samples were centrifuged and 1 ml of the clear supernatant was taken for liquid scintillation counting. The results of one of these experiments (**Fig. 1**) showed a remarkably rapid and similar biphasic type of equilibration in both control and experimental animals. Within 3 min, radioactivity increased to levels that were 60–80% of peak levels. Subsequently, the level of radioactivity decreased by 10–20% over a 3-min period and then rose, quickly at first, to full equilibration between 20–40 min after intraperitoneal injection. In two experiments, the total areas under the Triton curves were only 4% and 6% less than under the saline curves.

RESULTS

Plasma and hepatic lipid levels (Fig. 2)

Plasma triglyceride levels in rats killed 75 min and 285 min after Triton WR 1339 injection increased to levels that were, respectively, 4 and 13 times greater

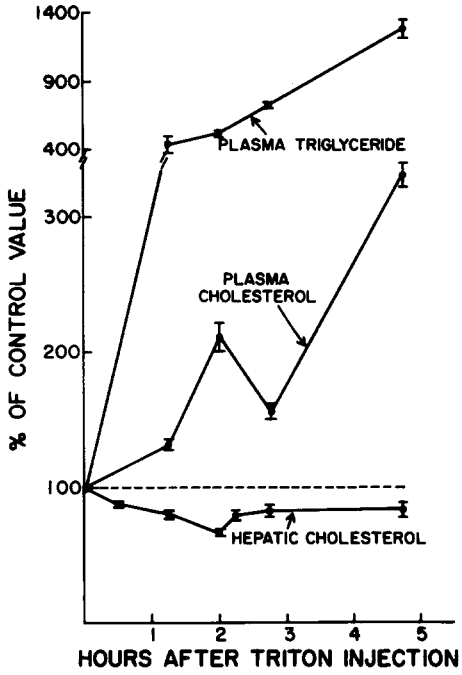

Fig. 2. Hepatic cholesterol, plasma triglyceride, and plasma cholesterol concentrations in groups of rats killed at intervals after intravenous injection of Triton WR 1339, expressed as percentage of control value. The range of values at each point is the SEM.

than control values, confirming the previously reported rapid increase (7). Plasma cholesterol increased much less dramatically and was only elevated by 30% after 75 min; however, it continued to rise, fluctuating slightly, and reached a 250% elevation after 285 min. Values for different control groups were between 94 and 180 mg/dl for plasma triglyceride, while plasma cholesterol concentrations were between 45 and 69 mg/dl. In one of the experiments in which in vivo cholesterogenesis was determined (Section C, Experiment 1), an increase in plasma unesterified cholesterol after Triton injection was confirmed (6). The ratio of unesterified to total cholesterol (mg/ml) 2.5 hr after injection was 0.23 (0.12/0.53) for saline-injected rats and 0.65 (0.56/0.86) for Triton-injected rats. A consistent, modest, but significant decrease in hepatic cholesterol was noted in all experimental groups (**Table 1** and Fig. 1). Hepatic cholesterol was 88% of control values after 30 min and reached its lowest level of 66% of control values after 2 hr. Mean values for hepatic cholesterol ranged between 2.04 and 2.90 mg of cholesterol for different control groups.

Microsomal HMG-CoA reductase activity (Fig. 3)

HMG CoA reductase activity 30 min after Triton WR 1339 injection was the same as that in control rats. However, by 105 min there was a 7-fold increase ($P < 0.05$) and the activity continued to rise, showing a 14-fold increase 18 hr after injection ($P < 0.01$).

TABLE 1. Hepatic cholesterol after intravenous injection of Triton WR 1339

Time After Injection	Saline-Treated	Triton-Treated	*P* Value
hr	*mg/g*	*mg/g*	
0.5	2.04 ± 0.06[a] (5)[b]	1.81 ± 0.06 (6)	$0.02 > P > 0.01$
1.25	2.66 ± 0.11 (5)	2.15 ± 0.12 (5)	$0.02 > P > 0.01$
2.0	2.85 ± 0.04 (5)	1.93 ± 0.11 (5)	$0.001 > P$
1.25	2.28 ± 0.06 (6)	1.82 ± 0.04 (6)	$0.001 > P$
2.75	2.11 ± 0.10 (5)	1.76 ± 0.09 (5)	$0.05 > P > 0.02$
4.75	2.22 ± 0.27 (4)	1.88 ± 0.16 (4)	Not significant

[a] Each value is the mean ± SEM.
[b] The number of rats is indicated in parentheses.

In vivo hepatic cholesterogenesis (Table 2)

Since HMG CoA reductase had increased by 7-fold in less than 2 hr after injecting Triton WR 1339, it was decided to determine the level of ^3H_2O incorporated into hepatic sterols at approximately the same time. Two and one-half hr after Triton injection and 1 hr after ^3H_2O injection (Exp. 1), the hepatic tissue contained 2.76 μmol of ^3H_2O/g in digitonin-precipitated sterol, a level almost 3 times greater than in the saline controls. A slightly lower but still significantly increased level of incorporation was noted in rats killed 15 min earlier (Exp. 2). In both experiments, the amount of newly synthesized cholesterol that appeared in the plasma was appreciable, representing at least 10–20% of that in the liver. The accumulation of ^3H-labeled cholesterol in the plasma was

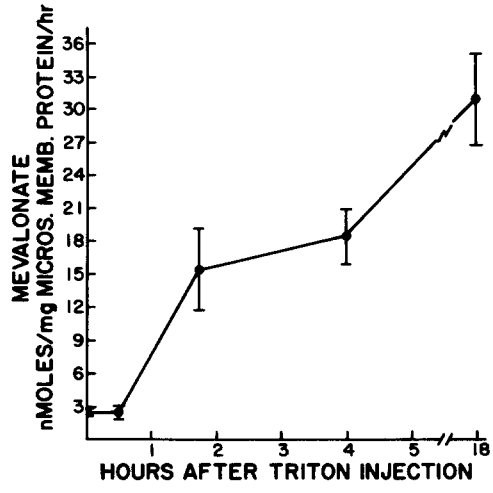

Fig. 3. Hepatic microsomal HMG CoA reductase activity of rats killed at different times after intravenous injection of Triton WR 1339. The range of values at each point is the SEM.

TABLE 2. Incorporation of ^3H_2O into [^{3}H]cholesterol after intravenous injection of Triton WR 1339

	Injection	Liver		Plasma	
		Cholesterol	$^3H_2O \rightarrow$ [^{3}H]Cholesterol	Cholesterol	$^3H_2O \rightarrow$ [^{3}H]Cholesterol
		mg/g	*μmol/g*	*mg/ml*	*μmol/ml*
Exp. 1	Triton (6)[b]	2.53 ± 0.14[a,c]	2.76 ± 0.71[d]	0.86 ± 0.03[f]	0.48 ± 0.13[e]
	Saline (6)	2.91 ± 0.23	0.95 ± 0.13	0.53 ± 0.01	0.18 ± 0.05
Exp. 2	Triton (7)	2.60 ± 0.08[d]	2.29 ± 0.26[e]	1.48 ± 0.08[f]	0.27 ± 0.04[d]
	Saline (6)	3.05 ± 0.21	1.08 ± 0.12	0.70 ± 0.09	0.08 ± 0.02

[a] Each value is the mean ± SEM.
[b] The number of rats is indicated in parentheses.
[c] Significance of difference from control group $0.1 > P > 0.05$.
[d] Significance of difference from the control group $0.02 > P > 0.01$.
[e] Significance of difference from the control group $0.01 > P > 0.001$.
[f] Significance of difference from the control group $0.001 > P$.

For purposes of calculation, all 3β-hydroxysterols precipitated with digitonin are considered to be cholesterol. In Experiment 1, Triton was injected 2.5 hr prior to killing. In Experiment 2, Triton was injected 2.25 hr prior to killing. In all rats, 10 mCi of ^3H_2O was injected intraperitoneally 60 min prior to sacrifice.

about three times greater for Triton-injected than saline-injected rats in both experiments. Differences in hepatic and plasma cholesterol levels between control and Triton-injected rats were similar to those reported in Results (Fig. 2).

DISCUSSION

In the present study an increase of hepatic cholesterogenesis and HMG CoA reductase activity was induced much more rapidly by Triton WR 1339 injection than previously reported (5, 6). The data strongly suggest that the enzyme is rate controlling for cholesterol synthesis since a 7-fold increase in enzyme activity 105 min after injecting the detergent correlated with an almost 3-fold increase in hepatic cholesterogenesis during the interval between 1.5 hr and 2.5 hr after Triton injection. Since the enzyme induction was accompanied and even preceded by a significant reduction in hepatic cholesterol content, the depletion of hepatic cholesterol or a sterol derived from cholesterol is probably the specific event that releases the repression or inhibition of HMG CoA reductase synthesis. Interestingly, in a previous study (27), the early effect of Triton in decreasing hepatic cholesterol levels was missed, possibly because of an oversight in controlling the rats' feeding patterns. More recently, Kuroda et al. (6) presented data that in general support our findings (1). They too observed that injection of Triton WR 1339 caused a depletion of hepatic cholesterol that preceded stimulation of HMG CoA reductase activity and cholesterogenesis; however, the time course of the changes was much longer in their study than in ours and statistical validation of these observations was lacking. The hypothesis that a sterol regulates cholesterogenesis is consistent with other studies showing an inverse relationship between hepatic HMG CoA reductase activity and microsomal (28) or hepatocellular (29) cholesterol levels. The precise nature of the cholesterol-derived inhibitor and its mechanism of action are not yet known, but the inhibition of HMG CoA reductase activity by oxygenated cholesterol derivatives (30) and the increase in concentration of free cholesterol bound to chromatin immediately prior to the diurnal decrease in enzyme activity (31) are exciting developments in both of these areas.

In an earlier publication, Goldfarb and Pitot (32) speculated that at least two different mechanisms for regulation of hepatic cholesterogenesis, one hormonal and the other nonhormonal, were suggested by studies that demonstrated a loss of dietary cholesterol-mediated "feedback" control but retention of diurnal rhythmicity of HMG CoA reductase in Morris hepatomas. Since that time, studies with diabetic and nondiabetic rats have suggested that insulin might be the humoral regulator of the diurnal rhythm of hepatic HMG CoA reductase (33). In view of the rapidity with which HMG CoA reductase can be stimulated in vivo after depletion of hepatic cholesterol, serious consideration should now be given to the hypothesis that some hormones mediate cholesterogenesis by effecting intracellular redistribution of cholesterol or its metabolites.

The rate of cholesterol synthesis described here and expressed as μmol of ^3H_2O incorporated in digitonin-precipitated sterols per gram of liver is considered a valid measurement of cholesterogenesis for the following reasons. First, protons from water are incorporated into covalent carbon–hydrogen

bonds in a number of steps in the sterol's synthesis in a fixed ratio of water: cholesterol molecules (34). Second, the level of 3β-hydroxysterols, other than cholesterol, in the liver or blood can be considered insignificant under most conditions. Third, intra- and extracellular pools of water in small animals equilibrate very rapidly (35). The highly reproducible, rapid rise, then abrupt fall, secondary rise, and plateauing of ^3H_2O concentration in the plasma, demonstrated in this study, most probably reflects the movement of water from the peritoneal cavity to the blood compartment, then to intracellular water compartments, and finally equilibration in the whole animal. The similarity in flux of ^3H_2O in control and Triton-injected animals was reassuring and served to validate the use of a single plasma ^3H_2O determination at the time of killing for the determination of hepatic cholesterogenesis.

The technique described in this paper for measuring in vivo hepatic cholesterogenesis has certain advantages over previously described in vitro methods. For example, the method may be superior to liver slice techniques (36) which measure less than 10% of the hepatic cholesterol synthesized in the intact animal. In addition [^{14}C]acetate has been frequently used, and its rate of uptake, acylation to acetyl-CoA, and distribution among different intracellular pools may vary under different conditions (37). The present method is also considerably simpler than the in vitro method employing [^{14}C]octanoate (37).

The drawbacks to the in vivo method for measuring cholesterogenesis include the high levels of radioactivity which require special precautions for disposal, possible differences between plasma and intracellular ^3H_2O concentrations, and difficulties in assessing the degree of equilibration of newly synthesized cholesterol in hepatic and extrahepatic pools. The last of these is the most serious problem. Recently, it was shown that in rats infused with Triton WR 1339 for 3 days, in vitro cholesterol synthesis significantly increased in six organs in addition to the liver (36). Although these nonhepatic tissues might have contributed slightly to the increase in plasma [^{3}H]cholesterol that was observed in the present study, it is much more probable, considering its mass and potential for cholesterogenesis (38), that the liver was the major source of the newly synthesized cholesterol. This conclusion is also supported by the observation that hepatectomy followed by Triton injection did not cause an increase in plasma cholesterol concentration (7). Since more [^{3}H]cholesterol accumulated in the plasma of Triton-injected rats than in the control animals, and since physical exchange of cholesterol in vitro (39) and in vivo (40) are known to occur rather rapidly, it is most unlikely that the increased levels of hepatic [^{3}H]cholesterol merely reflected its preferential retention due to a toxic effect of the detergent. Thus, while the in vivo technique cannot provide absolutely accurate data, it can provide very useful approximations of hepatic cholesterogenesis.

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