Stereochemical Importance of Fucosterol Epoxide in the Conversion of Sitosterol into Cholesterol in the Silkworm *Bombyx mori*†

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ABSTRACT: Studies on the stereochemistry of 24,28-epoxy-24-ethylcholesterol, a key intermediate in sitosterol to cholesterol conversion in insects, were undertaken. In nutritional experiments, the 24*R*,28*S* and 24*S*,28*R* stereoisomers (isofucosterol epoxides) were unable to support growth and development of silkworm larvae. Incubation of 3α-^{3}H-labeled (24*R*,28*R*)- and (24*S*,28*S*)-epoxide (fucosterol epoxides) with a cell-free preparation from silkworm guts resulted in effective conversion into desmosterol and cholesterol; (24*R*,28*R*)-epoxide was the slightly better substrate. Fucosterol incubation yielded (24*R*,28*R*)-epoxide and the 24*S*,28*S* isomer in approximately equal amounts, while slightly preferential formation of the 24*R*,28*R* isomer was observed in in vivo experiments. These results indicate that both the formation of the epoxide from fucosterol and its conversion to desmosterol proceed with a low degree of stereospecificity.

Since phytophagous insects lack the capacity of de novo sterol biosynthesis, they require exogenous phytosterols such as sitosterol (**1**), stigmasterol, and campesterol for their normal growth and development. In insects, these phytosterols undergo side-chain dealkylation at the C-24 position to afford cholesterol (**5**) which serves as a precursor for ecdysone and is an important component of the cell membrane (Svoboda et al., 1975).

The observations that fucosterol epoxide (**3**) upon boron trifluoride etherate treatment yields desmosterol (**4**) (Ikekawa et al., 1971; Ohtaka et al., 1973) and [3α-^{3}H]fucosterol epoxide is converted to cholesterol in the silkworm (Morisaki et al., 1972) led us to propose that, in insect, fucosterol epoxide is a key intermediate in the conversion of sitosterol (**1**) into cholesterol (**5**) (Figure 1). The data subsequently reported (Randall et al., 1972; Allais et al., 1973; Fujimoto et al., 1974a,b; Pettler et al., 1974; Morisaki et al., 1974; Awata et al., 1975, 1976; Allais & Barbier, 1977) supported the biogenetic pathway shown in Figure 1.

Subsequently we incubated the chemically synthesized 3α-^{3}H-labeled (24*R*,28*R*)-epoxide[1] **3a** and the 24*S*,28*S* isomer **3b** with a cell-free enzyme system (Awata et al., 1975) prepared from the midguts of silkworm and found that only the former isomer was effectively converted into desmosterol (Chen et al., 1975). This suggests the (24*R*,28*R*)-epoxide to be the actual intermediate of sitosterol dealkylation. In contrast, the in vivo experiments by Awata (1976) revealed no significant difference between these two stereoisomers in the conversion to cholesterol.

In the present investigation, the two stereoisomers **3a** and **3b** were incubated under varying conditions with cell-free preparations of silkworm guts to compare the conversion yields to desmosterol (**4**), and the relative yields of **3a** and **3b** from fucosterol (**2**) were also examined in vivo and in vitro. No absolute stereospecifity will be demonstrated both in the formation of the epoxide from fucosterol and in its conversion to desmosterol.

Experimental Section

Materials. (24*R*,28*R*)- and (24*S*,28*S*)-24,28-epoxy-24-ethylcholesterol (fucosterol epoxide **3a** and **3b**) were prepared as described elsewhere (Fujimoto et al., 1980). A diastereoisomeric mixture (1:1) of (24*R*,28*S*)- and (24*S*,28*R*)-24,28-epoxy-24-ethylcholesterol (isofucosterol epoxide **3c**; mp 134–138 °C) was prepared by oxidation of isofucosterol acetate (Dusza, 1960), followed by saponification. [3α-^{3}H]-Fucosterol epoxide (diastereoisomeric mixture, 1:1; 2.4 mCi/mmol) and [3α-^{3}H]fucosterol (4.15 mCi/mmol) were prepared as reported previously (Awata et al., 1975). The corresponding [3α-^{3}H]fucosterol epoxide benzoate was resolved by thin-layer chromatography (TLC) on silica gel, developed 10 times with benzene–hexane (2:1), and further purified with high-pressure liquid chromatography (LC) (for details, see the legend of Figure 5). Saponification of the resolved benzoates gave the 3α-^{3}H-labeled (24*R*,28*R*)-epoxide (**3a**) (0.85 mCi/mmol) and the 3α-^{3}H-labeled (24*S*,28*S*)-epoxide (**3b**) (0.64 mCi/mmol).

Boron Trifluoride Etherate Treatment of the Epoxide Isomers. The three acetates of the epoxides (5 mg each of **3a**, **3b**, and **3c**) were reacted for 2 min at room temperature with BF_3-etherate (10 μL) in benzene (2 mL) (Ohtaka et al., 1973), and the reaction products were analyzed by gas chromatography using a GC–MS system (Shimadzu-LKB 9000S; 1.5% OV-17; 280 °C).

Nutritional Experiments. Silkworm (*Bombyx mori*) larvae (20 species in one group), reared as already described (Morisaki et al., 1974) on a semisynthetic diet containing 0.2% of the epoxide isomers, were weighed 7 and 15 days after hatching (Table I). Insect sterols were analyzed as described previously (Fujimoto et al., 1974a).

Cell-Free Preparation. Cell-free extracts from silkworm midguts were prepared by the slightly modified method of Awata et al. (1975). Sea sand (20–30 mesh; 10 g) was used to grind the midguts from 25 larvae of the fifth instar at a temperature below 4 °C in a mortar containing 10 mL of Bucher's medium (Bucher & McGarrahan, 1965). The homogenate was centrifuged at 1500*g* for 10 min at 0 °C, and the supernatant was diluted with Bucher's medium to obtain preparation A, having a protein concentration of 12.5 mg/mL. Further centrifugation of the supernatant at 20000*g* for 10 min afforded preparation B, having a protein concentration

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[1] The previous stereochemical assignment is incorrect. The (24*R*,28*R*)-epoxide and (24*S*,28*S*)-epoxide in the previous paper (Chen et al., 1975) should be read as the (24*S*,28*S*)-epoxide and (24*R*,28*R*)-epoxide, respectively (Fujimoto et al., 1980).

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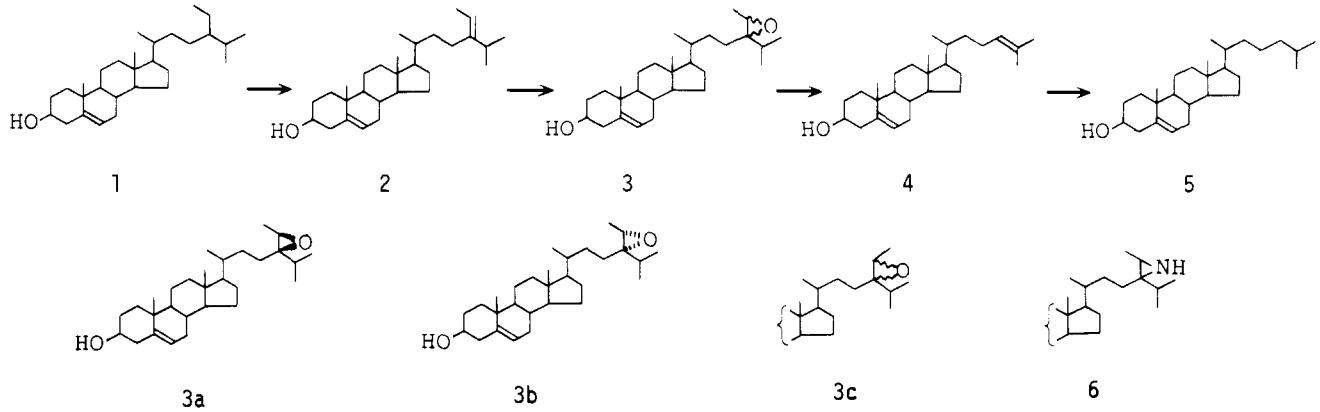

FIGURE 1: Dealkylation pathway (upper) from sitosterol (**1**) to cholesterol (**5**) through fucosterol (**2**), fucosterol epoxide (**3**), and desmosterol (**4**) in insects and chemical structures (lower) of (24*R*,28*R*)-fucosterol epoxide (**3a**), fucosterol (24*S*,28*S*)-epoxide (**3b**), isofucosterol epoxide (1:1 diastereoisomeric mixture) (**3c**), and 24,28-iminofucosterol (**6**).

Table I: Growth and Development of Larvae of Silkworm Reared on a Semisynthetic Diet

sterol source	no. of larvae (day 7) first instar	no. of larvae (day 7) second instar	av wt (mg/head)	no. of larvae (day 15) second instar	no. of larvae (day 15) third instar	av wt (mg/head)
sitosterol	0	20	6.3	0	20	41
(24*R*,28*R*)-epoxide (**3a**)	0	20	8.8	0	20	35
(24*S*,28*S*)-epoxide (**3b**)	0	20	7.9	0	20	41
3a plus **3b** (1:1)	0	20	5.9	0	20	34
isofucosterol epoxide (**3c**)	3[a]	17[a]	3.9	0	0	

[a] These larvae died on the seventh or eighth day.

of 1.0 mg/mL. The protein content was estimated by the Lowry–Folin method.

Incubation of Fucosterol Epoxide and Product Determination. The tritium-labeled epoxides (**3a** plus **3b**, 2.1×10^5 cpm, 50 μg; **3a**, 1.1×10^5 cpm, 50 μg; **3b**, 8.3×10^4 cpm, 50 μg) were incubated at 37 °C with preparation A or B (4 mL). When necessary, NADPH (2 mg) was added to the incubation medium. An aliquot of the incubation mixture was extracted with ethyl acetate and was saponified for 1 h with 5% methanolic potassium hydroxide at reflux. The ether extract of the unsaponifiable fraction was acetylated with acetic anhydride–pyridine, and the acetates were chromatographed on a silica gel column to obtain the sterol, epoxide, and polar fractions which were eluted with hexane–benzene (2:1), hexane–benzene (1:4), and benzene–ethyl acetate (4:1), respectively. Separation of cholesterol acetate from desmosterol acetate in the sterol fraction was done by TLC on 2% silver nitrate impregnated silica gel. Resolution of the 24,28 stereoisomers of the epoxide was carried out by LC as their benzoates. The radioactivity of each fraction was determined before or after several recrystallizations with carrier on a Packard 3320 liquid scintillation counter.

Incubation of Fucosterol. [3α-^{3}H]Fucosterol (4.4×10^5 cpm, 58 μg in 20 μL of dimethylformamide) was introduced into 4 mL of preparation A containing 2 mg of NADPH, oxygen gas was bubbled into the mixture, and it was incubated with shaking for 3 h at 37 °C in an oxygen atmosphere. After 10 mL of 5% KOH–methanol was added, the mixture was heated at reflux for 1 h. The unsaponifiable fraction was converted to benzoate and then chromatographed on a silica gel column. The epoxide fraction was rechromatographed on a silica gel plate with benzene–ethyl acetate (30:1) to give the pure epoxide fraction, and the conversion ratio was calculated based on recovered radioactivity of the unsaponifiable material.

In another run, [3α-^{3}H]fucosterol in the presence of 0.5 mg of 24,28-iminofucosterol (Fujimoto et al., 1974a) was incubated as described above, and a portion of the obtained benzoate was applied on a 2% $AgNO_3$-impregnated silica gel plate and developed twice with hexane–benzene (1:1). The bands corresponding to cholesterol (R_f 0.7), fucosterol (R_f 0.53), and desmosterol benzoate (R_f 0.41) were eluted with ethyl acetate, and a portion of each extract was recrystallized with carrier to obtain constant specific activity. Another portion of the benzoate was separated as described above to give the epoxide fraction, which was further purified by LC. A portion of the separated (24*R*,28*R*)- and (24*S*,28*S*)-epoxide was recrystallized 4 times with carrier to obtain constant specific activity. For further confirmation of these structures, the two epoxide fraction were saponified and separately reincubated with 4 mL of preparation A in the presence of 2 mg of NADPH. The incubation products were acetylated and separated on a silica gel column.

In Vivo Conversion of Fucosterol into Fucosterol Epoxide. Each of five 3-day-old larvae of the fifth instar perorally received 300 μg of (24*R*,28*R*)-epoxide, (24*S*,28*S*)-epoxide, or a 1:1 mixture thereof, in 20 μL of dimethylformamide, by using a microsyringe. Two hours later, [3α-^{3}H]fucosterol (43.5 μg, 3.3×10^5 cpm) in 15 μL of dimethylformamide solution was administered. After 5 h, the larvae were homogenized in a blender with 50 mL of $CHCl_3$–methanol (2:1). Filtration and evaporation of the solvent in vacuo gave a green residue which was saponified with 10 mL of 5% KOH–methanol at 60 °C for 1 h. The unsaponifiable fraction was benzoylated with benzoyl chloride–pyridine, and the benzoates were purified on TLC and analyzed by LC as described.

Results

Reaction of the Epoxide Isomers with Boron Trifluoride Etherate (BF_3-etherate). Treatment of **3a** or **3b** acetate with BF_3-etherate yielded desmosterol acetate (35%), 24-acetylcholesterol acetate (45%), and 24-formyl-24-methylcholesterol acetate (20%). The identical treatment of the **3c** acetate gave the three compounds in yields of 15, 60, and 25%, respectively.

Nutritional Effect of Fucosterol and Isofucosterol Epoxide. Like the controls, larvae fed **3a** or **3b** grew to the third instar within 15 days. However, those fed **3c** died on the 7th or 8th day after hatching (Table I). Cholesterol was the major sterol component in 15-day-old **3a**-fed larvae with desmosterol rep-

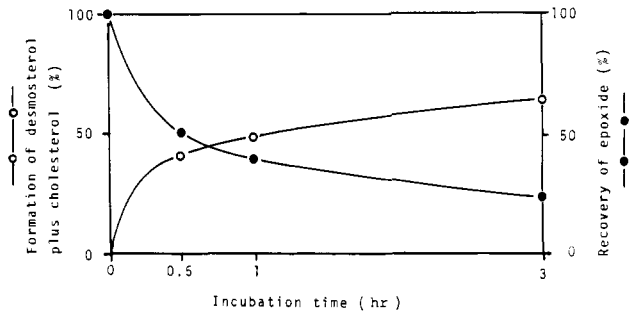

FIGURE 2: Production of desmosterol plus cholesterol (○) and disappearance of fucosterol epoxide (●). [3α-^{3}H]Fucosterol epoxide (diastereoisomeric mixture) was incubated with preparation A in the presence of NADPH as described under Experimental Section. An aliquot of the incubation medium taken at 0.5, 1, and 3 h was acetylated and chromatographed on a silica gel column to isolate sterols and the epoxide.

resenting approximately 5%. The sterol pattern of **3b**-fed larvae was similar, while the sterols in those fed **3c** could not be characterized because all of them died by the 8th post-hatching day.

Incubation of the (24R,28R)- and (24S,28S)-Epoxide Mixture and Effect of NADPH Addition. When a 1:1 mixture of **3a** and **3b** was incubated with preparation A, the epoxide concentration declined and there was a concomitant formation of cholesterol and desmosterol (Figure 2). On LC analysis of the recovered epoxide benzoate, the **3a** to **3b** ratio was 1:8 at 0.5-h incubation and 1:6 at 3-h incubation.

When [3α-^{3}H]fucosterol epoxide was incubated with preparation A, recovered radioactivity was 57 and 77% in the sterol fraction, 15 and 7% in the epoxide fraction, and 28 and 16% in the polar fraction in the absence and presence of NADPH, respectively. In the sterol fraction, the ratio of cholesterol/desmosterol production was 1:1 in the absence and 7:1 in the presence of NADPH. Repeated crystallization of the corresponding acetates with the carriers cholesterol acetate and desmosterol acetate gave constant specific activity. The observed effect of NADPH coincided with the findings of Svoboda et al. (1969) that in cell-free preparations from the guts of the tobacco hornworm *Manduca sexta*, the conversion of desmosterol to cholesterol is catalyzed in the presence of NADPH.

Incubation of (24R,28R)- and (24S,28S)-Epoxide. Figure 3 shows the time course of desmosterol formation by incubation with preparation B. It can be seen that **3a** was a little more effectively converted to desmosterol then **3b**. Similar incubation of [3α-^{3}H]-**3a** or [3α-^{3}H]-**3b** with preparation A in the presence of NADPH gave the sterol fraction in yields of 19, 37, and 67% at 1, 5, and 40 min, respectively, for **3a** and 8, 34, and 65% at 1, 5, and 40 min, respectively, for **3b**.

Incubation of Fucosterol. Incubation of [3α-^{3}H]fucosterol with preparation A afforded the epoxide fraction in a 3.1% yield; when imine or fucosterol epoxide was added, it increased to 9.5 and 8.9%, respectively. The recovery of radioactivity in the unsaponifiable fraction was approximately 70%.

Figure 4 is a TLC radiochromatogram of the products (in the form of benzoate esters) derived from [3α-^{3}H]fucosterol incubation in the presence of 24,28-iminofucosterol. It shows that fucosterol was converted into the epoxide, desmosterol, and cholesterol. The latter two fractions were converted to the benzoates and crystallized with the respective carrier to give constant specific activity. The estimated yields from these values were as follows: desmosterol, 30%; cholesterol, 9%.

The epoxide fraction was cochromatographed on LC with authentic (24*R*,28*R*)-epoxide (**3a**) and (24*S*,28*S*)-epoxide

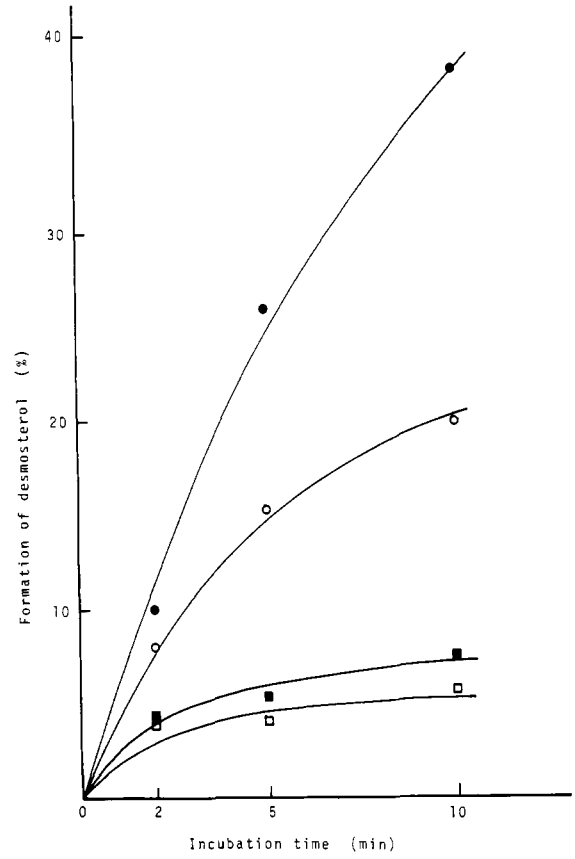

FIGURE 3: Time course of the production of desmosterol by incubation of [3α-^{3}H]fucosterol epoxide isomers: (●) (24*R*,28*R*)-epoxide plus NADPH; (○) (24*R*,28*R*)-epoxide; (■) (24*S*,28*S*)-epoxide plus NADPH; (□) (24*S*,28*S*)-epoxide. 3α-^{3}H-Labeled (24*R*,28*R*)-epoxide and (24*S*,28*S*)-epoxide were incubated with preparation B with or without NADPH as described under Experimental Section.

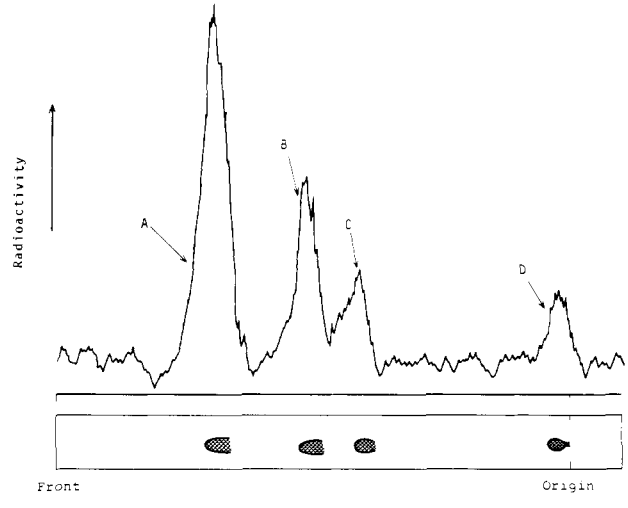

FIGURE 4: Radiochromatogram on an $AgNO_3$ (2%) impregnated silica gel plate of the product obtained by incubation of [3α-^{3}H]fucosterol with preparation A in the presence of 24,28-iminofucosterol. Marker: (A) cholesterol benzoate; (B) fucosterol benzoate; (C) desmosterol benzoate; (D) fucosterol epoxide benzoate.

(**3b**). Figure 5 clearly shows that the radioactivity was equally distributed in the two stereoisomers. The fractions from **3a** and **3b** were converted to the benzoates and recrystallized with the respective carrier from methanol to give the constant specific activity. Incubation of biologically produced (24*R*,28*R*)- and (24*S*,28*S*)-epoxide gave the sterol fraction in 75% yields from both of the stereoisomers (data not shown).

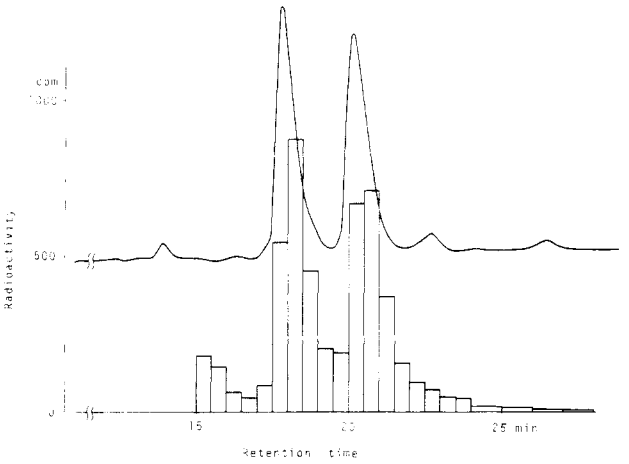

FIGURE 5: LC separation of the fucosterol epoxides (benzoates) obtained by incubation of [3α-^{3}H]fucosterol with preparation A. Retention time of the (24*R*,28*R*)-epoxide was 17.7 min and that of the (24*S*,28*S*)-epoxide was 20.1 min. A Shimadzu-Du Pont 830 liquid chromatograph with a Zorbax SIL column was used with a solvent of hexane–methylene dichloride (1:1).

Incubation of [3α-^{3}H]fucosterol in the presence of fucosterol epoxide as a trapping agent gave similar results: the radioactivity of the epoxide was almost equally distributed in the two isomers (data not shown).

In Vivo Conversion of Fucosterol to the 24,28-Epoxides. Silkworm larvae perorally administered with (24*R*,28*R*)-epoxide (A), (24*S*,28*S*)-epoxide (B), or a 1:1 mixture thereof (C) were given [3α-^{3}H]fucosterol. The epoxides contained 2–4% trapped radioactivity. LC analysis indicated that the radioactivity ratios of **3a** to **3b** were 3:1 for A, 5:1 for B, and 2:1 for C.

Discussion

Since our preliminary work on the stereochemistry of fucosterol epoxide in insects (Chen et al., 1975), studies on this subject have been greatly facilitated by the development of a facile synthesis of (24*R*,28*R*)-epoxide (**3a**) and (24*S*,28*S*)-epoxide (**3b**) (Fujimoto et al., 1980), LC separation of **3a** and **3b**, and a cell-free preparation which not only catalyzes the conversion of the epoxide to desmosterol but also catalyzes the formation of the epoxide from fucosterol.

In the present study, we first investigated the stereochemical influence of the epoxide reaction with BF_3-etherate. While **3a** and **3b** afforded similar desmosterol yields (35%), isofucosterol epoxide (**3c**) yielded only 15%. In an apparent agreement with these chemical behaviors, the nutritional experiment revealed that while silkworms reared on **3a** or **3b** grew normally, **3c** failed to support growth and development. These results suggest that the stereoisomers of (24*R*,28*S*)- and (24*S*,28*R*)-epoxide (isofucosterol epoxide) have no intermediate role in sitosterol dealkylation. The findings that isofucosterol satisfied the sterol requirement in silkworms (Morisaki et al., 1974) and that it was converted into cholesterol in insects (Morisaki et al., 1972) suggest that the transformation takes place via a pathway different from the epoxide intermediate. Thus, the remaining candidate(s) for the true intermediate of sitosterol dealkylation should be the (24*R*,28*R*)-(**3a**) and/or (24*S*,28*S*)-(**3b**) isomers.

In line with the previous results (Chen et al., 1975), a slightly preferential formation of desmosterol from **3a** over **3b** has been observed when incubated with preparation B (Figure 3) and preparation A, and it was also found (Figure 2) that, on incubation of a mixture of **3a** and **3b** (1:1) with preparation A, **3a** was consumed at a faster rate than **3b**. However, the observed discrimination between **3a** and **3b** seemed not enough for excluding **3b** as the intermediate of sitosterol dealkylation.

In spite of the low substrate specificity of the epoxide metabolism, it may be expected that formation of the epoxide could occur stereospecifically to afford a single stereoisomer, e.g., the (24*R*,28*R*)-epoxide (**3a**). Actually, however, incubation of fucosterol furnished **3a** and **3b** in an approximately 1:1 ratio (Figure 5). Although a slightly better yield of **3a** than **3b** was observed with in vivo experiments, it must be also taken into account that the once-formed epoxides were further metabolized to desmosterol and cholesterol under these conditions.

We are now inclined to conclude that stereospecificity, both in the formation and in the metabolism of the epoxide, is not strict.

Three reasons come to mind when attempting to explain this phenomenon. The substrate specificity of the enzymes may be low, the silkworms may have evolved two enzyme systems specific for the 24*R* or 24*S* compound, and/or the insects may have an isomerase of the C-24 stereoisomers. To shed some light on this matter, we are currently attempting to isolate fucosterol epoxide(s) from insects and to determine the stereochemistry. Examination of the stereospecificity in the dehydrogenation of 24-ethylcholesterol is also under way.

In this connection it will be worthy to note that both stereoisomers of 24-ethylcholesterol (sitosterol and clionasterol) completely satisfied the silkworm sterol requirement (Fujimoto, unpublished observation), and low stereospecificity in utilizing 24-methylcholesterols (campesterol and dihydrobrassicasterol) was demonstrated with *M. sexta* (Svoboda & Robbins, 1968). Ikan et al. (1970, 1971) found that in *Dermestes macultus* there was a preference for (24*R*)-alkylsterols over 24*S* isomers. It was also reported that *Drosophila melanogaster* utilizes both (24*R*)- and (24*S*)-methylcholest-5,7-dien-3-ol, while *Drosophila pachea* prefers the 24*S* isomers and *Drosophila mojavensis* prefers the 24*R* isomers (Kircher & Rosenstein, 1974).

Acknowledgments

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Stable RNA–DNA–RNA Polymerase Complexes Can Accompany Formation of a Single Phosphodiester Bond†

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ABSTRACT: Incubation of RNA polymerase with poly[d(A-T)$_n$] template results in a binary enzyme–DNA complex. Further addition of the dinucleotide UpA and [α-^{32}P]UTP results in catalytic formation of the labeled trinucleotide UpApU until substrate exhaustion. In contrast, incubation of binary enzyme–DNA complexes with ApU and [α-^{32}P]ATP results in labeled ApUpA formation to an extent that is stoichiometric with the amount of enzyme present despite an excess of substrates. The occurrence of ApUpA in a stable DNA–enzyme–RNA ternary complex is shown by gel exclusion chromatography, Millipore filtration, and the ability of ternary complexes to support subsequent RNA chain elongation. Radioactivity is not bound to Millipore filters when purified, labeled ApUpA is added to enzyme–DNA binary complexes. Hence, phosphodiester bond formation is required for stable ternary complex formation. The absence of the σ subunit of RNA polymerase or the addition of rifampicin to the reaction before ribonucleotide substrates results in catalytic ApUpA formation instead of stable ternary complexes.

DNA-dependent RNA polymerase is possibly one of the most extensively studied enzymes in *Escherichia coli*; for example, see Chamberlin (1976). Nevertheless, uncertainty remains as to how RNA chain initiation occurs and whether this step in DNA transcription can be regulated at a level beyond promoter recognition.

Kinetically the formation of the first phosphodiester bond is much faster than the formation of open promoter complexes by RNA polymerase (Rhodes & Chamberlin, 1975) even in instances in which there are relatively high rates of dissociation from open promoter complexes (Seeburg & Schaller, 1975). This difference in rates has been used to argue that RNA chain initiation is an almost certain event after proper promoter recognition and therefore unlikely to be a point at which regulation might occur (Chamberlin et al., 1976; Seeburg et al., 1979).

Recently, a phenomenon termed abortive inititation has been discovered in which RNA polymerase has been shown to form and release short oligonucleotides rather than to continue to elongate RNA chains (Johnston & McClure, 1976). These events occur when RNA polymerase is bound to natural promoters either in the presence or in the absence of the initiation inhibitor rifampicin. It is evident that the formation of a stable RNA–DNA–RNA polymerase ternary complex is not obligatory after promoter complex formation in the presence of ribonucleoside 5′-triphosphates even though such complexes can be formed after many rounds of phosphodiester bond formation (Rhodes & Chamberlin, 1974). The process of abortive inititation is of interest to us because of the potential regulatory implications accompanying formation of the first phosphodiester bond as well as the possible priming activities anticipated for the abortive oligoribonucleotides produced in this fashion.

It is experimentally difficult to dissect out determinants of the rate of formation of the first phosphodiester bond from those related to steps just before initiation and those that occur just after initiation (Chamberlin, 1974; Krakow et al., 1976). Early efforts to deduce properties of RNA chain initiation were based upon indirect assays which often used rifampicin as an indicator of the first phosphodiester bond formation since the drug could be shown to inhibit RNA chain initiation but not elongation. These studies led So & Downey (1970) to suggest that a stable ternary complex can accompany the formation of a single phosphodiester bond. Analogous rifampicin challenge experiments (Mangel & Chamberlin, 1974) have been complicated by the finding that rifampicin not only does not prevent phosphodiester bond formation but also stimulates the abortive variant of this reaction to the extent that such oligoribonucleotides are the major reaction product (Johnston & McClure, 1976; McClure & Cech, 1978). Evidently a direct assay is needed for the formation of the first phosphodiester bond in RNA chain initiation. Direct measurements

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