Inactivation and activation of various membranal enzymes of the cholesterol biosynthetic pathway by digitonin

Haviva Eilenberg, Ety Klinger, Fiorenza Przedecki, and Ishaiahu Shechter

Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

Abstract The activity of rat liver microsomal squalene epoxidase is inhibited effectively by digitonin. Concentrations of 0.8 to 1.2 mg/ml of digitonin cause total inhibition of microsomal (0.75 mg protein/ml) squalene epoxidase either in microsomes that were pretreated with digitonin and subsequently washed and subjected to epoxidase assay or when digitonin was added directly to the assay. The inhibition of squalene epoxidase by digitonin is concentration-dependent and takes place rapidly within 5 min of exposure of the microsomes to digitonin. Octylglucoside, dimethylsulfoxide, CHAPS, as well as cholesterol or total microsomal lipid extract were ineffective in restoring the digitonin-inhibited squalene epoxidase activity. Epoxidase activity in digitonin-treated microsomes was fully restored by Triton X-100. The reactivation by Triton X-100 displays a concentration optimum with maximal reactivation of the epoxidase (0.7 mg protein/ml) occurring at 0.2% Triton X-100. Microsomal 2,3-oxidosqualene-lanosterol cyclase is also inhibited by digitonin. Higher concentrations of digitonin are required to obtain full inhibition of the cyclase activity and only 40% inhibition of cyclase activity is observed at 1 mg/ml of digitonin. Solubilized (subunit size 55 to 66 kDa) and microsomal (subunit size 97 kDa) 3-hydroxy-3-methylglutaryl CoA reductase are totally unaffected by the same concentration of digitonin. Squalene synthetase, another microsomal enzyme in the biosynthetic pathway of cholesterol, is activated by digitonin. A 2.2-fold activation of squalene synthetase is observed at 0.8 mg/ml of digitonin. The results agree with a model in which squalene, and to a lesser degree 2,3-oxidosqualene, are segregated by digitonin into separate intramembranal pools. Formation of such pools by digitonin prevents lateral intramembrane mobility or accessibility of squalene and 2,3-oxidosqualene to the microsomal enzymes. The results also indicate a practical and an efficient assay for microsomal squalene synthetase. —**Eilenberg, H., E. Klinger, F. Przedecki, and I. Shechter.** Inactivation and activation of various membranal enzymes of the cholesterol biosynthetic pathway by digitonin. *J. Lipid Res.* 1989. **30:** 1127–1135.

Supplementary key words squalene epoxidase • cholesterogenesis • 2,3-oxidosqualene-lanosterol cyclase • HMG-CoA reductase • squalene synthetase • supernatant protein factor • carrier protein

Digitonin is a steroid glycoside that forms equimolar insoluble complexes with cholesterol (1). It has been used for the precipitation and quantitation of 3β-hydroxysterols (2). The molar ratio of cholesterol to phospholipid in eucaryotic plasma membranes is several fold greater than in intracellular membranes (3). Thus, digitonin, administered under appropriate conditions, can perforate the cholesterol-rich plasma membrane of cells and make it permeable to ions (4), metabolites (5), and proteins (6, 7). Due to the low cholesterol content of the endoplasmic reticulum (ER) and other intracellular membranes (1, 8), digitonin binds to and affects mainly the cellular plasma membrane without significantly affecting inner cell membranes. Under appropriate conditions, digitonin treatment results in a cell ghost that retains above 90% of its intact mitochondria, peroxisomes, and ER (7). The binding specificity of digitonin makes it useful in studying various types of cell membranes differing in their cholesterol content (9).

All enzymes of the cholesterol biosynthetic pathway following and including squalene synthetase reside in the ER. Apart from the structural importance of the ER, this membrane contains all the lipophilic intermediates in the later part of the cholesterol biosynthetic pathway. Squalene and 2,3-oxidosqualene, the two first hydrophobic intermediates of this pathway, have been suggested to undergo lateral movement between different membrane pools (10–14). This intramembranal transfer is suggested to be facilitated by a supernatant protein factor (SPF) (12). SPF specifically enhances the activities of both squalene epoxidase and 2,3-oxidosqualene-lanosterol cyclase, whereas other activating proteins are reported to be functional with other enzymes in lipid metabolism (15–18).

Abbreviations: S_{105}, cytosolic fraction; SPF, supernatant protein factor; MVA, mevalonate; PG, phosphatidylglycerol; FPP, *trans*-farnesyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; CHAPS, 3[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate; TMD, 4,4,10-trimethyl-*trans*-decal-3-ol; AMO 1618, 2'-isopropyl-4'-(triethyl ammonium choloride)-5'-methylphenyl-piperidine-1-carboxylate.

In this report we show that digitonin is a potent inhibitor of microsomal squalene epoxidase (EC1.14.99.7, squalene 2,3-monooxigenase) activity while it exerts only a partial inhibition of the activity of 2,3-oxidosqualene-lanosterol cyclase (EC 5.4.99.7, 2,3-oxidosqualene lanosterol-cyclase), no inhibition of HMG-CoA reductase (EC1.1.1.34, mevalonate: $NADP^+$ oxidoreductase (CoA-acylating)), or even a stimulatory effect on squalene synthetase (EC 2.5.1.21, farnesyldiphosphate: farnesyldiphosphate farnesyl transferase). Possible mechanisms for the inhibition are discussed.

MATERIAL AND METHODS

Materials

DL-[2-^{14}C]-Mevalonate (51 mCi/mmol) was purchased from the Radiochemical Center, Amersham. [^{14}C]-Squalene was biosynthesized in our laboratory from [^{14}C]mevalonate according to the method of Popják (19) with slight modifications (20). [^{14}C]2,3-Oxidosqualene was biosynthesized in our laboratory by the same procedure as [^{14}C]squalene, except that the plant growth retardant Amo 1618 (2-isopropyl-4-[trimethylammonium chloride]-5-methylphenyl-piperidine-1-carboxylate) was added to inhibit 2,3-oxidosqualene-lanosterol cyclase (12, 21). Specific radioactivity for both labeled squalene and 2,3-oxidosqualene was determined experimentally by gas–liquid chromatography. *Trans*-[^{3}H]farnesol (32.6 mCi/mmol) was prepared according to Shechter and Bloch (22). Pyrophosphorylation was according to Popják (19) and purification to yield *trans*-[^{3}H]farnesyl pyrophosphate was according to Gafni and Shechter (23). Mevalonate, cholesterol, and digitonin were from Sigma. All chemicals were of analytical grade.

Preparation of microsomes and supernatant fractions

Rat liver microsomes and supernatant fractions were prepared according to Yamamoto and Bloch (15) except that the working buffer was 10 mM Tris-HCl, pH 7.4, containing 0.3 M sucrose. The soluble fractions of livers (S_{105}) as well as the corresponding microsomal preparations were stored in liquid N_2.

Preparation of digitonin-treated microsomes

Microsomes, suspended in phosphate-buffered saline (PBS) containing 0.25 M sucrose and 3 mM EDTA, were incubated at room temperature with digitonin at concentrations and time periods as indicated for each experiment. The microsomes were precipitated by Airfuge centrifugation (160,000 *g* for 10 min at room temperature) and washed once with 10 mM Tris-HCl containing 0.3 M sucrose. The digitonin-treated microsomes were resuspended in the same buffer and assayed for enzyme activity.

Preparation of total lipid extract of rat liver microsomes

Total lipids were extracted from rat liver microsomes by the method of Bligh and Dyer (24) with some modifications. In brief, microsomes were mixed at a volume ratio of 1:4 with chloroform–methanol 1:2. The mixture was mixed thoroughly and left for 45 min at 37°C. The homogeneous phase was then separated into a chloroform and a methanol phase by the addition of water and chloroform. The chloroform phase was made to float by the addition of excess hexane and was separated from the methanol phase. This organic phase was evaporated to dryness and the residue was suspended by mixing and sonication. Aliquots of this micellar suspension were added to the assay.

Preparation of PG-cholesterol liposomes

Increasing concentrations of cholesterol (up to 600 μg/ml) were sonicated with 250 μg/ml of PG until a clear suspension was obtained. Aliquots of these suspensions, each containing 50 μg of PG, were used for enzyme assays.

Assay of squalene synthetase

Squalene synthetase assays were in 0.1 M Tris-HCl, pH 7.4, and contained NADPH, 1.0 mM; $MgCl_2$, 5 mM; G6P, 3 mM; G6PD, 0.7 I. U.; and 100,000 dpm of *trans*-[^{3}H]farnesyl pyrophosphate in a final volume of 0.5 ml. Incubations were carried out at 37°C for 60 min. Incubations were stopped by addition of 0.5 ml of 10% methanolic-KOH; 10 μl of 0.5% (v/v) nonlabeled carrier squalene in ethanol was then added. Nonsaponifiable lipids were extracted 3 × with 2 ml each of hexane–acetone 1:1. The extract was evaporated under N_2 and chromatographed on silica gel thin-layer chromatography plates (0.2 mm) developed with hexane to 10 cm. Squalene standards were detected with iodine vapor and the zones containing the radiolabeled squalene (R_f 0.5) were scraped and assayed for radioactivity.

Assay of squalene epoxidase

The squalene epoxidase assay was essentially the same as that described by Tai and Bloch (25) except for some modifications. The standard assay mixture contained, in a total volume of 500 μl, the following reagents: Tris-HCl buffer, 0.1 M, pH 7.4; NADPH, 1 mM; EDTA, 1 mM; AMO 1618, an inhibitor of squalene 2,3-oxide lanosterol cyclase (21), 0.4 mM; FAD, 0.01 mM; phosphatidylglycerol (PG), 0.1 mg/ml; 50,000–100,000 dpm [^{14}C]squalene (sp act 100 Ci/mol) dispersed in 40 μl of 0.3% Tween 80 in acetone, and enzyme as indicated in the legends of the tables and figures.

Unless otherwise noted, the incubations were agitated in open test tubes at 37°C for 1 h, and stopped by addition of 0.5 ml of 10% methanolic KOH. After 30 min, 20 μg each of squalene, lanosterol, and cholesterol were added to the tubes as nonlabeled carriers. Acetone (0.5 ml) was added and the nonsaponifiable material was extracted three times from the mixture by 1 ml of hexane. The combined extracts were evaporated under a stream of N_2. The residue, taken up in a samll volume of hexane and ethylacetate, was spotted on thin-layer silica gel plates which were then developed to a height of 10 cm with 0.5% ethyl acetate in benzene as the solvent system. Zones of 0.5 cm were scraped and counted. The amount of Amo 1618 used in the reaction did not completely inhibit the conversion of 2,3-oxidosqualene to sterols; the sterols formed varied between 0–10% of total products. Epoxidase activity is, therefore, expressed in terms of the sum of dpm of 2,3-oxidosqualene and sterols formed per h.

Preloading of microsomes with[^{14}C]squalene

Rat liver microsomes (3.6 mg protein) were washed twice with 0.1 M Tris-HCl, pH 7.5. The washed microsomes were suspended and incubated, in a total volume of 480 μl, with 703,000 dpm of [^{14}C]squalene for 15 min at 37°C in the presence of 0.6 mg PG and 5.0 mg S_{105} under N_2. The microsomes were then centrifuged and washed with 0.1 M Tris-HCl and resuspended in 80 μl of the same buffer.

Assay of 2,3-oxidosqualene-lanosterol cyclase

2,3-Oxidosqualene-lanosterol cyclase activity was determined according to the method of Shechter, Sweat, and Bloch (26) with some modifications. In short, the assay mixtures contained, in a total volume of 0.6 ml, the following reagents: Tris-HCl buffer, 0.1 M, pH 7.4; PG, 0.1 mg/ml; [^{14}C]2,3-oxidosqualene, 10,000 dpm (~100 Ci/mol), dispersed in 40 μl of 0.3% Tween 80 in acetone; and 0.4 mg microsomal protein. The incubations were started by addition of enzyme and carried out as for squalene epoxidase. Extraction of lipids and application on silica gel thin-layer chromatography plates was done as described above for squalene epoxidase. The plates were developed to a height of 10 cm with 10% ethyl acetate in benzene. Zones of 0.5 cm were scraped and counted. Radiolabeled lanosterol migrated to R_f = 0.5.

Assay of HMG-CoA reductase

HMG-CoA reductase activity was assayed according to the method of Shapiro et al. (27) using 60,000 dpm (4.5 μCi/μmol) [^{14}C]HMG-CoA as substrate.

Assay of NADPH-cytochrome P-450 reductase

NADPH-cytochrome P-450 reductase activity was determined, as NADPH-cytochrome C reductase, in a 1-ml reaction mixture containing 17.5 μg of microsome protein, 25 μg cytochrome C, and 0.1 M PBS, pH 7.5. The reaction was started by addition of 0.2 μmol NADPH. The cytochrome C reduction was detected spectrophotometrically at 550 nm over 10 min.

Protein determination

Protein was determined by the method of Lowry et al. (28).

RESULTS

Inhibition of rat liver squalene epoxidase activity by digitonin

Fig. 1 shows the inhibition of rat liver microsomal squalene epoxidase in microsomes that were pretreated with various concentrations of digitonin. Complete inhibition of squalene epoxidase can be observed in microsomes that were preincubated with 0.8 mg/ml digitonin. Similar inhibition of epoxidase activity was observed

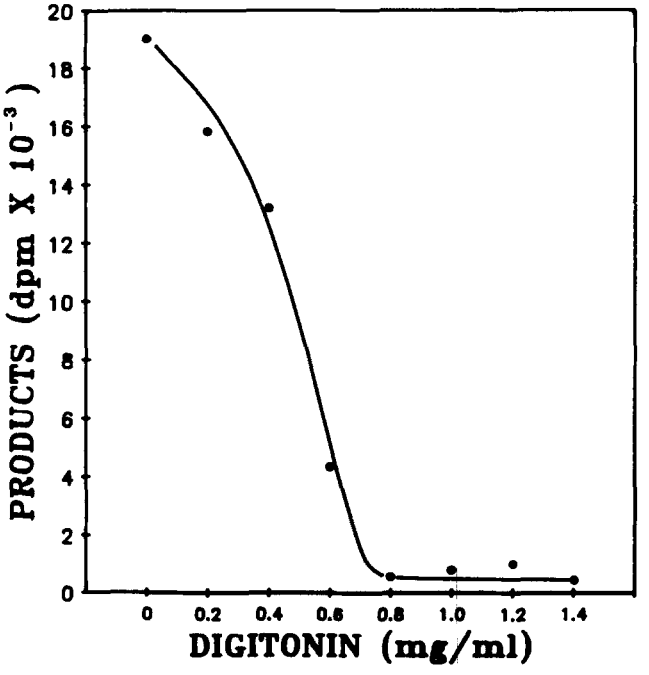

Fig. 1. Inhibition of rat liver microsomal squalene epoxidase activity by digitonin. Rat. liver microsomes (0.75 mg protein/ml) were incubated for 5 min at room temperature with increasing concentrations of digitonin as indicated. Thereafter, the microsomes were precipitated in an Airfuge at 160,000 *g* for 7 min and the pellet was washed with 10 mM Tris-HCl, pH 7.4, containing 0.3 M sucrose. The washed pellet was resuspended in the same buffer and assayed for epoxidase activity using the standard assay mixture as described in Methods. Each assay contained 0.6 mg/ml microsomal protein. The results are expressed as dpm products (2,3-oxidosqualene and sterols) formed per h.

when instead of pretreating the microsomes, digitonin was added directly to the enzyme assay. The inhibition of microsomal epoxidase occurs rapidly. Microsomes preincubated with 1.0 mg/ml digitonin at 37°C for various time periods showed a 94% inhibition of rat liver squalene epoxidase activity at very short preincubation periods with digitonin (**Fig. 2**). A similar inhibitory effect by digitonin on Chinese hamster ovary cell-derived squalene epoxidase was also observed (data not shown). Preincubation of microsomes with up to 1.0 mg/ml of cholesterol, as cholesterol-PG liposomes, did not significantly prevent the subsequent inhibition by digitonin. Poor reproducibility of results was encountered in these experiments probably due to nonreproducible loading of the microsomes with cholesterol and removal of the added digitonin by the cholesterol left in the liposomes. The squalene epoxidase activities observed in microsomes pretreated with cholesterol-PG liposomes and subsequently with digitonin, both up to 1 mg/ml, never exceeded 10% of that of microsomes treated with digitonin alone.

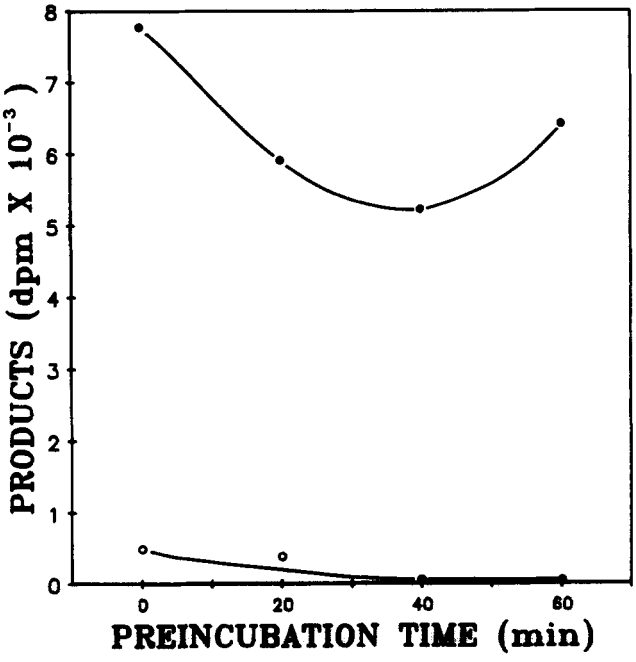

Fig. 2. Squalene peoxidase activity in rat liver microsomes pretreated for various time periods with digitonin. Rat liver microsomes (1.7 mg protein/ml), suspended in PBS containing 0.25 M sucrose and 3 mM EDTA, were preincubated for various time periods either in the presence (○) or absence (●) of 1 mg/ml digitonin at 37°C. At various time periods, as indicated in the figure, the microsomes were precipitated by Airfuge centrifugation (160,000 *g* for 7 min at room temperature) and washed once with 10 mM Tris-HCl, pH 7.4, containing 0.3 M sucrose. The microsomes were resuspended in the same buffer and assayed for squalene epoxidase activity as described in Methods using 0.6 mg/ml microsomal protein. Enzyme activity is expressed as dpm products (2,3-oxidosqualene and sterols) formed per h.

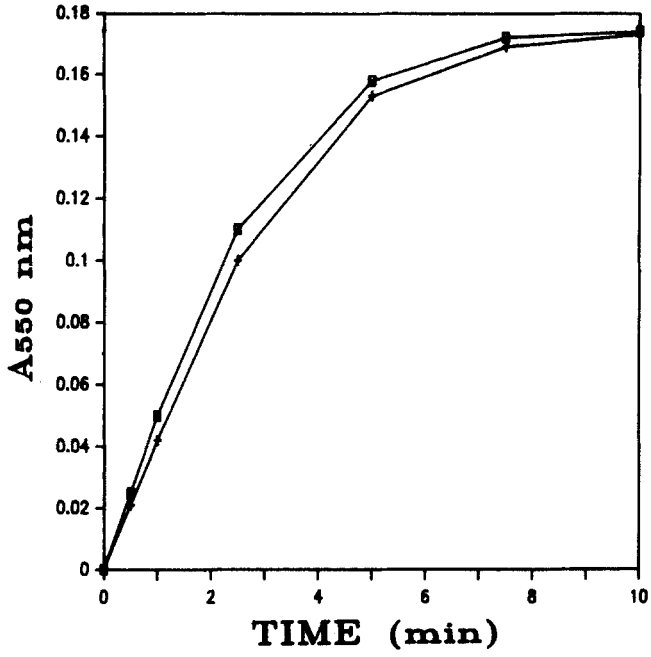

Fig. 3. NADPH-cytochrome P-450 reductase activity in rat liver microsomes pretreated with digitonin. Rat liver microsomes (1.3 mg/ml) were pretreated for 5 min at room temperature with 1 mg/ml digitonin as described in Methods. The digitonin-treated, washed microsomes were resuspended in 0.1 M PBS, pH 7.5. NADPH-cytochrome P-450 reductase activity was determined in 17.5 μg of these pretreated microsomes (+) as well as in control microsomes (□) which were not exposed to digitonin. The P-450 reductase activity was determined as described in Methods by monitoring the reduction of cytochrome C spectroscopically at 550 nm over a period of 10 min.

Effect of digitonin on NADPH-cytochrome P-450 reductase

The rat liver squalene epoxidase system is known to be comprised of NADPH-cytochrome P-450 reductase, detected as NADPH-cytochrome C reductase, and squalene epoxidase embedded in the endoplasmic reticulum (29). Our first aim was to determine whether the observed inhibition of the epoxidase system was due to inactivation of the NADPH-cytochrome P-450 reductase, electron transport system component. **Fig. 3** shows the activity of the cytochrome C reductase in microsomes pretreated with 1 mg/ml digitonin. It can be seen that the reductase activity in the pretreated microsomes is identical to that observed in control microsomes that underwent the same treatment but without digitonin. Squalene epoxidase activity was completely inhibited in these digitonin-treated microsomes. Since the NADPH-cytochrome P-450 reductase activity is unaffected under conditions at which squalene epoxidase activity is strongly inhibited, it should be concluded that the inhibition of squalene epoxidase by digitonin is not due to an effect on the electron transport component of the epoxidase system.

Reactivation of squalene epoxidase digitonin-treated microsomes

To obtain further information on the inhibition of microsomal squalene epoxidase by digitonin, we attempted to restore the enzyme activity in rat liver digitonin-treated microsomes with various substances. The assumption was made that by specifically relieving the inhibition we would be able to identify the essential component of the enzyme complex at which the inhibition occurs and perhaps rule out other possible inhibition sites. Therefore, the following questions were put to test: *a*) does digitonin specifically affect the mobility of squalene, which is mediated by cytosolic carrier protein within the membrane? *b*) could the solubilizing properties of Triton X-100, octylglucoside, or DMSO result in a release of membrane-bound digitonin and reactivation of the enzyme? *c*) could CHAPS release microsomal-bound digitonin by the combination of its detergent properties and/or ligand exchange due to its 3β-hydroxysterol structure and, thus, relieve the inhibition? and finally *d*) could added cholesterol, or a total microsomal lipid extract, relieve the inhibition by the release of microsomal-bound digitonin or replacement of a digitonin-bound essential 3β-hydroxysterol? From **Table 1** it is clear that Triton X-100 effectively restores the activity of squalene epoxidase in digitonin-treated microsomes (see also Fig. 4). Rat liver cytosolic fraction (S_{105}), DMSO, CHAPS, and octylglucoside were totally ineffective in restoring the inhibited epoxidase activity in the indicated range of concentrations. Cholesterol, added in DMSO or as PG-cholesterol liposomes, was also ineffective in restoring squalene epoxidase activity. In addition, no significant increase in activity above that exerted by Triton X-100 was noted when cholesterol was supplied in this detergent. We also measured the effect of a wide range of cholesterol concentrations (up to 240 μg/ml) and were not able to observe reactivation of epoxidase in these microsomes. Similarly, addition of total lipid extract of rat liver microsomes, dissolved in the detergents and substances indicated in Table 1, to the inhibited enzyme was totally ineffective in reactivation (data not shown). Triton X-100 reactivates the digitonin-inhibited enzyme in a concentration-dependent manner. As shown in Table 1, 0.15% of Triton X-100 restored total squalene epoxidase activity in comparison to only 30% recovery of the activity in the presence of 0.08% Triton X-100. Despite the fact that we did not carry out a detailed study of the effect of Triton X-100 on the reactivation process, it is possible that the extent of activation by Triton X-100 is related to the ratio of the detergent to the amount of microsomes in the assay rather than its absolute concentration. Thus, at various concentrations of digitonin-treated microsomes, different concentrations of detergent will cause maximal reactivation. **Fig. 4** shows that the activation of squalene epoxidase by Triton X-100 is concentration-dependent with maximal activation obtained at 0.2% detergent.

TABLE 1. Reactivation of squalene epoxidase activity in digitonin-treated microsomes

Additions to Assay	% Reactivation
S_{105} (up to 16 mg protein/ml)	0
Triton X-100 (0.08%)	30.0
Triton X-100 (0.15%)	101.7
DMSO (up to 6%)	0
CHAPS (up to 0.16%)[a]	0
Octylglucoside (up to 0.4%)[a]	0
Cholesterol (50 μg/ml)	
a) Solubilized in 5% DMSO	0.7
b) Solubilized in 0.08% Triton X-100	33.0
c) Given as PG-cholesterol liposomes	0.5

Rat liver microsomes (1.5 mg protein/ml) were incubated with 1 mg/ml digitonin for 5 min at room temperature. Thereafter the microsomes were precipitated by Airfuge (160,000 *g*) and washed once with 10 mM Tris-HCl buffer containing 0.3 M sucrose. The pellet was suspended in the same buffer and squalene epoxidase activity was determined in digitonin-treated and nontreated microsomes (0.6 mg/ml protein) using the standard assay mixture in the presence of the various additions to the assay. Liposomes (PG-cholesterol) were prepared as described in Methods. The epoxidase activity is expressed as % reactivation of the digitonin-treated microsomes compared to microsomes which were treated the same except that digitonin was omitted (100% activity equals 10,725 dpm 2,3-oxidosqualene and sterols). Cholesterol was first solubilized in Triton X-100 or DMSO and then added to the assay to give the appropriate cholesterol and detergent concentrations.

[a]Inhibition of squalene epoxidase activity by this concentration did not exceed 30%.

Inhibition of squalene epoxidase activity in rat liver squalene-preloaded microsomes

The data presented above indicate that digitonin does not cause an irreversible destruction of squalene epoxidase and complete activity of the inhibited enzyme can be restored in the presence of Triton X-100. One possible mechanism by which digitonin may exert inhibition of squalene epoxidase activity is by sterically hindered uptake and/or incorporation of squalene into the microsomal membrane (see Discussion below). In order to rule out the possibility of steric hindrance of squalene incorporation into the microsomes, rat liver microsomes were preloaded with [^{14}C]squalene by the method of Chin and Bloch (30) prior to the exposure to digitonin. These preloaded microsomes were then subjected to digitonin treatment. Thereafter, squalene epoxidase activity was determined in the microsomes without further addition of substrate. **Table 2** shows the results obtained in such an experiment. Less than 4% of the epoxidase activity, expressed in the [^{14}C]squalene-preloaded microsomes, can by seen in microsomes which were treated with digitonin prior to the assay. Control experiments indicated that there was no conversion of the [^{14}C]squalene to nonsaponifiable lipids following the preloading the during the digitonin treatment. In addition, loss of total radioactivity during digi-

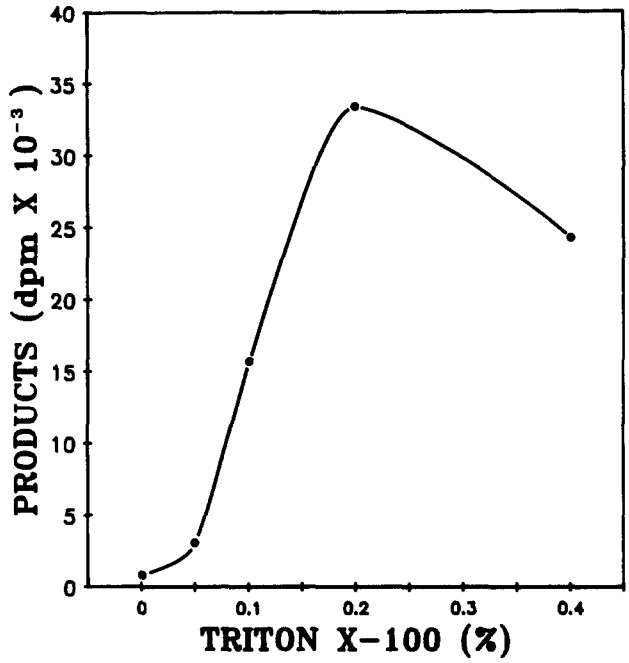

Fig. 4. Reactivation of digitonin-inhibited squalene epoxidase by Triton X-100. Rat liver microsomes were pretreated for 5 min at room temperature with 1.2 mg/ml digitonin as described in Methods. The digitonin-treated, washed microsomes were resuspended in 10 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose (11.7 mg protein/ml), and assayed for squalene epoxidase activity in the presence of increasing concentrations of Triton X-100. The assay was performed as described in the Methods section using 0.7 mg/ml microsomal protein. Epoxidase activity is expressed as dpm products (2,3-oxidosqualene and sterols) formed per h.

tonin treatment was relatively low and never exceeded 18%, indicating that digitonin does not cause any significant mobilization of squalene out of the membrane. The presence of increasing concentrations of radiolabeled squalene during the digitonin treatment of the microsomes did not prevent or reverse the inhibition of the epoxidase. Similarly, increasing concentrations of digitonin, up to a molar ratio of 1400 (digitonin/squalene), did not affect the incorporation of the radiolabeled squalene into the microsomes. The above results rule out a mechanism by which the inhibition of squalene epoxidase by digitonin is due to either direct interaction of digitonin and squalene or steric hindrance of squalene uptake and incorporation into the microsomal membrane.

Effect of digitonin on other membrane enzymes of the cholesterol biosynthetic pathway

To obtain information on the specificity of the inhibition by digitonin, we studied the effect of digitonin on three other membranal enzymes of the cholesterol biosynthetic pathway: squalene synthetase, 2,3-oxidosqualene-lanosterol cyclase, and HMG-CoA reductase. Cyclase, which catalyzes the cyclization of 2,3-oxidosqualene to lanosterol, is also known to be stimulated by the same supernatant protein factor (SPF) and phospholipids as squalene epoxidase (12). Squalene synthetase and HMG-CoA reductase are both membranal enzymes that utilize water-soluble substrates and, thus, no intramembranal movement of substrates is required. **Fig. 5** depicts the effect of digitonin on the activities of these microsomal enzymes as well as on solubilized HMG-CoA reductase. It can be seen that digitonin is more effective in inhibiting epoxidase activity than it is in the inhibition of the activity of 2,3-oxidosqualene-lanosterol cyclase. Total inhibition of epoxidase activity is observed at 1.2 mg/ml of digitonin (see also Fig. 1) whereas 2,3-oxidosqualene-lanosterol cyclase is inhibited under the same conditions only to about 40%. In comparison, HMG-CoA reductase is completely unaffected by the presence of digitonin in the assay and inhibition of either microsomal or solubilized HMG-CoA reductase was not observed. Perhaps the most surprising observation was that of the effect of digitonin on squalene synthetase. As seen, not only was this enzyme not inhibited by digitonin, as were the two enzymes that follow it in the biosynthetic pathway, but its activity was even enhanced. About 2.2-fold stimulation of squalene synthetase activity was observed at high concentrations of digitonin (0.8 to 2.0 mg/ml).

DISCUSSION

We have found that digitonin, which is known to specifically interact with 3β-hydroxysterols, inhibited microsomal squalene epoxidase activity in hepatic microsomal preparations. A 5-min preincubation of microsomes with 0.8–1.0 mg/ml digitonin results in a 100% inhibition of both the Chinese hamster ovary (data not shown) and rat liver enzymes (Fig. 1). Such inhibition has not been previously reported. One mechanistic explanation to the inhibition was that digitonin causes steric or chemical

TABLE 2. Inhibition of squalene epoxidase activity in rat liver microsomes preloaded with [^{14}C]squalene

Enzyme[a]	Squalene Epoxidase Activity[b] (dpm products/h)
Preloaded microsomes	5689
Digitonin-treated preloaded microsomes	210

[a]Microsomes, preloaded with [^{14}C]squalene (1.8 mg protein suspended in 400 μl), were incubated either in the presence or in the absence of 0.4 mg digitonin for 5 min at room temperature. The microsomes were then centrifuged, washed once, and resuspended in 0.1 M Tris-HCl. Enzyme assays were done on 0.9 mg of both digitonin-treated and nontreated microsomes. Each assay contained 57,000 dpm of [^{14}C]squalene when assayed.

[b]The enzyme activity is expressed as dpm products (2,3-oxidosqualene and sterols) formed per h.

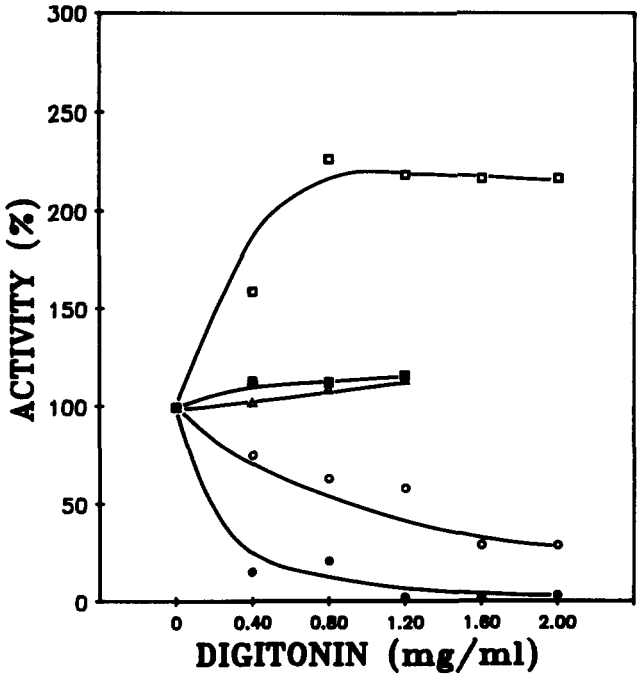

Fig. 5. The effect of digitonin on HMG-CoA reductase, squalene synthetase, squalene epoxidase, and 2,3-oxidosqualene-lanosterol cyclase activities. Rat liver microsomes were treated with increasing concentrations of digitonin as described in the Methods section. After the washing and resuspension of the microsomes in 10 mM Tris-HCl, pH 7.4, containing 0.3 M sucrose, squalene synthetase (□), squalene epoxidase (●), and cyclase (○) activities were determined in these digitonin-treated microsomes (0.8 mg protein/ml) as described in the Methods section. Soluble (△) and microsomal (■) (20 μg protein/ml) HMG-CoA reductase activities were determined in the presence of increasing concentrations of digitonin in the assay. The assay was performed as described in the Methods section. The enzyme activities in the presence of digitonin are expressed as per cent enzyme activity in its absence.

hindrance of [^{14}C]squalene incorporation into the microsomal membrane which results in reduction of substrate availability to the enzyme. In order to study this possibility, we preloaded microsomes with [^{14}C]squalene. These preloaded microsomes were exposed to digitonin, washed, and then assayed for squalene epoxidase activity without further addition of substrate. The finding that digitonin still inhibited epoxidase activity in these preloaded microsomes (Table 2), rules out the possibility of hindrance, by digitonin, of [^{14}C]squalene incorporation/uptake into the microsomes. Considering the specificity of digitonin to 3β-hydroxysterols, additional possible explanation for the observed inhibition of squalene epoxidase can be put forward. If a postulated 3β-hydroxysterol, essential for squalene epoxidase activity, exists in the vicinity of the enzyme, formation of a digitonin complex with such putative sterol would then result in a loss of enzyme activity. If this, indeed, was the reason for the inhibition, then replenishment of exogenous 3β-hydroxysterols or the removal of the bound digitonin by ligand-exchange would restore epoxidase activity. Cholesterol content of rat liver ER is known to be very low (0–5 % of the total membranal cholesterol) (1, 8). Therefore, the strong inhibition caused by digitonin could hint that if an essential 3β-hydroxysterol exists, it is concentrated in the proximity of the epoxidase in the microsomes. To test this hypothesis, we assayed the digitonin-treated microsomes in the presence of increasing concentrations of cholesterol or microsomal lipid extract in order to replenish any digitonin-bound sterol. The amount of cholesterol added ranged up to 20-fold the amount reported to exist in rat liver microsomes (1, 8). In addition, we attempted to restore the enzyme activity with various detergents (Triton X-100, DMSO, or CHAPS), with the assumption that these agents might restore activity by the release of the bound digitonin. We found that Triton X-100 could fully restore squalene epoxidase activity in rat liver digitonin-treated microsomes, whereas other detergents and addition of sterols had no reactivation effect (Table 1). Since digitonin-inhibited epoxidase activity could be recovered completely in the presence of Triton X-100, we conclude that digitonin does not cause the removal of the enzyme from the microsomes. The possibility that Triton X-100 releases the membrane-bound digitonin, thus re-exposing an es sential sterol, also seems unlikely. This conclusion is based on the observation that neither the presence of cholesterol nor the presence of microsomal lipid extract in the assay resulted in any reactivation of the enzyme regardless of the way it was administered. The fact that we could not observe significant difference in epoxidase activity by pretreating the microsomes with cholesterol-PG liposomes supports this conclusion as well. Therefore, it seems most likely that the inhibition phenomenon might be due to disruption of structural intactness, or change in the physical properties of the membrane, rather than to a shortage of a certain specific sterol necessary for the enzyme activity. This conclusion also seems logical in light of the findings by Friedlander et al. (31), that squalene undergoes inter- and intramembranal transfer. This transfer is facilitated by the cytosolic protein factor and PG and requires an intact membrane.

Triton X-100, on the other hand, has been shown to be able to activate both membrane-bound and solubilized epoxidase (32). Our results support previous observations that only squalene present in certain membranal pools can be used as substrate for epoxidation (31, 33). This conclusion is further supported by the results obtained from a study of three additional membranal enzymes of the cholesterol biosynthetic pathway: 2,3-oxidosqualene-lanosterol cyclase, squalene synthetase, and HMG-CoA reductase. The observed inhibition of cyclase and lack of inhibition of reductase is in accordance with our hypothesis, since cyclase, like epoxidase, requires intramembranal movement of its lipophilic substrate whereas the substrates, as well as products, of HMG-CoA reductase are soluble. The structural details of HMG-CoA reductase

indicate that although it is associated with and embedded in the ER, its catalytic site is located in an hydrophilic domain of the protein which protrudes into the cytosol (34). Thus, it is not surprising that variations in the properties of the microsomal membrane which are caused by digitonin do not significantly affect the activity of the reductase since access of the substrates and release of the products of the reaction should not be hindered. This is also in agreement with reports that treatment of Chinese hamster ovary cells with digitonin resulted in release of cytosolic proteins but activity of HMG-CoA reductase was retained (35). Squalene synthetase is in many ways a unique case. Its substrates, *trans*-farnesyl pyrophosphate and NADPH are water-soluble and produced by cytosolic enzymes similar to the substrates of HMG-CoA reductase. Therefore, it is reasonable to assume that the catalytic site of the synthetase is exposed to the cytosolic side of the ER to allow access of the substrates. On the other hand, squalene, one of the reaction products, is a non-water-soluble hydrocarbon and its release from the catalytic site should be either mediated by a carrier protein or by direct insertion into the hydrophobic inner part of the membrane. Whatever the mechanism, changes in the properties of the membrane would probably have their pronounced effect on the release of the products from the enzyme rather than on the uptake of the substrates by it. An interesting observation is the effect itself. Unlike the inhibitory effect of digitonin on the epoxidase and the cyclase, squalene synthetase is actually activated to a large extent by digitonin. A 2.2-fold activation is observed at about 0.8 mg/ml digitonin (Fig. 5). One possible explanation of these results might be that digitonin facilitates removal of squalene from the enzyme catalytic site, thus releasing product inhibition of the enzyme.

One practical aspect of this work is that the presence of digitonin allows a more precise assay of squalene synthetase. To prevent further conversion of squalene in the assay of squalene synthetase, the reaction has to be done under anaerobic conditions with no molecular oxygen in order to suppress squalene epoxidase activity. Digitonin, on the other hand, is effective in the complete inhibition of the epoxidase and the same concentration of digitonin causes the activation of squalene synthetase (see Fig. 5). At concentrations of digitonin above 1 mg/ml in the assay, squalene epoxidase is completely inhibited whereas a plateau of activation is obtained for squalene synthetase, thus assuring a block to further conversion of squalene.

This work was supported by Grant 1426AR1 from the Council for Tobacco Research-U.S.A., Inc.

Manuscript received 28 April 1988, in revised form 3 January 1989, and in revised form 21 March 1989.

REFERENCES

1. Amar-Costesec, A., M. Wibo, D. Thines-Sempoux, H. Beaufay, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. IV. Biochemical, physical, and morphological modifications of microsomal components induced by digitonin, EDTA, and pyrophosphate. *J. Cell Biol.* **62:** 717–745.
2. Tabacik, C., S. Aliau, and A. Crastes de Paulet. 1983. Digitonin-precipitable sterols as a measure of cholesterol biosynthesis: contradictory results. *Lipids.* **18:** 641–649.
3. Colbeau, A., J. Nachbaur, and P. A. Vignais. 1971. Enzymic characterization and lipid composition of rat liver subcellular membranes. *Biochim. Biophys. Acta.* **249:** 462–492.
4. Ronning, S. A., G. A. Geatley, and T. F. S. Martin. 1982. Thyrotropin-releasing hormone mobilizes Ca^{2+} from endoplasmic reticulum and mitochondria of GH_3 pituitary cells: characterization of cellular Ca^{2+} pools by a method based on digitonin permeabilization. *Proc. Natl. Acad. Sci. USA.* **79:** 6294–6298.
5. Ucker, D. S., and K. R. Yamamoto. 1984. Early events in the stimulation of mammary tumor virus RNA synthesis by glucocorticoids. *J. Biol. Chem.* **259:** 7416–7420.
6. MacKall, J., M. Meredith, and M. D. Lane. 1979. A mild procedure for the rapid release of cytoplasmic enzymes from cultured animal cells. *Anal. Biochem.* **95:** 270–274.
7. Katz, J., and P. A. Wals. 1985. Studies with digitonin-treated rat hepatocytes (nude cells). *J. Cell. Biochem.* **28:** 207–228.
8. Beaufay, H., A. Amar-Costesec, D. Thines-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. III. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. *J. Cell Biol.* **61:** 213–231.
9. Lang, Y., and T. L. Steck. 1985. Cholesterol-rich intracellular membranes: a precursor to the plasma membrane. *J. Biol. Chem.* **260:** 15592–15597.
10. Saat, Y. A., and K. Bloch. 1976. Effect of a supernatant protein on microsomal squalene epoxidase and 2,3-oxidosqualene-lanosterol cyclase. *J. Biol. Chem.* **251:** 5155–5160.
11. Gavey, K. L., and T. J. Scallen. 1978. Studies on the conversion of enzymatically generated, microsome-bound squalene to sterol. *J. Biol. Chem.* **253:** 5476–5483.
12. Caras, I. W., and K. Bloch. 1979. Effects of a supernatant protein activator on microsomal squalene-2,3-oxide-lanosterol cyclase. *J. Biol. Chem.* **254:** 11816–11821.
13. Nakamura, M., and R. Sato. 1979. The roles of soluble factors in squalene epoxidation by rat liver microsomes. *Biochem. Biophys. Res. Commun.* **89:** 900–906.
14. Fuks-Holmberg, D., and K. Bloch. 1983. Intermembrane transfer of squalene promoted by supernatant protein factor. *J. Lipid Res.* **24:** 402–408.
15. Yamamoto, S., and K. Bloch. 1970. Studies on squalene epoxidase of rat liver. *J. Biol. Chem.* **245:** 1670–1674.
16. Scallen, T. J., M. W. Schuster, and A. K. Dhar. 1971. Evidence for a noncatalytic carrier protein in cholesterol biosynthesis. *J. Biol. Chem.* **246:** 224–230.
17. Ritter, M. C., and M. E. Dempsey. 1973. Squalene and sterol carrier protein: structural properties, lipid-binding, and function in cholesterol biosynthesis. *Proc. Natl. Acad. Sci. USA.* **70:** 265–269.

18. Trzaskos, J. M., and J. L. Gaylor. 1983. Cytosolic modulators of activities of microsomal enzymes of cholesterol biosynthesis. Purification and characterization of a non-specific lipid-transfer protein. *Biochim. Biophys. Acta.* **751:** 52–65.
19. Popják, G. 1969. Enzymes of sterol biosynthesis in liver and intermediates of sterol biosynthesis. *Methods Enzymol.* **15:** 393–454.
20. Shechter, I., A. M. Fogelman, and G. Popják. 1980. A deficiency of mixed function oxidase activities in cholesterol biosynthetic pathway of human granulocytes. *J. Lipid Res.* **21:** 277–283.
21. Douglas, T. J., and L. G. Paleg. 1972. Inhibition of sterol biosynthesis by 2-isopropyl-4-dimethylamino- 5-methylphenyl-1-piperidine carboxylate methyl chloride in tobacco and rat liver preparations. *Plant Physiol.* **49:** 417–420.
22. Shechter, I., and K. Bloch. 1971. Solubilization and purification of *trans*-farnesyl pyrophosphate-squalene synthetase. *J. Biol. Chem.* **246:** 7690–7696.
23. Gafni, Y., and I. Shechter. 1979. An efficient method for the synthesis and purification of *trans*-[^{14}C]geranylgeranyl pyrophosphate. *Anal. Biochem.* **92:** 248–252.
24. Bligh, E. G., and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
25. Tai, H., and K. Bloch. 1972. Squalene epoxidase of rat liver. *J. Biol. Chem.* **247:** 3767–3773.
26. Shechter, I., F. W. Sweat, and K. Bloch. 1970. Comparative properties of 2,3-oxidosqualene-lanosterol cyclase from yeast and liver. *Biochim. Biophys. Acta.* **220:** 463–468.
27. Shapiro, D. G., J. L. Nordstrom, J. J. Mitschelen, V. W. Rodwell, and R. T. Schimke. 1974. Micro assay for 3-hydroxy-3-methylglutaryl-CoA reductase in rat liver and in L-cell fibroblasts. *Biochim. Biophys. Acta.* **370:** 369–377.
28. Lowry, O. H., N. H. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
29. Ono, T., K. Nakazono, and H. Kosaka. 1982. Purification and partial characterization of squalene epoxidase from rat liver microsomes. *Biochim. Biophys. Acta.* **709:** 84–90.
30. Chin, J., and K. Bloch. 1984. Role of supernatant protein factor and anionic phospholipid in squalene uptake and conversion by microsomes. *J. Biol. Chem.* **259:** 11735–11738.
31. Friedlander, E. J., I. W. Caras, L. F. H. Lin, and K. Bloch. 1980. Supernatant protein factor facilitates intermembrane transfer of squalene. *J. Biol. Chem.* **255:** 8042–8045.
32. Ono, T., and K. Bloch. 1975. Solubilization and partial characterization of rat liver squalene epoxidase. *J. Biol. Chem.* **250:** 1571–1579.
33. Kojima, Y., E. J. Friedlander, and K. Bloch. 1981. Protein-facilitated intermembrane transfer of squalene. *J. Biol. Chem.* **256:** 7235–7239.
34. Liscum, L., K. L. Luskey, D. J. Chin, Y. K. Ho, J. L. Goldstein, and M. S. Brown. 1983. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase and its mRNA in rat liver as studied with a monoclonal antibody and a cDNA probe. *J. Biol. Chem.* **258:** 8450–8455.
35. Leonard, D. A., and H. W. Chen. 1987. ATP-dependent degradation of 3-hydroxy-3-methyl glutaryl coenzyme A reductase in permeabilized cells. *J. Biol. Chem.* **262:** 7914–7919.