家蚕の代謝特性とその有効利用

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はしがきー研究の目的と成果の概要

昆虫などの無脊椎動物は現在の地球上で最も繁栄している生物群であり、脊椎動物、植物、微生物とは異なった特殊な生活史を営んでいる。この特異性の解明を本研究の基本的な目標にした。昆虫の中でも家蚕は古くから多面的に研究されており、かつ生産動物として活用されている。カイコガを家畜化し、その絹糸生産能を拡大させた知恵は今後の無脊椎動物を生物資源として開発利用する上の大きな指針となる。

家蚕は強大な絹タンパク質生合成能を保障すべく種種の生理機能を選択的に発達させている。これらの機能発現に与る代謝系なり、代謝産物の同定並びにその発現調節機構の解明は家蚕のタンパク質合成能の一層の強化に与ると共に家蚕の持つ潜在機能の開発利用にも貢献する。更にその成果は家蚕に留まるものではなく、昆虫、無脊椎動物の資源化につながる。

本研究班はこれまで家蚕の代謝調節について一貫して研究を進め、既にここに掲げた目標に取り組みつつある研究者によって組織された。それぞれの研究者が開発した実験系を有効に用い、指標とした代謝系をまづ一般生物学の知見と比較検討し、その上に立って家蚕や昆虫に特異的な代謝について深く検討した。

本研究では昆虫のペプチドホルモンの作用特性について、休眠ホルモンを対象にして検討した。ペプチドホルモンは環状ヌクレオチドを介してその作用を発現している。休眠ホルモンは環状ヌクレオチド代謝を調節しているが、その作用は第二情報物質としての作用ではなくて、むしろ環状ヌクレオチドの貯蔵量を調節していた。カルシュウムは環状ヌクレオチドと並んで細胞外の情報を細胞内へ伝達する機能を果している。この過程にカルモジュリンが関与していることは哺乳動物でよく知られている。昆虫にはカルモジュリンは存在しないとも言われていたが、本研究の結果は、家蚕には明らかにカルモジュリンが存在し、しかもその構成アミノ酸にメチルリジンを欠損している特異なものであることを明らかにした。

胚発生期における情報発現系は遺伝子発現機構の解析のモデルとして有効である。昆虫卵の多くはモザイク卵であるので、脊椎動物の発生とは異なり、母性mRNAを含めた情報物質の卵内での存在部位によって発生の運命の一部が決定されている可能性がある。この細胞質による細胞分化の決定の機構の解明は発生生物学の中心課題でもある。家蚕卵は胚発生の初期に休眠に入るので、休眠性を人為的に操作することにより胚発生のパターンを実験的に変更することもできる。これらを組み合わせた結果、胚の器官分化はmRNAとリボソームの利用増によること、また特異的なタンパク質の合成が一時的に見られることが明らかになった。

休眠は遺伝情報の発現停止のみでなく、特異的な代謝系の出現を伴っている。家蚕卵で見られるグリコーゲン　　ソルビトール　　グリコーゲン系はその代表的なものである。ソルビトールからグリコーゲンへの変換は低温刺激によってもたらされるが、5'と0°Cでは全く異なった結果を与えた。つまり5°Cでの一定期間の保護は休眠覚醒と同時にソルビトール脱水素酵素の誘導によるソルビト

ールのグリコーゲンへの変換をもたらしたが、0°Cはいずれに対しても無効で合った。温度環境情報による生体制御はこれまで多くの生物で知られているが、本研究の結果は、酵素誘導がきわめて特異的な低温刺激によってもたらされることを明らかにしたものであり、温度刺激による代謝調節の機構解明に新たな視点を与えた。

昆虫はその免疫機構を脊椎動物とは異にしている。プロテアーゼインヒビター系が生体防御系に関与していることは最近の研究から明らかにされつつある。

本研究では家蚕の血中で強い活性を示すキモトリプシンインヒビターを取り上げ、その精製と化学的同定を行った。血中のインヒビターは脂肪体で生合成されること、この生成をエクジステロイドが促進していることを明らかにした。

さらに、血中に存在するプロテアーゼインヒビターの一種であるカイコアンチトリプシンの全アミノ酸配列をcDNAのヌクレオチド配列より決定した。この配列をすでに解明されている人の血清中の1ーアンチトリプシン、1ーアンチキモトリプシン及びアンチトロンビンのアミノ酸配列と比較し、本インヒビターが無脊椎動物で同定された最初のセルピンであると確認された。トリプシントの反応部位はLys 343-Val 344であることが判明したが、Arg 340-Ser 341配列が近傍に見られたので家蚕体内での標的プロテアーゼとの関係が今後の課題となった。

昆虫の発育は脱皮、変態によって完了する。ステロイドホルモンはこれらの発育制御にかけがえのない作用をしている。卵巣や卵にもエクジステロイドは存在している。卵エクジステロイドの同定の結果、側鎖のC－22位に水酸基を持たない2種が発見された。そのうちの1つは、(20S)-cholesta-7,14-diene-3β,5α,6α,20, 25 pentanolで、これは家蚕卵で初めて発見されたものであり、ボンビコステロンと命名された。また卵巣中では多くの結合型エクジステロンは22-phosphateであったが、ボンビコステロン等は3-phosphateとして存在した。

精子の成熟に与る一連の生体反応の解析は昆虫の生殖機能の全体像を知る上で重要な課題である。精包の形成と機能について検討したところ、精包では細胞外解糖系が作動してエネルギー供給が行われている。これは高等動物における細胞外解糖系の存在を示した最初の報告である。また、この解糖系と供役したアルギニン分解カスケードが存在し、イニシャトリンと命名した特異的なプロテアーゼとカルボキシペプチダーゼによってアルギニンは供給されている。イニシャトリンは無核精子の運動性の獲得、有核精子束の解離、カルボキシペプチダーゼの活性化等に当たっている。

以上が本研究班が3か年間に渡って得た研究成果の概要である。個別成果の詳細は以下の報告に詳細に述べられている。家蚕を対象としてもその特異性が多くの場面で認められった。この種の研究をさらに進めることによって家蚕の潜在機能の開発の理論化および昆虫、無脊椎動物の代謝特性はさらに体系化されるであろう。本研究がこの分野の研究の発展の礎となることを願っている。

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Delayed Action of Diapause Hormone on Cyclic GMP Metabolism in Developing Ovaries of the Silkworm, *Bombyx mori*

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INTRODUCTION

Diapause hormone (DH) is known to be a neuropeptide hormone which is responsible for induction of embryonic diapause in silkworm, *Bombyx mori* (Yamashita and Hasegawa,1985). Although the chemical structure of this hormone has not yet been fully elucidated, its biochemical actions are successively elucidated using the highly purified preparations. Enhancement of trehalase activity in developing ovaries of silkworms is one of the mosr conspicious action of this hormone (Yamashita et al.,1981).

Expression of peptide hormone information is mediated through the action of cyclic nucleotides in many biologica; systems. In insects, some peptide hormones are known to express their functions through the enhanced levels of cyclic AMP (cAMP) and cyclic GMP (cGMP) (Steele, 1985). Thus, it seems resonable to presume that DH affects the cyclic nucleotide metabolism to regulate the following biochemical events leading to diapause initiation of silkworm eggs.

Here, we describe that cAMP and cGMP behave quite differently during ovarian development and DH exerts its regulatory function on their metabolisms.

MATERIALS AND METHODS

Animals: Two silkworm races were used. One was a diapause

type (Kinshu and Showa) which was induced by incubating their embryonic life at a high temperature and the other was a non-diapause type (N_4). The diapause nature was further experimentally controlled either by ablivation or implantation of suboesophageal ganglion (SG) or injection of DH preparation.

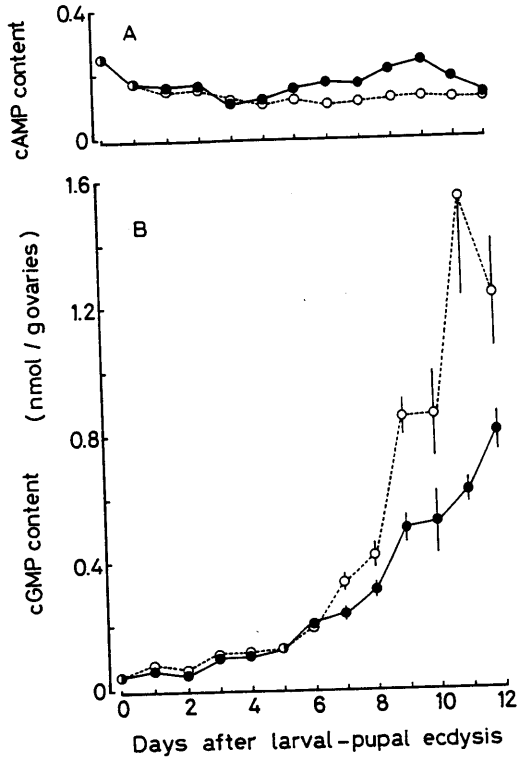
Determination of cyclic nucleotides: The developing ovaries were dissected throughout the pupal-adult development. Cyclic nucleotides were extracted with a cold 5 % TCA three times. After washing with ether to remove the acid, an aliquot of the extracts was used to measure cyclic nucleotides by the radioimmunoassay using respective antiserum. In some experiments, the same ovary preparation was used to determine trehalase activity and content of 3-hydroxykynurenie and glycogen according to Azuma and Yamashita (1982).

RESULTS

Effects of SG on developmental changes in cyclic nucleotides

Fig. 1 shows that cAMP and cGMP changed differently during the ovarian development. cAMP was present at a relatively high level in the young ovaries but declined slightly in the following few days. In the actively vitellogenic ovaries it increased and followed a decrease toward ovarian maturation. In contrast, a continmuous increase was found in cGMP and attaained the maximum level in the matured ovaries.

SG-removal on the day of pupation affected on these developmental profile; for cAMP it inhibited the increase in the vitellogenic ovaries but a great increase in cGMP was induced by it at the later stages of ovarian development. Further, SG-removal from the different ages of a hibrid animals exhibited the different effects on accumulation of cGMP in ovaries. The earlier the SG extirpated, the higher



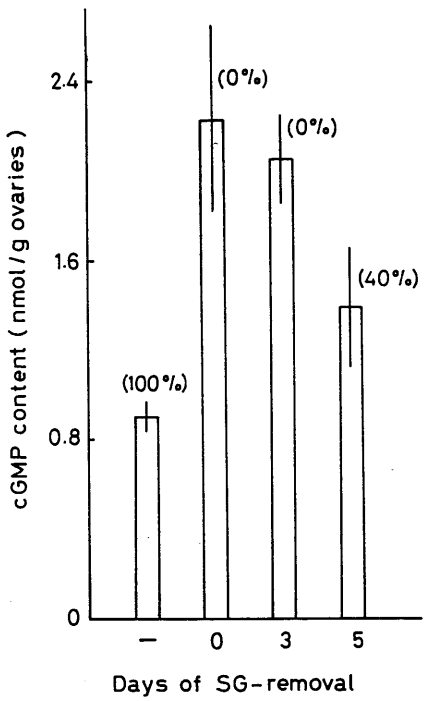


Fig. 1 (left). Effect of SG-removal on developmental changes in cAMP (A) and cGMP (B) in ovaries. (o), controls; (o),SG-removed.
Fig. 2 (right). Effect of SG-removal at different stages on cGMP content in ovaries. -, controls; Figures in parenthesis shows percentages of diapause eggs.

accumulation of cGMP occurred. The levels of cGMP apparently paralleled to the incidence of diapause eggs (Fig. 2).

SG implantation into N_4 pupae clearly decreased the content of cGMP in mature eggs. However, there was no change in cGMP content after SG-removal from N_4 (Fig. 3).

Effect of Diapause hormone on cyclic GMP content

Injection of highly purified DH preparation into day 4 N_4 pharate adults brought about a clear decrease in cGMP

content (Fig. 3). To compare the DH action on cGMP with the other matters, we injected DH prepartion into day 5 pharate

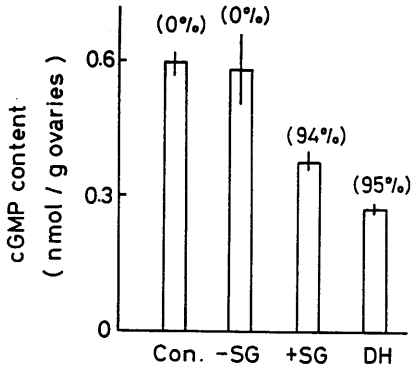


Fig. 3. Effects of SG-removal, SG implantation and DH injection into non-diapause pupae (N_4) on cGMP content. Figures in parenthesis show the percentage of diapause eggs induced.

adults whose SG had been extirpated on day 0. The effects were surveyed on cAMP, trehalase activity, 3-hydroxykynurenine and glycogen as well as cGMP at the various times after injection (Table 1). Hormones clearly decreased cGMP content in mature ovaries. Furthermore, a significant increase in cAMP on day 9, in trehalase activity on day 6, in glycogen and 3-hydroxykynurenine on day 11 was noted along with the incidence of diapause eggs.

Table 1. Effect of DH on cyclic nucleotides, trehalase activity, glycogen content and 3-hydroxykynurenine content in ovaries

	SG-removed	-SG + DH injection
cAMP (pmol/g)	120	145
cGMP (pmol/g)	1952	1555
Trehalase (nmol/g.min)	7.7	16.5
Glycogen (mg/g)	14.5	16.4
3-hydroxy-kynurenine (ug/g)	450	895
Diapause eggs (%)	0	60

Although data are not given, any significant changes in cGMP was observed in ovaries within 24 hr after DH injections, during which period trehalase activity rose more than two times.

DISCUSSION

The present results have clearly demonstrated that DH acts to regulate cyclic nucleotide metabolisms in developing ovaries of the sillworm, *Bombyx mori*. Although the more advanced effect is reflected on cGMP metabolisms, cAMP is also under the control of DH. Thereis a quite different response of these nucleotides to the hormone from view points of direction and times. Thus, DH exterts the different regulatory functions on cyclic nucleotide metabolism in ovaries.

However, it is not conceivable that the hormone function on biochemical shift providing the diapause induction is sequentially mediated through the changes in cyclic nucleotide metabolisms. Within 3 hr after hormone administration, a significant rise of trehalase activity is induced, which corresponds to the following accumulation of glycogen in oocytes. While, there is no change in cyclic nucleotide levels and it takes a few days to bring about an obvious change. Thus, the present effects of DH on cyclic nucleotides are likely due to the different action from those observed in trehalase stimulation, although the precise mechanisms remain to be resolved.

Several physiological functions of cGMP are proposed in various biological system (Deguchi,1984). Among them is the very intriguing the stimulatory action of cGMP on cell proliferation (Zeilig and Goldberg, 1977). The higher accumulation of cGMP in non-diapause eggs would be correlated with the continuous cell cleavage in the laid eggs. Our preliminary experiment indicates that the higher level of cGMP is maintained through the early stages in embryogenesis. An increase in post-vitellogenic follicles is comparable with the accumulation of ecdysteroids in the ovaries. Although the roles of ecdysteroids in embryonic development remaine unknown, they are proposed to play some

regulatory functions on cell division and organogenesis in embryos as the informational agents. There, DH is responsible for the incorporation of these informational substances into oocytes by which the following embryonic development will be controlled.

Anyhow, the present studies throw some new inshights on the peptide hormone action in insect system which is not included in the pathway of cyclic nucleotides as the sencondary messengers. In this sense, DH studies will develop the new model of the peptide hormone action which are mediated by the delyaed action.

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家蚕の発育卵巣の環状ヌクレオチド代謝に及ぼす休眠ホルモンの遅延効果

休眠性サナギから食道下神経節（ＳＧ）を摘出すると、卵巣中のcAMP及びcGMPの発育変動のパターンが変化した。cAMPは一時的に減少し、cGMPは卵成熟にともなって増加した。ＳＧの摘出時期を遅らせるとcGMPの増加割合は減少した。非休眠サナギへのＳＧの移植はcGMP量の減少をもたらした。休眠ホルモンの注射はcGMP量の減少、cAMP量の増加ならびにトレハラーゼ活性の上昇、３－ヒドロキシキヌレニン量、グリコーゲン量の増加をもたらした。以上の結果は、休眠ホルモンの環状ヌクレオチド代謝調節機構の特異性を示している。

家蚕のカルモデュリン並びにカルモデュリン依存性サイクリックヌクレオチド代謝酵素系に関する研究

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【緒言】

細胞膜を透過できないホルモンや化学伝達物質は，細胞表面に存在する特異的な受容体を介して細胞内へ情報を伝達し，その情報はさらに細胞内情報伝達物質により個々の酵素に伝えられる．この細胞内情報伝達系としては，情報伝達物質としてサイクリックヌクレオチド（ｃＡＭＰ 又はｃＧＭＰ）を用いる系とCa^{2+}を用いる系が知られている．　Ca^{2+}は，カルシウム受容タンパク質であるカルモデュリンと結合することにより多種の酵素活性を調節しているが，哺乳動物の脳等で，ｃＡＭＰ合成酵素（Adenylate cyclase）とｃＡＭＰ分解酵素（Phosphodiesterase（ＰＤＥ））の両者の活性がCa^{2+}-カルモデュリンにより調節されていることが明らかにされ，Ca^{2+}-カルモデュリン系とｃＡＭＰ系の両方の情報伝達系が密接に絡み合っていることが示唆されている．　ところが，無脊椎動物殊に昆虫においては，これらの情報伝達系についてはあまり詳しい研究がなされておらず，昆虫の代謝特性を理解し，その有効利用をはかる為には，これら情報伝達系を明らかにすることが必須であると考えられる．　そこで，本研究では，家蚕脂肪体よりカルモデュリンを単離し，その性質を明らかにすると共に，ｃＡＭＰによる情報伝達系に対するCa^{2+}の調節的役割を酵素学的手法により明らかにする．

【材料と方法】

家蚕(Bombyx mori 支１０８）はヤクルト(株)より購入した人工飼料を用いて，２８℃，短日条件下（８時間明，１６時間暗）で飼育したものを用いた．　ただし，カルモデュリンの調製には自然条件下で桑葉により飼育した交雑種を用いた．

カルモデュリン量は，ブタ脳より調製したカルモデュリン依存性ＰＤＥの活性化により求めた．この際，ブタ脳又は家蚕脂肪体より単離したカルモデュリンを標準として用いた．　アデニルシクラーゼ活性は[^{3}H]ＡＴＰを基質とし，生じたｃＡＭＰを既に述べた方法（Morishima, 1978）で単離し，その放射能を測定することにより求めた．　ＰＤＥ活性は[^{3}H]ｃＡＭＰあるいは[^{3}H]ｃＧＭＰを基質とし，既に述べた方法（Morishima, 1975）で測定した．

カルモデュリンの単離．　サナギ脂肪体５００ｇを１２００mlの４％トリクロル酢酸と共に磨砕し，遠心により沈殿を集め，５mM ＥＤＴＡ と１mM ＰＭＳＦを含む２０mM Tris-HCl,pH 7.5，にけん濁した．　遠心により不溶物を除いた後，DEAE-Sepharose CL-6B 及び，Phenyl-Sepharose によるクロマトグラ フィーを行い精製標品を得た．

【結果及び考察】

1．脂肪体カルモデュリンの性質

サナギ脂肪体より精製したカルモデュリンはＳＤＳ電気泳動及びｎａｔｉｖｅ電気泳動において単一のバンドを与えた．　その水溶液の紫外吸収スペクトル（図１）は２８０ｎｍに吸収極大を持たず，トリプトファンを含まないことを示している．　このカルモデュリンのアミノ酸分析の結果（表１）は，既に知られている種々の動物のカルモデュリンのものと類似していたが，トリメチルリジン（ＴＭＬ）が全く含まれていなかった（Bodnaryk & Morishima, 1984）．これは，それまでに分析された全ての動物由来のカルモデュリンにはＴＭＬが１分子含まれているという事実から考え，非常に興味のある点であり，このＴＭＬを欠くカルモデュリンが家蚕脂肪体にのみ特異的に存在するものであるかどうかを確かめるため，同じ鱗翅目昆虫の一種である Mamestra configurata のサナギ及び成虫頭部より同様の方法によりカルモデュリンを単離し，そのアミノ酸組成を調べたところ，ＴＭＬは含まれておらず，また，全体の組成は家蚕のものとほぼ同一であった（Morishima & Bodnaryk, 1985）．　一方，ショウジョウバエより単離されたカルモデュリンにはＴＭＬが１分子含まれている（Wandosell et al., 1986）ことから，ＴＭＬを欠くカルモデュリンは必ずしも昆虫界一般に分布するものではなく，一部の鱗翅目昆虫に特異的に見られるものではないかと考えられる．

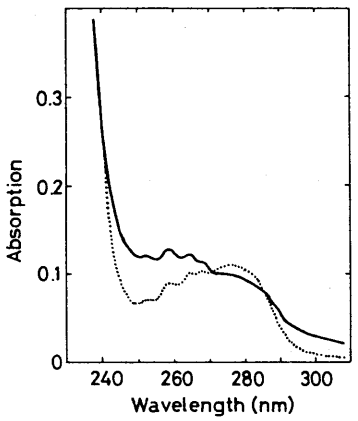

図１　Absorption spectra of calmodulins. Solid line, fat body calmodulin from pupae of *B. mori*, 1.0 mg/ml in 10 mM Tris–HCl buffer, pH 7.5, containing 0.1 mM $CaCl_2$; dotted line, bovine brain calmodulin, 0.5 mg/ml in 10 mM Tris–HCl buffer, pH 7.5, containing 0.1 mM $CaCl_2$.

2．家蚕の成長にともなうカルモデュリン量の変動

家蚕より摘出した種々の組織をホモジナイズし，105,000 x g で１時間遠心後，その上澄を沸騰水中で２分間加熱したものについてカルモデュリン量を測定した（Morishima, 1987）．　５令脱皮後羽化までの各時期における脂肪体，中腸，絹糸腺，精巣及び卵巣のカルモデュリン量を求めた結果を図２に示す．　脂肪体のカルモデュリン量は５令幼虫期では 0.5 - 0.6 μg/mg protein とほぼ一定であるが，蛹化に伴い急激に低下し，蛹期では幼虫期の 1/10 - 1/40 のレベルとなった．一方，中腸では，幼虫から蛹までの間ほぼ一定で，1 μg/mg protein 程度の比較的高い値を維持していた．　幼虫期の精巣は 1.5 - 1.7 μg/mg protein と最も高い値を示したが，蛹化と共に 1 μg/mg protein に低下し，再び一定のレベルを維持した．　これら組織のカルモデュリン量は，組織湿重当たりで比較した場合，ラットの種々の組織で報告されている値（Kakiuchi et al., 1982）とほぼ同レベルであった．

家蚕では，このようにカルモデュリン量は組織によって異なるが，各組織では幼虫期及び蛹期でそれぞれ固有の一定値を保っているように思われる．　これは，もう一つの情報伝達物質である

表 1 Amino acid composition of calmodulins from the fat body of the silkworm, *B. Mori* and from bovine brain

Amino acid	Fat body calmodulin: Hydrolysis (Hr) 24	48	72	Mean or extrapolated value	Nearest integer	Bovine brain calmodulin (Watterson *et al.*, 1980) residues/molecule
Asx	22.8	22.8	23.2	22.9	23	23
Thr	12.5	12.2	11.8	12.8*	13	12
Ser	5.3	4.8	4.3	5.8*	6	4
Glx	25.8	26.0	26.2	26.0	26	27
Pro	1.9	2.0	1.9	1.9	2	2
Gly	11.7	11.9	11.9	11.8	12	11
Ala	10.2	10.2	10.3	10.2	10	11
Cys	0	0	0	0	0	0
Val	7.1	7.2	7.3	7.2	7	7
Met	8.1	8.1	8.3	8.2	8	9
Ile	7.6	8.1	8.1	7.9	8	8
Leu	9.1	9.4	9.5	9.3	9	9
Tyr	0.9	0.8	0.8	0.8	1	2
Phe	9.2	9.2	9.3	9.2	9	8
Lys	8.0	8.1	8.0	8.0	8	7
His	1.0	1.0	1.0	1.0	1	1
Arg	6.0	6.0	6.0	6.0	6	6
Trp	0	0	0	0	0**	0
$Lys(Me)_3$	0	0	0	0	0	1
Total					149	148

*After extrapolation at zero time of hydrolysis. **Tryptophan was determined in methanesulphonic acid hydrolysates.

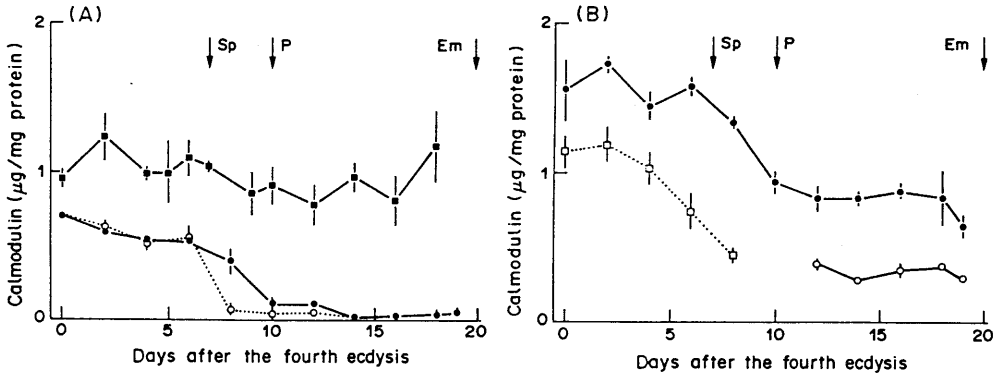

図 2 Calmodulin in various silkworm tissues during development.

Results are the mean +SD from duplicate assays on three to five individual insects. Sp, P and Em represent the periods of start of spinning, pupation and emergence of moth, respectively.

(A) ■——■, midgut; ●——●, fat body (male); ○·····○, fat body (female).
(B) ●——●, testis; ○——○, ovary; □·····□, silk gland.

ｃＡＭＰ及びその代謝酵素活性が昆虫の成長変態に伴って大きく変動すること（Bodnaryk, 1983）と対照的である が，情報を直接伝えるのはCa^{2+}イオンであり，カルモデュリンはその運搬役であるので，このことは伝えられるべき情報量の差を反映しているのかも知れない．

3．カルモデュリン依存性ホスホジエステラーゼ

カルモデュリンは，そもそも脳のｃＡＭＰ－ＰＤＥ活性化因子として見い出されたものであり，カルモデュリン依存性ＰＤＥはその後脊椎動物一般に広く分布することが認められているが，昆虫組織からは検出されず，昆虫には存在しないのではないかと考えられてきた（Bodnaryk, 1983）．しかし，前述の様に家蚕各組織には，哺乳動物に匹敵する量のカルモデュリンが存在することから，昆虫にのみカルモデュリン依存性ＰＤＥが存在しないというのは不自然であり，抽出法に問題があるのではないかと考え，ＰＭＳＦ，ペプスタチン等の種々のプロテアーゼ阻害剤を含む緩衝液を用いて，サナギ脂肪体をホモジナイズし 100,000 xg, 60分間遠心後の上澄より，DEAE-Sepharose CL-6B さらに Calmodulin-Sepharose 4B のアフィニティークロマトグラフィーにより，カルモデュリン依存性ＰＤＥを約5000倍に精製した（Morishima et al., 1985）．このＰＤＥの50％活性化に必要なCa^{2+}濃度は約 1×10^{-6}M，カルモデュリン濃度は約 1 nM であり，これらの値は哺乳動物のものとほぼ同じであった．

このＰＤＥは精製直後はCa^{2+}－カルモデュリンによって約4倍に活性化されたが，これを4℃で保存すると表2に示すように，カルモデュリンに対する感受性が徐々に失われた． また，精製したＰＤＥに5 μg/mlの trypsin を加え，0℃でインキュベートするとカルモデュリン無添加時の活性が上昇し，カルモデュリン依存性が完全に喪失した（図3）． これらのことから，このＰＤＥはプロテアーゼによる部分分解によってカルモデュリン結合部位が損傷を受け，カルモデュリン感受性を失うものと考えられ，通常の方法で精製した場合には，抽出，精製の過程でカルモデュリン感受性が失われてしまい，このことがこれまで昆虫でカルモデュリン感受性ＰＤＥが検出されなかった原因であると考えられる． 精製したＰＤＥはｃＡＭＰの他にｃＧＭＰも分解し，その反応速度論的解析から，同一の酵素タンパク質が両基質に作用しているものと考えられた．

表2　Stability of calmodulin-dependent phosphodiesterase activity at 4°C

Incubation at 4°C (days)	Phosphodiesterase activity (munits/ml)		Stimulation by calmodulin (fold)
	−Calmodulin	+Calmodulin	
0	53	215	4.1
2	53	225	4.2
4	70	190	2.7
11	67	145	2.2

The purified phosphodiesterase in buffer *B* (see text) containing 1 mM EGTA and 1 mg/ml bovine serum albumin was kept at 4°C for the indicated number of days. The activity was measured with 1 μM cyclic GMP in the presence of 0.2 mM EGTA (−calmodulin) or saturating concentrations of calmodulin and Ca^{2+} (+calmodulin).

4．カルモデュリン依存性アデニルシクラーゼ

サナギ脂肪体のアデニルシクラーゼ（ＡＣ）はすべて膜画分に存在するが，この膜画分をＥＧＴＡで繰り返し洗浄したものに，Ca^{2+}と家蚕脂肪体カルモデュリンを添加するとＡＣ活性は約2倍に上昇した（図4）． そこで，0.25 mM ＥＧＴＡ存在下でCa^{2+}を種々の濃度に添加し，ＡＣ活性に対するCa^{2+}及びカルモデュリンの効果を調べたところ，カルモデュリン存在下では 0.2-0.3

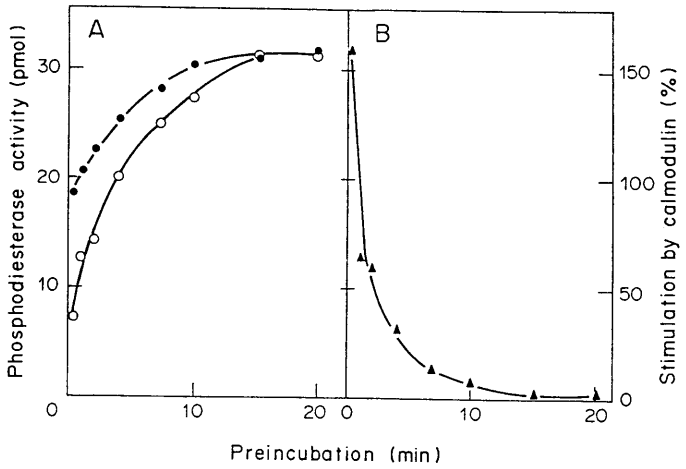

図 3 Trypsin-treatment of phosphodiesterase. Purified phosphodiesterase was preincubated at 0°C with 5 μg/ml of trypsin in buffer *B* containing 1 mg/ml bovine serum albumin. At the indicated time, trypsin inhibitor was added to 25 μg/ml and an aliquote was used for the measurement of phosphodiesterase activity in the presence of saturating concentrations of calmodulin and Ca^{2+} or 0.2 mM EGTA with 1 μM cyclic GMP as substrate. (A) Stimulation of phosphodiesterase activity by trypsin. (●——●), Calmodulin-stimulated activity; (○——○) basal activity. (B) Desensitization to calmodulin. Results are expressed as percentage stimulation of the basal activity.

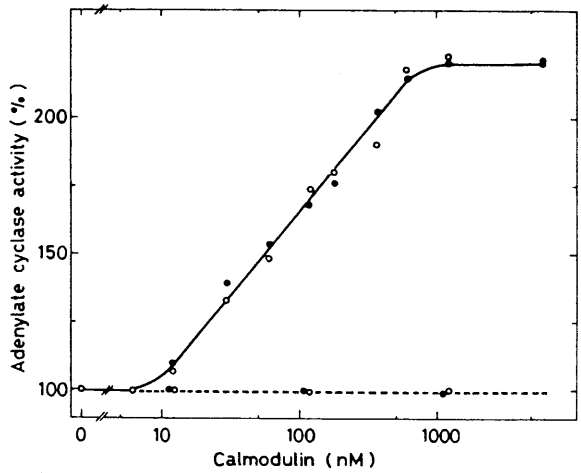

図 4 Dose-dependent effect of calmodulin on adenylate cyclase activity. Washed particulate fractions from fat body of the fifth instar larvae (230 μg protein) were incubated with varying concentrations of calmodulin from silkworm fat body (●) or porcine brain (○) in the presence (solid line) or absence (dashed line) of 0.2 mM $CaCl_2$. EGTA at 0.25 mM was included in all incubations. Calmodulin concentrations were expressed in nM assuming the molecular weight of both calmodulins is 17,000 (Bodnaryk and Morishima, 1984).

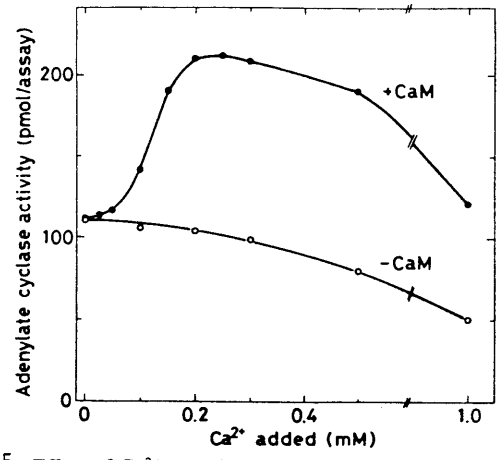

図 5 Effect of Ca^{2+} on adenylate cyclase activity. Washed particulate fractions from fat body of the fifth instar larvae (190 μg protein) were incubated under standard conditions with 0.25 mM EGTA and added $CaCl_2$ of the indicated concentrations in the presence (●——●) or absence (○——○) of fat body calmodulin (0.6 μM).

mM のＣａ$^{2+}$添加（遊離Ｃａ$^{2+}$ = 10^{-6} M 程度）で活性は最大になり，それ以上のＣａ$^{2+}$を添加すると，活性が逆に低下した（図５）．　これに対し，カルモデュリン無添加ではＣａ$^{2+}$は阻害効果のみを示した．　このことから，Ｃａ$^{2+}$はＡＣに対し 10^{-6} M 程度で促進，10^{-4} M 以上で阻害と言う biphasic な効果を示すことが明らかになった（Morishima, 1984）．　また，この活性化に必要なカルモデュリン濃度（５０％活性化濃度）は８０ mM で，これはＰＤＥの場合の約８０倍であった．

この様に，家蚕脂肪体において，哺乳動物組織のものに匹敵するカルモデュリン依存性ＰＤＥ及びＡＣの存在が確認されたことから，脂肪体においても，サイクリックヌクレオチド系及びＣａ$^{2+}$－カルモデュリン系の両者の情報伝達系が密接に絡み合って種々の酵素系を調節しているものと考えられる．

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Changes in pattern of protein synthesis in relation to diapause of *Bombyx mori* embryos

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INTRODUCTION

Silkworm eggs enter the diapause at the gastrula stage if they have been conditioned to hibernate. During diapause the rate of protein synthesis is suppressed (Saito *et al.*, 1983), but it rises 3 to 4 times after the onset of post-diapause development (Saito *et al.*, 1982). Most of the increase is during the first 20- to 24-hr period (Saito *et al.*, 1982), which is morphologically a preparatory stage preceding the burst of organogenetic processes (Takami and Kitazawa, 1960). This rise cannot be explained by the increases of ribosomes and translatable mRNAs in content, which augmented only 30 and 10%, respectively, during the period (Saito *et al.*, 1982, 1985). This suggests that the availability of mRNAs and ribosomes to make polysomes is an important factor in the the control mechanisms which determines the protein synthesis rate during the critical period of the post-diapause development. The principles of the data are summarized schematically in Fig. 1.

When total RNAs were extracted at the onset of, and at 10 to 20 hr of, the post-diapause development and their translation products in an *in vitro* system were compared, no marked differences in size of the products were observed, indicating that the higher rate of protein synthesis is

established without overall changes of translatable mRNA species (Saito *et al.*, 1985). However, this does not exclude the possibility of temporal appearance of a specific mRNA population. This report describes a translation product with a size of 73 kDa that is detected when RNAs are extracted within a limited duration of post-diapause development. A material with the same size was also found in proteins labeled *in vivo*.

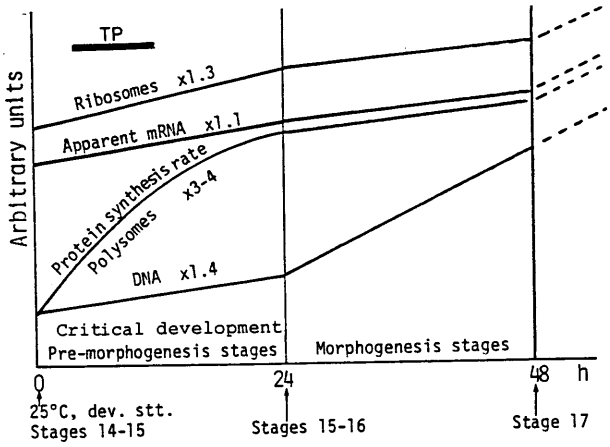

Fig. 1. Schematic representation of the changes in content of polysomes, ribosomes, mRNAs and in rate of protein synthesis during the early period of the post-diapause development. Zero hr stands for the time of hot HCl treatment. The first 24 hr has been named the critical development (Takami and Kitazawa, 1960), which precedes the burst of organogenesis processes. For TP, see text.

MATERIALS AND METHODS

Eggs of a Chinese-Japanese hybrid (N124 x C124) had diapause ended and post-diapause development started by long-term chilling followed by hot-HCl treatment (spec. grav. 1.10, 48°C, 6 min). Total RNAs were extracted and translated *in vitro* in a rabbit reticulocyte lysate cell-free system mixed with L-[^{35}S]methionine (1,100 Ci/mmol, Amersham). The radioactive products, as well as marker proteins, were

electrophoresed on 10% polyacrylamide slab gels under denaturing conditions and located by fluorography. All the above procedures were as detailed previously (Saito *et al.*, 1985). To analyze newly synthesized proteins by *in vivo* labeling, embryos were cut through the antero-posterior axis and cultured in a medium (Saito *et al.*, 1982) mixed with L-[^{35}S]methionine (1,100 μCi/10 embryos) at 25°C for 2 hr. Then the embryos were washed with fresh medium, collected by centrifugation at 1,800 rpm for 5 min, and extracted with the sodium dodecyl sulfate sample buffer (Saito *et al.*, 1982). The solution was electrophoresed and fluorographed as above.

RESULTS AND DISCUSSION

The results of fluorographies of the proteins obtained by the *in vitro* translation of embryonic RNAs and by the *in vivo* labeling of the embryos were summarized in Fig. 2.

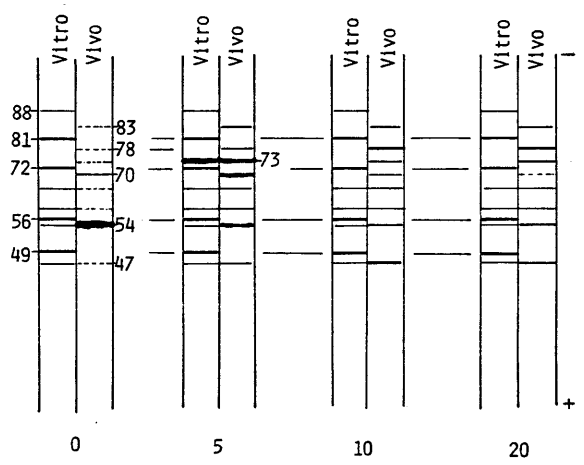

Fig. 2. Schematic representation of fluorography after gel electrophoresis. "Vitro" indicates the bands obtained by *in vitro* translation of RNAs and "Vivo" shows the bands of newly synthesized proteins labeled *in vivo*. Electrophoresis was made from top to bottom. Resulting bands are only partly drawn (see text). The numerals along abscissa, age in hr after the onset of post-diapause development (cf. Fig. 1); those along the lanes, the sizes in kDa of the bands.

Here, only several products out of 40 to 50 bands detected are presented. At 0 hr, before or shortly after the acid treatment, *in vivo* products were very few except for some bands, e.g. the one with a size of 54 kDa, whereas the RNA translation products were distinctly detected. The basic patterns of these products at 0, 10 and 20 hr of post-diapause development were similar to each other. However, an additional band the molecular mass of which was estimated to be 73 kDa was consistently seen when 3- to 9-hr eggs were used as samples. This was seen both in the RNA translation products and in the newly synthesized proteins. Fig. 2 shows typical patterns at 5 hr. A band with this size was scarcely detected at other ages. For the *in vivo* labeling products, co-migrating 73 kDa band was weakly seen also in the samples at 0, 10 and 20 hr (Fig. 2). Whether this is the same molecule or not is unclear.

On the whole, it can be concluded that at least one protein species, and its mRNA, appear (or increase) only during a limited stage after the onset of post-diapause development. This substance was named TP (a temporal protein). In Fig. 1, the duration when this band appears is drawn by a horizontal solid bar.

It was recently reported that a molecule which was similar to this 73-kDa protein in size and in mode of appearance has also been found in the eggs of European races of the silkworm and that its production was dependent upon the heat treatment; thus this substance may be a kind of heat-shock protein (M. Coulon, personal communication). Inducibility of the heat-shock genes is dependent upon the ability of embryonic transcription system (e.g. Bienz, 1984). Thus the period when TP appears, or possibly the whole of the critical development, might be the stage of de novo transcription of embryonic genes. Unpublished results

in our laboratory showed that the incorporation of labeled precursors into RNAs is rapid after the acid treatment. It is not until the post-diapause period that the rate of RNA synthesis rises significantly in the hibernating eggs; the rate scarcely increases before the onset of, and during, the diapause (Kurata *et al.*, 1979). Taken together, it can be supposed that the critical period of the post-diapause development is the time when the maternal messages are replaced by the zygotic molecules. During diapause the maternal transcripts may be kept stable. Details for the mechanisms underlying these processes remain to be investigated.

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カイコ卵休眠をめぐるタンパク質合成の変動

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和 文 要 旨

カイコ休眠性卵の休眠前・中・後全体でのタンパク質合成速度の変化を見渡すと，最もクリティカルなのは後休眠期の器官形成に先立つ一時期であり，ここでは，24時間の間にタンパク質合成速度が約３倍上昇する．ところが，これに伴うリボソームとmRNA含量の増加は全タンパク質合成速度の上昇を説明するほど著しくはない．このことから，リボソームおよびmRNAの利用度の増大による調節が考えられる． 一方，in vivoタンパク質合成パターンと mRNAのin vitro翻訳産物の分析から，この時期には寿命の短い，大きさ73 kDa のタンパク質が一時的に出現する． つまり転写のレベルでもなにか新しいことが起っている．これらの事実，および休眠性卵では休眠後まで RNAの著しい合成が見られないという観察結果から，後休眠24時間に母性／胚性mRNA切換えが起こるのではないか，したがって，休眠中は母性メッセージの保持の機構が存在するのではないかと推定される．

カイコ卵のＮＡＤ－ソルビトール脱水素酵素

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緒言

カイコ卵では、胚休眠と密接に関連して生じる現象としてグリコーゲンとソルビトールとの相互変換がある。　休眠開始期に生じるグリコーゲンからソルビトールへの系はグリコーゲンホスホリラーゼａによって（Yamashita et al, 1975)、休眠覚醒期に生じるソルビトールからグリコーゲンへの系は NAD-ソルビトール脱水素酵素（NAD-SDH）によって（Yaginuma and Yamashita, 1979）調節されると考えられている。　NAD-SDH 活性は、休眠卵には低く、長期冷蔵（５°Ｃ）処理や短期冷蔵（５°Ｃ）とＨＣｌ処理による休眠覚醒期に、誘導される。　ところが、０°Ｃに近い温度に休眠卵を保護するとソルビトールからグリコーゲンへの変換が認められない（Furusawa et al, 1982)。　このことは、０°Ｃに近い温度によっては NAD-SDH 活性が誘導されないことを示唆している。

従って、ここでは５°Ｃと０°Ｃに近い温度に対する NAD-SDH の応答性について検討し、次にこの２種の低温を用い NAD-SDH 活性を人為的に調節し、酵素活性とソルビトール量とを比較することによって、初期の結果、NAD-SDH がソルビトールの利用系に関与していることを再確認した。

材料と方法

（１）　カイコ蚕品種としては春嶺ｘ鐘月と錦秋ｘ鐘和を用いた。　休眠卵は産下後３時間以内のものを集め、一定の重さに測定し保護した。

（２）　酵素活性の測定：　酵素標品の調製及び活性の測定法は Yaginuma and Yamashita (1979) の方法に従った。　酵素活性の測定はすべて３０°Ｃ下で行った。

（３）　ソルビトール、グリセロール、グリコーゲンの抽出及び定量：　ポリオールとグリコーゲンとは各々エタノールとＫＯＨによって抽出し、各々酵素法（Beutler, 1984; Eggstein and Kuhlmann, 1974）と比色法とによって定量した。

（３）　ふ化率の測定：　通常３００個の卵を用いた。

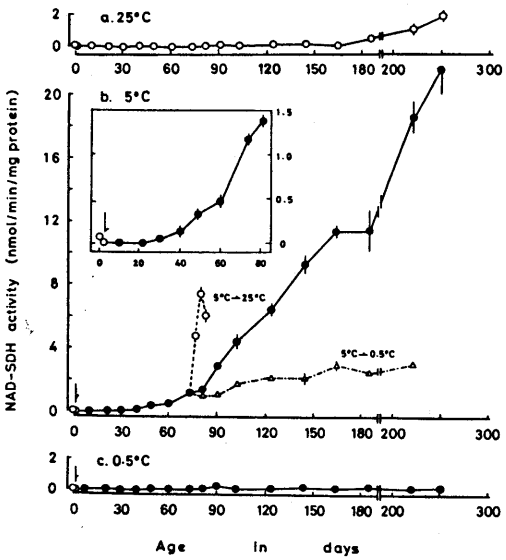

図１．産下後４８時間に低温処理した休眠卵における NAD-SDH 活性の変動

結果と考察

（１）　産下後４８時間に低温処理した休眠卵：

まず、休眠状態が完全に確立される前に卵を低温処理した。　５°Ｃ保護卵の場合、NAD-SDH 活性は産下後３０日まで認められず、４０日以降から明らかに増加し、２５０日までほぼ直線的に増加し続けた（Fig. 1c)。この活性の出現時期は、低温処理の後２５°Ｃに保護し胚子発育を再開させた卵のふ化が斉一化し始める時期と一致していた（Fig. 3a)。　５°Ｃ処理後７１日に２５°Ｃ、０．５°Ｃに卵を移した場合、前者では急激な活性の上昇が、後者では活性上昇の抑制が各々に認められた（Fig. 1bc)。　産下後２日から０．５°Ｃに保護した卵では、この実験期間中（２５０日）、　有意な NAD-SDH 活性は認められなかった（Fig. 1c)。　ふ化能力から見ると、１４２日間０．５°Ｃ処理で辛うじて２５°Ｃ催青後１７日に蟻蚕がふ化し始め、約３５％のふ化率を示したにすぎない。　その後の０．５°Ｃ処理はふ化率を減少させた(Fig. 3b)。　２５°Ｃに産下後連続保護した卵では、９０日まで活性は検出されず、その後僅かの活性（ca. 0.1 nmol/min/mg protein 前後）が認められ、１８０日以降２５０日まで明らかに活性が上昇した(Fig. 1a)。　２５°Ｃ連続保護卵でも２２０日位から３００日にかけて６％弱のふ化を見ることから、後半の NAD-SDH 活性の上昇は２５°Ｃ下でも胚子発育し始めた卵に由来するものと考えられる。　このような温度処理によって様々な NAD-SDH 活性変動型が生じたが、その同じ卵のソルビトールの変動を見るとほぼ酵素活性と鏡像関係の変動型を示していた(Fig. 2)。　このことは初期の結果を再確認したことになる。　ただし、２５°Ｃ連続保護卵では９０日以後ソルビトールの緩慢な減少が生じたのに対して、それに見合ったグリコーゲンの回復が認められなかった。　産下後１２３日における NAD-SDH 活性は約　9.4 umol/day/g eggs（３０°Ｃ）であるが、in vivo でのソルビトールの減少率は約　0.6 umol/day/g eggs（２５°Ｃ）であり、酵素活性の約 1/10 でこの減少は賄えることになる。　しかし、グリコーゲン量の回復の生じない理由については不明である。

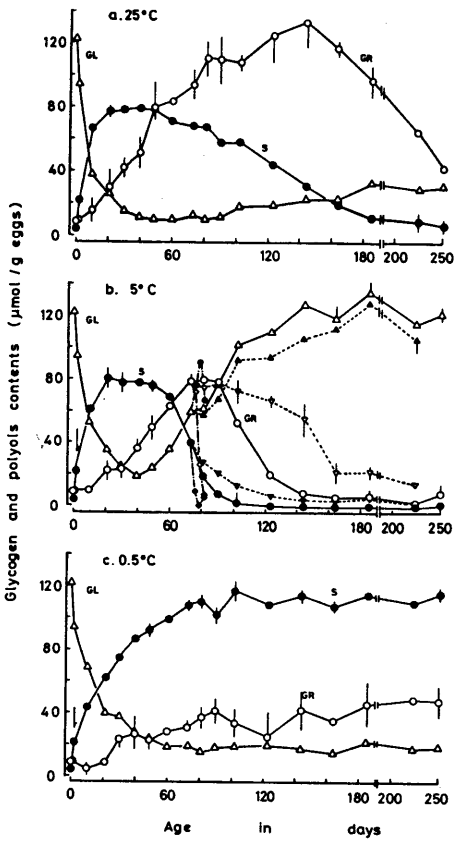

図２・産下後４８時間に低温処理した休眠卵における グリコーゲン、ポリオール量の変動

（２）　産下後３０日に低温処理した休眠卵：

次に、NAD-SDH 活性とふ化能力との誘導に対する低温の効果をさらに検討するため、完全に休眠状態が確立されている卵を５° C、０．５° Cに処理した。 基本的には（１）とほぼ同じ結論を得た。 ただし、５° C接触期間が NAD-SDH 活性及びふ化能力の誘導には６０日以上を要した (Figs. 4 and 6)。 また、０．５° C保護卵においては NAD-SDH 活性の上昇及びソルビトールの減少は認められなかったが、ふ化率は１９２日、３３６日 冷蔵で有意に増加した (Fig. 6)。 しかし、この長期冷蔵にも係わらず、初めて蟻蚕のふ化するのは２５° C催青後１６日であり、またそのふ化の斉一化は５° C冷蔵には及ばない（長期５° C冷蔵では２５° C催青後１０日に蟻蚕が出現するようになる。 これは非休眠卵本来の胚子発育期間に等しい）。

以上の結果は５° C は NAD-SDH 活性の出現、増加をもたらすが、０．５° Cはその出現、さらなる増加を抑制することを示している。 ふ化能力の点では、その誘導に５° Cは効果的であるが、０．５° Cは効果に劣る。 NAD-SDH 活性とふ化能力の誘導に対するこれら低温による差異については次のように理解できる。 卵は NAD-SDH 活性の誘導に至る過程 (covert phase) の情報として５° C、０．５° C両者を利用することが出来る。 ただし、 NAD-SDH 活性の出現という、目に見えるようになる過程 (overt phase) は０．５° Cでは進行せず、５° C以上の温度を必要とする。 このような異なる低温によるポリオールの蓄積への異なる効果は、 Gall fly の越冬幼虫で知られている。 １０° C－０° Cの温度はグリセロールの、０° C－－２０° Cの温度はソルビトールの蓄積を各々促す (Storey et al, 1981)。 これらの低温は解糖系の鍵酵素を含めた他の数種の酵素活性を上昇させる (Storey and Storey,

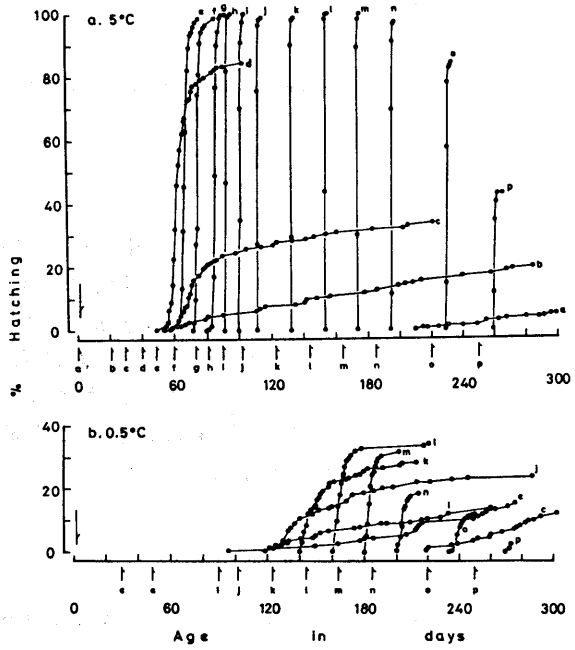

図３．産下後４８時間に低温処理した休眠卵におけるふ化率の変動．矢印の日から２５° Cに 催青した。

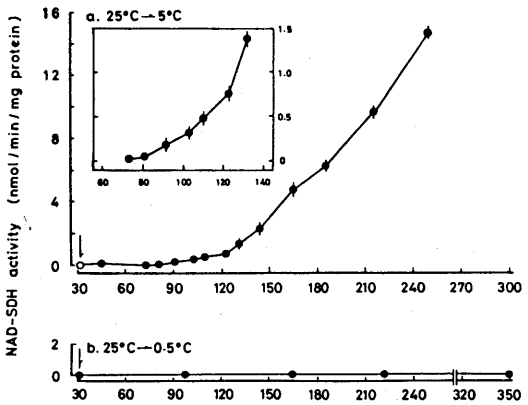

図４．産下後３０日に低温処理した休眠卵における NAD-SDH 活性の変動

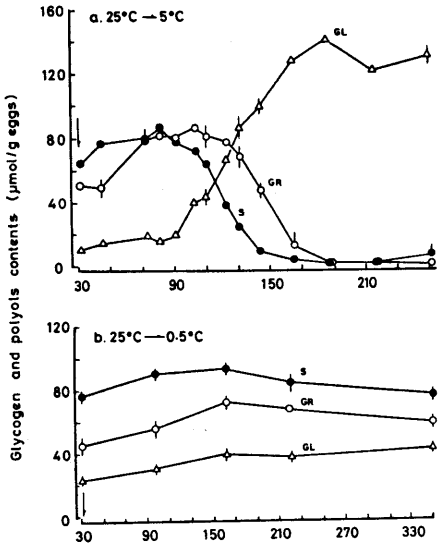

図５．産下後３０日に低温処理した休眠卵におけるグリコーゲン、ポリオール量の変動

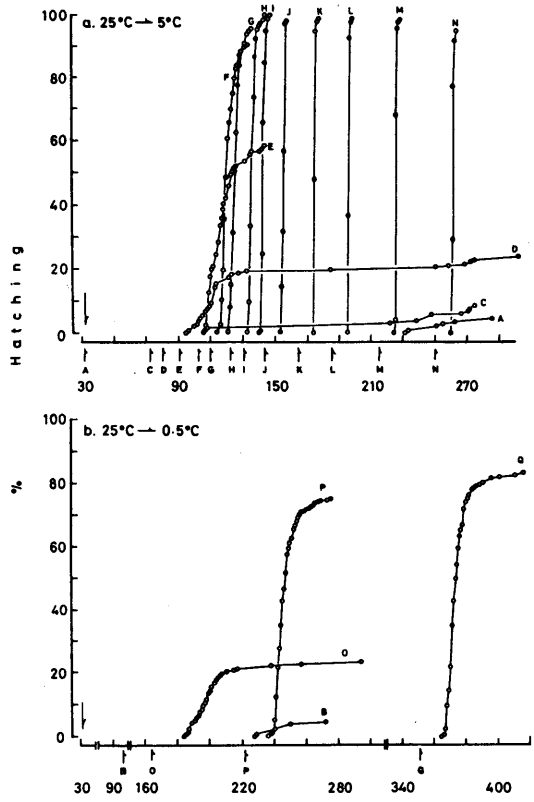

図６．産下後３０日に低温処理した休眠卵におけるふ化率の変動．矢印の日から２５° Cに催 青した。

1981; Hochachka and Somero, 1984)。 また、低温によるグリセロールの蓄積（セクロピア蚕休眠蛹、Ziegler and Wyatt, 1975)、トレハローズの蓄積（シンジュ蚕休眠蛹、Hayakawa and Chino, 1982）促進の例も報告されているが、いずれの場合も、低温の調節はすでに存在する酵素の modulation によるものである。 カイコ卵に見られる NAD-SDH 活性の低温による誘導がこれらの低温調節の仕組みの範疇に入るものであるのか否かを含めて、カイコ休眠卵での５° C低温による NAD-SDH 活性誘導の mechanism および休眠、非休眠の違いによる NAD-SDH 活性出現の差異の mechanism を明らかにすることは急務の課題である。

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カイコの脂肪組織及び血液中のキモトリプシンインヒビターの精製と性質

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筆者らは主としてカイコの血液を用い，プロテアーゼインンヒビターについて，生理・遺伝・生化学・病理などの立場から研究を進めてきた（江口，1987）。血液中には活性の強い多くのキモトリプシンインヒビターがあり，これらのアイソインヒビターは，電気泳動やカラムクロマトグラフィーなどによって分離することができる。また我々はキモトリプシンインヒビターについての多数の変異系統を発見した（Eguchi et al.,1984）。

最近我々は脂肪組織にも，かなり強いキモトリプシンインヒビター活性があることを見い出した。このことは脂肪組織がインヒビターの合成部位の一つではないかという示唆を与える。

一方，昆虫においては，精製したインヒビターを用いた研究は非常に少ないので，活性の強い低分子インヒビターｅ，ｇ（血液）及びｇ′（脂肪組織）を精製し，それらの性質を比較した。また血液中の高分子インヒビターｄについては精製法の改良を行った。さらに，脂肪組織と血液のインヒビターの関係を知るために２，３の実験を試みたので，その結果についても報告する。

材料及び方法

材料としては，桑葉育を行った支124-Nを用いた。脂肪組織は５齢幼虫から取り出し，冷0.8% NaCl で洗浄後，濾紙上で水分を除き，ガラスホモジナイザーで磨砕した。10% 磨砕液（0.1Mトリス塩酸緩衝液，pH 7.4）を6,000gで10分間遠心分離し，上清を測定に用いた。血液は主として熟蚕の腹脚を切り，氷冷試験管中に採取した。

インヒビターの定量には，プロテアーゼとして牛スイ臓由来の精製キモトリプシンを使用した。キモトリプシン活性はベンゾイルチロシン－ρ－ニトロアニリドを基質として比色法によって定量した(Eguchi and Shomoto,1984; Eguchi et al.,1986)。インヒビター活性は，プロテアーゼ活性を50% 減少させるインヒビター量を１ユニット（１U）として表した。

電気泳動は既報（Eguchi et al.,1984）に準じて行い，泳動後のインヒビターの検出法は Uriel and Berges (1968)の方法を基に，少し改変したものである。

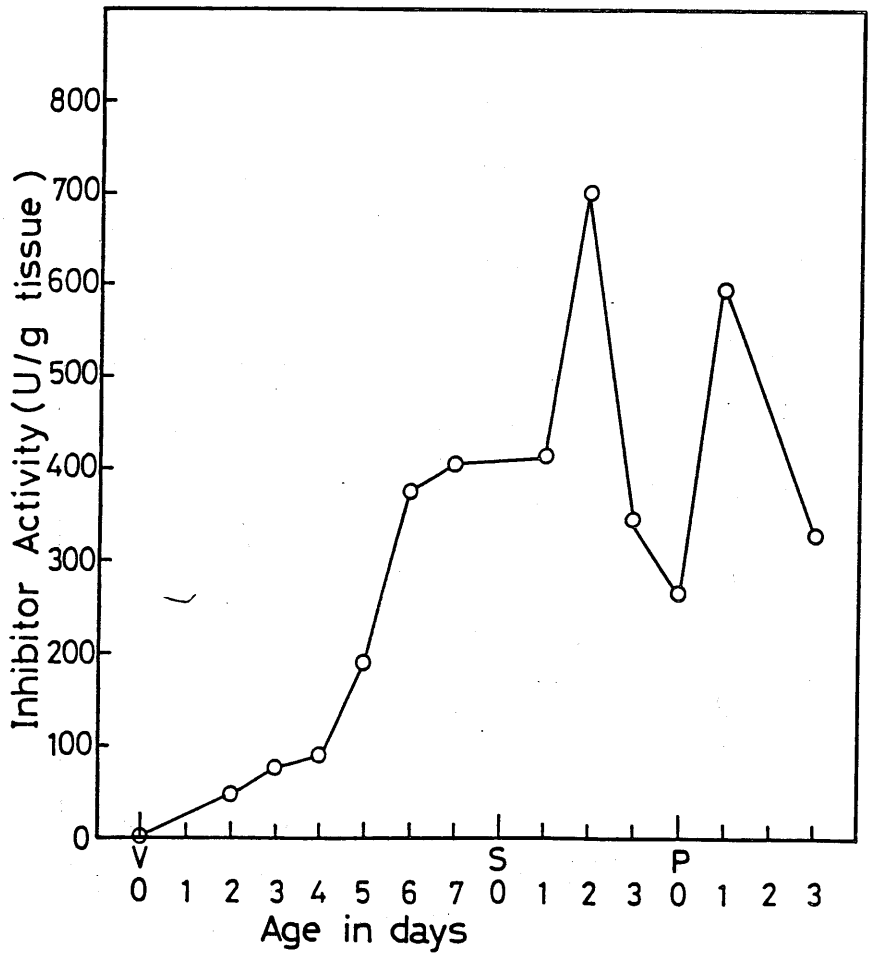
結果と考察

脂肪組織のインヒビター活性及び電気泳動像の発育変動

まず，脂肪組織，皮膚及び血液中のインヒビター活性を吐糸前後のカイコについ

て調べると，皮膚については非常に低い活性しか認められなかったが，脂肪組織には強い活性がみられた。そこで，５齢起蚕から蛹初期までの活性の変化を調べたところ，第１図にみられるように，活性は５齢幼虫の発育に伴って急激に増加し，吐糸２日後にピークとなり，一旦低下した後第二のピークが化蛹１日後にみられた。血液中のインヒビターの発育変動については既に報告した（Eguchi et al.,1986）が，そのピークの時期よりも脂肪組織の方はやや後期にずれていることが分かった。

一方，電気泳動像を調べると，血液には検出されないｇ′バンドは熟蚕期以後活性が非常に強まり，ｅバンドも吐糸前期を除いて強い活性を示した。血液中には明瞭な活性が認められるｄバンドは脂肪組織には検出されなかった。



第１図　発育に伴う脂肪組織のキモトリプシンインヒビター活性の変化

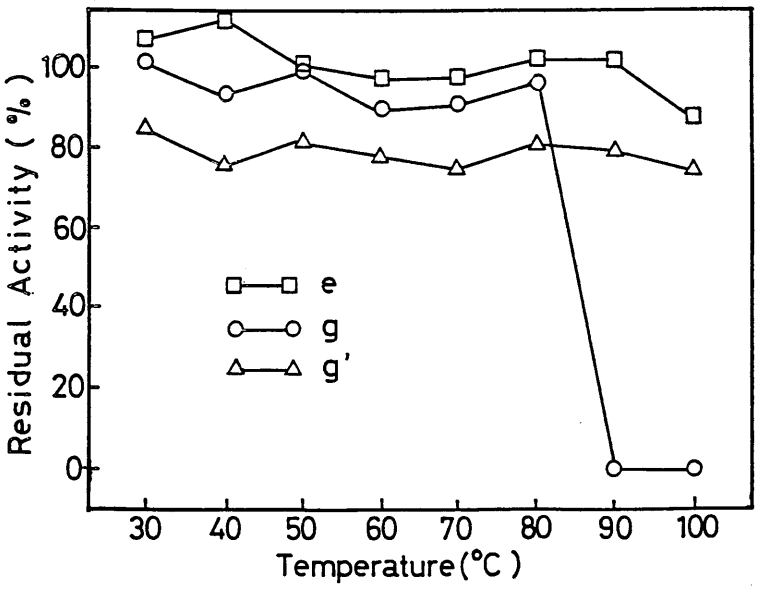
脂肪組織及び血液のインヒビターの精製

脂肪組織に特異的なインヒビターg′と血液中で活性の強いe及びg型の精製を行った。まず，g′については，35～65%硫安分画後，DEAE-Sephacel 及びUltrogel AcA 54カラムを用いて精製し，約 530倍に精製された標品が得られた。e及びgについても，上と同じ手順で精製を進めたが，e型の場合は DEAE-Sephacelカラムクロマトグラフィー後，CM-Celluloseカラムを通す方が有効であった。e，gそれぞれ，約 5,100倍及び 1,100倍に精製された。これらの精製標品は電気泳動によって，一本のタンパクバンドであることが確認された。

精製インヒビターe，g及びg′の性質

Sephadex G-50, Ultrogel AcA 54カラムクロマトグラフィー及びSDS-ポリアクリルアミドゲル電気泳動法により推定した平均分子量は，e，g，g′型それぞれ約 7,200, 8,400及び7,700 という値が得られた。

続いてインヒビター活性に対する温度の影響を調べると（第２図），e型は最も安定で，100 ℃,15 分間の処理によっても，約90%の活性を保っていた。g′もこの条件で対照区の80%弱の活性を示した。一方，g型は80℃までは非常に安定であったが，90℃で失活した。この点，既報の部分精製標品による結果（Eguchi and Shomoto, 1984）と異なっている。



第２図　精製キモトリプシンインヒビターの熱安定性

さらに，インヒビター有無の場合の基質濃度と酵素活性との関係から反応様式を推定した。Lineweaver-Burk の逆数プロットをとり，直線の交叉の位置から，３種のインヒビターは拮抗型阻害を示すと考えられる。

３種のインヒビターを用い，阻害スペクトルを調べるとこれらのインヒビターは共にキモトリプシン活性を強く，トリプシン活性を弱く阻害した。カイコの消化液プテアーゼ6B-3及びAspergillus melleus 由来の精製プロテアーゼはほとんど阻害しなかった。また，e型はサブチリシンを弱く抑えた。

脂肪組織及び血液のインヒビターの関係

脂肪組織は血液のインヒビターの合成部位の一つと考えられるが，この組織に特異的で活性の強いg′型は血液にはみられず，電気泳動ではe及びg型の中間に移動する。また，カラムクロマトグラフィーによる挙動も両インヒビターと似ているので，このg′型が血液中のeやgに変化するのではないかと想像される。

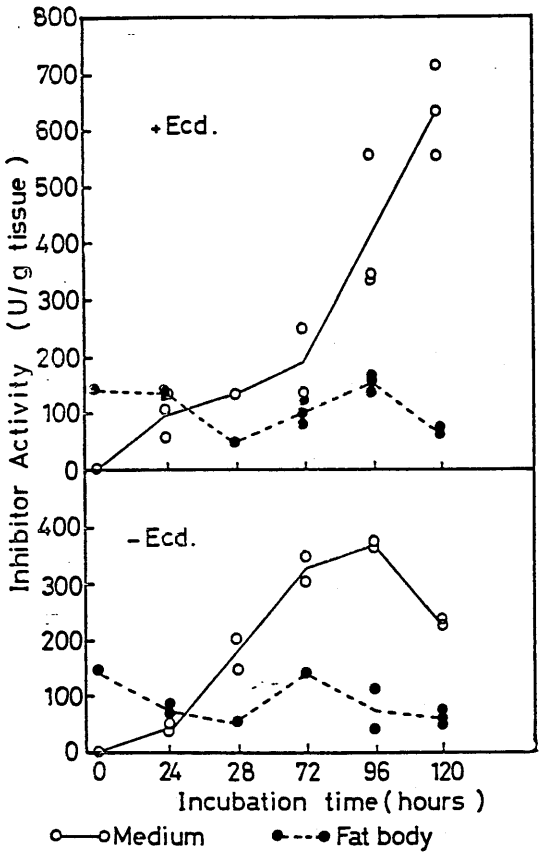
脂肪組織をSDS,Triton X-100，ノイラミニダーゼあるいはpH 8.0の緩衝液と磨砕後，５℃で20時間保温後電気泳動すると，かなり強い活性を持つe型が検出された。無処理のものではe型はほとんど認められなかったので，この結果はg′からe型への転換を示唆している。この外，分子量及び熱安定性の面でもg′はgよりもe型との類似性が強い傾向が認められる。

一方，血液中には活性の強い高分子インヒビターdは脂肪組織には認められないが，d型に対する抗血清を用いた免疫電気泳動から，抗体と反応するタンパク質が脂肪組織中に存在することが分かった。このタンパク質はd型の前駆体ではないかと考えられる。

培養された脂肪組織のインヒビターに対する20-ハイドロキシエクダイソン の影響

血液及び脂肪組織中のキモトリプシンインヒビターは，カイコの発育に伴い大きな変化を示し，血液インヒビターについては，ホルモンによる制御を受けていることも分かっている（Eguchi et al.,1986）。さらに，血液中のインヒビターの多くは脂肪組織で合成・分泌されるものと推論されるので，次のような実験を試みた。

すなわち，４齢４日後と吐糸３日後のカイコから取り出した脂肪組織を，Graceの培地で培養し，20-ハイドロキシエクダイソン の影響を培養５日後まで調べた。４齢４日後のものについては，脂肪組織中のインヒビター活性の変化は比較的小さかったが，培養液中のインヒビターについては，特に20-ハイドロキシエクダイソン 添加区で，時間と共に活性が著しく増大した（第３図）。



吐糸３日後の脂肪組織を用いた場合には，組織中のインヒビター活性は，１日以後，時間の経過と共に激減する傾向が認められた。培養液中のインヒビターは，ホルモン添加区で培養４日以後活性が増加した。計算の結果，４齢期の場合特に20－ﾊｲﾄﾞﾛｷｼｴｸﾀﾞｲｿﾝ添加によってインヒビターの新たな合成あるいは活性化が行われているように推論される。

第３図　in vitroでの脂肪組織のインヒビター活性に対する20-ﾊｲﾄﾞﾛｷｼｴｸﾀﾞｲｿﾝ の影響

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Amino Acid Sequence of Silkworm Antitrypsin Deduced from Its cDNA Nucleotide Sequence

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INTRODUCTION

Silkworm larval hemolymph contains several kinds of serine proteinase inhibitors (Sasaki,1978; Sasaki and Kobayashi,1984). They are classified into two groups based on their physico-chemical characteristics. Lower molecular weight inhibitors (7kDa) which are named SCIs (silkworm chymotrypsin inhibitors) can inhibit bovine chymotrypsin reversibly with dissociation constant $K_D < 10^{-9}$M (Sasaki,1978). Amino acid sequence analysis of one of SCIs (SCI-III) revealed that it should belong to kunitz-type serine proteinase inhibitor represented by bovine pancreatic trypsin inhibitor (Kassell et al.,1965), though one amino acid was inserted in the first half cystine frame (Sasaki,1984). Higher molecular weight inhibitors (42-43kDa) are separated into at least two species, that is, silkworm antitrypsin (sw-AT) and antichymotrypsin (sw-Achy). Silkworm-AT can inhibit bovine trypsin and silkworm digestive juice proteinase P-II (Sasaki and Suzuki,1982). Silkworm-Achy, on the other hand, can inhibit bovine chymotrypsin and silkworm digestive juice proteinase P-III (Sasaki and Suzuki,1982; Sasaki and Itou,1985). Both inhibitors make covalent, alkali labile linkage with each

target proteinase at their COOH-terminal region (Sasaki,1985; Sasaki et al.,1987). In addition, complex formation accompanies cleavage of the peptide bond between P_1-P_1' (Schechter and Berger,1967) corresponding to the reactive site. This irreversibility in inhibition is the distinctively different characteristic from that of SCI group. Silkworm-AT and sw-Achy seem to resemble inhibitor group called serpin (Carrell and Boswell,1986) in their inhibition mechanism (Sasaki,1985; Sasaki et al.,1987). It is necessary to determine the amino acid sequence of these inhibitors in order to clarify the homology between these and serpins.

In this study, the amino acid sequence of sw-AT is determined from the nucleotide sequence of cloned sw-AT complementary DNA (cDNA). As a result, it is revealed that sw-AT certainly belongs to serpin group and that the reactive site against trypsin is Lys_{343}-Val_{344} peptide bond of sw-AT.

MATERIALS AND METHODS

Fragmentation of sw-AT and Amino Acid Sequence Analysis

Silkworm-AT was prepared according to Sasaki and Kobayash(Sasaki and Kobayashi,1984). Chemical fragmentation of sw-AT was performed by two reagents, cyanogen bromide (Gross and Witkop,1962) or iodosobenzoic acid (Fontana et al.,1981). Gel filtration with Sephadex G-50 was the first step for rough fractionation. Further separation of peptide was done with high performance liquid chromatography using reversed phase ODS-120T column (Toso Co.Ltd,) by linear gradient elution increasing acetonitrile concentration containing 0.1% trifluoroacetic acid. Automated amino acid sequence analysis was performed using JEOL JAS-47K sequence analyzer. Phenylthiohydantoin amino

acid derivatives were identified with high performance liquid chromatography according to Sasaki et al. (Sasaki et al.,1987).

Preparation of mRNA and in vitro Translation

Total RNA was extracted from silkworm larval fat body in the 5th day of the 5th larval instar (strain Kinshu x Showa) by guanidinium thiocyanate-hot phenol method (Maniatis et al.,1982). Poly(A)$^+$RNA was isolated with oligo(dT)-cellulose. *In vitro* translation was performed using rabbit reticulocyte lysate in the presence of ^{35}S-methionine and the product was trapped specifically by rabbit anti-(sw-AT) antibody. Then, protein A-Sepharose CL-4B was mixed and the components bound to this resin were eluted with 125mM Tris-HCl,pH6.8, containing 1% SDS and 2% 2-mercaptoethanol. The eluent was analyzed by SDS-PAGE and the products were visualized by fluorography.

Construction of cDNA Library

Complementary DNA library of fat body poly(A)$^+$RNA was constructed using expression vector pKEN602 by Okayama-Berg method (Okayama and Berg,1982; Nakamura et al.,1986). The constructed cDNA transformed *E.coli* strain JM83. Number of colonies formed on solid medium containing ampicillin were 4 x 10^4. Isolated plasmid DNA was electrophresed on 0.5% agarose and DNA larger than pKEN602 were recovered from gels. These DNA retransformed *E.coli* strain JM83.

Screening of sw-AT cDNA Clone

Clones expressing sw-AT were surveyed by immunoscreening technique (Helfman et al.,1983) using anti-(sw-AT) antiserum adsorbed with cell lysate of *E.coli* strain JM83. Colonies reacted with anti-(sw-AT) antibody were identified by ^{125}I-labeled protein A. Positive clones by immunoscreening were further ascertained by Sourthern hybridization. Plasmid DNA from positive

clones were cut by BamHI and electrophoresed on 0.7% agarose. Synthetic 23-mer oligonucleotide mixture corresponding to the amino acid sequence of sw-AT, Lys_{10}-Asn-Gly-Asn-Asp-Asn-Phe-Thr_{17}, synthesized with DNA synthesizer model 380A, Applied Biosystem, and was used as probe after labeling at 5'-end with ^{32}P according to Maxmam and Gilbert method (Maxam and Gilbert,1977). Colony hybridization was performed using EcoRI-PstI fragment (750bp) of one of the sw-AT cDNA clones (pSWAT1) as a probe after labeling with ^{32}P by random oligonucleotide primer method (Feinberg and Vogelstein,1983) using oligonucleotide kit (Pharmacia).

Northern Hybridization

To asses the size of mRNA of sw-AT, Northern hybridization was performed. Poly(A)$^+$RNA from fat body was electrophoresed and then transfered to nylon membrane. EcoRI-PstI fragment (750bp) of sw-AT cDNA clone (pSWAT1) was used as a probe.

Sequencing of cDNA

BamHI-XbaI fragment of sw-AT cDNA clone (pSWAT1) was subcloned to Bluescript KS M13$^+$ and M13$^-$ (Stratagene Cloning System Co.Ltd.) and after transformed *E.coli* strain MV1184, plasmid DNA was recovered. Deleted cDNA fragments were obtained by exonucleaseIII digestion for 2, 4, 6, 8 and 10min and after the treatment by exonucleaseVII, prtoducts were ligated to Bluescript. To obtain overlapping, BamHI-EcoRI and EcoRI-PstI fragment of cDNA pSWAT1 were subcloned to Bluescript. The dideoxy method was employed for DNA sequencing (Sanger et al.,1977). Amino acid sequence corresponding to cDNA nucleotide sequence was determined by using computer program GENETYX (SDC Software Develop.Co.Ltd.). Hydropathy defined by Kyte and Doolittle (Kyte and

Doolittle,1982) was calculated using program DNASIS (Hitachi Software Engineering Co.Ltd.).

RESULTS AND DISCUSSION

SDS-PAGE pattern of *in vitro* translation products revealed the incorporation of ^{35}S-methionine into 42kDa component immunoreactive with anti-(sw-AT) antibody (Fig.1). This size is identical with taht of sw-AT and it is concluded that sw-AT is synthesized in the fat body. Preform of sw-AT is not recognized clearly. A 30kDa component also reactive with anti-(sw-AT) antibody. This component is also recognized even after the translation products are preliminary treated by anti-(hemolymph 30K protein) antibody. The character of this component is not clear untill now.

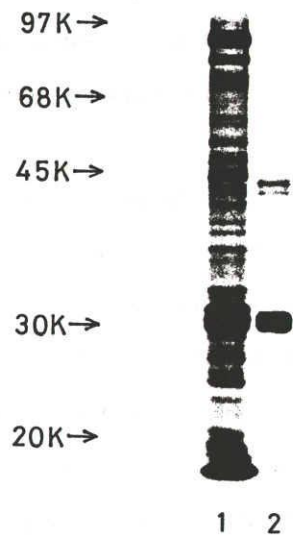

Fig.1 Fluorography after SDS-PAGE of in vitro translation products of fat body poly(A)$^+$RNA from silkworm. poly(A)$^+$RNA prepared from silkworm fat body was translated in a cell-free system of rabbit reticulocyte lysate with ^{35}S-methionine. Lane 1, total products; lane 2, products reacted with anti-(sw-AT) antiserum; lane 3, products reacted with normal rabbit serum. Molecular weight standards are as follows: 97K, phosphorylase b; 68K, bovine serum albumin; 45K, ovalbumin; 30K, carbonic anhydrase; 20K, soybean trypsin inhibitor.

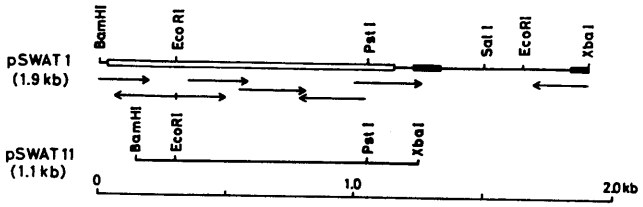

Fig.2 Sequencing strategy and restriction map about the clones pSWAT1 and pSWAT11. The arrows towards right and left indicate the lengths of nucleotide sequences determined from 5' and 3'-end, respectively. Open box indicates the coding region in the pSWAT1 clone (1.9kb cDNA). Closed boxes indicate the poly(A) site.

Immunoscreening of the library could identify two positive clones (pSWAT1 and pSWAT2) among 2.4 x 10^4 colonies. Southern blotting of BamHI fragment of plasmid DNA from these clones by synthetic oligonucleotide could estimate both inserts as 1.9kb long.

DNA sequencing was performed about cDNA of clone pSWAT1. ExonucleaseIII digest of pSWAT1 DNA gave 1.5kb, 1.25kb, 1.0kb, 0.55kb and 0.2kb fragments. As the result of sequencing, this cDNA, though partly lacking 5'-portion of mRNA, contained nucleotide sequence corresponding to the NH_2-terminal amino acid sequence of mature sw-AT. On the other hand, curiously, this cDNA had two poly(A) regions and any amino acid sequence identical with that of sw-AT was not found between them.

Colony hybridization using EcoRI-PstI fragment of pSWAT1 cDNA could pick up a clone (pSWAT11) having cDNA of 1.1kb long. This cDNA was 150bp shorter than that of pSWAT1 at 5'-end and had only one poly(A) region. The chain length of sw-AT mRNA was estimated as 1.4kb by Northern hybridization and it became clear that pSWAT1 cDNA had artifactual 0.6kb portion at its 3'-end. Sequencing strategy of sw-AT cDNA was summarized in Fig.2. The result of nucleotide sequence of sw-AT cDNA and amino acid sequence deduced from it was shown in Fig.3. Also in Fig.3, amino acid sequences determined from peptides derived from sw-AT were included and undrlined.

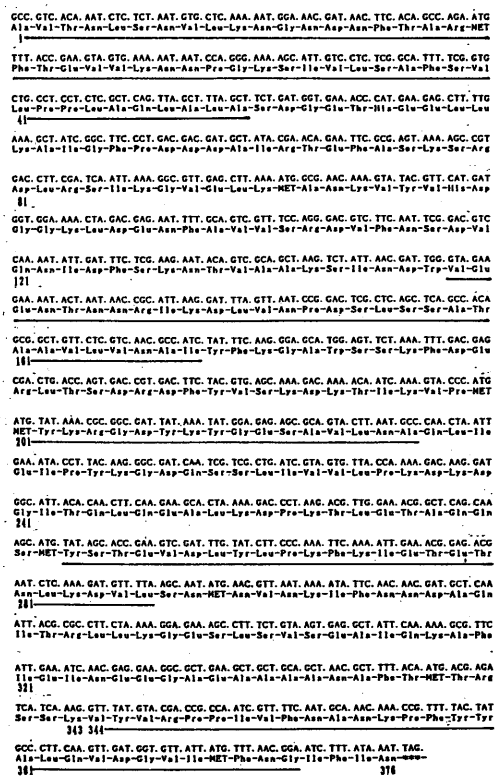

Fig.3 Nucleotide sequence of mature sw-AT cDNA and amino acid sequence deduced from it. The mature sw-AT is constructed with 376 amino acids. The amino acid sequences underlined are determined by peptide analysis. The reactive site of sw-AT with trypsin is Lys_{343}-Val_{344}.

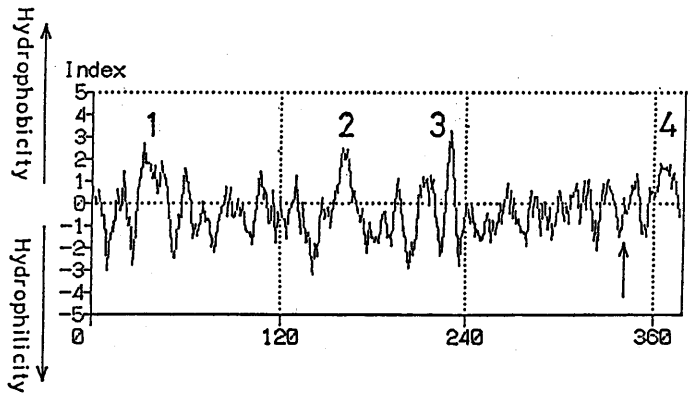

Fig.4 Hydropathy profile of sw-AT. Hydropathy was calculated according to Kyte and Doolittle using DNASIS program (Hitachi Software Engineering). The numbered four regions show relatively higher hydrophobicity. The vertical arrow indicates the position of the reactive site of sw-AT with trypsin.

It is already revealed that sw-AT can make complex with bovine trypsin and that, at that time, new NH_2-terminal amino acid sequence beginning with Val-Tyr-Val-Arg-Pro-Pro---- is produced (Sasaki et al.,1987). Amino acid sequence of sw-AT can identify the position of this peptide (Fig.3). As expected, it begins with Val_{344} in the COOH-terminal region of sw-AT. Also it is clarified that bovine trypsin specifically reacts at Lys_{343}-Val_{344} peptide bond and is inhibited. The degree of hydropathy indicated that the region around this site is hydrophilic, or exposed on the molecular surface (Fig.4).

Amino acid sequence of sw-AT is compared with that of a member of serpin, that is, human α_1-proteinase inhibitor (Long et al.,1984), α_1-antichymotrypsin (Chandra et al.,1983a) or antithrombinIII (Chandra et al.,1983b)(Fig.5). The positions in which the identical amino acid is found for three serpin members are also occupied by almost the same amino acid in the case of sw-AT. It is clear that sw-AT belongs to serpin family. It is interesting that Arg-Ser bond is found at the position just corresponding to the reactive sites in three serpins cited. In silkworm, there may exist a trypsin-like proteinase that reacts and is inhibited at this site.

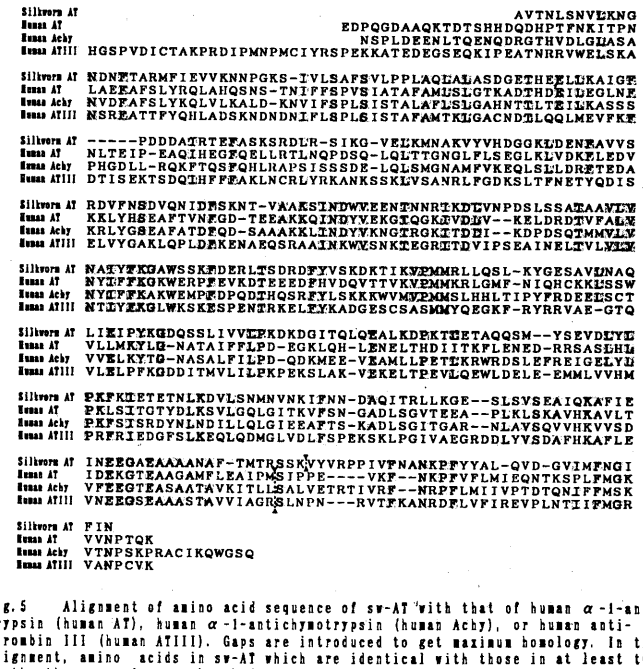

Fig.5 Alignment of amino acid sequence of sw-AT with that of human α-1-antitrypsin (human AT), human α-1-antichymotrypsin (human Achy), or human antithrombin III (human ATIII). Gaps are introduced to get maximum homology. In the alignment, amino acids in sw-AT which are identical with those in at least two of the three serpins are hatched. The reactive site of sw-AT with trypsin is indicated by closed triangles. Reactive sites of three serpins with each target proteinase are indicated by open triangles.

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《和文要旨》

家蚕幼虫体液中に存在するプロテアーゼインヒビターの一種、カイコアンチトリプシン（ｓｗ－ＡＴ）は、そのタンパク化学的性質やトリプシンに対する阻害機能から、高等脊椎動物の血清中などに見いだされるセルピンと呼ばれる一群のプロテアーゼインヒビターの一員であることが予測されていた。これを確認する目的でその全アミノ酸配列を相補ＤＮＡ（ｃＤＮＡ）のヌクレオチド配列より決定した。ｓｗ－ＡＴのｃＤＮＡは、家蚕脂肪体のｍＲＮＡより発現ベクターｐＫＥＮ６０２を用いて作製したｃＤＮＡライブラリー中から抗ｓｗ－ＡＴ抗体と反応するコロニーを捜し、ｓｗ－ＡＴの部分的アミノ酸配列に基づいて合成したＤＮＡプローブとのハイブリダイゼーションにより同定した。そのｃＤＮＡ（ｐＳＷＡＴ１）のヌクレオチド配列を決定し、成熟ｓｗ－ＡＴのＮ末端からＣ末端までの３７６個のアミノ酸を決定した。ヒト血清中のα－１－アンチトリプシン、α－１－アンチキモトリプシンおよびアンチトロンビンIIIとのアミノ酸配列の比較から、ｓｗ－ＡＴが無脊椎動物由来の初めてのセルピンの一員であることが確認された。ｓｗ－ＡＴとトリプシンの反応部位はLys_{343}－Val_{344}であることが判明したが、すぐ近傍にArg_{340}－Ser_{341}が存在することも明らかになり、未だ不明である家蚕体内でのｓｗ－ＡＴの標的プロテアーゼの特異性との関連が注目された。

22-Deoxyecdysteroids : specific ecdysteroids found in the ovaries of Bombyx mori

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Introduction

Ecdysteroids are the group of steroid compounds having the molting hormone activity. Since the isolation of ecdysone from Bombyx pupae in 1954, more than 15 molecular species have been isolated from insects and their structures have been identified. The common features of their structures are hydroxyl groups at C-3,C-14,C-22,C-25, together with 6-one-7-ene structure. According to the classical scheme of insect post-embryonic development, ecdysteroids in immature stages (larvae and pupae) are synthesized in and secreted from the prothoracic glands. In a past decade, ovaries (and testes in some insects) are shown to be the second organ(s) which are capable of ecdysteroid biosynthesis (1). Whereas in immature stages of insects, the major ecdysteroids are generally 20-hydroxyecdysone or ecdysone, in ovaries or eggs occur multiple molecular species, including several 2-deoxyecdysteroids (2). In the ovaries of Bombyx mori, we have isolated and identified two species of ecdysteroids, lacking hydroxyl group at C-22. This kind of ecdysteroids has not been found so far in insects. The meaning of the occurrence of these unusual ecdysteroids in Bombyx ovaries is discussed.

Materials and Methods

Animals and isolation procedure

Matured ovaries, isolated from pupae of hybrid strains, were the source of ecdysteroids. Frozen ovaries were crushed and ground in a mortar under liquid nitrogen. Pulverized samples were then extracted first with 5 volumes of ethanol and with 80 % aq.ethanol several times.

Details of the isolation of free and conjugated ecdysteroids from Bombyx ovaries have been described elsewhere (2,3). The method is essentially as follows. The ethanolic extract of the ovaries was concentrated under reduced pressure and precipitate produced was filtrated. The filtrate was further concentrated to a small volume and was partitioned between 70% aq.methanol and petroleum ether. The methanolic layer was concentrated and applied to the column of Sephadex G15. The eluate was then applied to the column of silicic acid. Free ecdysteroids were eluted from the column with a mixture of benzene-methanol (9:1, v/v), while the conjugates were eluted with methanol. The free ecdysteroids were further purified by thin-layer chromatography (TLC) on silica gel and high performance liquid chromatography (HPLC), using a reversed-phase column. The conjugated ecdysteroids in the eluate of silicic acid column were further purified by Sephadex LH-20 column, using methanol as a developer, and then by HPLC using gradient elution system.

Characterization and identification of ecdysteroids

Characterization and identification of the isolated ecdysteroids were done in collaboration with Drs.Ikekawa and Fujimoto of Department of Chemistry, Tokyo Institute of Technology. The methods consisted of enzyme (snail juice, alkaline phosphatase) hydrolysis, UV- and IR-spectrometry, mass spectrometry and proton magnetic resonance.

Results

Isolation and identification of Bombycosterol

In the chromatogram of HPLC of the ovarian extract, ecdysteroids eluted from the reversed-phase column in an order of polarity. Namely, 20-hydroxyecdysone came out first,following ecdysone, 2-deoxy-20-hydroxyecdysone,2-deoxyecdysone and 2,22-dideoxy-20-hydroxyecdysone, although peaks of 20-hydroxyecdysone and ecdysone are sometimes consealed by impurities mostly appeared at the breakthrough. However, long after the elution of 2,22-dideoxy-20-hydroxyecdysone, another UV-absorbing peak appeared. The material ehxibiting the Uv-absorption was collected and subjected to UV-spectrometry. It showed absorption maximum in ethanol at 243 nm, indicating that it is ecdysteroid. From about 6000 pairs of the ovaries, 600 μg of this material were obtained and were subjected to physico-chemical analysis. From mass spetrometry, molecular weight of this material was assumed to be 448, and it has the same side chain structure as 2,22-dideoxy-20-hydroxyecdysone. Mostly from the chemical shift and coupling patterns of NMR charts, the structure was deduced to be (20S)-cholesta-7,14-diene-3β,5α, 6α,20,25-pentaol (4). Since it is not the derivative of ecdysone, and has no keto-group at C-6 position, the name "bombycosterol" was coined.

Isolation and identification of bombycosterol 3-phosphate and 2,22-dideoxy-20-hydroxyecdysone 3-phosphate

From the methanolic eluate of silicic acid column, ecdysteroid comjugates could be isolated. prior to the application to HPLC, chromatography on Sephadex LH-20 was quite efficient.From unknown reason, the conjugates were eluted just after the breakthrough. In the chromatogram of HPLC, six major peaks could be recognized. These UV-absorbing materials were collected and subjected to

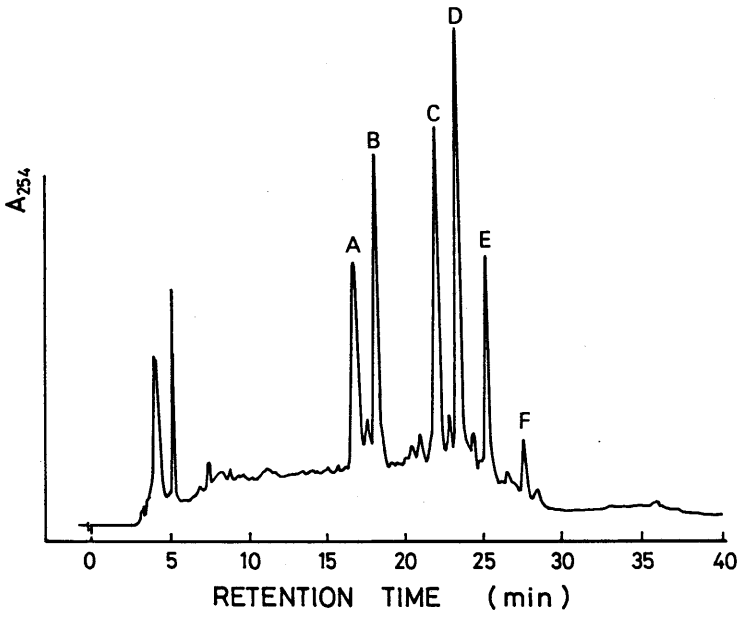
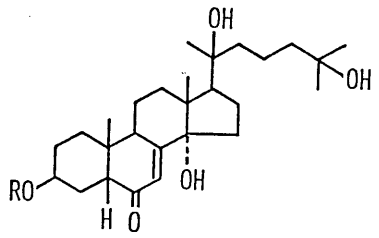
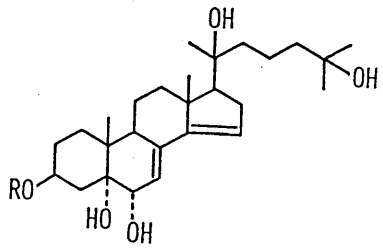


Fig.1. HPLC profile of ecdysteroid conjugates in Bombyx ovaries. A: 20-hydroxyecdysone 22-phosphate, B: ecdysone 22-phosphate, C: 2-deoxy-20-hydroxyecdysone 22-phosphate, D: 2-deoxyecdysone 22-phosphate, E: 2,22-dideoxy-20-hydroxyecdysone 3-phosphate, F: bombycosterol 3-phosphate



(1) $R=PO_3H_2$
(1a) R=H



(2) $R=PO_3H_2$
(2a) R=H

Fig.2. Structures of 2,22-dideoxy-20-hydroxyecdysone (1a) and its 3-phosphate (1), of bombycosterol (2a) and its 3-phosphate (2).

enzymatic and physico-chemical analyses. The results revealed that these substances were the phosphate esters of 20-hydroxyecdysone (A), ecdysone (B), 2-deoxy-20-hydroxyecdysone (C), 2-deoxyecdysone (D), 2,22-dideoxy-20-hydroxyecdysone (E), and bombycosterol (F) (the alphabet in the parentheses corresponds to those marked in Fig.2). Among these conjugates, 4 were identical to the previously reported ones, but 2 were previously unknown. They were exceptional in that phosphate radical is bound to the hydroxyl group at C-3. These structures are shown with their corresponding ecdysteroids in Fig.2.

Discussion

Ecdysteroids accumulated in the ovaries of insects vary markedly among insect species. In Bombyx mori, 6 major ecdysteroids accumulate as the free and conjugate forms. This pattern is in contrast to that found in the ovaries of Manduca sexta (5), where 26-hydroxyecdysone 26-phosphate is the predominant species, accompanied with 26-hydroxyecdysone 2-phosphate as a minor component. Among the 6 ecdysteroids in Bombyx ovaries, 2 have exceptional structures lacking hydroxyl group at C-22, but have them at C-20. More than 50 ecdysteroids have been isolated and identified from various plants and animals up to now. However, only few of them lack hydroxyl-group at C-22. This fact is partly attributable to the screening methods used. Usually, ecdysteroids have been detected by bioassay, which make use of the molting hormone activity, and by radioimmunoassay, in which ecdysone or 20-hydroxyecdysone was used as haptene of the antigen. Since hydroxyl group at C-22 is indispensable for the molting hormone activity and also for the antibody-antigen reaction, ecdysteroids lacking hydroxyl group at C-22 usually escape the

detection. Recently, 2 ecdysteroids lacking C-22 hydroxyl have been found in the pycnogonid, Pycnogonum litorale (6). There remains a possibility that many 22-deoxyecdysteroids would be found if we use the screening methods which could detect these compounds. Since 22-deoxyecdysteroids have no or quite weak molting hormone activity, we have to find out the function(s) of these new class ecdysteroids. The relation of 22-deoxyecdysteroids to 22-hydroxyecdysteroids rather resembles to that between glucocorticoids and mineral corticoids or between androgen and estrogen in vertebrates.

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２２－デオキシエクジステロイド：カイコ卵巣に見出された特異的エクジステロイド

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カイコ卵巣から６種のエクジステロイドが遊離型および結合型として得られた。　このうちの２種は側鎖のＣ－２２位に水酸基の無い特異な構造をしている。　このようなエクジステロイドは他の昆虫からは得られていない。　現在までに５０種以上のエクジステロイドが動植物から得られているが，Ｃ－２２に水酸基の無いものは僅かしかない。　これは，主としてエクジステロイドのスクリーニングの方法によると思はれる。　Ｃ－２２に水酸基の無いエクジステロイドはおそらくスクリーニングの方法を変えればかなり見出される可能性があると思われる。この新しいタイプのエクジステロイドは殆ど脱皮ホルモン活性が無いので，新しい生理活性を見出さねばならない。

ARGININE DEGRADATION CASCADE OF THE SPERMATOPHORE RELATED TO THE SPERM MATURATION OF SILKMOTH, BOMBYX MORI

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INTRODUCTION

In the silkworm, Bombyx mori, the complicated process from sperm maturation to fertilization is distinguished by the following five stages: 1. spermatogenesis in the testis, 2. transfer of apyrenes and eupyrene bundles to the vesicula (v.) seminalis, 3. ejaculation and spermatophore formation, 4. migration of both apyrenes and eupyrenes to spermatheca and 5. fertilization of eggs in the vestibule (Osanai et al., 1987a). I principally studied the stages 2 and 3. The sperm maturation, the acquisition of apyrene motility and the dissociation of eupyrene bandles followed to the acquisition of eupyrene motility, occurs in the spermatophore consisting of secretions of male reproductive tracts (Kasuga et al., 1987). Accompanied with these physiological changes, various biochemical reactions related to the sperm maturation must also be caused in the spermatophore as a closed recotor, since the male secretions containing separately many sorts of enzymes and their substrates are mixed, for the first time, to each other by ejaculation.

MATERIALS AND METHODS

Two hybrids of commercial strains of the silkworm, Bombyx mori, Fuyo X Tokai and J124 X Ch124, were used. The glandula (g.) lacteola, g. spermatophorae, g. prostatica and v. seminalis were each excised from female moths 30 min after the beginning of copulation, arginase activity and free amino acid contents in these male reproductive glands and the b. copulatrix were measured as described previously (Aigaki and Osanai, 1985; Osanai and Yonezawa, 1985). Metabolites of glycolysis in the spermatophore and release of carbon dioxide were analyzed by use of [1-^{14}C]glucose-1-phosphate, [1-^{14}C]pyruvate and [U-^{14}C]-compounds of 2-oxoglutarate, ornithine, pyruvate and glucose-1-phosphate (Osanai et al., 1987b). Enzyme activity of a specific prostatic endopeptidase, BAEEase or initiatorin was determined toward a synthetic substrate, Nα-benzoyl-L-arginine ethyl ester (BAEE) (Aigaki et al., 1987). Male reproductive tracts and b. copulatrix (or spermatophore) were microscopically and electronmicroscopically observed (Osanai et al., 1987a;d) to compare their histological and morphological changes with biochemical changes.

RESULTS

Sperm mature after transfer to the spermatophore: apyrenes acquire motility and eupyrenes dissociate due to digestion of their bundles by initiatorin or trypsin. Their dissociation is promoted mechanically by the rotating movement of the active apyrenes. With time after the

spermatophore formation, the regions and granules staining with hematoxylin and PAS in the spermatophore decrease, while eosinophilic substances appear and accumulate. The PAS-positive granules derived from secretions of the male g. lacteola and the proximal part of the v. seminalis were identified as glycogen by treating them with α-amylase.

During and after mating, a large amount of urea accumulates in the spermatophore, indicating active hydrolysis of arginine (Fig. 1.). Arginase, which is present in high activity in the male v. seminalis is transferred to the spermatophore without loss of activity during ejaculation. In the spermatophore, the level of ornithine remains much lower than that of urea formed in the equimolar amounts. The levels of proline and glutamine also increase

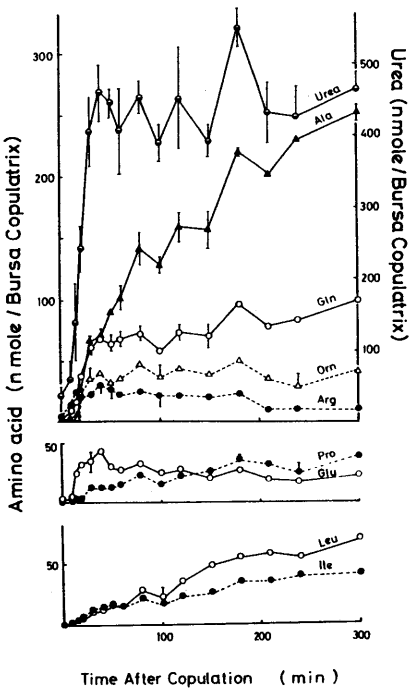

Fig. 1. Changes in amino acid pools in the bursa copulatrix during and after mating.

slightly, but there is a marked increase in the level of alanine in the spermatophore. On the contrary, the concentrations of all free amino acids are very low, in the exocrine glands of the male reproductive system. No urea is detectable in the secretions of any male glands. The changes in levels of these free amino acids thus strongly suggested the presence of a cascade reaction in the spermatophore from arginine via glutamate for the active formation of 2-oxoglutarate with consumption of pyruvate produced by extracellular glycolysis. High alanine-glutamate aminotransferase activity was found in the v. seminalis as well as spermatophore.

This cascade reaction was confirmed by showing that [^{14}C]glucose-1-phosphate and [^{14}C]pyruvate were metabolized

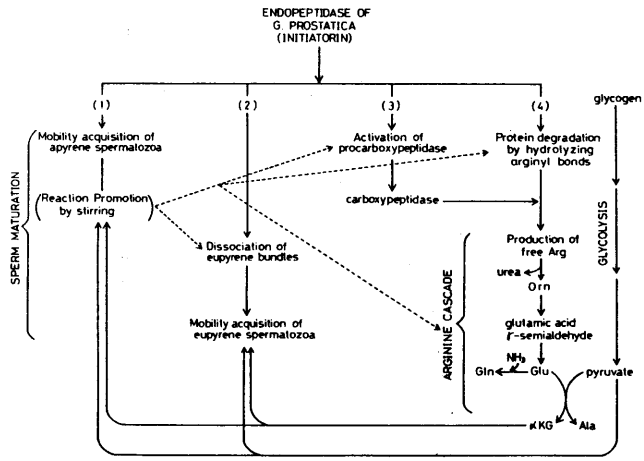

Fig. 2. Initiatorin and its regulation mechanism of arginine degradation cascade in the spermatophore of Bombyx mori.

with the formation of radioactive alanine. Moreover, in the spermatophore containing spermatozoa, $^{14}CO_2$ was released not only from these radioactive glycolytic intermediates but also from both labelled ornihtine and 2-oxoglutarate which was a preferred respiratory substrate for the sperm. Thus, the arginine degradation cascade coupled with glycolysis serves as an energy-yielding system for spermatozoa.

For this supply of so much free arginine, a specific proteolytic system composd of a specific endopeptidase of g. prostatica, initiatorin, and a carboxypeptidase acts in the spermatophore. By this system, only arginine is specifically produced. Unlike trypsin, initiatorin (30 KDalton) showed marked hydrolytic activity on BAEE, and slight activity on Nα-p-tosyl-lysine methyl ester, but none on Nα-p-tosyl-lysine methyl ester. Inhibition specificity with many endopeptidase inhibitors showed that initiatorin must belong to a serine protease.

CONCLUSION

Only the fragmentary information is available about other insects for comparison with these findings on the spermatophore of Bombyx mori. In this series of experiments, the following phenomena were newly discovered or elucidated.

1. Enzymes and their substrates are separately localized in the secretions of the male reproductive tracts and the mixing of them at the ejaculation causes, for the first time, various reactions for sperm maturation in the spermatophore.

2. Physiological functions of the spermatophore are the site for sperm maturation as well as the metabolic reactor necessary for it.

3. Role of apyrene sperm is to stir the viscous spermatophore contents to promote reactions.

4. Arginine degradation cascade occurs in the spermatophore.

5. Free arginine is supplied by a specific proteolytic system composed of initiatorin and a carboxypeptidase. Initiatorin contained only in the secretion of the proximal part of g. prostatica is, therefore, the important key substance acting as an initiator: (a) in inducing for motility of apyrenes, (b) in dissociating eupyrene bundles, (c) in activating carboxypeptidase, and (d) in the arginine degradation cascade (Fig. 2). Namely, initiatorin is a new type of protease, since this extracellular protease functions for the appearance of physiological cell (sperm) actions and their regulations like a intracellular protease.

6. Extracellular glycolysis proceeds in the spermatophore. This occurrence has never been reported in higher animals.

7. Glycogen is utilized as a energy source for sperm maturation. Hitherto, glycogen has not appeared to be a common constituent of secretions of male reproductive tracts of insects (Leopold, 1976; Lai-Fook, 1982).

8. Protein is primarily utilized as an energy source.

9. 2-Oxoglutarate is a preferred respiratory substrate

for the silkworm sperm: it is twice more available than pyruvate.

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要　約

カイコガの交尾、射精によって、次のように、極めて特異な代謝系が作動し、精子の成熟を活性化する。

1．酵素と基質が、雄生殖輸管の各分泌物に別々に局在しており、射精による混合が精包における精子成熟のための様々な反応を惹起する。

2．精包の生理的機能は精子成熟のための場であると共に、そのために必要な代謝の反応槽である。

3．運動性を獲得した無核精子は、高粘性の精包内容物を攪拌して、諸反応を促進させる。

4．精包では、細胞外解糖と、それと共役したアルギニン分解カスケードが起こる。昆虫の生殖輸管中のグリコーゲンの存在は、これまで否定的であった。高等動物における細胞外解糖も初めての知見である。

5．遊離アルギニンは、イニシャトリンとカルボキシペプチダーゼとからなる特異な蛋白分解系によって供給される。イニシャトリンは、生殖輸管の最末端に位置を占める前立腺にのみ存在するエンドペプチダーゼで、（1）無核精子の運動性獲得、（2）有核精子束の解離、（3）カルボキシペプチダーゼの活性化、（4）アルギニン分解カスケードの引金としての働きを有する。

6．蛋白質が積極的な呼吸基質になりうる。

7．2－オクソグルタル酸がカイコガ精子にとって好適なエネルギー生成の基質となる。