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111. Nobuo Ikekawa,*1 Mieko Suzuki (née Saito), Masatoshi Kobayashi,*2 and Kyosuke Tsuda*3 : Studies on the Sterol of *Bombyx mori* L. IV.*4 Conversion of the Sterol in the Silkworm.

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Recently, the conversion of sterol in insects using labelled compounds have been studies by several workers. In the house fly, there is no evidence of the conversion of β-sitosterol in diets to cholesterol,[1,2] while in Virginia pine sawfly, β-sitosterol is converted to cholesterol,[3] and in German cockroach, β-sitosterol to cholesterol[4] and ergosterol to 22-dehydrocholesterol.[5] In the Silkworm, Bergmann has suggested that cholesterol might originate from sitosterol in diet from an evidence that the larva reared with a diet containing only phytosterol as a source of sterol contained cholesterol in the body.[6] In order to confirm this suggestion, we studied the conversion of β-sitosterol to cholesterol in silkworm larva and pupa using ^{3}H-β-sitosterol.

^{3}H-β-Sitosterol dissolved in olive oil was ingested twice in 42 larvae. These larvae were sacrificed one week after ingestion, extracted with methanol, and then reextracted with ether. The ether extract was fractionated with alumina column and radioactivity of the fractions obtained was counted. ^{3}H-β-Sitosterol dissolved in olive oil was also injected in 22 pupae and these pupae were treated by the same procedure as for larva one week after injection. The results obtained are presented in Table I.

Table I. Radioactivity of the Fractions from Ether Extracts of the Silkworm injected with ^{3}H-β-Sitosterol

Expt. No. / Fractions	I		II	
	Larva (mμc.)	Pupa (mμc.)	Larva (mμc.)	Pupa (mμc.)
Hexane (hydrocarbon)	319	368	13	0
Benzene (sterol ester)	7,223	5,800	5,250	3,900
Ether (free sterol)	1,122	4,414	1,275	4,450
Methanol (others)	460	586	975	0

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The radioactivity of both sterol ester and free sterol fractions obtained from ether extracts of larva and pupa was very strong, while that of other fractions was very weak, if present. Moreover, in the larva the activity of ester sterol fraction was stronger than that of free sterol, while the activity of both fractions, ester and free, was about same in the pupa. Cholesterol and β-sitosterol were separated effectively by gas chromatography from both ester and free sterol fractions. Radioactivity of each collected fraction was counted by liquid scintilation counter. The results are listed in Table II.

Table II. Radioactivity of Cholesterol and β-Sitosterol Fractions from the Silkworm injected with ^{3}H-β-Sitosterol

Expt. No.	Trapping fraction	Larva sterol		Pupa sterol	
		Free fraction (mμc.)	Ester fraction (mμc.)	Free fraction (mμc.)	Ester fraction (mμc.)
I	A (Cholesterol)	—	25.0	—	1.4
	B	—	14.5	—	4.5
	C (β-Sitosterol)	—	21.2	—	17.5
II	A (Cholesterol)	6.09	9.16	14.58	6.09
	B	3.58	4.02	8.99	5.31
	C (β-Sitosterol)	16.8	26.01	20.01	10.50

As shown in Table II, radioactivity of larval cholesterol was strong. This fact suggested that larva is able to convert β-sitosterol in the diet to cholesterol, showing larval cholesterol originates from β-sitosterol in mulberry leaf. However, different results were obtained on the radioactivity of cholesterol between free and ester fractions in the pupa. In the pupa, incorporation of ^{3}H-β-sitosterol into cholesterol in free sterol fraction was observed, while that in sterol ester fraction was not. As reported in the previous paper,[7] the slight incorporation of ^{14}C-acetate into digitonide fraction was recognized in both prepupal and pupal stages, though that was not in larval stage. These results suggest that the sterol metabolism in silkworm pupa might be differed from that in larva.

Experimental

Material——The larva immediately following the 4th moulting and pupa immediately after pupation were used as the material. These silkworms were taken from F_1 hybrid between two races, J. 124 and C. 124.

Preparation of ^{3}H-β-Sitosterol——^{3}H-β-Sitosterol used in the first experiment presented in Tables I and II was prepared by the Wilzbach procedure[8]. β-Sitosterol (100 mg.) was tritiated with 3 c. tritium gas in a dark place for 30 days at room temperature. Radioactivity of tritiated sterol was 2 mc./mg. after removal of excess tritium gas. In order to remove labile tritium, the tritiated sterol was dissolved in ethanol, precipitated as the digitonide with addition of digitonine, and was reisolated after liberation of digitonide with pyridine. Radioactivity of the recrystallized sterol from acetone-ethanol (1:1) was 271 μc./mg.

By-product, ^{3}H-dihydro-β-sitosterol, was removed by the following method[9,10]. Tritiated sterol was converted to chloride with phosphorus pentachloride and the chloride was dissolved in glacial acetic acid containing anhydrous potassium acetate. By this procedure, 3-β-chlorine of ^{3}H-β-sitosteryl chloride was converted to 3-β-acetoxy group, while 3-β-chlorine of ^{3}H-dihydro-β-sitosteryl chloride was inverted to α acetoxy group. After hydrolysis using 4% KOH solution for 2 hr., β-sitosterol without inclusion of dihydro-

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compound was obtained as its digitonide. The radioactivity of ^{3}H-β-sitosterol was 121 μc./mg. ^{3}H-β-sitosterol used in the second experiment presented in Tables I and II was a gift from Dr. Thompson.

Preparation of Silkworm Sterol Labelled with Tritium——First, 4.5 μc. of ^{3}H-β-sitosterol in 0.015 ml. of olive oil was ingested in each of 42 larvae. Second, 3.3 μc. of ^{3}H-β-sitosterol in 0.01 ml. of the oil was ingested in each animal after rearing with mulberry leaves for one day and then the silkworm larvae were reared for 1 week on mulberry leaves. The larvae were sacrificed, homogenized and extracted with methanol. Radioactivity of the extract was 29,400 mμc. The residue was reextracted with ether. Radioactivity of the extract was 21,000 mμc. The ether extract was fractionated with alumina (neutral Woelm) chromatography.

Further, each of 25 pupae was injected with 5 μc. of ^{3}H-β-sitosterol in 0.125 ml. of olive oil. One week after the injection, these pupae were treated by the same procedure as the larva, and the radioactivity of methanol and ether extracts from pupae were 26,000 mμc. and 20,000 mμc., respectively.

In the second experiment using the same method mentioned above, obtained almost similar results. In this case, pupae were sacrificed 10 days after injection.

Separation of Cholesterol and β-Sitosterol——For collection of cholesterol and β-sitosterol, both ester and free sterol fractions, separated by the column from each extract of silkworm larva and pupa, were fractionated by gas chromatography. The sterol ester fraction was hydrolyzed prior to the chromatography.

Collection method was much the same as the procedure of Fales, *et al.*[11] Shimadzu Gas Chromatograph Model GC-1C instrument with hydrogen flame ionization detector was used in this study. The glass column (180 cm. × 6 mm., i.d.) was packed with the 1.5% SE-30 on Gas Chrom P (80~100 mesh). The outlet was connected with a T-type joint, which was designed to split the flow of carrier gas in the ratio of 1:9 with two stainless steel capillary tubing of different diameters in column oven. The smaller branch of the joint was connected with the hydrogen flame detector and larger tube with a U-shaped glass trap using a silicone stopper tightly fitted to the glass tube with a hole allowing the tube of the joint to pass through. The trap was immersed in liquid nitrogen during collection of the sample. Argon was used as carrier gas. Sterols were condensed along with argon which was liquidfied due to the low temperature. The temperature of the column, detector and sample heater were 240°, 260° and 250°, respectively. Carrier gas flow rate was 100 ml./min. Each 500 μg. sample in acetone was repeatedly injected into the column with Hamilton microsyringe. The peaks of the chromatogram were rather broad due to the introduction of a large amount of samples. Three fractions, A (cholesterol), B and C (β-sitosterol) were separately collected as shown in Fig. 1. Radioactivity of each fraction was counted by liquid scintilation counter (Table II).

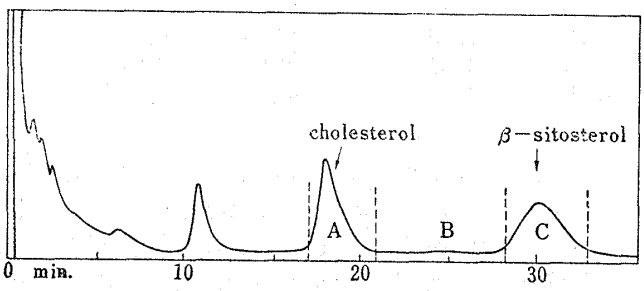

Fig. 1. Separation of Larval Sterols after Injection of ^{3}H-β-Sitosterol with a Preparative Column

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Summary

Conversion of β-sitosterol to cholesterol in the silkworm was demonstrated using ^{3}H-β-sitosterol. After ingestion of ^{3}H-β-sitosterol in the larva, the ether extract of homogenized larva was fractionated on alumina. The sterol fraction was separated into cholesterol and β-sitosterol by gas chromatography and the radioactivity of each collected fraction was counted. The strong radioactivity of the larval cholesterol fraction suggested that larva is able to convert β-sitosterol in the diet to cholesterol. But in the pupa incorporation of ^{3}H-β-sitosterol into cholesterol in free fraction was observed, while that in sterol ester fraction was not.

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