Induction of HMG CoA reductase by the administration of 20,25-diazacholesterol

Ronald Langdon, Souheir El-Masry, and Raymond E. Counsell

Laboratory of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109

Abstract This paper describes the direct examination of HMG CoA reductase activity in rats treated with 20,25-diazacholesterol. Conversion of acetyl CoA and HMG CoA to mevalonate increased to over 200% of control values in the microsomes and in the 12,000 *g* supernatant of liver homogenates after 5 days of treatment. The time course of induction coincided with the development of hypocholesterolemia. Animal weights, liver weights, and microsomal protein content did not vary significantly between animal groups. Incubations to which the compound was introduced in vitro in concentrations as great as 0.5 mM produced no significant difference from control incubations. Similar treatment of the animals with 7-ketocholesterol, a cholesterol derivative reported to repress HMG CoA reductase activity in tissue cultures, produced no appreciable difference in reductase activity or serum steroid levels in vivo.

Supplementary key words feedback repression · rate-limiting step · cholesterol biosynthesis · rat liver microsomes · 7-ketocholesterol · hypocholesterolemic agent · desmosterol

The ability of 20,25-diazacholesterol to lower serum sterol levels in rats and humans has been known for some time (1,2). It is also well known that this compound causes an abnormal accumulation of Δ^{24}-sterol precursors of cholesterol by inhibition of Δ^{24}-sterol reductase (3–5). In addition, there is indirect evidence that this compound exhibits a minor inhibition of cholesterolgenesis at conversion sites preceding the Δ^{24}-bond reduction (4, 6). Although the demonstration of inhibition of Δ^{24}-bond reduction has accounted for the appearance of desmosterol in the tissues and sera of treated animals and humans, the ability of 20,25-diazacholesterol to reduce total serum sterol levels (cholesterol plus desmosterol) has remained unexplained. If a metabolic blockade is responsible for this effect, it seems logical that it must occur prior to the cyclization of squalene, since inhibition after this step would result in the appearance of sterol precursors of cholesterol, such as desmosterol, without a reduction in the total quantity of sterol synthesized.

From studies comparing [^{14}C]acetate and [^{14}C]-mevalonate incorporation into cholesterol by cell-free homogenates from treated and untreated rats, Ranney and Cook (7) and Ranney (8) concluded that a blockade was occurring between these two early intermediates and they suggested that 20,25-diazacholesterol and other azasterols may have an inhibitory effect on HMG CoA reductase. As the feedback repression of dietary cholesterol became known (9, 10), it then seemed reasonable to hypothesize a "cholesterolmimetic" function for 20,25-diazacholesterol; that is, the azasterol, like cholesterol, functions to shut off the synthesis and replacement of HMG CoA reductase as it is degraded. In 1973, Kandutsch and Chen (11) and Kandutsch and Packie (12) demonstrated precisely such an effect in tissue cultures with 7-ketocholesterol, and with a number of other cholesterol analogues that were not azasterols. Furthermore, previous studies have shown that the hypocholesterolemic activity of diazasterols largely depends on their similarity in structure to cholesterol (1, 13, 14).

In the intervening 13 years since the initial synthesis and biological testing of 20,25-diazacholesterol, more reliable and direct methods of assaying HMG CoA reductase have been developed by a number of investigators (15–18). It was the purpose of this present study to use these more recent methods to directly examine the effect of treatment with this compound on HMG CoA reductase, using a dose and a mode of administration that have been demonstrated to be effective in depressing total serum sterol levels (7). In addition, 7-ketocholesterol, one of the most potent analogues investigated by Kandutsch et al (11, 12) in tissue cultures, was tested for biological activity as a hypocholesterolemic agent and

Abbreviations: CoA, coenzyme A; HMG CoA, β-hydroxy-β-methylglutaryl CoA; MVA, mevalonic acid; TLC, thin-layer chromatography; dpm, disintegrations per minute. "Microsomal reductase" or simply "reductase" in this article refers to HMG CoA reductase. Trivial names used are: cholesterol, cholest-5-en-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; 7-ketocholesterol, 3β-hydroxycholest-5-en-7-one.

Downloaded from www.jlr.org by guest, on June 19, 2012

a repressor of microsomal reductase activity in the intact animal.

EXPERIMENTAL METHODS

Materials

[1-^{14}C]Acetyl CoA, DL-[5-^{3}H]mevalonic acid (dibenzolethylenediamine salt) and DL-[3-^{14}C]HMG CoA were obtained from New England Nuclear Corp., Boston, MA. Nonradiolabeled HMG CoA and dithiothreitol were purchased from P & L Biochemicals, Milwaukee, WI. Mevalonic acid, acetyl CoA, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, niacinamide, and NADP were obtained from Sigma Chemical Co., St. Louis, MO. Disodium EDTA was purchased from Mallinckrodt, St. Louis, MO. 20,25-Diazacholesterol was obtained from Searle Laboratories, Skokie, IL. Cellulose and silica gel G TLC plates were purchased from Eastman Kodak Co., Rochester, NY. PCS scintillation cocktail was purchased from Amersham-Searle Corp., Arlington Heights, IL.

Animals and dosage

Male Sprague-Dawley rats, 250–300 g, were maintained on Teklad mouse and rat diet (4% fat) ad libitum, under controlled illumination, for at least 10 days. The manufacturer estimated the cholesterol content of the diet as about 0.05%. Animals from the different experimental groups received either: *a*) 20,25-diazacholesterol, 2 mg/kg per day, *b*) 7-ketocholesterol, 2 mg/kg per day, or *c*) 7-ketocholesterol, 10 mg/kg per day. The vehicle for these water insoluble compounds was Mazola corn oil, at a dose of 1 ml/kg per day. The vehicle and compounds were injected subcutaneously into the back of the neck 1–2 hr before the onset of the dark phase. Control animals received the vehicle only.

Preparation of microsomes

Microsomal pellets were prepared following the procedure of Brown (15), except that the homogenizing medium was 0.30 M sucrose, 0.010 M Na_2EDTA, 0.005 M dithiothreitol, adjusted to pH 7.2 with 10% NaOH, and the microsomal pellets were prepared without washing. The pellets were stored frozen for up to 5 days.

Preparation of microsomes with cytosol

The procedure was as above, with the following exceptions. The livers were homogenized in a medium containing 0.10 M potassium phosphate, pH 7.2, 0.030 M niacinamide, 0.10 M Na_2EDTA, and 0.001 M dithiothreitol. For analysis of the conversion of acetyl CoA to MVA, the livers from the respective animal groups were pooled. After ultracentrifugation, the cytosol portions, like the microsomal pellets. were stored frozen for up to 5 days.

Assays

Conversion of HMG CoA and acetyl CoA into mevalonate was assayed by modifications of the procedures of Goldfarb and Pitot (16), and White and Rudney (17). Microsomal pellets were resuspended in 2.0 ml per original gram of tissue in 0.10 M potassium phosphate buffer, pH 7.2, containing 0.005 M dithiothreitol and 0.010 M Na_2EDTA. For analysis of the conversion of HMG CoA to MVA, assays were carried out in this same buffer containing, in addition, 20 μl of microsomal suspension, 0.42 mM DL-MVA, 16.7 μM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, and 5.3 mM NADP in a final volume of 0.24 ml. Blanks contained no NADP. Assays were performed at 37.5°C in a Dubnoff shaker set at 40 cycles/min. After 10 min preincubation, 65 nmoles of DL-[3-^{14}C]HMG CoA (3310 dpm/nmole) were introduced. After 30 min incubation, the reaction was stopped by the addition of 50 μl of 10% HCl.

The same procedure was followed to assay conversion of acetyl CoA to MVA, with the following exceptions. The incubations included microsomes plus 20 μl of thawed cytosol. The buffer for these incubations was 0.001 M dithiothreitol, 0.030 M niacinamide, 0.010 M Na_2EDTA, and 0.10 M potassium phosphate, pH 7.2. After 15 min of preincubation, 360 nmoles of [1-^{14}C]acetyl CoA (665 dpm/nmole) was introduced, and incubation was allowed to proceed for 20 min.

In some assays in which the test compound was added to the incubations in vitro, the incubations contained 0.10 M potassium phosphate, pH 7.2, 0.005 dithiothreitol, 1.25 mM NADP, 4.0 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 20 μl of microsomal suspension, 0.5 or 0.05 mM 20,25-diazacholesterol, and 0.0092 mM (9.2 nmole) DL-[3-^{14}C]HMG CoA (5,430 dpm/nmole) in a final volume of 1.0 ml. This larger assay volume was used to allow for greater product formation at lower substrate levels without significantly distorting the substrate concentration. These vials were preincubated for 20 min, incubated for 4.5 min, and the reaction was stopped by the addition of 0.2 μl of 10% HCl.

Determination of [^{14}C]MVA

The product was separated from the substrate and quantitated by the dual-label TLC procedure of

Downloaded from www.jlr.org by guest, on June 19, 2012

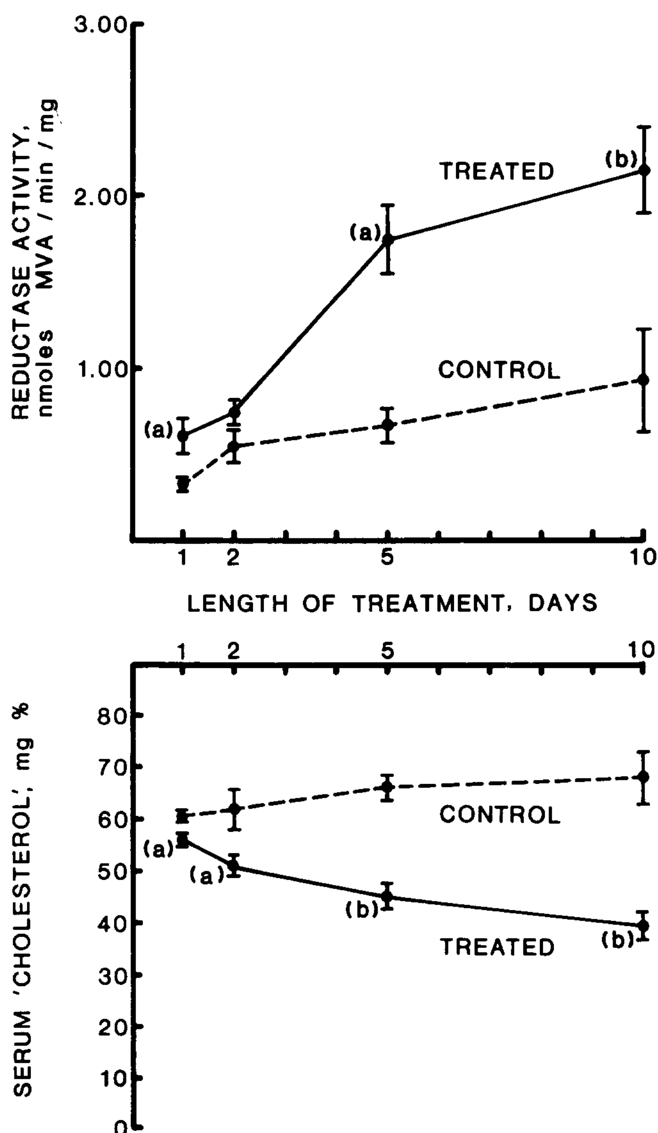

Fig. 1. HMG CoA reductase activity and serum cholesterol levels, determined as described in the text, as a function of length of treatment. There were six animals per group. Experimental group animals received 2 mg/kg per day 20,25-diazacholesterol in 1.0 ml/kg per day corn oil. Control group animals received only corn oil. Vertical bars indicate 1 SEM. (a), $P < 0.05$ by a one-tailed Student test; (b), $P < 0.01$ by a one-tailed Student test.

Shapiro, Imblum, and Rodwell. (18). Aliquots of approximately 3×10^5 dpm of DL-[5-^{3}H]MVA were introduced into each assay vial as the internal standard. After plate development, all radioactivity was located in two areas, one at the origin, and one at R_f 0.5–0.7. Sections of the plates containing the latter peak were excised and transferred to scintillation vials containing 10 ml of PCS cocktail. Dual isotope analyses were made in a Beckman LS-150 liquid scintillation counter using Automatic Quench Correction, adjusted so that tritium spillover into the ^{14}C channel was less than 0.1%, which was considered negligible. ^{14}C spillover into the tritium channel, which was 8.4%, was corrected for in calculation.

The purity of the [^{3}H]MVA was determined by TLC on cellulose plates developed in *n*-propanol–ammonium hydroxide 7:3. The plates showed a single band at R_f 0.65–0.75, from which 75% of the spotted standard was recovered. From samples in which the standard was dried directly in a scintillation vial, and then counted, it was shown that most of the impurity was volatile, and was presumed to be tritium-exchanged water. It was observed that it was necessary to wet cellulose or silica gel strips with 1% NaOH in order to quantitatively elute isotopic mevalonic acid (but not mevalonolactone) into PCS cocktail. The presence of volatile tritium was corrected for. [^{3}H]MVA recovery was calculated for each sample. The figures given for MVA formation have been corrected for losses during the separation procedure.

The mean recovery of [^{3}H]MVA in a typical experiment was 54.5 ± 7.73% (SD). Occasional samples in which recovery was less than 25% were discarded. Sixteen percent of the tritium was recovered from the origin, and this was taken to represent mevalonic acid that had not lactonized, but had partitioned into the ether phase during extraction. By counting the level of ^{14}C at the origin of blank incubations, it was determined that approximately 96% of the unreacted substrate remained in the aqueous phase during ether extraction.

Determination of serum sterols

Serum sterol levels were measured on a Technicon Autoanalyzer (Technicon Instruments, Tarrytown, NY) according to the method of Block (19). It was determined that the autoanalyzer method is 87% as sensitive to desmosterol as to cholesterol. It has been demonstrated previously (3, 4) that these two sterols account for essentially all of the total sterols in either control or 20,25-diazacholesterol-treated animals. For this reason, these determinations were taken to be a reasonable estimate of the total sterol levels.

Protein determinations

Protein contents were determined by the method of Lowry et al. (20) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

The previously drawn conclusion that treatment with 20,25-diazacholesterol causes a metabolic blockade of the early steps of cholesterol biosynthesis

Downloaded from www.jlr.org by guest, on June 19, 2012

was observed to be false. Treatment of animals with this compound resulted in an increase in the activity of HMG CoA reductase rather than repression of this enzyme, as was earlier hypothesized. This increase was relatively slow in developing, requiring an interval of 5–10 days to approach an asymptote ranging between 210 and 255% of control values (**Fig. 1**). Simultaneously, serum sterol levels of the treated animals underwent a significant depression to 68% of control values.

The microsomes from untreated animals or corn oil-treated control animals typically gave a specific enzymatic activity of approximately 0.75 nmole/min per mg of microsomal protein (7.5 nmole/min per g of liver). The Day 1 control group gave a mean activity that was abnormally low, according to our experience. If this control value is aberrant, then the figure of 180% greater activity in treated animals is an exaggeration of the actual effect of treatment at that point in time. Whether or not the Day 1 value is aberrant, however, a trend toward increased reductase activity in the control animals was apparent over the 10 day period. It is possible that the daily stress of restraint and injection disrupted the animals' physiological states, or feeding patterns, and created this trend. The possibility that the corn oil, rather than the injection process, was stimulating reductase activity was discounted when no difference was observed between corn oil and saline-injected animals (**Table 1**).

Recent work by Clinkenbeard et al. (21) and Sugiyama et al. (22) has shown that the conversions of acetyl CoA to acetoacetyl CoA and HMG CoA are feedback-regulated by dietary cholesterol, as is the previously studied conversion of HMG CoA to MVA, with the difference that the effect was observed over a period of days, rather than hours. It was, therefore, suggested that these earlier steps might also function as sites of the control of cholesterol biosynthesis. When our work demonstrated an induction rather than a blockade of microsomal reductase upon treatment of animals with 20,25-diazacholesterol, the focus of investigation shifted to an examination of the entire sequence of acetyl CoA to mevalonate, in order to determine whether a blockade before HMG CoA was the mechanism responsible for the observed depression in serum sterols. Evaluation of this possibility showed, however, that drug treatment caused an induction of activity over the larger pathway of conversion of acetyl CoA to MVA that was similar to the specific conversion of HMG CoA (Table 1). About 70% as much MVA was produced in the same time period when acetyl CoA was supplied as the substrate, as when HMG CoA was supplied.

TABLE 1. Mevalonate synthesis by liver preparations from animals treated with 20,25-diazacholesterol, 7-ketocholesterol, and control groups[a]

	MVA Formed	
Treatment	Microsomes Alone HMG CoA Substrate	Microsomes and Cytosol Acetyl CoA Substrate[b]
	nmoles/min/mg microsomal protein	
Isotonic saline 1 ml/kg/day	0.879	
Corn oil 1 ml/kg/day	0.786	0.526
20,25-Diazacholesterol 2 mg/kg/day in corn oil	1.66	1.09
7-Ketocholesterol 2 mg/kg/day in corn oil	0.855	
7-Ketocholesterol 10 mg/kg/day in corn oil	0.725	

[a] The animals were treated for 5 days. The enzyme system was as described in the text. Livers from groups of five animals each were pooled. Figures given are the averages of duplicate assays, for which the overall coefficient of variation was 4.0%.

[b] It was assumed that three molecules of [^{14}C]acetyl CoA were incorporated into one of mevalonic acid.

The availability of microsomal reductase may well have been rate-limiting, so the data do not disclose whether all of the conversion activities were induced, or just the reduction of HMG CoA. Assuming, however, that the assay provides a reasonable reflection of the in vivo state, the data indicate that if 20,25-diazacholesterol exerts a depressive effect on total sterol biosynthesis, it must do so after the mevalonate stage.

For reasons given previously, three other experimental groups were included in this same study (Table 1). Corn oil-injected animals in this experiment showed activity no different from saline-injected controls. It was also observed that 7-ketocholesterol was inactive as a hypocholesterolemic or a cholesterol-mimetic repressor of HMG CoA reductase when administered subcutaneously at dosages of 2 and 10 mg/kg per day. The inactivity of 7-ketocholesterol in vivo does not necessarily discount the importance of 7-substituted cholesterol derivatives as repressors of HMG CoA reductase. The activity of a compound in the intact animal ultimately depends on its ability to avoid catabolism and excretion. It is most likely that 7-ketocholesterol did not survive in this respect.

Three additional parameters were followed in the experimental groups. Earlier work by Linn (10) and Regen et al. (23) showed that fasting dramatically decreases reductase activity. In order to monitor any change in dietary intake, the animals were weighed daily, and a normal weight gain pattern was observed in all but one animal, which was discarded from the experiment. Liver weights and microsomal protein

Downloaded from www.jlr.org by guest, on June 19, 2012

contents were also examined to indicate whether toxicity or a generalized microsomal induction had occurred. No significant difference was detected between experimental and control animals in any of the three above-mentioned parameters.

Variation between individual animals was analyzed by comparing activities obtained from separate assays of portions of the same liver, and with activities obtained from other animals in the same treatment group. The coefficient of variation between assays of the same liver was ± 6.34%, whereas the coefficient of variation between different animals was ± 56.9% (data not shown). The large variation between animals was not taken, therefore, to be inherent in the experimental design or execution, but as one that existed in the animals themselves. Most probably, it was the result of asynchronicity within each group of the sharp spike of enzyme activity that naturally occurs nightly, in the first six hours of darkness (24–28). Variations between duplicate assays of the same microsomal preparations were nearly always within 4% of the means.

Recent progress in the understanding of lipid metabolism has produced at least two good reasons why the earlier studies of 20,25-diazacholesterol and related azasterols may be invalid. Dietschy and Brown (29) have demonstrated the inaccuracy inherent in using [^{14}C]acetate in whole cell systems to monitor cholesterol synthesis. Just as important, the earlier studies were performed without regard to the diurnal reductase cycle. Unless the earlier investigators happened to kill their animals in the midpoint of the dark phase, the measured activities of conversion of acetate to cholesterol represent only one-seventh to one-tenth of the animals' peak converting capability (24–28).

Numerous possible explanations exist for the observed increase in HMG CoA reductase activity upon treatment with 20,25-diazacholesterol, and each explantion must be dealt with separately. An apparent induction of activity may not be a reflection of change in enzyme activity, but a reflection of change in the endogenous pool of substrate. Thus, the "induction" demonstrated may actually be a sharp decrease in the level of endogenous HMG CoA available to the microsomes. However, this explanation is unlikely for two reasons. *a*) Isolated microsomes possess no available source of acetyl CoA, the substrate for synthesis of HMG CoA. Furthermore, microsomes possess only a small amount of HMG CoA synthetase activity, as determined in this laboratory and elsewhere (21). Pre-existing HMG CoA should have largely separated into the cytosolic fraction; however, the induction was exhibited both by isolated microsomes and by microsomes combined with cytosol. *b*) Furthermore, the concentration of exogenous substrate used was well beyond that found to be necessary for enzyme saturation, so competition from endogenous substrate is thought to have been insignificant.

Similarly, if some of the [^{14}C]MVA produced in the control assays was further metabolized during incubation, an alteration in the extent of MVA metabolism due to the test compound could be misinterpreted as a change in reductase activity. In this case, the data could be indicating a drug-induced slow-down in the rate of MVA metabolism rather than an increase in MVA production. However, the phosphorylations and polymerizations of mevalonate into isoprenoids and eventually squalene require the cytosolic fraction, as well as Mg^{2+} or Ca^{2+} and ATP (30, 31), factors that were not included in this incubation. As a final safeguard, a relatively large quantity of exogenous MVA was included in the incubation after it was determined that this product did not inhibit the action of reductase.

The possibility was also considered that 20,25-diazacholesterol was functioning as an allosteric inhibitor of HMG CoA reductase in vivo, but that the effect went undetected because the process of isolating and resuspending a small quantity of microsomes would greatly dilute the concentration of the compound initially present in the hepatocytes. To see if the compound had a direct effect on the enzyme, in vitro studies were performed using the same assay conditions used to evaluate the microsomes from treated animals, and the compound was introduced into the assays at concentrations of 0.5 and 0.05 mM (**Table 2**). The concentration of D-HMG CoA in some of these assays was 0.069 mM. Because recent work in this laboratory has indicated that the K_m of HMG CoA reductase for D-HMG CoA is approximately 0.001 mM (32), rather than near 0.012 mM as previously reported (15), an in vitro study was also performed using a D-HMG CoA concentration of 0.005 mM and somewhat modified conditions (see Experimental Methods). In no case was a significant difference observed between control and experimental samples. The lack of inhibitory activity of this close structural analogue of cholesterol is consistent with the lack of evidence for allosteric activity of cholesterol itself, and the evidence that regulation of this enzyme comes via regulation of both genetic expression (24–28, 33) and degradation (34).

After making the above considerations, it was concluded that the increase in [^{14}C]MVA formation by microsomes from treated animals that we observed reflects an increase in the activity of HMG CoA reductase in vivo. Although no mechanism for this

Downloaded from www.jlr.org by guest, on June 19, 2012

effect is clearly demonstrated, it does not appear to involve a direct action of the drug on the regulatory mechanism because responses of this kind are much more rapid (25–28). Cholesterol feeding, for example, will result in a near-total cessation of cholesterol synthesis within 24 hr (26, 27). Any explanation of the induction observed in the present study must therefore account for its laggard development.

Examination of the time course of enzyme induction, and development of the depression in total serum sterols shows that these two events occurred concurrently (Fig. 1). The simplest explanation for the induction may be that it is a homeostatic response to the depression of serum sterols. It is also known that the hypocholesterolemic effect of this agent is accompanied by the partial replacement of cholesterol by desmosterol, (3–5), a sterol precursor of cholesterol that may not be as effective as cholesterol in triggering the feedback response. In studies in which the agent was administered orally at doses of 1–5 mg/kg per day, 34–61% of the total serum sterols was present as desmosterol after 10 days, at which time total serum sterol levels were reduced from 11 to 47% (4, 5). The potency of desmosterol as a feedback inhibitor of HMG CoA reductase is not known, and would be difficult to ascertain, because normal living animal tissue will readily convert desmosterol to cholesterol. If desmosterol is less potent as a feedback repressor than cholesterol, then this also could account for the induction of HMG CoA reductase observed in this study. Our results tend to indicate that desmosterol is no more potent than cholesterol in this respect.

Studies in our laboratory in which [7-^{3}H]20,25-diazacholesterol was injected subcutaneously have indicated that compound taken up by the liver persists there, with only a 25% loss of tracer over 6 days.[1] Therefore, repeated administration may result in an accumulation of the compound, which may be having a direct effect, such as displacing cholesterol from binding sites that initiate feedback regulation.

What remains unexplained, however, is the mechanism by which this compound reduces serum sterol levels. Induction of the enzyme indicates that the liver is actively responding in an attempt to maintain homeostasis of serum sterol levels. Whatever the mode of action of this compound, in addition to producing a significant reduction in serum sterol levels, it must also resist the liver's homeostatic response.

[1] Johnson, M., A. Buswink, and R. E. Counsell, unpublished observations.

TABLE 2. Mevalonic acid formation in the presence of 20,25-diazacholesterol, in vitro

	Mevalonate Formation[a]		
	Microsomes plus Cytosol	Microsomes Alone	
Concentration of 20,25-Diazacholesterol[d]	1.50 mM [1-^{14}C]Acetyl CoA[b]	0.138 mM D,L-[3-^{14}C]HMG CoA[b]	0.0092 D,L-[3-^{14}C]HMG CoA[c]
	Percent of Controls		
5.0×10^{-4} M	92.1%	108%	108%
5.0×10^{15} M	96.1%	113%	107%

[a] The values indicated are the means of duplicate and triplicate determinations for which the overall coefficient of variation was 8.2%. The differences observed were not significant at a level of $P < 0.05$ with a one-tailed Student test. The substrate levels and tissue fractions present are indicated.
[b] Total assay volume was 0.24 ml, as described in the text.
[c] Total assay volume was 1.0 ml, as described.
[d] Introduced as the Cl^- salt.

The depression of serum sterol levels in the face of a 2.5-fold induction of HMG CoA reductase may supplement the argument against the concept of this conversion as the sole rate-limiting step in cholesterol biosynthesis. If there is a blocked step later in the pathway to sterols, then one might expect to see an accumulation of the sterol precursor directly prior to that blocked conversion. The 250% increase in reductase activity increases the anticipated size of this accumulation. However, in previous studies of this agent, there is little evidence for such an accumulation. It is quite possible then that 20,25-diazacholesterol does not function as an inhibitor of sterol syntheses. Other possible sites of action for this drug could be in the metabolism of cholesterol into bile acids, in the esterification of cholesterol with fatty acids, or in the movement of cholesterol or bile acids across body membranes, particularly those that absorb these sterols from the gut. It is not unlikely that the liver is accumulating this compound and secreting it into the gut via the bile. There, it may inhibit the absorption of exogenous and endogenous sterols.

In this last regard, cholestyramine and β-sitosterol both interfere with the absorption of sterols from the gut, and have been shown to induce HMG CoA reductase activity in rats to a degree similar to that observed here for 20,25-diazacholesterol (35, 36).

We are grateful to Larry Branstrom for care of the animals, to Dr. Marc Longino for assistance in the preparation of the enzymes, and to Helen Palmer for analysis of serum sterol levels. We appreciate also the helpful comments of Dr. James Miller, Searle Laboratories, during the course of this study. This research was supported by grants from

Downloaded from www.jlr.org by guest, on June 19, 2012

JOURNAL OF LIPID RESEARCH
ASBMB

the National Institutes of Health (HL-11274) and the Michigan Heart Association.

Manuscript received 24 March 1976 and in revised form 4 August 1976; accepted 23 September 1976.

REFERENCES

1. Counsell, R. E., P. D. Klimstra, and R. E. Ranney. 1962. Hypocholesterolemic agents. III. *N*-Methyl-*N*-(dialkylamino)-alkyl-17β-aminoandrost-5-en-3β-ol derivatives. *J. Med. Pharm. Chem.* **5:** 1224–1233.
2. Sachs, B. A., and L. Wolfman. 1965. 20,25-Diazacholesterol dihydrochloride. *Arch. Intern. Med.* **116:** 366–372.
3. Thompson, M. J., J. Dupont, and W. E. Robbins. 1963. The sterols of liver and carcass of 20,25-diazacholesterol-fed Rats. *Steroids.* **2:** 99–104.
4. Ranney, R. E., and E. G. Daskalakis. 1964. Appearance of cholesterol precursors as a result of treatment with 20,25-diazacholesterol. *Proc. Soc. Exp. Biol. Med.* **116:** 999–1001.
5. Fumagalli, R., and R. Niemiro. 1964. Effect of 20,25-diazacholesterol and triparanol on sterols, particularly desmosterol, in rat brain and peripheral tissues. *Life Sci.* **3:** 555–561.
6. Niemiro, R., and R. Fumagalli. 1965. Studies on the inhibitory mechanism of some hypocholesterolemic agents on 7-dehydrocholesterol Δ^7-bond reductse activity. *Biochim. Biophys. Acta.* **98:** 624.
7. Ranney, R. E., and D. L. Cook. 1965. The hypocholesterolemic action of 20,25-diazacholesterol. *Arch. Int. Pharmacodyn. Ther.* **154:** 51–62.
8. Ranney, R. E. 1967. The effects of azasterols on sterol metabolism. *Prog. Biochem. Pharmacol.* **2:** 50–55.
9. Siperstein, M. D. 1960. The homeostatic control of cholesterol synthesis in liver. *Amer. J. Clin. Nutr.* **8:** 645–650.
10. Linn, T. C. 1967. The effect of cholesterol feeding and fasting upon β-hydroxy-β-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **242:** 990–993.
11. Kandutsch, A. A., and H. W. Chen. 1973. Inhibition of sterol synthesis in culture mouse cells by 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol. *J. Biol. Chem.* **248:** 8408–8417.
12. Kandutsch, A. A., and R. M. Packie. 1970. Comparison of the effects of some C_{27}-, C_{21}-, and C_{19}-steroids upon hepatic sterol synthesis and hydroxymethylglutaryl-CoA reductase activity. *Arch. Biochem. Biophys.* **140:** 122–130.
13. Counsell, R. E., P. D. Klimstra, R. E. Ranney, and D. L. Cook. 1962. Hypocholesterolemic agents. 1. 20α-(2-Dialkylaminoethyl)-aminopregn5-en-3β-ol derivatives. *J. Med. Pharm. Chem.* **5:** 720–729.
14. Counsell, R. E., P. D. Klimstra, L. N. Nysted, and R. E. Ranney. 1965. Hypocholesterolemic agents. V. Isomeric azacholesterols. *J. Med. Chem.* **8:** 45–48.
15. Brown, M. S., S. E. Dana, J. M. Dietschy, and M. D. Siperstein. 1973. 3-Hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **248:** 4731–4738.
16. Goldfarb, S., and H. C. Pitot. 1971. Improved assay of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res.* **12:** 512–515.
17. White, L. W., and H. Rudney. 1970. Biosynthesis of 3-hydroxy-3-methylglutarate and mevalonate by rat liver homogenates in vitro. *Biochemistry.* **9:** 2713–2725.
18. Shapiro, D. J., R. L. Imblum, and V. W. Rodwell. 1969. Thin-layer chromatographic assay for HMG-CoA reductase and mevalonic acid. *Anal. Biochem.* **31:** 383–390.
19. Block, W. D. 1966. An improved automated determination of serum total cholesterol with a single color reagent. *Clin. Chem.* **12:** 681–689.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–272.
21. Clinkenbeard, K. D., T. Sugiyama, J. Moss, W. D. Reed, and M. D. Lane. 1973. Molecular and catalytic properties of cytosolic acetoacetyl coenzyme A thiolase from avian liver. *J. Biol. Chem.* **248:** 2275–2284.
22. Sugiyama, T., K. D. Clinkenbeard, J. Moss, and M. D. Lane. 1972. Multiple cytosolic forms of heaptic β-hydroxy-β-methylglutaryl synthase: Possible regulatory role in cholesterol synthesis. *Biochem. Biophys. Res. Comm.* **48:** 255–261.
23. Regen, D., C. Riepertinger, B. Hambrecht, and F. Lynen. 1966. The measurement of β-hydroxy-β-methylglutaryl CoA reductase in rat liver; effect of fasting and refeeding. *Biochem. Z.* **346:** 78–84.
24. Goldfarb, S., and H. C. Pitot. 1971. The regulation of β-hydroxy-β-methylglutaryl coenzyme A reductase in Morris hepatomas 51230,7800, and 9618A. *Cancer Res.* **31:** 1879–1882.
25. Shapiro, D. J., and V. W. Rodwell. 1971. Regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol synthesis. *J. Biol. Chem.* **246:** 3210–3216.
26. Shapiro, D. J., and V. W. Rodwell. 1969. Diurnal variation and cholesterol regulation of hepatic HMG CoA reductase activity. *Biochem. Biophys. Res. Comm.* **5:** 867–872.
27. Edwards, P. A., and R. G. Gould. 1974. Dependence of the circadian rhythm of hepatic β-hydroxy-β-methylglutaryl coenzyme A reductase on ribonucleic acid synthesis. *J. Biol. Chem.* **249:** 2891–2906.
28. Edwards, P. A., and R. G. Gould. 1972. Turnover rate of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase as determined by use of cycloheximide. *J. Biol. Chem.* **247:** 1520–1524.
29. Dietschy, J. M., and M. S. Brown. 1974. Effect of alterations of the specific activity of the intracellular acetyl CoA pool on apparent rates of hepatic cholesterolgenesis. *J. Lipid Res.* **15:** 508–516.
30. Tchen, T. T. 1963. Preparation and determination of intermediates in cholesterol synthesis. *Methods Enzymol.* **6:** 509–510.
31. Johnson, R. C., and S. M. Shah. 1974. Hepatic cholesterol synthesis from mevalonate and squalene in rats: Effect of feeding cholesterol supplemented diet during weaning and following starvation. *Lipids.* **9:** 962–970.
32. Langdon, R. B., and R. E. Counsell. 1976. Determina-

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

tion of the Michaelis-Menten constant for β-hydroxy-β-methylglutaryul coenzyme A reductase. *J. Biol. Chem.* **251:** 5820–5823.

33. Kandutsch, A. A., and S. E. Saucier. 1969. Prevention of cyclic and Triton-induced increases in hydroxymethylglutaryl coenzyme A reductase and sterol synthesis by puromycin. *J. Biol. Chem.* **244:** 2299.

34. Higgins, M., and H. Rudney. 1973. Regulation of rat liver β-hydroxy-β-methylglutaryl-CoA reductase activity by cholesterol. *Nature (London) New Biol.* **246:** 60–61.

35. Goldfarb, S., and H. C. Pitot. 1972. Stimulatory effect of dietary lipid and cholestyramine on hepatic HMG CoA reductase. *J. Lipid Res.* **13:** 797–801.

36. Raicht, R. F., B. I. Cohen, S. Shefer, and E. H. Mosbach. 1975. Sterol balance studies in the rat. Effects of dietary cholesterol and β-sitosterol on sterol balance and rate-limiting enzymes of sterol metabolism. *Biochim. Biophys. Acta.* **388:** 374–384.

JOURNAL OF LIPID RESEARCH
ASBMB
Downloaded from www.jlr.org by guest, on June 19, 2012