The Squalene Dioxide Pathway of Steroid Biosynthesis

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Cholesterol has been, and continues to be, the subject of an enormous amount of research for the obvious reason of its physiological importance. Over the last six decades at least 13 Nobel Prizes have been awarded to scientists who made discoveries concerning the structure, biosynthesis, and metabolic regulation of this essential, but sometimes too abundant, substance.[1] The cholesterol biosynthetic pathway, shown in abbreviated form in Figure 1, comprises at least 30 enzymatic reactions which have been studied in exquisite mechanistic and stereochemical detail. The elucidation of this biosynthetic pathway constitutes one of the most beautifully complete chapters in biochemistry.[2] Yet, despite all this continuing intensive effort, it was realized only recently that there is a natural bifurcation in steroid biosynthesis at the stage of squalene oxide (1), as indicated in Figure 1. Almost all of the naturally biosynthesized squalene oxide is indeed converted in cells to lanosterol (2) and then to cholesterol (3), but a small amount, under normal metabolic conditions, is transformed instead into squalene dioxide (4) and then, through a series of conversions which parallels the steps in cholesterol biosynthesis, into 24(*S*),25-oxidolanosterol (5) and 24(*S*),25-epoxycholesterol (6).[3] This Account describes the discovery of this alternate, squalene dioxide pathway of steroid biosynthesis, describes the natural incidence of its products, and discusses what its biological significance may be.

Determination That Squalene 2,3(*S*):22(*S*),23-Dioxide Is Converted to 24(*S*),25-Epoxycholesterol. In 1967 Corey reported in a footnote that "2,3,22,23-dioxidosqualene" accumulated along with larger amounts of squalene 2,3-oxide when the cyclization of the latter was inhibited by treatment of partially purified hog liver oxidosqualene cyclase with 2,3-iminosqualene.[4] Shortly thereafter, Corey used "squalene dioxide" as a substrate termed "factitious" and "unnatural" in anaerobic incubation with the same kind of cyclase preparation and found that "24,25-oxidolanosterol" was formed.[5] This result with hog liver enzyme was subsequently confirmed by Suga.[6] Access to squalene dioxide as a synthetic mixture, presumably a 1:2:1 ratio of *R,R*, *R,S*, and *S,S* stereoisomers, had been provided by the ingenious 1962 procedure of van Tamelen and Curphey[7] for selective bromohydrin formation at the terminal double bonds of squalene, and it was this material that was used exclusively in cyclization studies for some time.[8] Since a 2,3(*S*)-oxide is required for enzymatic cyclization,[9] Corey's observation that after two cycles of incubation ca. 50% conversion to "oxidolanosterol" could be achieved indicated that the configuration of the 22,23-oxide "is not critical to cyclization".[5]

Investigation of plant enzyme cyclization of synthetic squalene dioxide followed shortly. In 1970 it was shown that this substrate is converted by bramble microsomes to "24,25-epoxycycloartenol".[10] A different type of cyclization, from both ends of squalene dioxide, was reported in 1971 by Rowan, Dean, and Goodwin,[11] who demonstrated conversion of the synthetic mixture to α-onocerin by a cell-free plant enzyme system. In 1977, Field and Holmlund[12] showed, analogously to Corey,[4] that an inhibitor of oxidosqualene cyclase, in this case 3β-(β-(dimethylamino)ethoxy)androst-5-en-17-one, causes an accumulation of "squalene dioxide" in yeast. These authors further showed that when the inhibitor was

Thomas A. Spencer was born in 1934 in New Jersey and grew up there until he went to Amherst College, followed by graduate work at the University of Wisconsin under the direction of E. E. van Tamelen. After frequent early trips to the showers had made it clear that throwing baseballs was not going to provide a living, chemistry teaching and research became his professional goal, and upon receiving the Ph.D. degree in 1960 he accepted a position at Dartmouth, where he has been ever since, becoming New Hampshire Professor of Chemistry in 1972. His research interests have broadened from their base in natural product synthesis to include synthetic methodology, kinetic studies of enzyme model systems, and several aspects of steroid biochemistry, including currently the subject of the Account.

(1) Earlier recipients are listed by Brown and Goldstein in their Nobel Prize lecture: Brown, M. S.; Goldstein, J. L. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 583–602.

(2) Reviews: (a) Nes, W. R.; McKean, M. L. *Biochemistry of Steroids and Other Isopentenoids*; University Park Press: Baltimore, 1977. (b) Gibbons, G. F.; Mitropoulos, K. A.; Myant, N. B. *Biochemistry of Cholesterol*; Elsevier Biomedical: Amsterdam, 1982.

(3) A note concerning nomenclature: The author's practice (begun in ref 21) of naming 5 as an oxide of lanosterol (*cf.* oxidosqualene) vs 6 as an epoxide of cholesterol (based on the *Chemical Abstracts* name for 6, "cholest-5-en-3-ol, 24,25-epoxy-, (3β,24*S*)-") is continued in this Account. Most authors have not bothered to try to distinguish between the saturation levels of the "parent" compounds related to 5 vs 6 and have used either "epoxy" or "oxido" for both 5 and 6 or related oxiranes. Panini and Sinensky (ref 38) prefer the acronyms OLAN and EC for 5 and 6, respectively. Usage of the terms "steroid" and "sterol" to refer to essentially the same type of compounds also varies, with the latter gaining favor as the background of the user becomes more biological. Nes (ref 2a) and Gibbons (ref 2b) both discuss this weighty matter. Nes defines a sterol as "any hydroxylated steroid that retains some or all of the carbon atoms of squalene in its side chain and partitions almost completely into the ether layer when it is shaken with equal volumes of water and ether".

(4) Corey, E. J.; Ortiz de Montellano, P. R.; Lin, K.; Dean, P. D. G. *J. Am. Chem. Soc.* **1967**, *89*, 2797–2798.

(5) Corey, E. J.; Gross, S. K. *J. Am. Chem. Soc.* **1967**, *89*, 4561–4562.

(6) Shishibori, T.; Fukui, T.; Suga, T. *Chem. Lett.* **1973**, 1289–1292.

(7) van Tamelen, E. E.; Curphey, T. J. *Tetrahedron Lett.* **1962**, 121–124.

(8) Cyclization studies required radiolabeled dioxide obtained according to ref 7 from radiolabeled squalene prepared biosynthetically or by the procedure of Nadeau and Hanzlik: Nadeau, R. G.; Hanzlik, R. P. *Methods Enzymol.* **1969**, *15*, 346–349.

(9) This is evidenced by the one-half efficiency of cyclization observed with squalene 2,3(*R,S*)-oxide and by the 3*S* configuration in the sterol products formed in mammalian systems. Plant cyclases exist, however, which transform squalene 2,3(*R*)-oxide to sterols (Schroepfer, Jr., G. J. *Annu. Rev. Biochem.* **1982**, *51*, 555–585).

(10) Heintz, R.; Schaefer, P. C.; Benveniste, P. *J. Chem. Soc. D.* **1970**, 946–947.

(11) Rowan, M. G.; Dean, P. D. G.; Goodwin, T. W. *FEBS Lett.* **1971**, *12*, 229–232. Rowan, M. G.; Dean, P. D. G. *Phytochemistry* **1972**, *11*, 3111–3118.

(12) Field, R. B.; Holmlund, C. E. *Arch. Biochem. Biophys.* **1977**, *180*, 465–471.

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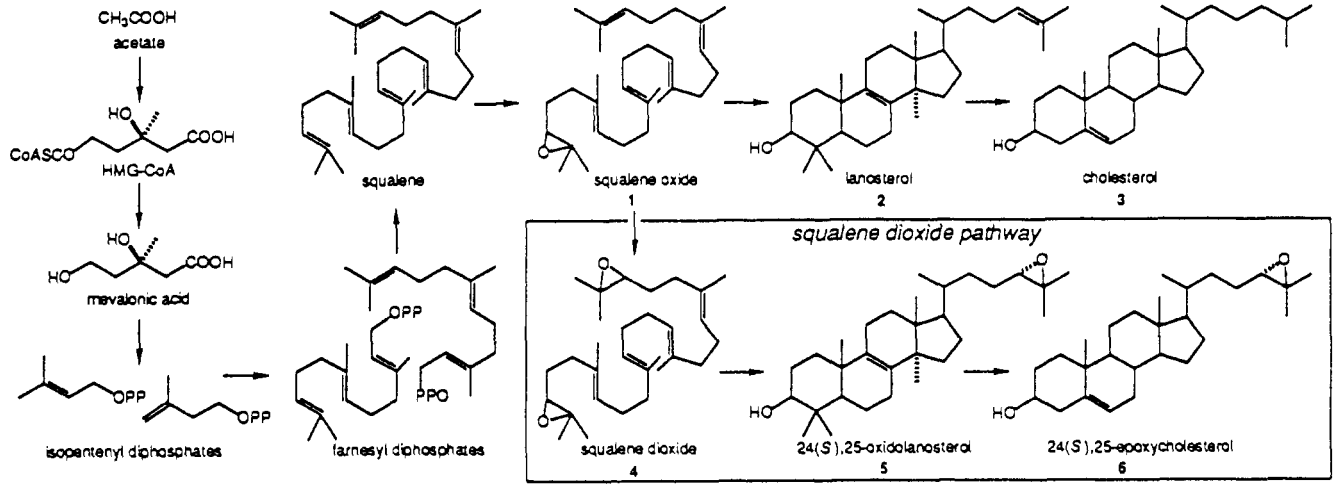


Figure 1. Biosynthesis of cholesterol and the alternate squalene dioxide biosynthetic pathway.

removed, the yeast cells converted the squalene dioxide that had accumulated to "24,25-oxidolanosterol".[12]

Our interest in squalene dioxide was stimulated by reports[13] that sterols bearing a second oxygen atom (oxysterols) are potent repressors of the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the formation of mevalonic acid in what is generally regarded as the rate-limiting step in cholesterol biosynthesis (Figure 1). Such findings led A. A. Kandutsch to propose[14] that oxysterols are natural regulators that serve as the messenger molecules to trigger repression of transcription of the gene for HMG-CoA reductase, a primary mechanism by which that enzyme's activity, and therefore cholesterogenesis, is regulated naturally. This oxysterol regulatory hypothesis[14] stood in contrast to the then prevailing view, which has yet to be completely discounted and which is still reflected in biochemistry textbooks,[15] that cholesterol itself, accumulating in cells after endocytosis of LDL (containing cholesteryl esters), is the regulatory messenger molecule.[16]

One oxysterol that attracted considerable early attention was 25-hydroxycholesterol (**7**), a particularly potent repressor of HMG-CoA reductase activity,[13] and it was speculation concerning the possible biosynthetic origin of **7** that led us to investigate more thoroughly the biochemistry of squalene dioxide. If squalene dioxide were formed naturally and were to undergo the same enzymatic reactions that convert squalene oxide (**1**) to cholesterol (**3**), plus reduction of the epoxide group at C24, then 25-hydroxycholesterol (**7**) would be produced. Had we known then that there were no recorded examples of enzymatic reduction of simple aliphatic epoxides,[17] we might not have found this hypothesis for the biological origin of **7** so chemically plausible and appealing, and might never have embarked on any of the research described in this Account. As it was, however, we were not alone in entertaining this hypothesis, first suggested in print in 1980 by Werthessen et al.,[18] whose conjecture was that **7** might induce the formation of atherosclerotic lesions, rather than participate in the natural regulation of cholesterol metabolism.

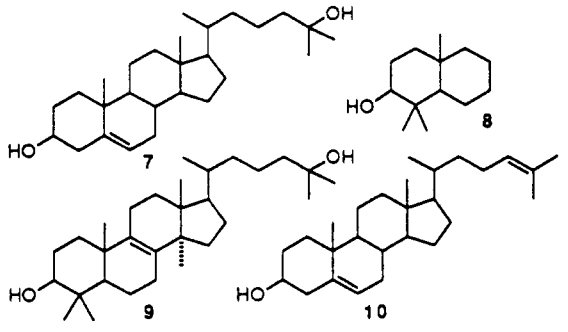
Our experimental entry into this field was facilitated by our discovery in 1978 that 3β-hydroxy-4,4,10β-trimethyl-*trans*-decalin (TMD) (**8**) is an efficient, specific inhibitor of oxidosqualene cyclase.[19,20] Use of TMD allowed us conveniently to biosynthesize radiolabeled squalene dioxide for use in studying its further enzymatic conversions, because TMD, like other cyclase inhibitors,[4,12] causes accumulation of the dioxide as well as squalene oxide (**1**). When [2-^{3}H]mevalonic acid was treated with rat liver homogenate (RLH) in the presence of TMD, the resulting lipid extract contained 63% squalene oxide and 15% squalene dioxide.[21] The latter was purified chromatographically and used in the experiments described next, which established for the first time that the enzymatically produced material is the 3*S*,22*S* stereoisomer **4**.

When this tritiated **4** was incubated with a standard RLH under anaerobic conditions, to prevent metabolism beyond the lanosterol (**2**) stage, it was converted in 59% yield to a product shown to be "24,25-oxidolanosterol" via its conversion to 25-hydroxy-24,-25-dihydrolanosterol (**9**) by $LiAlH_4$ reduction and comparison with an authentic sample.[21] Thus, as anticipated, incubation of the putatively "natural" stereoisomer of squalene dioxide led to more efficient conversion to sterol. When this same **4** was incubated with RLH instead in air, it was converted in 65% yield to a single product which was clearly different in its chromatographic behavior from 25-hydroxycholesterol (**7**).[21] This product did have essentially the same TLC R_f value as 25-hydroxy-24,25-dihydrolanosterol (**9**), but isotopic dilution with authentic **9** readily established that this also was not the correct structure of the metabolite.[21] Analysis of the TLC behavior observed for a number of related steroids indicated that this metabolite might be "24,25-epoxycholesterol", and this

(13) Kandutsch, A. A.; Chen, H. W. *J. Biol. Chem.* **1973**, *248*, 8408–8417; **1974**, *249*, 6057–6061; *J. Cell Physiol.* **1975**, *85*, 415–424.
(14) Kandutsch, A. A.; Chen, H. W. Heiniger, H.-J. *Science* **1978**, *201*, 498–501.
(15) *E.g.*: Stryer, L. *Biochemistry*, 3rd ed.; Freeman: New York, 1988; p 562.
(16) Gould, R. G. *Am. J. Med.* **1951**, *11*, 209–227.
(17) The following reference lists no enzyme which effects this conversion: Dixon, M.; Webb, E. C. *Enzymes*, 3rd ed.; Academic Press: New York, 1979.
(18) Imai, H.; Werthessen, N. T.; Subramanyam, V.; LeQuesne, P. W.; Soloway, A. H.; Kanisawa, M. *Science* **1980**, *207*, 651–653.
(19) Nelson, J. A.; Czarny, M. R.; Spencer, T. A.; Limanek, J. S.; McCrae, K. R.; Chang, T. Y. *J. Am. Chem. Soc.* **1978**, *100*, 4900–4902.
(20) Chang, T. Y.; Schiavoni, E. S., Jr.; McCrae, K. R.; Nelson, J. A.; Spencer, T. A. *J. Biol. Chem.* **1979**, *254*, 11258–11263.
(21) Nelson, J. A.; Steckbeck, S. R.; Spencer, T. A. *J. Biol. Chem.* **1981**, *256*, 1067–1068.

next structural hypothesis turned out to be correct. Reduction of the tritiated metabolite with $LiAlH_4$ yielded 78% of 25-hydroxycholesterol (**7**), identified again by isotopic dilution.[21] Confirmation that this metabolite had the 24*S* configuration, as it presumably would if the precursor **4** had the 22*S* configuration, was obtained by its conversion to its benzoate derivative and comparison with an authentic synthetic sample of 24(*S*),25-epoxycholesterol benzoate.[22] The squalene dioxide that accumulates when oxidosqualene cyclase is inhibited had thus been shown to be converted by rat liver enzymes to 24(*S*),25-epoxycholesterol (**6**) with an efficiency comparable[23] to that of the conversion of squalene oxide (**1**) to cholesterol (**3**) in the "normal" steroid biosynthetic pathway. In recent work that helps to complete the picture, Prestwich[24] has shown that **1** is converted to **4** by partially purified pig liver squalene epoxidase, and Schuber[25] has reported that partially purified rat liver oxidosqualene cyclase actually cyclizes **4** with a modest preference over **1**.

No 25-hydroxycholesterol (**7**) could ever be detected in any of our RLH incubation products from [^{3}H]-squalene 2,3(*S*):22(*S*),23-dioxide (**4**). Subsequently, incubation of 24(*S*),25-epoxycholesterol (**6**)[26] or desmosterol (**10**)[27] in cultured cells likewise led to no detectable formation of **7**. All the evidence is consistent with the view that 25-hydroxycholesterol (**7**) is formed by enzymatic hydroxylation of cholesterol.[28] Our original idea concerning the origin of oxysterol **7** had proved to be incorrect, but exploration of that hypothesis had led to the discovery of a novel type of steroid metabolite, 24(*S*),25-epoxycholesterol (**6**). Our attention understandably turned to investigation of the possible natural incidence and biochemical importance of **6**.



Natural Incidence of Sterol 24(*S*),25-Oxides. The first question was whether 24(*S*),25-epoxycholesterol (**6**) is formed under normal metabolic circumstances or only from a nonnatural substrate, squalene dioxide (**4**), produced when sterol biosynthesis is perturbed, as by use of an inhibitor. "24,25-Epoxycholesterol" had at that time recently been reported as a constituent of extracts from red sea algae,[29] but it was not known whether this material, of unspecified C24 stereochemistry, was an enzymatically generated compound or an autoxidation product of desmosterol (**10**). Our initial experiment to determine if 24(*S*),25-epoxycholesterol (**6**) were generated under "normal" biochemical circumstances involved incubation of [^{14}C]acetate with RLH in the absence of inhibitor to afford the anticipated mixture of squalene (18%), lanosterol (15%), and "cholesterol" (66%).[30] This cholesterol fraction was shown to contain 24,25-epoxycholesterol by its reduction with $LiAlH_4$ to afford 6% of 25-hydroxycholesterol (**7**), and by its benzoylation to afford 24(*S*),25-epoxycholesterol benzoate.[22] Control experiments demonstrated that no **6** was formed from lanosterol (**2**) under the same conditions.[30] Thus it was clear that, at least in RLH, no artificial factors, such as inhibitors, were necessary for **6**, and therefore presumably **4**, to be produced enzymatically. It remained, however, to be demonstrated whether or not 24(*S*),25-epoxycholesterol (**6**) is formed intracellularly.

During this same period, Kandutsch was continuing to explore his oxysterol regulatory hypothesis by investigating whether the types of oxysterols he had shown to act as HMG-CoA reductase repressors[13] were naturally occurring metabolites within cells. In these studies, analysis of the lipid extracts from cultured Chinese hamster lung (Dede) cells had led to the identification of two chromatographically purified fractions that had reductase repressor activity.[31] One of these fractions was readily identified as 25-hydroxycholesterol (**7**); the other was not identical with any of the numerous other oxysterols to which Kandutsch had access.[32] Mutual New England reticence undoubtedly contributed to the considerable period of time which then elapsed before the discoverers of this material with biological activity in need of structural identification came into contact with the discoverers of a novel metabolite in search of biological significance. After communication finally was established, it was quickly found, of course, that the compound responsible for the second band of HMG-CoA reductase repressor activity in the cultured cells was 24(*S*),25-epoxycholesterol (**6**),[31] and an enjoyable and fruitful collaboration with Kandutsch was established. The concentrations of **7** and **6** found in the Dede cells were in the range required for natural regulation of HMG-CoA reductase,[31] as measured by the potency of these compounds in Kandutsch's assay.[32]

At the same time as this work was being concluded, samples of human liver, obtained from donors in the Stanford University School of Medicine Heart Transplant Program through the collaboration of S. K. Erickson, were being analyzed in our laboratory for the presence of **6**. This was our introduction to the rigors of analysis for trace amounts of oxygenated sterols. Early work with GC had indicated that the sterol 24,-

(22) Seki, M.; Koizumi, N.; Morisaki, M.; Ikekawa, N. *Tetrahedron Lett.* **1975**, 15–18.
(23) In an experiment in which the same RLH preparation was used, **1** was converted to **3** in 62% yield and **4** was converted to **5** in 56% yield.
(24) Bai, M.; Xiao, X.-y.; Prestwich, G. D. *Biochem. Biophys. Res. Commun.* **1992**, *185*, 323–329.
(25) Boutaud, O.; Dolis, D.; Schuber, F. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 898–904.
(26) Taylor, F. R.; Kandutsch, A. A.; Gayen, A. K.; Nelson, J. A.; Nelson, S. S.; Phirwa, S.; Spencer, T. A. *J. Biol. Chem.* **1986**, *261*, 15039–15044.
(27) Saucier, S. E.; Kandutsch, A. A.; Gayen, A. K.; Nelson, J. A.; Spencer, T. A. *J. Lipid Res.* **1990**, *31*, 2179–2185.
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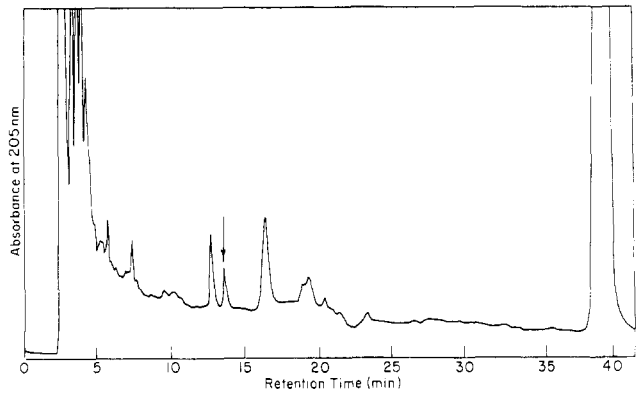

Figure 2. Reverse-phase HPLC analysis as described in ref 34 of the portion of the nonsaponifiable extract from human liver which has the chromatographic mobility of cholesterol on normal-phase preparative TLC. The solvent system was 19:1 CH_3OH/0.1% K_2CO_3 in H_2O. The arrow indicates the peak shown to be 24(*S*),25-epoxycholesterol (**6**). Cholesterol has a retention time of 39.3 min.

25-epoxides decomposed at the temperatures required, so use of HPLC was mandated, even though oxysterols contain only the $\Delta^{5,6}$ double bond as a chromophore for UV detection.[33] Experience taught us that the use of three different solvent systems is often advisable when attempting to quantitate, by use of their weak absorbance at 205–210 nm, the minute amounts of oxysterols that exist among a plethora of highly varied and often highly chromophoric substances in complex biological materials such as liver. Confirmation of structure by time-consuming collection and pooling of appropriate fractions for mass spectrometric analysis has also proved essential.

This initial foray into analysis for oxysterols in complex mixtures was rewarded, however, by identification of an HPLC peak in the nonsaponifiable extract from human liver, as shown in Figure 2, which was demonstrated by mass spectrometric analysis and conversion to 25-hydroxycholesterol (**7**) to be 24(*S*),25-epoxycholesterol (**6**), present at a concentration about 10^{-3} relative to that of cholesterol.[34] Subsequently, 24(*S*),25-oxidolanosterol (**5**) was also identified in human liver,[28] and the presence of **6** in normal human serum was also demonstrated, although in a much lower concentration.[35]

These results document clearly that the squalene dioxide pathway of steroid biosynthesis functions in different types of cells under normal metabolic conditions. One might wonder why, given the vast amount of research devoted to cholesterol, 24(*S*),25-epoxycholestrol (**6**) had gone undetected as a natural product for so long. A probable reason is that, upon normal phase chromatography (TLC or HPLC), **6** remains buried in the eluting tail of the enormously greater amount of cholesterol (**3**). However, upon reverse phase chromatography, applied only more recently, the epoxide group exhibits quite polar properties, and **6** is elutable distinctly separated from and, importantly, earlier than **3** (Figure 2).[34]

Metabolism of the 24(*S*),25-Epoxysterols. For studies of the biochemical behavior of epoxides **5** and **6**, radiolabeled samples of these compounds had to be synthesized.[34,36] This necessitated development of means of separation of the 24*S* from the 24*R* epimers of the epoxides produced by peracid treatment of desmosterol (**10**) (to afford **6**) or lanosterol (**2**) (to afford **5**). Initially, chromatographic separation of derivatives, such as benzoates[21] or acetates,[36] was employed. Then, HPLC conditions were found that would, with care, separate **6** from 24(*R*),25-epoxycholesterol (**11**)[34] and **5** from 24(*R*),25-oxidolanosterol (**12**).[37] Eventually, the advent of chiral columns for HPLC provided by far the most convenient method for separation of **6** from **11**[27] and **5** from **12**,[38] as well as for separation of other oxysterol epimers.[39] This ability to resolve epimeric oxysterols by chiral column HPLC is of particular significance because it allows one to deal reliably and conveniently with the nagging question of whether these compounds are true, physiologically relevant metabolites or merely artifacts of autoxidation. The fact that trace amounts of oxysterols, *e.g.*, side-chain hydroxylated derivatives of cholesterol, are known to form autoxidatively[40] and are, in fact, very difficult to avoid when manipulating biological samples, made many investigators skeptical about the natural incidence or regulatory role of these compounds.[41] However, by use of chiral column HPLC it could readily be established, for example, that the 24,25-epoxycholesterol isolated from rat liver is exclusively the 24*S* epimer **6**,[42] indicating an enzymatic origin. On the other hand, the 24,25-epoxycholesterol isolated from incubations of desmosterol (**10**), in a study of its possible conversion to 25-hydroxycholesterol (**7**),[27] was approximately a 1:1 mixture of **6** and its 24*R* epimer **11**, indicating a nonenzymatic, autoxidative origin.

With respect to the actual metabolism of the sterol 24(*S*),25-oxides, the fact that **6** is not converted to 25-hydroxycholesterol (**7**) in RLH[21] or in cultured cells[26] was, of course, the first relevant observation. Incubation of **6** in cultured cells showed that this compound eventually does undergo conversion to more polar products,[26] but these have not yet been identified. Similar incubation of **5** resulted in rapid metabolism to **6**,[26] as expected. Recently, in an attempt to gain preliminary insight into its in vivo metabolic behavior, **6** was administered intragastrically to mice and its hepatic uptake and subsequent disappearance were measured.[43] The epoxysterol was taken up rapidly by the liver, with maximum concentrations being reached

(33) Schroepfer (Kudo, K.; Emmons, G. T.; Casserly, E. W.; Via, D. P.; Smith, L. C.; St. Pyrek, J.; Schroepfer, G. J., Jr. *J. Lipid Res.* **1989**, *30*, 1097–1111) later developed a radiolabeling derivatization procedure as an alternative method of oxysterol analysis.

(34) Spencer, T. A.; Gayen, A. K.; Phirwa, S.; Nelson, J. A.; Taylor, F. R.; Kandutsch, A. A.; Erickson, S. K. *J. Biol. Chem.* **1985**, *260*, 13391–13394.

(35) Beitz, L. O.; Mungovan, J. A.; Gayen, A. K.; Spencer, T. A. Unpublished results.

(36) Steckbeck, S. R.; Nelson, J. A.; Spencer, T. A. *J. Am. Chem. Soc.* **1982**, *104*, 893–895.

(37) Panini, S. R.; Sexton, R. C.; Gupta, A. K.; Parish, E. J.; Chitrakorn, S.; Rudney, H. *J. Lipid Res.* **1986**, *27*, 1190–1204.

(38) Panini, S. R.; Delate, T. A.; Sinensky, M. *J. Biol. Chem.* **1992**, *267*, 12647–12654.

(39) Saucier, S. E.; Kandutsch, A. A.; Gayen, A. K.; Swahn, D. K.; Spencer, T. A. *J. Biol. Chem.* **1989**, *264*, 6863–6869.

(40) Reviews: (a) Smith, L. L. *Cholesterol Autoxidation*; Plenum Press: New York, 1981. (b) Smith, L. L. *Chem. Phys. Lipids* **1987**, *44*, 87–125.

(41) *E.g.*: Chang, T. Y. *The Enzymes*; Academic Press: New York, 1983; Vol. XVI, pp 491–521.

(42) Erickson, S. K.; Lear, S. R.; Gayen, A. K.; Spencer, T. A. Unpublished results.

(43) Saucier, S. E.; Kandutsch, A. A.; Clark, D. S.; Spencer, T. A. *Biochim. Biophys. Acta* **1993**, *1166*, 115–123.

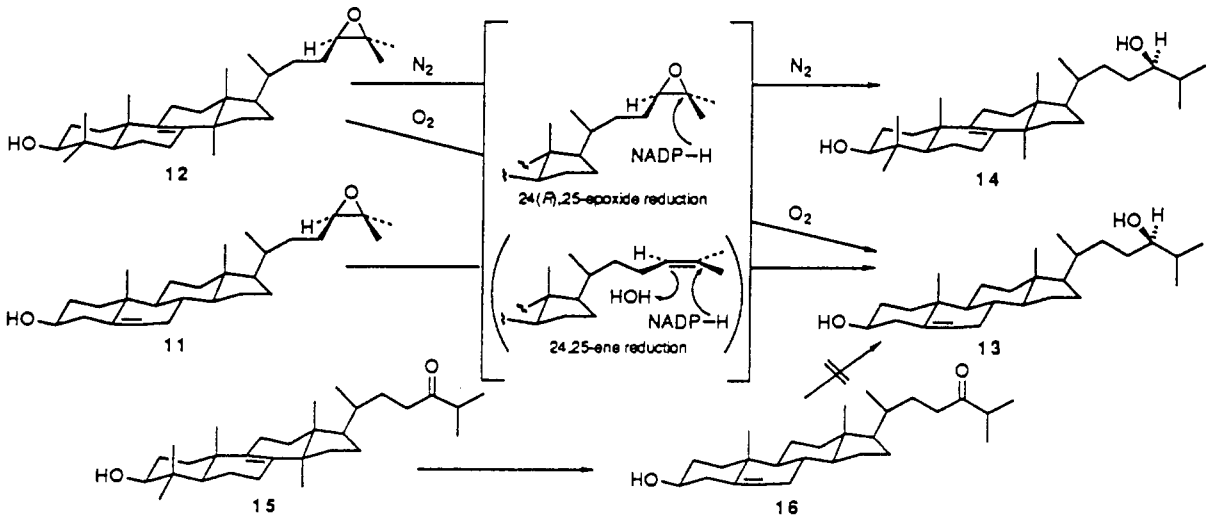

Figure 3. Results of metabolism of 24(*R*),25-epoxycholesterol (**11**), 24(*R*),25-oxidolanosterol (**12**), and 24-ketolanosterol (**15**) in rat liver homogenates and in Dede cells under nitrogen and in air. In brackets are shown schematic representations of the postulated mechanism for reduction by the $\Delta^{24,25}$-reductase system of the 24(*R*),25-epoxide group in comparison to that of the $\Delta^{24,25}$ double bond.

after about 4 h and then declining to control levels by 8 h.[43] Relatively rapid hepatic inactivation had previously been observed with other oxysterols,[44,45] but in no case, including **6**, has the precise metabolic pathway been elucidated.[46]

Metabolism of 24(*R*),25-Epoxysterols. The separation of C24 epimeric epoxides required to obtain radiolabeled **5** and **6** had also yielded samples of pure, labeled 24(*R*),25-oxidolanosterol (**12**) and 24(*R*),25-epoxycholesterol (**11**), so it was decided to test the behavior of these presumably "unnatural" isomers in biochemical media, especially since Corey[5] had earlier inferred that 3(*S*),22(*R*)-squalene dioxide is enzymatically cyclized (presumably to **12**). These experiments provided surprising and intriguing results,[36] summarized in Figure 3. Incubation of 24(*R*),25-oxidolanosterol (**12**) with RLH in air did not result in formation of 24(*R*),25-epoxycholesterol (**11**), but instead yielded 24(*R*)-hydroxycholesterol (**13**). When the incubation was conducted anaerobically, 24(*R*)-hydroxy-24,25-dihydrolanosterol (**14**) was the exclusive product. That the conversion of **12** to **14** or **13** was not occurring via rearrangement to a 24-keto steroid and subsequent reduction was demonstrated by incubation of 24-keto-24,25-dihydrolanosterol (**15**), which produced 24-ketocholesterol (**16**) (Figure 3). Appropriate control experiments indicated that the reduction of **12** to **14** is indeed enzyme mediated.

Our hypothesis[36] for the mechanism of this unprecedented[17] enzymatic reduction of **12** was that it is effected by the same enzyme system that reduces the $\Delta^{24,25}$ bond in the latter stages of cholesterol biosynthesis. This double-bond reduction has been shown to involve addition of one hydrogen from H_2O at C24 and one from NADPH at C25.[47] It is also known that the introduction of these hydrogens is cis, with the incoming H at C24 in the *pro-S* position (Figure 3).[48] These results require that if 24(*R*),25-oxidolanosterol (**12**) were enzymatically bound in the same manner as a $\Delta^{24,25}$ steroid, the back side of the C–O bond at C25 would be accessible to attack by NADPH. Conversely, if 24-(*S*),25-oxidolanosterol (**5**) were so bound, the epoxide oxygen would protrude toward the NADPH, and hydride transfer to C25 could not occur.

Further insight into this unusual reduction was provided by studies of the metabolism of **12** and **11** in cultured cells.[26] Two cell lines were employed: Dede, which has sterol $\Delta^{24,25}$-reductase activity; and L, which lacks this reductase, so that desmosterol (**10**) rather than cholesterol (**3**) is the sterol biosynthetic end product. Incubation of either **12** or **11** in Dede cells afforded 24(*R*)-hydroxycholesterol (**13**).[26] In L cells, however, **11** was recovered unchanged and **12** was converted to **11**.[26] (As expected, in both cell lines 24-(*S*),25-epoxycholesterol (**6**) was recovered unchanged and 24(*S*),25-oxidolanosterol (**5**) was converted to **6**.[26]) Finally, when an inhibitor of the sterol $\Delta^{24,25}$-reductase, triparanol, was used in incubations of 24(*R*),25-epoxycholesterol (**11**) in Dede cells, under conditions that caused 75% inhibition of the conversion of desmosterol (**10**) to cholesterol (**3**), a 60% inhibition of the conversion of **11** to **13** was observed.[26] All of this evidence is consistent with our original proposal that the 24(*R*),-25-epoxides are reduced by the Δ^{24} reductase system.[36] The only other examples of enzymatic epoxide reductions of which we are aware are two cases involving naphthoquinone 2,3-oxides,[49,50] which are structurally quite different from the simple aliphatic sterol side chain oxides in **11** and **12**. This facet of behavior of the

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(46) Metabolism of **6** could occur via epoxide hydrolase mediated conversion to diol. Hammock et al. (Hammock, B. D.; Gill, S. S.; Mumby, S. M.; Ota, K. In *Molecular Basis of Environmental Toxicity*; Bhatnagar, R. S., Ed.; Ann Arbor Science Pub.: Ann Arbor, MI, 1980; Chapter 10, pp 245–247) have reported that a "soluble epoxide hydrase is capable of slowly hydrating squalene oxide and more rapidly, squalene dioxide and apparently lanosterol epoxide". For a recent relevant reference, *cf.*: Grant, D. F.; Storms, D. H.; Hammock, B. D. *J. Biol. Chem.* **1993**, *268*, 17628–17633. The author thanks J. A. Watson and B. D. Hammock for helpful discussions.

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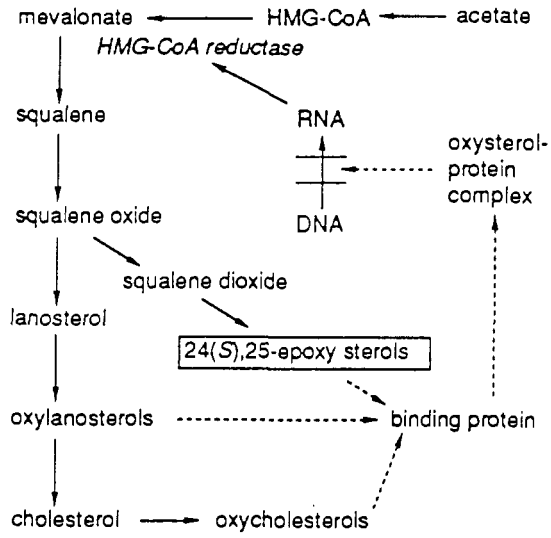

Figure 4. The Kandutsch model for the regulation of HMG-CoA reductase by oxysterol metabolites, adapted from ref 54.

sterol 24(*R*),25-oxides presumably has no direct relevance to natural biological phenomena, but it does represent a novel and intriguing aspect of enzymatic versatility.

Biological Significance of the Squalene Dioxide Pathway. Oxysterols as a class of natural products may still be relatively obscure, but they possess a striking range of biological activities, having been reported "to influence such vital matters as de novo sterol biosynthesis, membrane function, DNA synthesis, cell growth and proliferation, and aortal atherosclerosis".[51] However, relatively little progress has been made toward gaining understanding of these biological properties, and a "definitive or even satisfying viewpoint integrating (the effects of oxysterols) with human health interests has yet to emerge".[51] Much of the research to date on oxysterols has focused on their hypothesized involvement in the regulation of HMG-CoA reductase,[14] and it was in this context that **6**, formed via the squalene dioxide pathway, became recognized as an "oxysterol". As recounted earlier, Kandutsch had proposed the oxysterol regulatory hypothesis[14] after finding that pure cholesterol itself is ineffective at suppressing HMG-CoA reductase when autoxidation reactions are prevented, whereas numerous oxysterols are potent reductase repressors.[13] Among these oxysterols are oxidized derivatives of cholesterol, intermediates between lanosterol (**2**) and cholesterol (**3**), and, of course, **6**. By analogy to the mechanism of steroid hormone action,[52] Kandutsch postulated the existence of a cytosolic oxysterol binding protein and then searched for and identified such a protein.[53] A strong correlation was demonstrated between the binding affinity of a large number of oxysterols to this protein and their potency as repressors of HMG-CoA reductase.[32] All this evidence led to the model shown in Figure 4 for the regulation of HMG-CoA reductase by oxysterols.[54] In this model a complex is formed between an oxysterol (of one of the types just listed) and the cytosolic binding protein (receptor), followed by transport of that complex to the nucleus where it acts to suppress transcription of the gene for synthesis of HMG-CoA reductase.

During the same period, other workers were also conducting investigations that related to oxysterols as possible HMG-CoA reductase regulators. Most notably, Rudney showed that products of the squalene dioxide pathway, expecially 24(*S*),25-oxidolanosterol (**5**), accumulated in cultured cells when inhibitors of cholesterol biosynthesis, *e.g.*, of lanosterol 14α-demethylation, were employed, and he obtained evidence suggestive that these compounds are involved in cholesterol regulation.[37,55,56] Trzaskos[57] and Gibbons,[58] on the other hand, concluded that oxidized metabolites of lanosterol (**2**), particularly the 14α-formyl derivative (30-oxolanosterol, **17**), are the regulatory oxysterols which accumulate when lanosterol 14α-demethylation is similarly inhibited. Gibbons[59] and Aguilera[60] observed that use of cholesterol-supplemented diets to suppress cholesterogenesis resulted, in rats and chicks respectively, in accumulation of "polar sterols", also believed to be oxidized lanosterols.

Our own initial systematic attempt to correlate cellular oxysterol concentrations with variations in rates of cholesterogenesis involved administration to Dede cells of a concentration of mevalonic acid high enough to repress HMG-CoA reductase activity by 90%, whereupon there was indeed observed about a 30% increase in oxysterols.[61] However, this increase was not in the oxysterols previously identified in those cells (**6** and **7**),[31] but rather in 30-oxolanosterol (**17**) and 30-hydroxylanosterol (**18**),[61] the oxysterols being reported by others at about the same time to accumulate under different conditions.[57-60,62] Subsequent findings, most recently and incisively by Trzaskos,[63] have led to the conclusion that these oxidized lanosterols, induced by mevalonate or otherwise, are associated with a different, post-transcriptional mechanism of cholesterol regulation, distinct from that represented in Figure 4.

It was then decided to take on the major challenge of determining which oxysterols are present naturally in mammalian liver, where most cholesterol biosynthesis occurs, and whether the concentrations of these oxysterols increase when cholesterol biosynthesis decreases. Located at the Jackson Laboratory, Kandutsch naturally selected the mouse as the experimental animal. Laborious protocol was developed for separating an "oxysterol fraction" from mouse liver and assaying initially arbitrary chromatographic subfractions of it for repression of HMG-CoA reductase. Extensive

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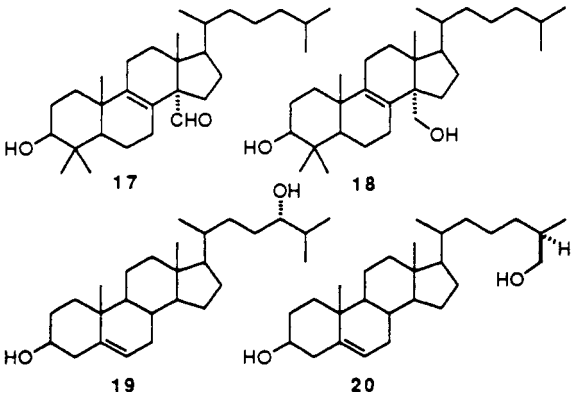
further chromatographic purification and chemical analysis eventually allowed identification of six oxysterols in the livers of control mice, and the total amount of reductase repression activity represented by the amounts of these oxysterols, particularly 24(*S*)-hydroxycholesterol (**19**), 25-hydroxycholesterol (**7**), and (25*R*)-26-hydroxycholesterol (**20**), did increase 2.5- and 6-fold after 1 and 2 days, respectively, of cholesterol feeding.[39] However, to our disappointment, 24(*S*),25-epoxycholesterol (**6**) was not detected in either the control or cholesterol-fed mice,[39] perhaps indicating that the squalene dioxide pathway is, for some reason, of less importance in the mouse than in other species. In a subsequent similar study of mouse liver by Björkhem, "the general pattern of oxysterols obtained . . . was similar," although the conclusion about the importance of hydroxycholesterols in regulation was different.[64]



Recently, since Kandutsch's unwelcome retirement, we have been continuing, in collaboration with S. K. Erickson, efforts to determine if there is a correlation between HMG-CoA reductase activity and the intracellular levels of oxysterols, especially **6**. For this work, the rat, whose liver (at least when homogenized) had been shown[21,31] to produce **5** and **6**, was selected. It was decided, in addition to studying the total concentrations of oxysterols in rat liver, to try to determine as well their subcellular distribution. Such an endeavor, not previously attempted by other research groups, seemed sensible because, if the model in Figure 4 is valid, then changes in specific oxysterol concentrations in liver cell nuclei, specifically in the chromatin, might be expected when cholesterol metabolism is altered. Four oxysterols were selected for study on the basis of their previous implication as possible regulators of cholesterol metabolism: 24(*S*)-hydroxycholesterol (**19**), 25-hydroxycholesterol (**7**), (25*R*)-26-hydroxycholesterol (**20**), and, of course, 24(*S*),25-epoxycholesterol (**6**). Analysis of homogenates prepared from livers of rats fed a standard rat chow (control) diet gratifyingly showed that all four oxysterols were indeed present,[42] in approximately the same amounts as the oxysterols in mouse liver.[39]

Attention was then turned to the determination of the subcellular distribution of the four selected oxysterols in control rats and in rats which had been fed a cholesterol-enriched diet for 2 weeks, a prolonged period of cholesterol feeding which causes 96% reduction in HMG-CoA reductase activity.[42] Analyses of liver subcellular fractions[65] from control rats showed that all four oxysterols were indeed present in the nuclei,[42] and in the cytosol, microsomes, and mitochrondria as well.[42] After 2 weeks of cholesterol feeding, there was a marked (approximately 10-fold) increase in the total (free plus esterified) concentrations of two of the oxysterols, 24(*S*)-hydroxycholesterol (**19**) and our sentimental favorite, 24(*S*),25-epoxycholesterol (**6**), but only in the nucleus.[42] The subcellular selectivity of the increases suggests preferential translocation of these two oxysterols to the nucleus.[66]

Although further work is needed to confirm and extend these results, this striking effect of a cholesterol-rich diet on the concentration of two specific oxysterols in rat liver nuclei is certainly strongly suggestive of involvement of 24(*S*)-hydroxycholesterol (**19**) and 24(*S*),25-epoxycholesterol (**6**) in regulation of cholesterol metabolism at the nuclear level. The results from the various studies cited above are consistent with Kandutsch's idea,[54] represented in Figure 4, that different types of oxysterols, *e.g.*, **19** and **6**, may function as regulators in different biological media. In the context of this Account, of course, the apparent involvement of 24(*S*),25-epoxycholesterol (**6**) in rat[30,42] and human[34] liver is of particular note, because it indicates that at least one metabolite of the squalene dioxide pathway, as well as compounds derived from cholesterol (**3**), takes part in the natural regulation of cholesterol metabolism. Since **6** is derived from a biosynthetic pathway that is completely different from any of the other putative oxysterol regulators, it could well function as a different sensing factor and interact preferentially with different proteins and/or gene elements to provide needed versatility in regulatory mechanisms. However, even if further research firmly establishes an inverse (and reversible) relationship between HMG-CoA reductase activity and the hepatic concentration of **6** or other specific oxysterols, such circumstantial evidence, although compelling, will not alone establish convincingly that there is indeed oxysterol regulation of cholesterol metabolism. Understanding must first be achieved of the mechanism by which these oxysterols exert their regulatory effect on HMG-CoA reductase, presumably through interactions with appropriate proteins and gene elements.

As mentioned above, Kandutsch found support for his regulatory model (Figure 4) by identifying a cytosolic protein that binds a wide variety of oxysterols, including **6**, with a strength proportional to their effectiveness at repressing HMG-CoA reductase.[32] But, despite extensive efforts, neither this nor any other oxysterol binding protein has been reliably identified in the nucleus,[67,68] and the known cytosolic protein does not have specific affinity for the identified sterol regulatory DNA sequence element.[67,68] Progress in elucidating the

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(65) Liver cell nuclei, microsomes, mitochondria, cytosol, and plasma membrane were prepared by standard literature procedures.

(66) Concern about the validity of these subcellular analyses, particularly with respect to the question of cross-contamination of fractions, led to our conducting extensive control experiments, including assaying of markers, including the enzymes succinate INT dehydrogenase (mitochondria), glucose-6-phosphatase (endoplasmic reticulum), and 5′-nucleotidase (plasma membrane), and DNA (nuclei). All assays indicated insignificant cross-contamination. Analysis of the plasma membrane for oxysterols showed no increase after cholesterol feeding.

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putative role of oxysterols in regulating HMG-CoA reductase transcription is currently stalled in attempts to elucidate the mechanism of signal transduction by identifying the oxysterol–receptor–gene interactions involved, and it seems reasonable to conclude that at least the nuclear "proteins that mediate the action of oxysterols remain to be identified".[69]

We have recently undertaken a novel approach to this problem by seeking to identify proteins from different cellular fractions that have oxysterols bound to them in vivo. By separating protein fractions from liver cytosol or nuclei by isoelectric focusing and analyzing these fractions for endogenously bound oxysterols, we hope to be able to identify new binding proteins. Initial experiments with cytosolic proteins are encouraging: whereas cholesterol is relatively randomly distributed among 20 separated protein fractions, the two oxysterols that cholesterol feeding experiments implicate in regulation in rat liver, **6** and **19**, are found in only two discrete fractions with isoelectric points of 5.4 and 8.2.[42] One or both of these fractions may contain a new, different cytosolic binding protein, which could turn out to be a participant in regulation by oxysterols. The potentially important analogous experiments with proteins from rat liver nuclei have yet to be conducted.

The evidence obtained to date certainly is consistent with the hypothesis that 24(*S*),25-epoxycholesterol (**6**) is indeed involved in the transcriptional regulation of HMG-CoA reductase. But is this the only role for products of the squalene dioxide pathway in cholesterol regulation? There is now direct or indirect evidence for interaction of oxysterols with a wide variety of additional elements related to regulation of cholesterol metabolism, including squalene synthase,[70] cholesterol 7α-hydroxylase,[71] LDL receptors,[72] and numerous other proteins.[73] The model shown in Figure 4 is clearly only part of the story. As noted earlier, Trzaskos[63] has recently presented evidence for post-transcriptional regulation of HMG-CoA reductase by oxidized lanosterols **17** and **18**. Panini and Sinensky[38] have also recently described results consistent with post-transcriptional regulation by 24(*S*),25-oxidolanosterol (**5**) and have suggested that there may be separate receptors for C27 and C30 sterols. In addition to participation in regulation of cholesterol metabolism, the possibility obviously also exists that products of the squalene dioxide pathway may have other biochemical roles. In collaboration with Sinensky, we observed that 24(*S*),25-epoxycholesterol (**6**) has a vital, but as yet completely undefined, role in maintaining viability of certain cell cultures,[74] and similar observations have recently been made for 24(*S*),25-oxidolanosterol (**5**).[38]

Concluding Remarks. Although the existence of an alternate steroid biosynthetic pathway via squalene dioxide **4** has clearly been established, much indeed remains to be learned about the biochemistry and possible physiological importance of its principal products, 24(*S*),25-oxidolanosterol (**5**) and 24(*S*),25-epoxycholesterol (**6**). For example, what factors determine how much squalene oxide is diverted to the squalene dioxide pathway from the dominant formation of cholesterol? And, are **5** and **6** simply "ordinary" oxysterols, or do they, as products of a different biosynthetic pathway, have distinct physiological functions? Progress toward complete understanding of the biological properties of **5** and **6**, as for any other oxysterol, will almost certainly come gradually. Elucidation of the possible role of the abundant cholesterol itself in regulation of its own metabolism has a long and as yet incomplete history. Oxysterols present an even greater challenge because, as already mentioned, they exist naturally in only minute amounts and are readily formed by autoxidation, so that care must be taken to be certain that observed biological effects are indeed caused by endogenous metabolites. Even with the advent of powerful molecular biological techniques, it seems likely that an understanding of how the products of the squalene dioxide pathway participate in cholesterol regulation or other biochemistry will be achieved only slowly and arduously.

Most of this research was supported by NIH Grant HL23083. The author is greatly indebted to Andy Kandutsch and Sandra Erickson for their very enjoyable collaborations and their personal generosity and graciousness. He is even more indebted to the small group of principal co-workers in this research, whose skill, dedication, and willingness to do things not usually undertaken in an organic chemistry laboratory are largely responsible for such progress as has been made: Sharon Steckbeck Nelson, Seloka Phirwa, Apu Gayen, Luigi Anzalone, Dawn Swahn Clark, and, especially, Jim Nelson, whose original ideas initiated this work and who successfully guided all of the earlier, noncollaborative portions of it.

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