References and Notes

1. E. T. Krebs and E. T. Krebs, Jr., U.S. patent 2,985,664, issued in 1961; British patent 788,855, issued in 1958.
2. E. Rottman, Ed., *FDA Consumer*, December 1976–January 1977, pp. 5-9.
3. R. Campbell and W. M. Haworth, *J. Chem. Soc.* **125**, 1337 (1924).
4. R. B. Conrow and S. Bernstein, *J. Org. Chem.* **36**, 863 (1971); L. R. Schroeder and J. W. Green, *J. Chem. Soc. C* (1966), p. 530.
5. I. Parikh, D. W. MacGlashan, C. Fenselau, *J. Med. Chem.* **19**, 296 (1976); C. Fenselau, S. Pallante, I. Parikh, *ibid.* p. 679.
6. A Nahrstedt, *J. Chromatogr.* **50**, 552 (1970).
7. S. Billets, P. S. Lietman, C. Fenselau, *J. Med. Chem.* **16**, 30 (1973).
8. NMR's were run in [$^2H_6$]dimethyl sulfoxide on a Perkin-Elmer R12B 60-megahertz instrument with a Nicolet Technology Corp. TTI Fourier-transform attachment.
9. Merck and Co., Inc., Rahway, N.J.
10. Varian MAT 112 mass spectrometer operating in the CI mode.
11. R. P. Batzinger and E. Bueding, *J. Pharmacol. Exp. Ther.* **200**, 1 (1977).
12. B. S. Ames, J. McCann, E. Yamasaki, *Mutat. Res.* **31**, 347 (1975).
13. W. F. Benedict, M. S. Baker, L. Haroun, E. Choi, B. N. Ames, *Cancer Res.* **37**, 2209 (1977).
14. A. Nahrstedt, *Arch. Pharm. Chemie* **308** (No. 12), 903 (1975).
15. "Commissioner's Decision on Status of Laetrile," signed by Donald Kennedy, commissioner of Food and Drugs, was published in the *Federal Register* [**42**(No. 151), 39768 (5 August 1977)]. Further information may be obtained from Tenny P. Neprud, Compliance Regulations Policy Staff (HFC-10), Food and Drug Administration, Department of Health, Education, and Welfare, 5600 Fishers Lane, Rockville, Md. 20857.

16. We thank E. Bueding and D. F. Covey for consultation. Supported in parts by NIH grants GM-21248, GM-70417, and GM-16492.

* Address correspondence to W.R.B. or address in (*15*).

20 June 1977; revised 29 August 1977

3β-Hydroxy-5α-cholestan-6-one: A Possible Precursor of α-Ecdysone Biosynthesis

Abstract. *The conversion of cholesterol into 3β-hydroxy-5α-cholestan-6-one has been demonstrated to occur in prothoracic glands of last instar larvae of the silkworm,* Bombyx mori. *Incubation of glands containing radiolabeled 3β-hydroxy-5α-cholestan-6-one results in the disappearance of this sterol from the glands and the concomitant appearance of radiolabeled α-ecdysone in the medium. The observations suggest that the sterol is an intermediate in the synthesis of α-ecdysone.*

Since the elucidation of the structures of α- and β-ecdysone (*1*), a number of synthetic cholesterol derivatives have been studied as possible intermediates in the conversion of cholesterol to ecdysone (*2*). However, no attempts have been made to identify the intermediates of α-ecdysone synthesis in the prothoracic gland, which is a major site of α-ecdysone production (*3, 4*). Organ culture of prothoracic glands facilitates biosynthetic studies of this type, and has been used in this investigation of α-ecdysone synthesis in the prothoracic gland of the silkworm, *Bombyx mori*.

We have demonstrated that *Bombyx* prothoracic glands produce a large amount of α-ecdysone when the glands are cultivated in medium containing hemolymph (*3*). This, together with related reports (*5, 6*) and the observed presence of cholesterol in hemolymph (*7*), has led to the general assumption that cholesterol is a precursor of ecdysone synthesis. To further substantiate the role of cholesterol in ecdysone synthesis, prothoracic glands (day 9, fifth instar) were cultured in Wyatt's insect culture medium in the presence and in the absence of cholesterol. The glands were maintained in culture for 5 days before α-ecdysone was extracted from the medium and bioassayed by Ohtaki's method (*8*). The results (Table 1) indicated that inclusion of cholesterol, emulsified with Tween 80, in the culture medium enhanced ecdysone production four- to fivefold to levels similar to those obtained in pure hemolymph. However, when the glands were cultured in medium to which only the emulsifying agent Tween 80 was added, a similar enhancement was observed. These data suggest that the major precursors for ecdysone synthesis are contained within the gland, and no extraneous supply is required during the 5-day incubation period. The role of Tween 80 in activating ecdysone synthesis is not understood.

The extent of cholesterol incorporation from the medium into α-ecdysone was investigated by incubating 30 pairs of prothoracic glands in Wyatt's medium containing [4-^{14}C]cholesterol (4.125×10^7 dis/min, 380 nmole) emulsified with Tween 80. After 5 days, α-ecdysone was extracted from the medium and purified by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) (*9*). The amount of α-ecdysone recovered was 7.6 nmole, estimated by the ultraviolet absorbance of the HPLC fraction (*10*). This represented 2 percent of the total cholesterol (380 nmole) available in the medium. The radioactivity associated with the α-ecdysone fraction was 3.4×10^3 dis/min, indicating conversion of 0.0082 percent of the labeled cholesterol into α-ecdysone. From these data it can be calculated that only 0.4 percent of the total α-ecdysone produced was derived from the cholesterol provided in the medium. Thus the major precursor of α-ecdysone was not the cholesterol contained within the incubation medium.

Table 1. Effect of cholesterol and Tween 80 on the synthesis of α-ecdysone by prothoracic glands. Glands were cultured in Wyatt's medium containing cholesterol (100 μg/ml) emulsified with Tween 80 (20 μg in 1 percent ethanol), or only Tween 80 (20 μg/ml in 1 percent ethanol). The amount of hormone is expressed as nanograms of α-ecdysone per pair of glands.

Medium	Gland cultures (pairs)	Amount of hormone
Wyatt's	23	15
Wyatt's + hemolymph*	22	81
Wyatt's + cholesterol + Tween 80	28	70
Wyatt's + Tween 80	28	67

*See (*3*).

When last instar larvae were injected with labeled cholesterol, an unknown labeled sterol was found to accumulate in the prothoracic glands. Incubation of such glands resulted in the disappearance of this compound from the glands and the concomitant appearance of labeled α-ecdysone in the culture medium (Fig. 1). The radioactivity associated with the α-ecdysone fraction was 118 dis/min per gland (*11*), which was 4 percent of that present in the labeled sterol fraction of the glands (2960 dis/min per gland) before incubation. This conversion ratio far exceeded that (0.0082 percent) obtained with cholesterol in the medium, and identified the unknown sterol as a possible precursor of α-ecdysone.

The unknown sterol was purified and chemically identified by a combination of chromatographic and mass-spectrometric procedures. Prothoracic glands (400) were removed from larvae (day 9, fifth instar) and extracted with chloroform and methanol (2:1), and the extract was applied to TLC plates (the developing solvent was benzene and acetone, 2:1). The labeled unknown steroid (*12*) was put through the procedure at the same time as a marker. The fraction corresponding to the unknown sterol was eluted and subjected to HPLC together with the labeled marker; it was then collected and analyzed by gas chromatography–mass spectrometry (GC-MS). The presence of peaks at 402 (M), 387 (M−15), 384 (M−18), 369 (M−15−18), 289 (M−113), and 271 (M−113−18) indicated that the unknown sterol has a cholesterol skelton with two oxygen functions. The unknown compound was silylated with trimethylsilylimidazole at

100°C for 1 hour and analyzed by GC-MS. The gas chromatogram of the silylated derivative revealed two peaks (I and II), whereas only one peak was evident before silylation. In the mass spectra of the silylated derivative, the presence of peaks at 546 (M), 531 (M−15), 517 (M−29), 456 (M−90), 441 (M−90−15), 416 (M−113−15), and 336 (M−90−90) in peak I and at 474 (M), 459 (M−15), 445 (M−29), 384 (M−90), 369 (M−90−15), and 331 (M−113−15−15) in peak II indicated that peak II corresponds to the monotrimethylsilyl and peak I to the enol-trimethylsilyl derivative of peak II. These data, together with the knowledge that α-ecdysone has the keto group situated at the C-6 position (*1*), suggested that the unknown sterol may be closely related to 3β-hydroxy-5α-cholestan-6-one or its 5β isomer. These compounds were synthesized (*13*) and compared with the unknown sterol by TLC, HPLC, gas-liquid chromatography (GLC), and GC-MS. The properties of the synthetic 5α isomer and its trimethylsilyl derivative were identical with those of the unknown sterol in each system. Furthermore, when the labeled unknown sterol was subjected to TLC (*14*) and HPLC (*15*) with the synthetic 5α and 5β isomers as markers, the peak of radioactivity coincided with the peak of the synthetic 5α compound. Thus the cumulative data clearly indicate that the unknown sterol is 3β-hydroxy-5α-cholestan-6-one.

The possibility that the 6-one is a precursor was further investigated by conversion of 3β-hydroxy-5α-[4-^{14}C]cholestan-6-one into α-ecdysone in vitro. Forty pairs of prothoracic glands were incubated for 5 days in 1 ml of Wyatt's medium containing the labeled 6-one (2.14×10^7 dis/min, 175 nmole) emulsified with Tween 80; the sterol was prepared from [4-^{14}C]cholesterol (*16*). After purification of α-ecdysone from the medium (*17*), the radioactivity associated with the α-ecdysone fraction was 3.00×10^3 dis/min, which indicated conversion of 0.014 percent. The incorporation ratio was only a little higher than that of [4-^{14}C]cholesterol (0.0082 percent), but this may be due to the dilution of the radioactive 6-one with the endogeneous sterol accumulated in the glands before cultivation (Fig. 1).

The demonstration that 7-[^{3}H]-dehydrocholesterol may be incorporated into β-ecdysone, together with the observation that in some insects cholesterol is metabolized to 7-dehydrocholesterol, has led to the proposal that 7-dehydrocholesterol is an intermediate in ecdysone biosynthesis (*18*). However, the significance of this pathway has not been clearly established (*6*). In the investigation reported here the metabolism of labeled cholesterol into 3β-hydroxy-5α-cholestan-6-one was demonstrated in vitro, and the disappearance of this compound from incubated prothoracic glands was correlated with the appearance of α-ecdysone. Thus it can be concluded that the 6-one is a possible precursor of α-ecdysone biosynthesis in prothoracic glands.

SYO SAKURAI
Biological Institute, University of Tokyo, Komaba, Meguro, Tokyo, Japan
NOBUO IKEKAWA
Laboratory of Chemistry for Natural Products, Tokyo Institute of Technology, Meguro, Tokyo
TETSUYA OHTAKI*
Department of Entomology, National Institute of Health, Shinagawa, Tokyo
HARUO CHINO
Biochemical Laboratory, Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan

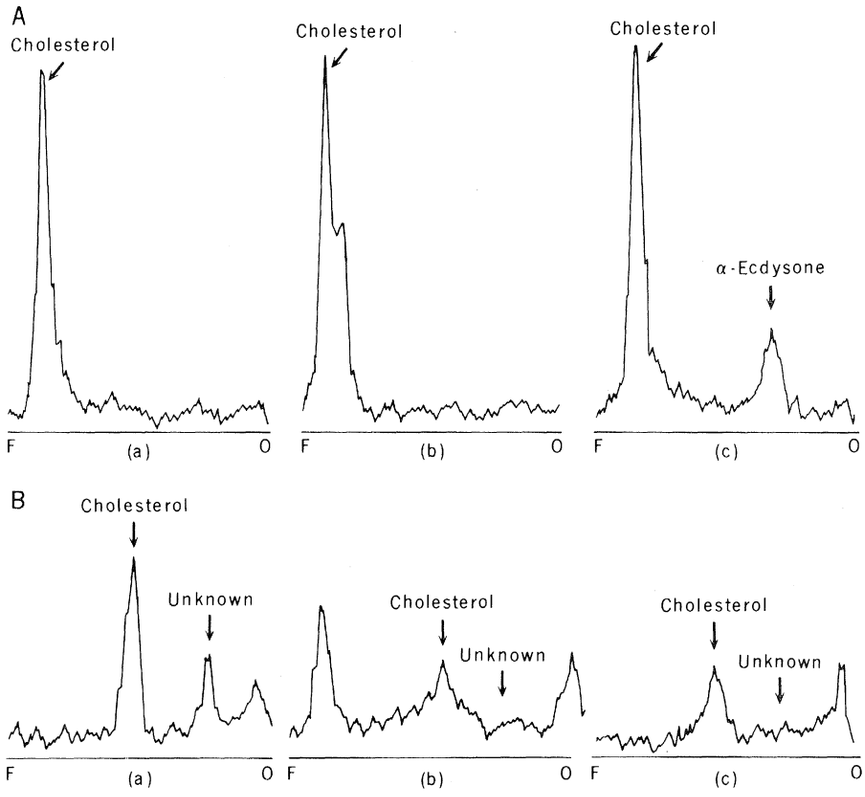

Fig. 1. Thin-layer radiochromatograms of extracts of prothoracic glands and incubation medium. Larvae were injected with [4-^{14}C]cholesterol (1×10^6 dis/min) emulsified with Tween 80 (1 μg in 5 μl of 10 percent isopropanol in water for each animal) between day 4 and day 8 (just spinning) of the last instar. On day 9, 39 glands were homogenized in chloroform and methanol (2:1) and extracted with the same solvent (a). Another 49 glands were cultured in hemolymph medium (*3*) for 5 days, and the glands (b) and medium (c) were extracted separately. These extracts were subjected to TLC (silica gel plate, Merck) and developed with chloroform and methanol (4:1) (A). The region corresponding to cholesterol was eluted with tetrahydrofuran and further separated by TLC using benzene and acetone (10:1) (B). *O*, origin; *F*, solvent front.

References and Notes

1. R. Huber and W. Hoppe, *Chem. Ber.* **98**, 2403 (1965); F. Hampshire and D. H. S. Horn, *Chem. Commun.* (1966), p. 37.
2. For general reviews of the metabolism of steroids in insects, see M. J. Thompson, J. N. Kaplanis, W. E. Robbins, J. A. Svoboda, *Adv. Lipid Res.* **11**, 219 (1974); D. H. S. Horn, in *Naturally Occurring Insecticides*, M. Jacobson and D. G. Crosby, Eds. (Dekker, New York, 1971); M. N. Galbraith, D. H. S. Horn, E. J. Middleton, J. A. Thomson, J. S. Wilkie, *J. Insect Physiol.* **21**, 23 (1975).
3. H. Chino, S. Sakurai, T. Ohtaki, N. Ikekawa, H. Miyazaki, M. Ishibashi, H. Abuki, *Science* **183**, 529 (1974).
4. D. S. King and E. P. Marks, *Life Sci.* **15**, 147 (1974); D. S. King, W. E. Bollenbacher, D. W. Borst, W. V. Vedeckis, J. D. O'Conner, P. I. Ittycheriah, L. I. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 793 (1974); D. W. Borst and F. Engelmann, *J. Exp. Zool.* **178**, 413 (1974).
5. P. Karlson and H. Hoffmeister, *Z. Physiol. Chem.* **331**, 298 (1963); A. Willing, H. H. Rees, T. W. Goodwin, *J. Insect Physiol.* **17**, 2317 (1971); K. Nakanishi, H. Moriyama, T. Okauchi, S. Fujioka, M. Koreeda, *Science* **176**, 51 (1972).
6. D. H. S. Horn, E. J. Middleton, J. A. Thomson, *J. Insect Physiol.* **20**, 2433 (1974).
7. H. Chino, S. Murakami, K. Harashima, *Biochim. Biophys. Acta* **176**, 1 (1969).
8. T. Ohtaki, *Jpn. J. Med. Sci. Biol.* **19**, 97 (1966).
9. The HPLC conditions for α-ecdysone were: instrument, DuPont 830 liquid chromatograph; column, Zorbax SIL (25 cm by 2.1 mm inside diameter); mobile phase, CH_2Cl_2 and CH_3OH (8:1); flow rate, 0.3 ml/min; column pressure, 1000 pounds per square inch; column temperature, 20°C; and detector, ultraviolet photometer at 254 nm.
10. The material of this fraction was identified as α-ecdysone by cochromatography with α-ecdysone and mass spectrometry. The cochromato-

gram showed a single peak. The prominent peaks at 464 (M), 446 ($M-H_2O$), 431 ($M-H_2O-CH_3$), 428 ($M-2H_2O$), 348 ($M-C_6H_{12}O_2$), and 330 ($348-H_2O$) coincided with those of α-ecdysone.

11. α-Ecdysone from the peak in Fig. 1A, part c, was further purified by HPLC (*9*) and the radioactivity associated with the α-ecdysone fraction was determined. Identification was carried out by the methods described above (*10*).
12. The labeled unknown steroid was prepared by incubating prothoracic glands with [4-^{14}C] cholesterol emulsified with Tween 80 in Wyatt's medium.
13. 3β-Hydroxy-5α-cholestan-6-one was synthesized by the method of E. A. Constantine and L. F. Fieser [*J. Am. Chem. Soc.* **76**, 532 (1954)]. The 5β isomer was prepared by treating the 5α isomer with 5 percent KOH in methanol for 1 hour. The 5β isomer was purified by use of a silicic acid column after several recrystallizations to remove the 5α isomer. The yield was less than 2 percent. The nuclear magnetic resonance spectrum ($CDCl_3$) showed the following shifts (parts per million): 0.64 [(C-18)H_3], 0.84 [doublet, *J* 5 hertz, (C-26/27)H_6], 0.89 [(C-19)H_3], 4.10 [multiplet, (C-3)H]. The retention times in GLC (1.5 percent OV-1, 180 cm by 4 mm inner diameter, 270°C) were 10.3 minutes for the 5β isomer and 11.8 minutes for the 5α isomer. Partial isomerization of the configuration at the C-5 position occurred in both isomers during GLC.
14. Thin-layer chromatograms were developed in three solvent systems: hexane and ethyl acetate (2:1), benzene and acetone (5:1), and chloroform and methanol (15:1). The R_F values of the 5α and 5β isomers were 0.16 and 0.21, 0.29 and 0.38, and 0.51 and 0.63, respectively.
15. The labeled unknown sterol was cochromatographed in HPLC with the 5α and 5β isomers and fractionated at each 0.5 minute. The conditions were as described above (*9*) except that the solvent system was CH_2Cl_2 and CH_3OH (99:1). Each fraction was analyzed by GLC to determine the amount of the 6-one, and the remaining part of each fraction was counted for radioactivity. The 5α isomer was eluted at 9.5 to 10.0 minutes and the 5β isomer at 8.0 to 8.5 minutes.
16. M. Morisaki, A. Saika, K. Bannai, M. Sawamura, J. Rubio-Lightbourn, N. Ikekawa, *Chem. Pharm. Bull.* **23**, 3272 (1975).
17. α-Ecdysone was extracted and partially purified by TLC (*3*). The α-ecdysone region from TLC was submitted to HPLC (*9*) and fractionated at 1-minute intervals to determine the radioactivity.
18. W. E. Robbins, J. N. Kaplanis, J. A. Thomson, J. S. Thompson, *Adv. Res. Entomol.* **16**, 53 (1971).
19. Supported by a grant from the Ministry of Education, which we greatly appreciate.

* Present address: Department of Biology, University of Kanazawa, Kanazawa, Japan.

13 June 1977

Visual Association Cortex and Vision in Man: Pattern-Evoked Occipital Potentials in a Blind Boy

Abstract. *In a 6-year-old child who had been blind since the age of 2 years, occipital potentials of normal amplitude and waveform could be evoked not only by diffuse light flashes but also by alternating checkerboard and sinusoidal grating patterns of low spatial frequency. Computerized tomography demonstrated destruction of the occipital lobes except of the primary visual projection area. Thus, in man, destruction of visual association cortices may result in loss of vision with partial preservation of pattern-evoked occipital potentials.*

The mammalian visual cortex is divided into anatomically distinct primary and associative areas (*1*). In primates, the striate cortex (area 17) is the primary receiving area of the geniculo-cortical visual pathways (*2*). Prestriate "association" cortices (areas 18 and 19) are supposed to have cognitive functions (*3*). When, in the monkey, areas 18 and 19 are ablated while area 17 is spared, visual acuity and the ability to sort objects is preserved (*4*). Thus, in the subhuman primate, some aspects of vision may be mediated by area 17 independently of the association cortices. Whether this is true for the human is unknown.

It is believed that the evoked potentials (EP's) that result in humans from repetitive stimulation with grating or checkerboard patterns originate from the occipital cortex (*5*). Grating patterns have been used extensively in physiological studies of elementary aspects of information processing in the human and subhuman visual systems (*6*). These patterns are optimal stimuli for width and orientation-selective neurons of both primary and association areas of the visual cortex (*7*). The variation of EP's with parameters of grating stimuli parallels their psychophysically determined visual detectability in normals and many patients with cerebral lesions (*8*). Whether pattern-evoked potentials can validly be used for assessing the competence of all areas of the visual cortex of man, or only the functions of area 17, is unknown. An unusual combination of circumstances provided a rare opportunity to explore these questions concerning (i) vision in a human without the association cortices and (ii) the diagnostic use of EP's when the association cortices are damaged.

A boy was left deaf and blind after an acute febrile illness when he was 2 years old. When he was examined at the age of 6 years, hearing and motor coordination had apparently returned to normal, but vision had not: observation revealed a child depending entirely upon auditory, tactile, and kinesthetic cues in coping with and moving around in the environment. When walking in a fully lit room, the patient did not avoid any obstacles. In a dark room he did not localize bright light. There was no blink reflex to light or threatening movements. These findings were in marked contrast to the behavior of patients with cerebral blindness, who often say they cannot see, but nevertheless are able to avoid obstacles and sometimes detect the presence of an object if it is in motion (*9*). Reexamination 6 months later showed no change (*10*). He is blind.

Neuro-ophthalmologic evaluation revealed that his eyes moved conjugatedly in all directions of gaze. Reliable optokinetic nystagmus (OKN) could not be elicited (*11*). Convergence was noted when his own finger was moved in front of him by the examiner; no convergence was noted when any other object was shown to him. His fundi were normal. Electroretinography and occipital EP tests with flash stimuli (Fig. 1A) yielded essentially normal results (*12*). The electroencephalogram (EEG) showed alpha-range activity in the usual distribution during eye closure. Furthermore, and quite unexpectedly, EP's of normal amplitude and waveshape were obtained when he was placed in front of alternating coarse checkerboard (*13*) or alternating vertical grating patterns (*14*) (Fig. 1B). The responses were no longer seen when he turned his eyes away from the screen or closed his eyes. Evoked potentials were also unrecordable when the patterns were made finer (that is, higher in spatial frequency).

Computerized tomography (CT scan) of the brain demonstrated that the occipital lobes had been almost entirely destroyed. Some tissue had been spared medially, mainly or entirely to the left of the midline; this preserved tissue corresponded to part of the optic radiation and area 17, or striate cortex (*15*) (Fig. 2). Destruction of areas 18 and 19 appeared complete on the right, whereas on the left there was some tissue preserved. No lesions were demonstrable anterior to the occipital lobes.

The almost complete absence of areas 18 and 19 suggests that the pattern-evoked potentials that were recordable over the scalp originated from area 17 (*16*). However, the spared striate cortex that was apparently sufficient to generate recordable electrical signals was not sufficient for vision (*17*). This dissociation between physiological and psychological functions (*18*) may be consistent with the classical concept that the visual association cortices are essential for seeing in the human (*3*). It is not consistent with most animal data, including that from primates. In adult monkeys, complete ablation of areas 18 and 19 does not lead to complete blindness, although it does prevent learning to discriminate between visual patterns, apparently because the pathway from the primary visual projection cortex to the inferotemporal cortex is interrupted (*19*). One conclusion from