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Bombykol biosynthesis from deuterium-labeled (*Z*)-11-hexadecenoic acid

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Summary. (*Z*)-[11,12d_2]-11-Hexadecenoic acid was applied topically to the pheromone gland of silkworm pupa. After eclosion, the gland components were analyzed by capillary GC-MS. (*Z*)-11-Hexadecenoic acid, a characteristic fatty acid of the gland, was found to be a precursor of bombykol, (*Z*)-11-hexadecenol and bombyk acid.

Biosynthesis of bombykol, the sex pheromone of the female silkworm moth, was studied by Inoue and Hamamura 10 years ago using radiolabeled (1-^{14}C) palmitic acid. They demonstrated that palmitic acid might be a precursor of bombykol[2].
Previously we reported the presence of a characteristic fatty acid, (*Z*)-11-hexadecenoic acid, [(*Z*)-11-HDA], in the pheromone gland of female silkworm pupae and moths and proposed a possible biosynthetic route for bombykol, from palmitic acid via (*Z*)-11-HDA[3].
We report here some experiments using deuterium-labeling and capillary GC and GC-MS which verify that (*Z*)-11-HDA is a precursor of (*Z*)-11-hexadecenol[4], bombyk acid, bombykol, and its (*E,E*) isomer[5].
Materials and methods. The female pupae were kept at 25 °C and 2 days before eclosion, the oviposition and pheromone gland were everted by pressing the abdomen with the fingers. d_2-(*Z*)-11-HDA (0.3 µl) was applied topically to the gland. The treated female pupae were kept at 25 °C. 8 h after eclosion, the pheromone glands of adult female moths were excised and extracted with ether. A half aliquot of the extract was subjected to capillary GC and GC-MS analysis (JEOL D-300 with HP-5740 GC attached; PEG 20 M g-SCOT 25 m×0.28 mm i.d.) to identify aliphatic alcohols. To another half aliquot including the glands, chloroform-methanol (2:1) was added, and the extract was subjected to acid methanolysis to generate methyl esters from acylglycerides. The resulting methyl esters were analyzed by the same methods as those used for the alcohols.
11d, 12d (*Z*)-11-HDA was synthesized by coupling 1-hexyne with decamethylenbromohydrin. The produced hexadecynol was treated with deuterium gas in catalytic partial reduction. The resulting d_2-hexadecenol was oxdized to the d_2-HDA.
Results and discussion. Fatty acids. Fatty acids of pheromone gland treated with 11*d*, 12*d* (*Z*)-11-HDA were analyzed by capillary GC-MS. 6 major and 1 minor peaks were observed on the total ion chromatogram and identified by gas chromatographic retention times and by mass spectrometry. Ions with m/z 268(M$^+$), 237(M-31), 236(M-32), and 194(M-74) which are the 'diagnostic ions of methyl hexadecenoate' and those with m/z 270(M$^+$), 239, 238, and 196 which are the 'diagnostic ions of d_2-methyl hexadecenoate' were observed on the same mass spectrum of the peak at tR. 10.0 min. Molecular ion of methyl bombykate was m/z 266, but ion m/z 268 which is on '(M$^+$) of d_2-methyl bombykate' was also present in the same mass spectrum of the minor peak at tR. 11.9 min. The change of m/z 266 to 268 on the spectrum was about 15%. Ion m/z 268 was not observed in the mass spectrum of methyl bombykate that originated from the pheromone glands of the untreated female. These data suggest that the bombyk acid is synthesized from (*Z*)-11-HDA in the pheromone gland.
Alcohols. The pheromone gland components of adult female moths were analyzed under the same conditions as those for fatty acid analysis. Four diagnostic peaks (peak A, tR. 9.0 min; peak B, tR. 9.5 min; peak C, tR. 11.6 min; peak D, tR. 12.1 min) were observed on the total ion chromatogram. On the mass spectrum of peak B, ion m/z 222 and 224 were observed. Ion m/z 222 was a (M-18) ion of (*Z*)-11-hexadecenol which was one of the usual components in the pheromone gland. The relationship between the retention times of peaks B and A (hexadecanol) on capillary GC indicated that peak B was (*Z*)-11-hexadece-

nol. The ions showing m/z 222 and 224 on the mass spectrum corresponded to the 2 ions eluted on the gas chromatogram with identical retention times. Thus peak B was considered to be composed of (*Z*)-11-hexadecenol and d_2-(*Z*)-11-hexadecenol. (*Z*)-11-Hexadecenol was found to be biosynthesized from (*Z*)-11-HDA.
Ion m/z 238($M^{+\cdot}$ of bombykol) and m/z 240($M^{+\cdot}$ of d_2-bombykol) were observed in the mass spectrum of peak C. The retention time on gas chromatography of peak C was identical with that of synthetic bombykol. Identical results were obtained by mass spectrometry of peak D. Peak D was identified as the (*E, E*) isomer of bombykol. Mass chromatogram using ion m/z 238, 239, 240 and 241 showed that these 4 ions had identical retention times on peaks C and D. The change of ion m/z 238 to 240 was about 15% on both spectra. These data indicate that bombykol and its (*E,E*) isomer are biosynthesized from (*Z*)-11-HDA. The conversion of bombyk acid into bombykol has not been proved yet. Dehydrogenase and reductases in the reduction of bombyk acid to the alcohol, bombykol, are presumed to exist but it remains unknown. Bombykol was produced by applying the D_2 precursor to the silkworm pheromone gland. But where biosynthesis occurs in or on the pheromone gland remains unknown.

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Effect of salt concentration on binding of proteins to a non-ionic adsorbent

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Summary. The effect of NaCl concentration on the adsorption of several proteins to palmityl-substituted Sepharose 4B has been investigated. It has been observed that the degree of adsorption first decreases and then increases with increasing salt concentrations, followed by total immobilization. The results are qualitatively explained by the simple theory of Debye-Hückel-Kirkwood, as applied to poly-electrolytes in the presence of salt.

The technique of hydrophobic chromatography[2] has been advanced and proved useful by several laboratories as a method for protein purification[2-7]. Until fairly recently, the most frequently used technique for the preparation of hydrophobic affinity adsorbents with agarose involved coupling of alkyl- or arylamines to CNBr-activated agarose[8]. This provides a matrix with partially ionic character[9]. Desorption of proteins from such gels may therefore be achieved by including salt in the medium[9-11]. More recently, use of neutral adsorbents for protein purification[12-16] and immobilization[9-11] has been demonstrated. It has further been argued that such purely non-ionic adsorbents are preferable to those having mixed ionic and non-ionic characters[17,18].
Recently, we reported on the use of palmityl-substituted Sepharose 4B as a non-ionic matrix for protein adsorption[20,21]. An important property of this gel is that it may bind proteins in the absence of any additional salt. However, some of the proteins adsorbed were found to desorb at intermediate concentrations of NaCl[20].
In the present investigation, binding affinity of the adsorbent for 6 proteins, arbitrarily chosen from those which are not normally immobilized[20], has been examined at different NaCl concentrations.
Materials and methods. All resins, chemicals and biochemicals were exactly as described previously[20]. Palmityl-substituted Sepharose 4B was prepared as reported[20], with the difference that dioxane was dried and distilled over sodium. The chromatographic profiles of the proteins tested were obtained using small columns and following our previously reported procedure[20], except in that the original buffer consisted of 5 mM sodium phosphate, pH 7.0, for all proteins.
Results and discussion. It is evident from the results presented in the figure and the table that including increasing concentrations of NaCl in the elution buffer frist decreases and then increases the binding affinity of the gel for the proteins examined. An important property of the gel used in this study is that it is essentially uncharged. Also, the ionic strength of the buffer used was such that no electrostatic interactions between these proteins and the few negatively charged groups known to occur in Sepharose[18] could take place. Therefore, desorption of the proteins which are normally immobilized on the matrix[20], and increased elution of the proteins reported here, both occurring at intermediate salt concentrations, may not be due to quenching of electrostatic interactions between the interacting components. On the other hand, the results may be explained in terms of free energy changes occurring in the association process.
The total free energy change ΔF for association of a protein molecule with Sepharose-lipid matrix may be written as[22-24]

$$\Delta F = \Delta F_o + \Delta F_h + \Delta F_{es} \quad (1)$$

Protein peaks obtained from the elution profiles of proteins at different concentrations of NaCl. Conditions are described in the legend to the figure. (a) no additional salt; (b) 0.05 M NaCl; (c) 0.1 M NaCl; (d) 1 M NaCl; (e) 2 M NaCl and (f) 4 M NaCl.

Protein	Protein peak*					
	a	b	c	d	e	f
Cytochrome c	7	22.5	23.5	8.5	0	0
α-Amylase	22	36	33	15	10	0
Peroxidase	10.5	30	34	27	17	0
Urease	14	24	26	14	0	0

* Percent of total applied.