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CHEMICAL STUDIES ON THE SILKWORM DIAPAUSE HORMONE

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DIAPAUSE EGGS INDUCED BY DIAPAUSE HORMONE

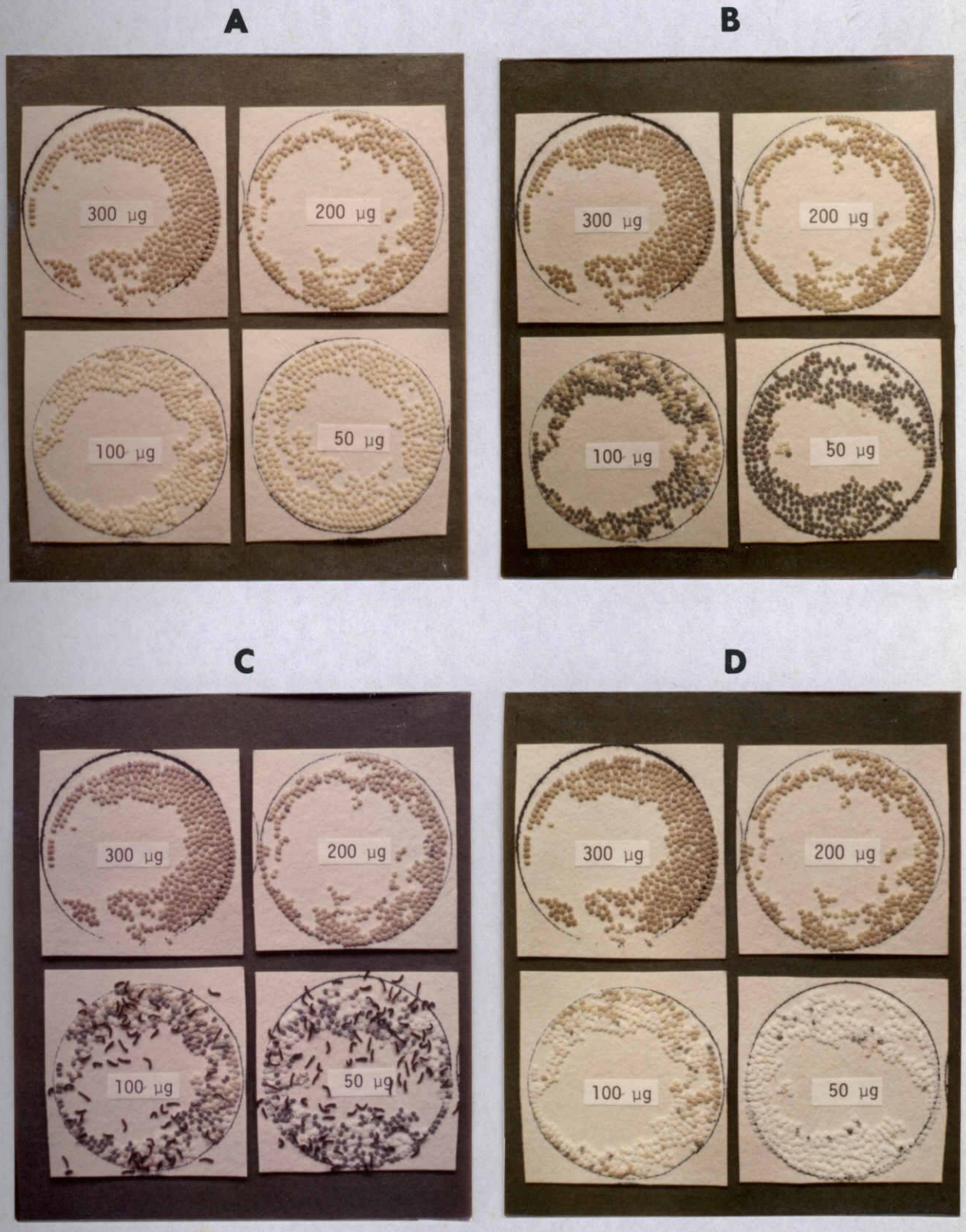

Fig. 1

CONTENTS

ABBREVIATIONS

DH	diapause hormone
DHE	diapause hormone extracts
DE%	diapause egg percentage
DH unit	diapause hormone unit
Glc-NH_2	glucosamine
Gal-NH_2	galactosamine
3-OHK	3-hydroxykynurenine
SG	suboesophageal ganglion

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CHAPTER I

ISOLATION OF THE SILKWORM DIAPAUSE HORMONE

INTRODUCTION

Insect Diapause

Diapause, a cessation of development at embryonic, larval, pupal or adult stage is one of spectacular phenomena in insects. It may be defined as a temporary interruption of development which would occur even if the insects were kept under optimum conditions for their growth, and a period of adverse conditions such as low temperature and/or long days is necessary before development can continue. Furthermore, diapausing insects can continue their arrested development even if environmental circumstances become adequate for their growth, whereas quiescent insects resume their development when the adverse conditions were removed.

The occurrence of diapause in the most unfavourable conditions for the development of the species, for example, the low temperatures of winter or summer drought, and its adaptive value for the species clearly show the role that environment has had in the evolution of a given species. Apart from this common 'hylogenetic' adaptation, in so-called 'facultative' diapause the ambient conditions concerned have been found to determine whether the genetically conditioned 'facultative' diapause will actually occur or not. On the other hand, obligatory diapause occurs always at a definite stage of the insect irrespective of external conditions. In other words, insects with obligatory diapause repeat only one generation a year and those with facultative

diapause repeat more than two generation according to the environmental conditions.

The termination of diapause is in general accomplished by exposure of the diapause insects to low temperature and/or long days. During this long period, physiogenesis is believed to proceed during in vivo which is called "diapause development" (Andrewartha, 1952).

The occurrence, maintenance and termination of diapause are also studied by endocrinological point of view and attracted great interest among research workers in the field of insect endocrines as well as insect biochemistry.

Silkworm Diapause

Some races of the silkworm, Bombyx mori, repeat many generations irrespective of environmental circumstances, which is called polyvoltinism. Other races two or only one generation a year, the former being bivoltine races and the latter univoltine races. Bivoltine races are of the facultative diapause and univoltine races the obligatory diapause, and the diapause occurs at embryonic stage in both.

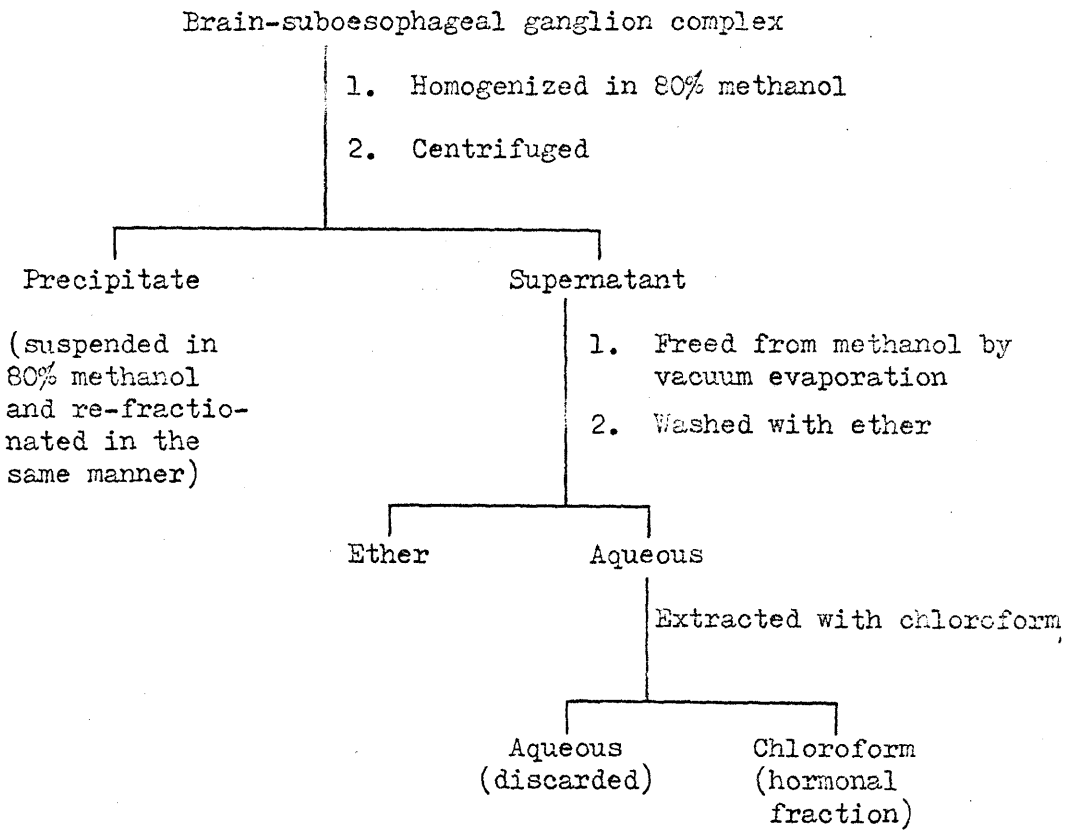
The studies on the voltinism of the silkworm was first reported by Toyama (1906) and since then profound results were obtained by only Japanese workers. Many hypotheses concerning the mechanism of diapause induction in silkworm eggs were put forwards: production of a substance inhibiting the development of embryos (inhibitory substance theory), low activity of an enzyme-like substance responsible for mobilization of yolk surrounding embryos (voltinism-

determiner theory), etc. But these theories are in fact working hypotheses and nobody knows these substances stated above (Hasegawa, 1959).

Hasegawa (1951) found that this hormone originates in the Suboesophageal ganglion (SG)——an observation which was confirmed almost simultaneously by Fukuda. The exterpation of SG at the beginning of the pupal stage causes eggs which were destined to diapause through their mother to develop without diapause. In the same way univoltine females can be caused to lay non-diapause eggs, a thing which they never do under normal conditions. Conversely, a female pupa which has been conditioned to lay non-diapausing eggs receives a SG from a female which would have laid diapausing eggs it also will lay diapausing eggs. The same effect can be produced by the implantation of SG from either females or males of a univoltine race.

The Diapause Hormone

Since the finding of the SG activity in diapause induction in silkworm eggs, Hasegawa engaged in extraction of substance secreted from the SG and succeeded in extraction of this substance which was called the diapause hormone (Hasegawa, 1957). The hormone was extracted from SG and brain complexes dissected out from about 15,000 silkworm pupae: the complexes were homogenized in 80% methanol and centrifuged. The supernatant fluid was evaporated in vacuo and washed with ether. The aqueous layer was then extracted with chloroform. The chloroform extract was evaporated to



PROCEDURE USED TO SEPARATE THE DIAPAUSE HORMONE FROM GANGLION TISSUES

from K. Hasegawa, Nature, 179, 1300 (1957).

give a solid which showed a high diapause inducing activity when it was injected into female pupae conditioned to lay non-diapausing eggs. Consequently, the diapause hormone secreted from SG is responsible for embryonic diapause in the silkworm.

Since this achievement of extraction of the hormone, its purification has been engaged using more than one million silkworms as the source every year.

Biological Significance of the Diapause Hormone

The diapause eggs of silkworms become dark a few days after oviposition. This pigment (ommochrome) originates from 3-hydroxykynurenine (3-OHK) accumulated in oocytes during pupal-adult development. In non-diapause eggs, however, 3-OHK less accumulates and hence no coloration in them was observed. Further, diapause eggs contain about 60% higher amount of glycogen than non-diapause eggs. These characteristics of diapause eggs were examined by Hasegawa and his-colaborators from the stand point of the mode of action of the diapause hormone.

Hasegawa and Yamashita (1963, 1965) have directly disclosed the effects of the hormone on the penetration of 3-OHK into the developing ovaries by injecting the diapause hormone into female pupae. They confirmed these effects using two egg-color mutants of silkworms: one has no enzymes to transform kynurenine into 3-OHK, the other has a selective permeability of 3-OHK in its ovaries (Yamashita and Hasegawa, 1966).

The effects of the diapause hormone on the glycogen content of the ovaries and the blood sugar level of the female pupae were studied (Yamashita and Hasegawa, 1965). It was, then, concluded that one of the actions of the hormone was to stimulate the penetration of blood sugars into the pupal ovaries and their deposition therein in the form of glycogen, thus decreasing their content in the haemolymph. Correlation of glycogen content with oocyte age and its synthesis in oocytes was demonstrated to explore the diapause hormone-action on pupal ovaries (Yamashita and Hasegawa, 1970).

Recently, they reported the effects of the diapause hormone on trehalase activity concerning the carbohydrate metabolism in the silkworm with the diapause (Yamashita and Hasegawa, 1967; Yamashita, Hasegawa and Seki, 1972). According to this paper, the trehalase activity in pupal ovaries decreased itself by extirpation of the SG secreting the hormone. Its activity can, however, be recovered in ca. 3 hr by injecting the diapause hormone into the SG-removed pupae: this hormone facilitate the accumulation of glycogen through enhancement of the trehalase activity in the ovaries.

In other recent investigation (Kai and Hasegawa, 1971, 1972) of the mode of the hormone affecting protein metabolism in the silkworm, "esterase A", a non-specific esterase involved in the embryonal development, disappeares or is less active in diapause eggs than in non-diapause eggs.

Thus, the diapause hormone has been known to play

important roles in the regulation in the mother organism of the silkworm together with the eggs.

Chemical Significance of the Diapause Hormone

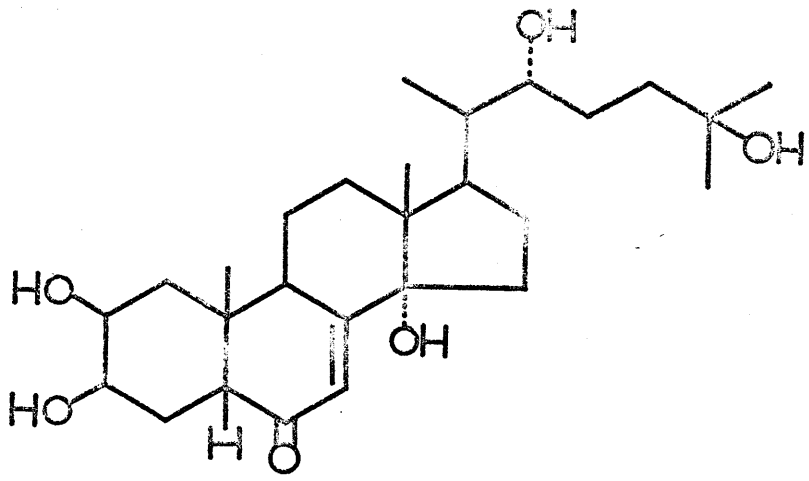
The insect hormones whose chemical structures are known to date are moulting hormone (MH) and juvenile hormone (JH) classified in a category of glandular hormone.

MH is secreted from prothoracic glands, acts in several tissues and induces insect moulting. Its structure was elucidated in 1965 by Hüber and Hoppe after X-ray crystallographic analyses as I (α-ecdysone). It is now known seven zoo-ecdysones isolated from animals together with ca. 30 phytoecdysone from plants.

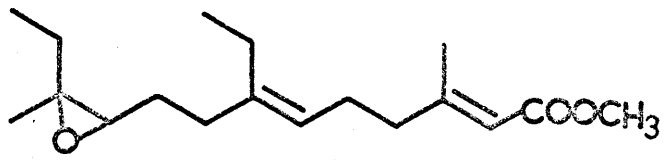
JH is secreted, on the other hand, from corpora allata and has morphologic, gonadotropic and prothoracotropic actions. In 1967, its structure was proved to be II (C_{18}-juvenile hormone) by Röller et al., and now C_{17}-JH is known to play JH activity.

The diapause hormone (DH) now isolated from the silkworm is secreted from suboesophageal ganglion, acts on pupal developing ovaries and induces diapause egg production. It is classified as neuro hormone together with brain hormone (or activation hormone: AH) which has been highly purified.

The life science of such insect hormones was initiated by biologists and then is in progress in the field of insect endcrinology, where the chemistry of the natural products plays also an important role. Elucidation of the chemical structure of ecdysone has stimulated investigations on



[I] α-Ecdysone



[II] Juvenile hormone

ecdysone-like substances from plants as well as other insects or animals. And these investigation has brought a great progress to insect physiology. And then the chemical research correlating the insect hormones takes part in the life science of the insect by its own way such as elucidating their chemical structures, total synthesis or biogenesis (biosynthesis) of them, etc. However, tremendously difficult problems are left unsolved, some of which should be elucidated chemically. Consequently, chemical approach toward the study of the silkworm diapause must be inevitable for further development of the study of the silkworm diapause.

ABSTRACT

The diapause hormone (DH), secreted from the suboesophageal ganglion is responsible for the embryonic diapause in the silkworm, Bombyx mori L. It was extracted by an efficient procedure from two millions of mated male adult heads of the silkworm. At least two species of DH were separated from the extracts (a complex lipid fraction) by gel permeation chromatography with Sephadex LH-20 by organic eluants. One of them was further purified by repeating gel permeation column chromatography with Merckogel^{R} Type OR 6000 in the cold and dark finally to give a single peak with high DH activity. This preparation seems to be chemically pure and its molecular weight is chromatographically estimated to be between 2,000 and 4,000. Other characteristics of DH are also presented. Biological activity of the final DH preparation is discussed with reference to other insect hormones such as juvenile hormone and α-ecdysone.

EXPERIMENTAL

1) Bioassay

The activity of DH in a test sample was directly estimated by observing whether or not diapause eggs would be laid by moths of non-diapause egg producers which had been injected with the sample at the pupal stage. So that available season for this assay is practically limited from April to December in a year (about ten times).

Silkworm pupae of a polyvoltine race (N_4) were mainly used for the bioassay, and sometimes those of a hybrid between Daizo (bivoltine race) and N_4 were also used. These non-diapause egg producers were exposed to a low temperature and a darkness during their embryonic life, so that the female adults should be destined to lay only non-diapause eggs but no diapause eggs.

Samples to be assayed were dissolved or suspended in water containing a small amount of ammonia, and was injected into 3-day old pupae with a finely tapered glass capillary. Each 5 to 30 µl of different concentrations of the solution was mainly injected into 9 pupae in the dorsum of their pregonadal segment avoiding the dorsal vessel.* The pupae injected with the test sample emerged

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* The injections of test samples are most effective in 3-day old pupae to produce diapause eggs owing to avoiding in vivo inactivation of the injected (to be continued)

4 or 5 days after the injection at 27-28°C.

In general, 3 days after oviposition, diapause eggs become dark brown in color owing to the pigment (ommochrome) formed in the serasa cells, while non-diapause eggs remain pale yellow up to the end of embryonal stage. Therefore, 3 days after oviposition, we can estimate the degree of DH activity of test samples by observing the color of the laid eggs. The degree of DH activity of a given sample, thus estimated, is expressed as follows:

+++ indicates the production of all or most diapause eggs, indicating that the injected sample has strong DH activity;

++ both diapause and non-diapause eggs, number of the former is less than the latter;

+ only a few diapause eggs; and

- no diapause eggs.

Intermediates among these degrees are tentatively presented as <+++, >++, <++, >+, <+ and ±.

Fig. 1-A was taken a few days after oviposition, indicating that egg color becomes reddish in 300 and 200 µg injections of DH fraction, whilst remains yellowish in less amount of the fraction. Colored eggs are diapause eggs

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samples. Purified DH preparations, however, were injected one day later when the compound eyes colored dark red, because by the usual method, the DH is completely inactivated before displaying its activity.

produced by the action of the DH injected, while remaining yellowish eggs non-diapause eggs produced without any effect of DH. Therefore, the embryos of the former eggs overwinters in this state, while those in the latter develop and hatch from egg shells as larvae with black pigment. Thus, in Fig. 1-B are shown those black non-diapause eggs with larvae just before hatching, C indicates larval hatching from non-diapause eggs and D the white egg shells after larval hatching and colored eggs remaining in diapause. In 200 and 300 μg injections of DH fractions, all or almost all eggs are of diapause type and in 50 μg injections of them, the eggs are mostly of non-diapause type though a few diapause eggs were produced. Thus, the hormonal activity of this sample is estimated as follows: +++ in both 300 and 200 μg/pupa, ++ in 100 μg/pupa, and + in 50 μg/pupa, respectively.

Silkworm larvae generally hatch from non-diapause eggs about 11 days after oviposition. Therefore, more than 2 weeks after oviposition of adults injected with a test sample, diapause eggs and egg shells of non-diapause eggs after larval hatching (just like the picture D-50 μg in Fig. 1) can be counted in each batch of eggs laid by each adult. To compare DH activity among the test samples in detail, some percent portions of the diapause eggs of about 9 adults was referred to as the DH activity of a given amount of the sample.

Furthermore, in order to estimate the relative

efficiency of extraction and purification of DH, a diapause hormone unit (DH unit) was tentatively established. One DH unit is defined as the amount of a test sample necessary to produce on an average 40-60% diapause eggs when injected in a non-diapause egg producer.

2) Materials

The most adequate source for the preparation of DH seems to be the complexes of brain and suboesophageal ganglion (SG), which, however, must be dissected out from silkworm pupae. Hence they can be used only in case of small amount preparation and they could be collected in impractically sufficient amount for the chemical studies on DH. The finding (Hasegawa, 1964) that DH content in the silkworm male adult heads is much higher than that in females made it promising for us to collect sufficient amount of the male heads as the DH source, since they are usually discarded in the last course of the silkworm seed production. In every year, more than one million adult heads were kindly supplied from the Nagano Silkworm Seed Production Institute, where the moths were dried below 80°C and their heads were cut off. The whole heads were throughly ground at room temperature with a mechanical pulverizer and used for the following investigation.

SEX DIFFERENCE IN CONTENT OF DIAPAUSE HORMONE IN MOTH HEADS

(Gimpaku, 'diapause' race)

Sex of donors	Amount of extract injected (mg)	Average numbers of eggs laid		Average percentage of diapause eggs
		Non-diapause eggs	Diapause eggs	
♀	0.8 (=23 heads)	289	0	0.0
♀	1.6 (=45 heads)	278	14	5.2
♂	0.8 (=18 heads)	229	31	12.6
♂	1.6 (=36 heads)	167	105	39.6*

* By exclusion of the moths showed low response (5.1 and 13.9), the percentage is on an average 49.6 ranging from 40.0 to 71.3.

from K. Hasegawa, J. Exp. Biol. 41, 855 (1964).

3) Extraction

The procedure originally used for extraction of DH from the dissected brain-SG complexes (Hasegawa, 1957) could not be applied for extraction of it from the whole adult heads in a large scale. After many trials, we have finally established extraction procedure as indicated in Fig. 2.

Thus, the pulverized one million heads (ca. 2 kg) were washed with acetone*(2.5 l x 5 times), and the residue was extracted three times with 2.5 l portions of a mixture of methanol and chloroform (1:1)* The extracts were evaporated under reduced pressure below 50°C to give an oily residue, which gave precipitates by addition of acetone. The precipitates were collected by centrifugation, rinsed with acetone and then partitioned between butanol and water. The upper (butanol) layer was separated and washed well with water (over five times) to become free from water-soluble materials and then evaporated in vacuo to dryness to give a dark brown solid (Diapause Hormone Extracts: DHE). The yield of the DHE from one million heads was 30 g, and 900 µg of this DHE which corresponds to about 30 heads was as active as 1 DH unit. Thus, the efficiency of this extraction procedure is comparable to that of the dissected brain-SG complexes as the DH source for the extraction.

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* Reproducibility of this procedure was confirmed during the course by monitoring the residual weight after evaporating every extract as shown in Fig. 3-a and Fig. 3-b.

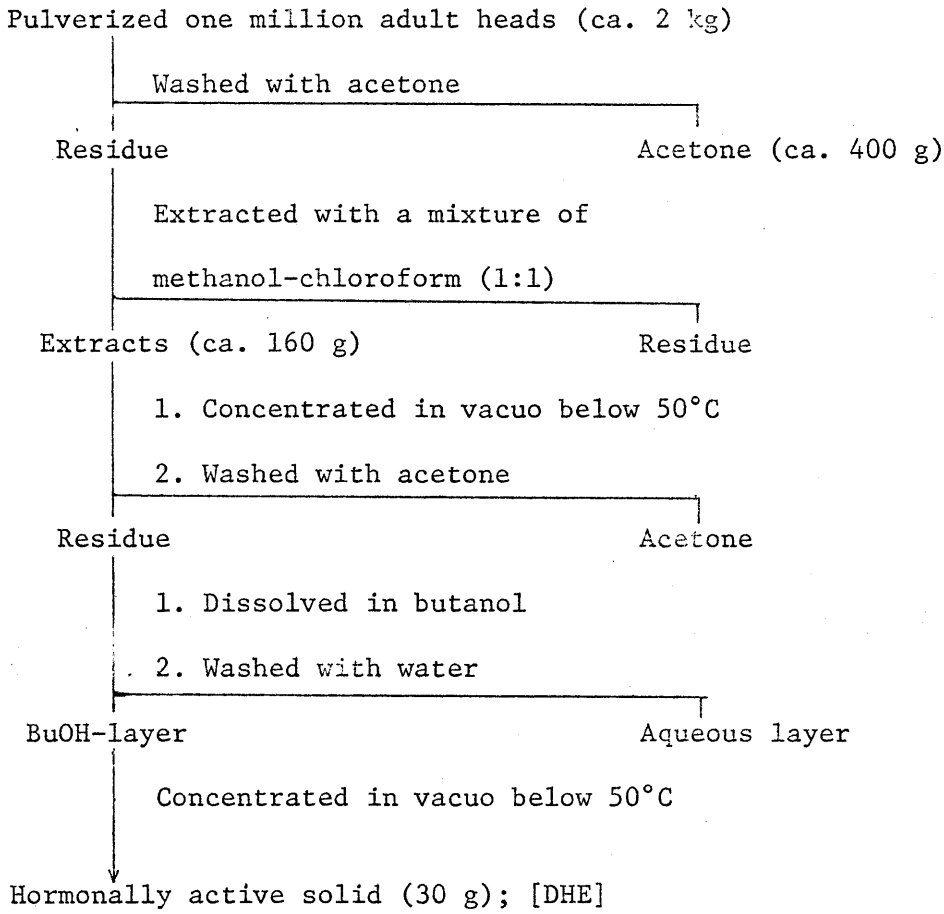

Fig. 2 Flow sheet of extraction of the diapause hormone from one million adult heads of mated males.

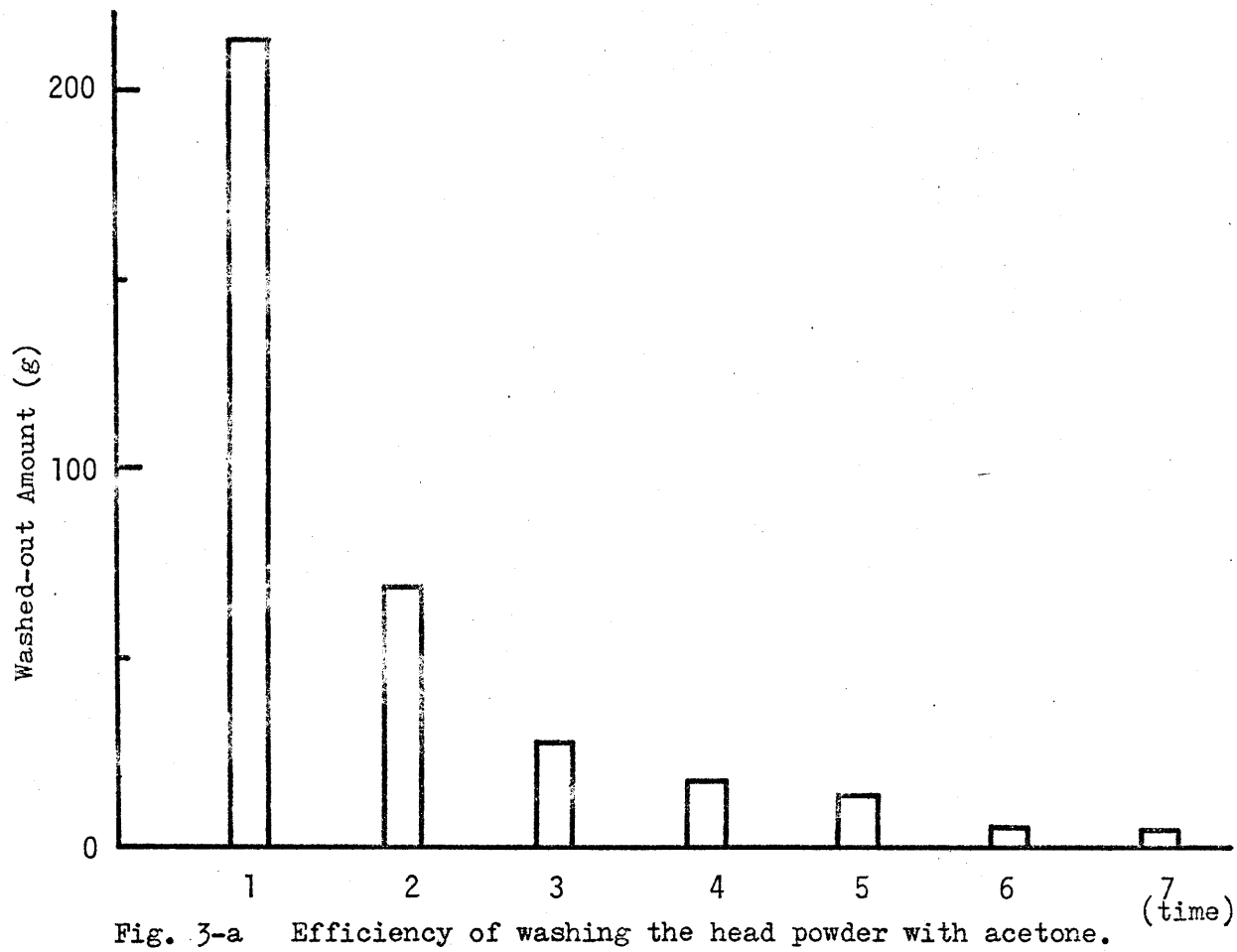

Fig. 3-a Efficiency of washing the head powder with acetone.

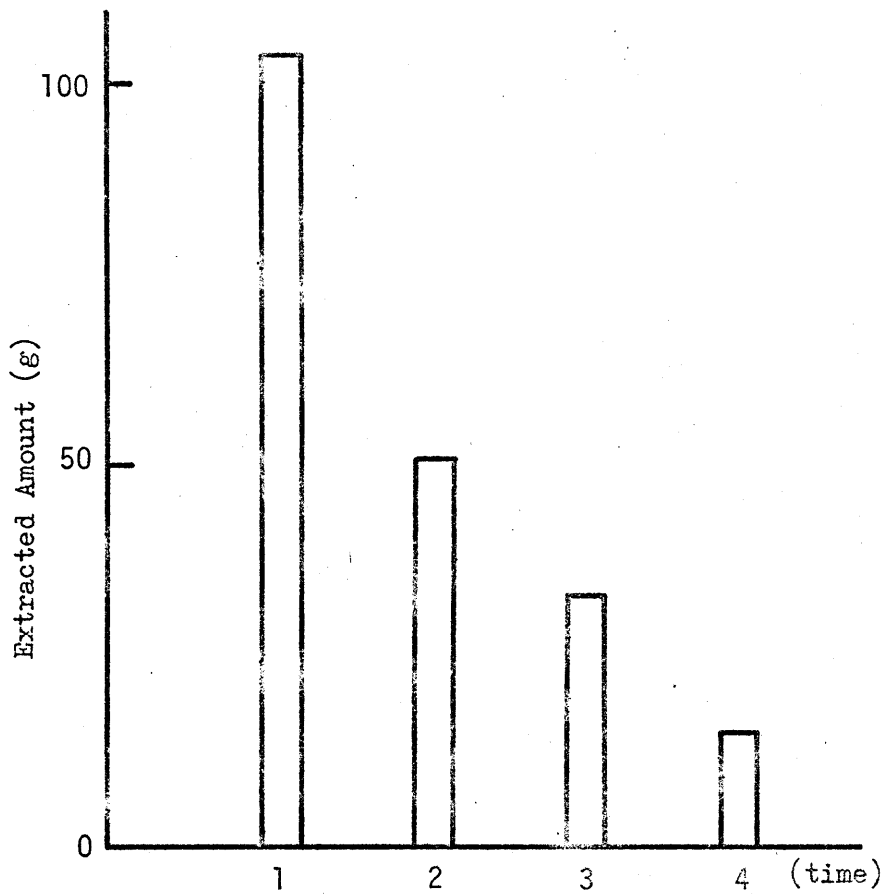

Fig. 3-b Extraction of the diapause hormone from the residual powder.

The yield of DHE is little different from sample to sample used every year so as to follow just the same procedure every time, and correspondingly the hormonal activity of it is almost in unity among them as illustrated in Fig. 4. The diapause egg percentage increases as the dose rises with drowing a curve.*

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* The reason why it does not correlate linearly lies in the biological fact that all eggs in the ovaries of a silkworm are not always sensitive to the hormone. Thus, some eggs are considered to be decided already as non-diapause when the hormone is injected, and some eggs do not grow up to a stage being destined to be diapause or non-diapause until the time when the hormone still exists therein.

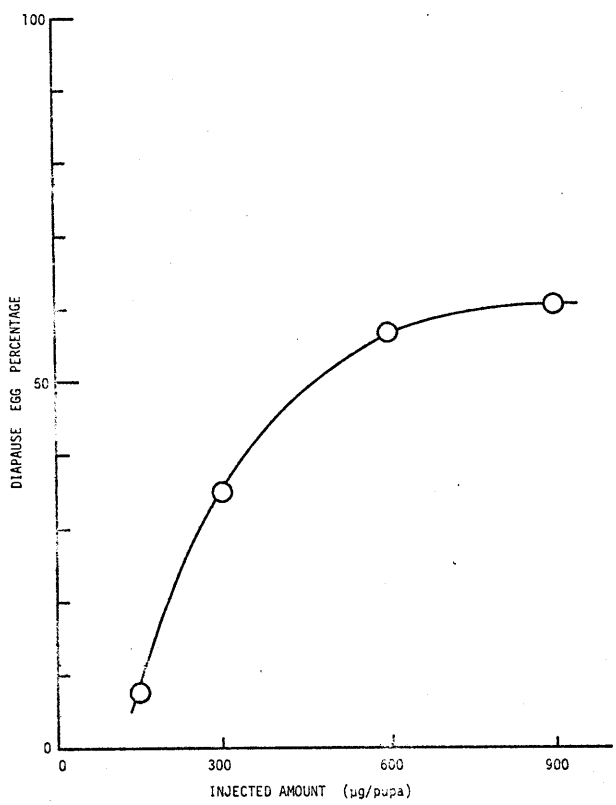

Fig. 4 An example of the hormonal activity of DHE.

4) Purification Step I

Gel permeation chromatography with Sephadex LH-20 (limited molecular weight to be permeated, 4,000) using organic solvents was applied to the first preparative scale of purification. Although DHE is sparingly soluble either in methanol or in chloroform, it dissolves easily in a mixture of methanol and chloroform (or dichloromethane), especially in a 1:1 mixture of the two solvents. The best solvent system for the chromatography of DHE was determined after analytical tests (Fig. 12 and Table 4) as methanol-dichloromethane (9:1). As DHE is fairly stable to heat and light (Table 3), this chromatography was carried out at room temperature.

Sephadex LH-20 (160 g) (Pharmacia Fine Chemicals, Uppsala, Sweden) was suspended in methanol-dichloromethane (9:1) for 2 days with occasional shaking and then packed into a glass column (2.5 x 150 cm) followed by washing with ca. 2 l of the same solvent mixture before use, the bed being 110 cm in length. About 1,000 mg of DHE was dissolved in 2 ml of the solvents (9:1) and the solution was centrifuged. The supernatant containing ca. 700 mg of DHE was placed on the column which was then eluted at a rate of 5 ml/hr for ca. 5 hr and then at 10 ml/hr. The fraction containing every 6 ml of the eluents from the column were collected and each fraction was evaporated in a 10-ml bial, which had been weighed beforehand, and dried in vacuo over phosphorus pentoxide. The chromatogram was monitored by weighing the residual material in every bial. Fig. 5 illustrates

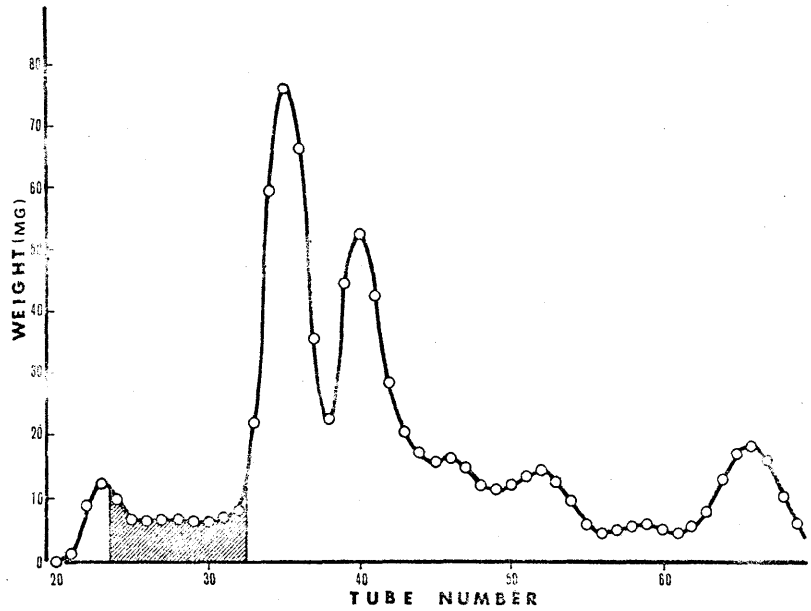

Fig. 5 First chromatography of the diapause hormone extracts (DHE).

Original sample, 700 mg of DHE (1 DH unit/900 µg) in 2 ml; gel, Sephadex LH-20, 160 g; column, 2.5 x 150 cm; elution, methanol-dichloromethane (9:1); flow rate, 5 to 10 ml/hr; fraction size, 6 ml/tube. Cross-hatched area, DH fraction with 1 DH unit by 90 µg/pupa injections.

a typical one of the first chromatograms.

In the first chromatogram (Fig. 5), a small peak on the left (tube number, 23) and the largest peak on the center (tube number, 35) consist of mainly peptides and dihydrosphingomyelin (III), respectively.* The hormonally active fractions, which show 1 DH unit by 90 μg/pupa injections, locate between the two peaks, i.e. in the cross-hatched area (tube numbers, 24 to 32). That is, DH activity of these fractions increased ten times to that of the original DHE. Therefore, this chromatography is very efficient to separate active component from DHE; the yield being almost quantitative.

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* The reproducibility of the 1st chromatogram is very high: dihydrosphingomyelin, the major peak, elutes into almost the same number of the tubes. Only the major peak (tube numbers, 34 to 36) amounting ca. 20% of DHE solidifies into white gel-like material when all of the tubes from the column were stored at -20°C for several hours. These facts helped us to find out exact tubes having dihydrosphingomyelin, and hence monitoring of the every chromatogram by weighing the eluents was not necessary in order to find out the hormonally active fractions.

The structure of dihydrosphingomyelin (III) was identified as follows: Rf=0.3 (silica gel G, $CHCl_3$-MeOH-H_2O, 65-35-5);

$$CH_3(CH_2)_{12}CH_2CH_2\underset{OH}{CH}-\underset{NHCO(CH_2)nCH_3}{CH}-CH_2O-P=O(O^-)-CH_2CH_2-\overset{+}{N}(CH_3)_3$$

(III) Dihydrosphingomyelin

The hormonally active fractions indicated in the cross-hatched area in Fig. 5 were combined* and re-chromatographed on a longer column (1.5 x 300 cm) with a minimum dead space, which was designed for more precise separation. To this column was packed Sephadex LH-20 (160 g) whose height was ca. 285 cm after washing with methanol-dichloromethane (9:1). About 150 mg of

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* The active fractions of the first chromatogram were combined by dissolving in methanol-dichloromethane (1:1), but they some times contained insoluble material when re-dissolved in the solvent system (9:1). Soluble fraction in this solvent system (ca. 80% of the collected amounts) was used for the second chromatography after centrifugation. The insoluble material had almost the same DH activity as the soluble fraction.

(continued from the forward page)

ν_{KBr} 3400, 2930, 2850, 1640, 1545, 1470, 1235, 1085, 1050, 965 (cm^{-1}); $\delta^{CDCl_3\text{-}CD_3OD}_{ppm}$ 1.85 (6H, t), 1.27 (ca. 40H, br s), 2.1 (6H), 3.18 (9H, s), 3.6 (2H), 4.0-4.3 (8H), 5.3-6.2 (ca. 3H); Dragendorf, +; treatment of an acid hydrolysate with diazomethane gave methyl behenate 53%, methyl arachidonate 38%, etc. by glc analysis on 12% DEGS column at 195°C; treatment of the alkaline hydrolysate with N,O-bis(trimethylsilyl)acetamide showed the same retention time (4.9 min at 150°C) on 2% SE-30 glass column as TMS-dihydrosphingosine prepared from bovine sphingomyelin hydrogenates on Pd-C and their mass spectra (m/e 313, etc.) were superimposable.

active substance (1 DH unit in 90 μg) obtained from the first chromatography was dissolved in 0.5 ml of the mixed solvent and the solution was poured on the column, which was developed with the mixed solvent at a rate of 1.1 to 1.5 ml/hr. The eluents were collected in every 200 drops (ca. 1.5 ml) per tube, and the chromatogram was monitored by weighing the residue of the eluents after evaporation. A typical diagram of the second chromatography is illustrated in Fig. 6, in which DH activities of some fractions are also shown by 20 μg per pupa injections.

In the second chromatogram (Fig. 6) are there a small (tube number, 51) and the largest peak (tube number, 82) which correspond, respectively, to the peptide- and the dihydrosphingomyelin-peak of the first chromatogram (Fig. 5). The half-height width of these two peaks are equivalent to about 4 tubes, indicating that the second chromatography was very efficient to separate these materials in a narrow range. On the other hand, fractions with high DH activity were widely spread over 13 tubes (tube numbers, 54 to 66). This fact suggests the presence of at least two types of DH; fractions in tube numbers 54 to 61 being tentatively called as Fraction A and those in tube numbers 63 to 66 as Fraction B. DH content in Fraction B is, however, changeable from sample to sample.

Repetition of Sephadex LH-20 chromatography of the active Fractions, A and B, further three times at room temperature gave finally one single peaks, respectively. The DH activity of them, however, did not increase more than that of the starting material. The reason why DH activity did

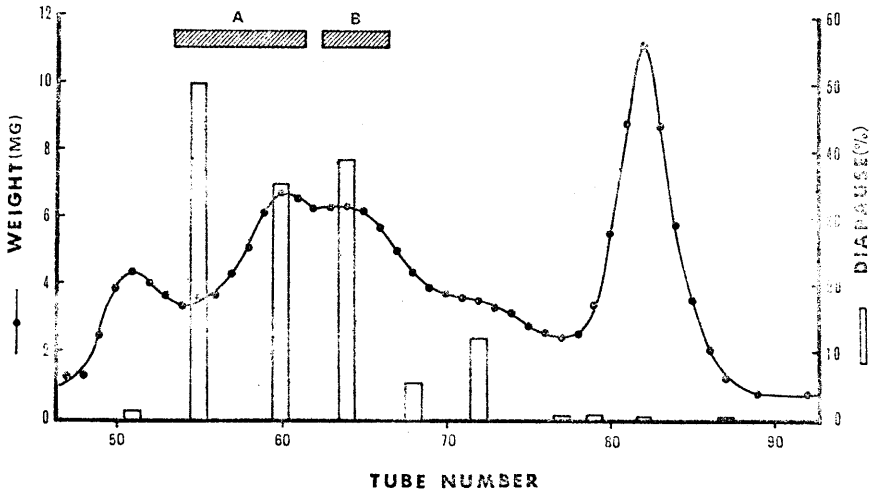

Fig. 6 Second chromatography of the diapause hormone.

Original sample, 150 mg (1 DH unit/90 μg) in 0.5 ml; gel, Sephadex LH-20, 160 g; column, 1.5 x 300 cm; elution, methanol-dichloromethane (9:1); flow rate, 1.1 to 1.5 ml/hr; fraction size, 1.5 ml/tube; assayed fractions (bars), average percentages of the diapause eggs produced by 20 μg/pupa injections. Cross-hatched area: A, Fraction A (tube numbers 54 to 61) and B, Fraction B (tube numbers 63 to 66).

not increase in such purification steps would be that purified samples containing more DH became more labile to heat and light.

5) Purification Step II

Further purification on the Fraction A had to be carried out in the dark at a low temperature. The best solvent system used in the purification step I, however, could not be used in this case because of its low solubility in the cold. We had to find out a new technique in order to perform further purification at a low temperature (practically below 5°C). New gels for permeation chromatography using organic solvents have become commercially available since 1971, i.e. Merckogel^{R} Type OR (E. Merck, Darmstadt, Germany). One of them, RO 6000 (limited molecular weight to be permeated, 4,000) exhibited satisfactory results for DH separation using 1:1 mixture of methanol and dichloromethane as the solvent.

Since the bed volume of the Merckogel column is extremely changeable with the pressure, we used a glass column (0.8 x 200 cm) equipped with a porous teflon filter at each end, and a mobile column stopper was placed on one end to adjust the filter on the gel bed. One end of this column was connected to a drop-counting fraction collector, and another end to a constant flow pump (JLC-P_2, JEOL, Tokyo, Japan) through a sampler (used for liquid chromatograph, JLC-3BC, JEOL) with a teflon tubing, respectively. Merckogel^{R} Type OR 6000 (45 g) was suspended in methanol-dichloromethane (1:1) for 2 days with occasional shaking, then washed by decantation and packed into the column. After letting the gel bed settle down by washing the column with the mixed solvent (1:1) at a constant pressure, about 40 mg of Fraction A was charged in the sampler and introduced into the column

without pressure change. The column was developed at a flow rate of ca. 1 ml/hr in a cold dark room (ca. 3°C). Fifty drops (ca. 0.4 ml) were collected in each tube. The chromatogram was monitored by weighing the eluents after evaporation in vacuo over P_2O_5. A typical diagram of the third chromatography is shown in Fig. 7 together with DH activity of some fractions tested by 9 µg/pupa injections.

As Fig. 7 indicates, high DH activities in the chromatogram locate in both sides of the largest peak, especially in the shoulder (tube numbers, 36 to 40), but the peak itself shows comparatively low activity. The active fractions before the peak seem to be derived from Fraction A of the second chromatogram, and those after the peak from Fraction B. Total DH units in 40 mg of original Fraction A are 2,000, whereas those in the assayed ten fractions in the 3rd chromatogram are calculated to be ca. 1750, i.e. 87% of DH are recovered in the ten fractions. Hence almost all DH was recovered without any decrement of the activity. Chromatography by the Merckogel at a low temperature was, thus, quite satisfactory for isolation of DH from highly active DH fractions.

Further purification by the fourth chromatography was performed on 25 mg of highly active fractions collected from tube numbers 36 to 39 of the third chromatography using methanol-dichloromethane (1:1) at a flow rate of ca. 1.1 ml/hr on a column (0.8 x 400 cm), which consists of

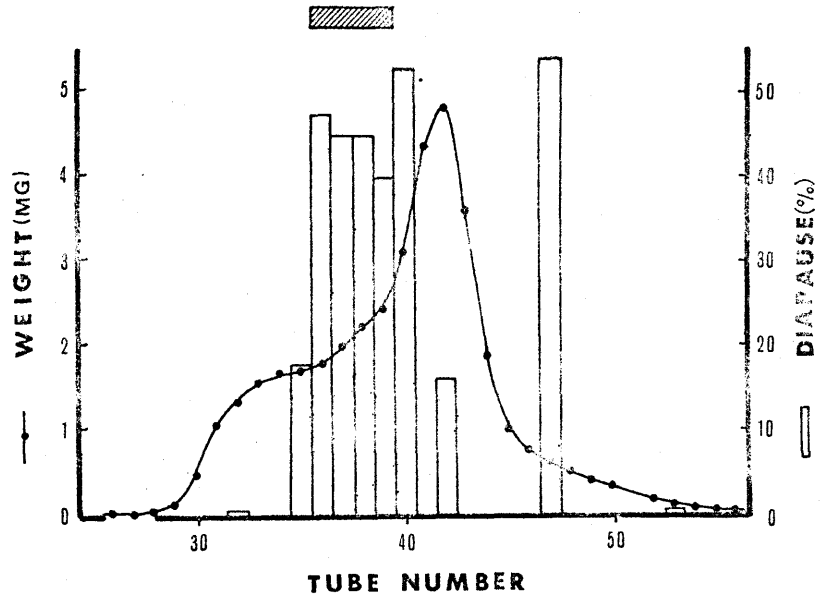

Fig. 7 Third chromatography of the diapause hormone.

Original sample, 40 mg of Fraction A (1 DH unit/20 μg) in 0.2 ml; gel, Merckogel^{R} Type OR 6000 (270-600 mesh), 45 g; column, 0.8 x 200 cm (see text); elution, methanol-dichloromethane (1:1); flow rate, 1 ml/hr; fraction size, 0.4 ml per tube; assayed fractions (bars), average percentages of the diapause eggs produced by 9 μg/pupa injections. Cross-hatched area: used for the following chromatography.

two 200 cm columns used for the 3rd chromatography jointed together with a teflon tubing. Fifty drops (ca. 0.4 ml) were collected in each tube. The chromatogram was also monitored by weighing the eluent by 1 µg-scale after evaporation. The fourth chromatogram shows only one single peak as illustrated in Fig. 8, where DH activities on five fractions were tested. Central three fractions of the peak show an equally strong activity (ca. 1 DH unit in 6 µg), but fractions in its both sides were less active. DH activity of the fraction at the center of the peak in the fourth chromatogram (tube number 57) was assayed in detail (Table 1-a and Table 1-b). In table 1-a are shown the actually produced diapause eggs and in Table 1-b, those summarized results. And the mean diapause egg percentage is illustrated against dose in Fig. 9. Thus, the higher the dose the much higher the percentage of diapause egg production, and 6 µg of the fraction were as active as to produce about 100 diapause eggs.

Eight mg of highly active fractions collected from tube numbers 53 to 61 of the fourth chromatogram was re-chromatographed on the same column used for the preceeding chromatography at a flow rate of 1.25 ml/hr collecting 0.6 ml per tube. There finally obtained one homogeneous peak as indicated in Fig. 10. Five µg of the central fraction of the peak was as active as to produce diapause eggs with an average of 27.7 per cent, which corresponds approximately to 1 DH unit in 6 µg (see Fig. 9).

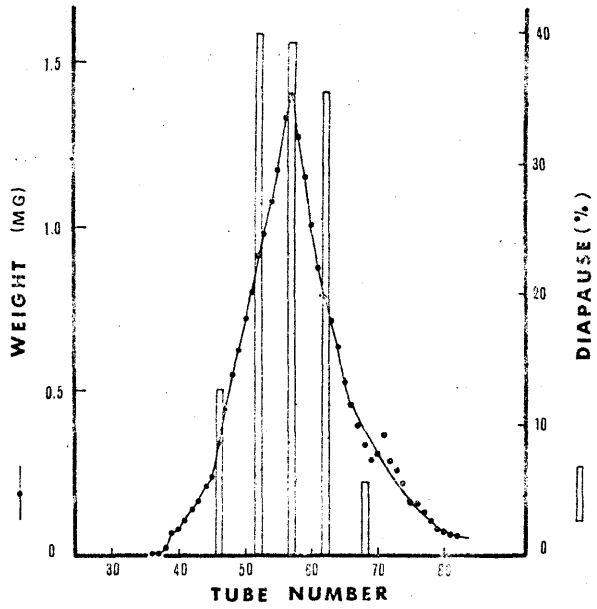

Fig. 8 Fourth chromatography of the diapause hormone.

Original sample, 25 mg of fractions collected from cross-hatched area in the 3rd chromatograms (1 DH unit/9 µg); gel, Merckogel^{R} Type OR 6000, 90 g; column, 0.8 x 400 cm; elution and fractionation, identical to the 4th chromatography; assayed fractions (bars), average percentage of the diapause eggs produced by 6 µg/pupa injections. Cross-hatched area: used for the following chromatography.

e 1-a Production of diapause eggs by the fraction at the top of the fourth chromatogram.

	Amount of the fraction injected (μg)							
	1		2		4		6	
	ND	D*	ND	D*	ND	D*	ND	D*
1	229	0(0.0)	254	8(3.1)	261	0(0.0)	213	85(28.5)
2	225	0(0.0)	232	12(4.9)	219	29(11.7)	173	85(32.9)
3	187	0(0.0)	169	10(5.6)	138	21(13.2)	134	71(34.6)
4	277	1(0.4)	247	15(5.7)	209	49(19.0)	179	119(39.9)
5	256	2(0.8)	169	12(6.6)	166	40(19.4)	165	112(40.4)
6	244	4(1.6)	176	14(7.4)	198	67(25.3)	154	109(41.4)
7	246	8(3.1)	180	21(10.4)	141	82(36.8)	139	106(43.3)
8	258	10(3.7)	140	19(11.9)	126	76(37.6)	147	165(52.9)
9	228	12(5.0)	166	45(21.3)	-	-	-	-
mean	239	4(1.6)	193	17(8.5)	182	46(20.4)	163	107(39.2)

Number of injected pupae, 9; ND, non-diapause eggs; D, diapause eggs;

* figures in the parentheses, percentages of diapause eggs.

Table 1-b Production of diapause eggs by the fraction at the top of the fourth chromatogram.

Amount of fractions injected (μg)	Number of Pupae injected	Number of Adults laying eggs	No. of adults laying non-diapause eggs alone	No. of adults laying diapause eggs	Average no. of eggs laid: non-diapause eggs	Average no. of eggs laid: diapause eggs	Percentage of diapause eggs: Min.	Percentage of diapause eggs: Max.	Percentage of diapause eggs: Mean
1	9	9	3	6	239	4	0.0	5.0	1.6
2	9	9	0	9	193	17	3.1	21.3	8.5
4	9	8	1	7	182	46	0.0	37.6	20.4
6	9	8	0	8	163	107	28.5	52.9	39.2

Recipients: pupae of N_4 (a polyvoltine race).

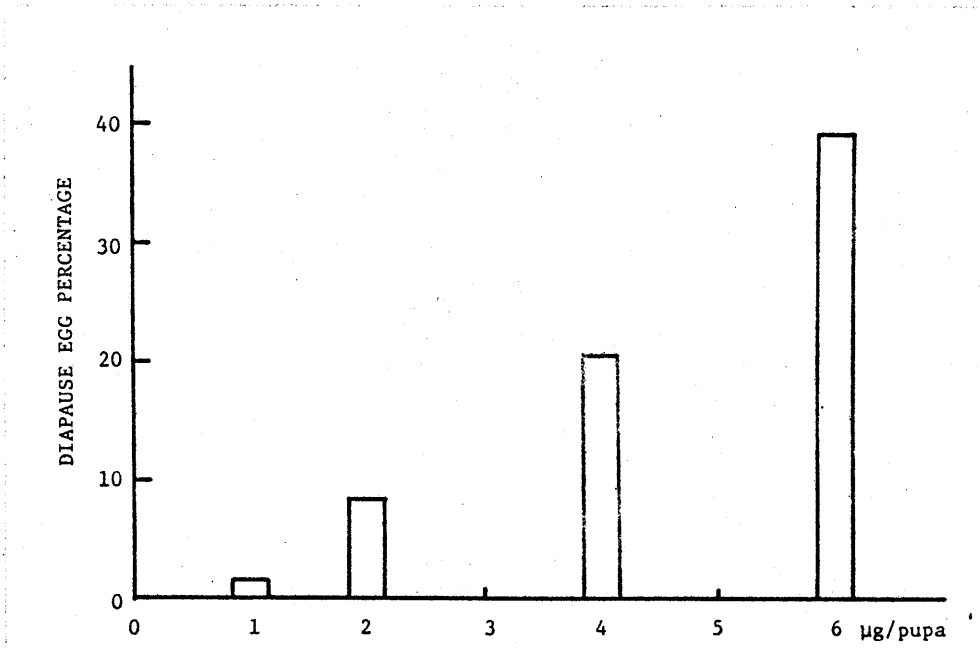

Fig. 9 Mean percentage of the diapause eggs induced by the fraction at the top of the fourth chromatogram (Fig. 8).

Diapause egg percentages were plotted against injected amount (µg/pupa); N_4 race was used.

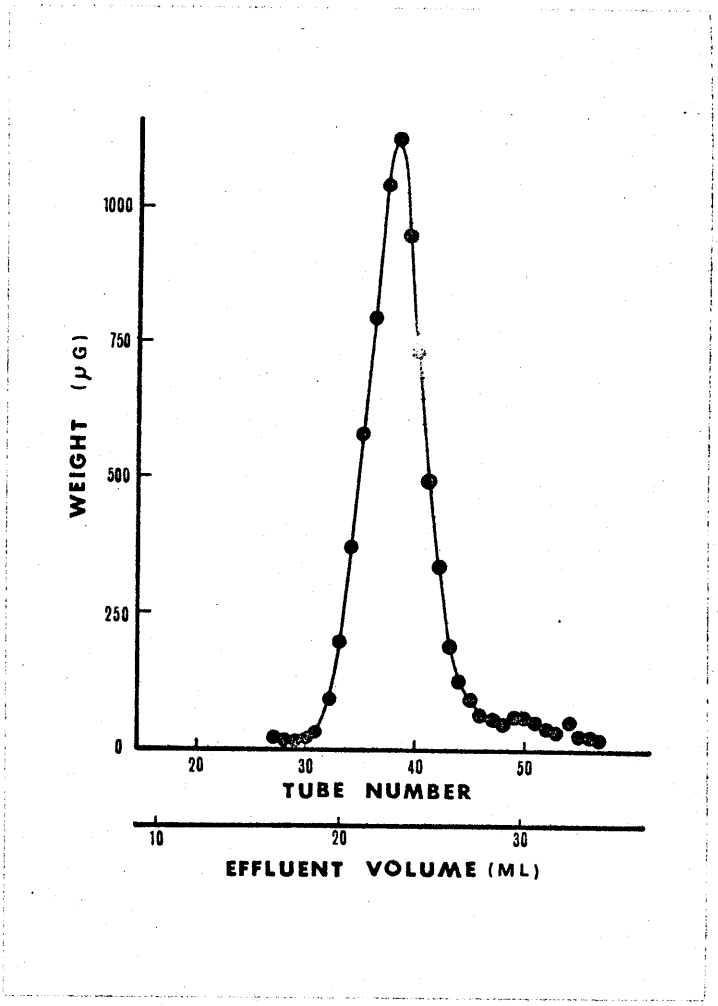

Fig. 10 Final chromatography of the diapause hormone.

Original sample, 8 mg of the fractions collected from tube numbers 53 to 61 of the 4th chromatography; gel, column and elution, identical to the 4th chromatography; flow rate, 1.25 ml/hr; fraction size, 0.7 ml/tube.

Table 2 is the summary of the purification sequence of DH starting from 2.18 million mated male adult heads of the silkworms. Thus, total DH units in finally purified DH preparation are 500, corresponding to less than 1% of those in the original heads. Work on the improvement of the yield is now in progress.

Table 2. Summary of purification of the diapause hormone from 2.18 million male adult heads.

Purification	Initial Weight (g)	Final Weight (g)	Activity* (μg/1DH unit)	Total DH units
MeOH-$CHCl_3$ extraction	4,260	327	-	-
BuOH-H_2O partition[DHE]	327	63	900	70,000
1st chromatography on Sephadex LH-20	38	2.1	90	23,000
2nd chromatography on Sephadex LH-20	0.93	0.167(Fr. A) 0.117(Fr. B)	20 20	8,300 5,800
3rd chromatography on Merckogel OR 6000	(Fr. A)0.165	0.029	9	3,200
4th chromatography on Merckogel OR 6000	0.025	0.012	6	2,000
5th chromatography on Merckogel OR 6000	0.008	0.003	(6)†	500

* This is defined as the amounts of test sample which gives 40-60% diapause eggs when injected into pupae of polyvoltine race (N_4); † 5 μg injections of this sample were as active as to produce diapause eggs with an average of 27.7 %, 6 μg injections being estimated to yield 1 DH unit.

DISCUSSION

1) Extraction

The first extraction of DH (Hasegawa, 1957) was achieved from 80% methanol homogenates of brain-SG complexes dissected out from silkworm pharate adults. In this case, the active principle was located in aqueous layer when washed with ether and it was extracted with chloroform from the aqueous layer. However, in case of whole adult heads used as the source, the active principle was located in the ether fraction when the first extraction procedure was followed and DH was precipitated by addition of excess acetone to the ether fraction. This difference may come from a high lipid content in the whole heads. In the case of small scale extraction from the whole heads a slight modification of the first procedure was necessary, but the procedure was not adequate in a large scale extraction.

One of the difficulties encountered during establishment of the new extraction procedure shown in Fig. 2 was that the pupae that had been injected with the methanol-chloroform extracts showed an abnormality in the metabolism and the resultant adults laid only very few eggs without any indication of DH effect. Therefore, the hormonal activity of the extracts was hardly estimated in the early stage of the extraction. After some trials, we have found it effective to partition the complex lipid fraction, methanol-chloroform (1:1) extracts, between butanol and water

to remove water soluble materials, especially proteins which might disturb the bioassay. No difficulties occurred in performing the bioassay on the remaining solid (DHE) thus obtained by evaporating the solvent from the butanol layer (Fig. 2). DHE was as active as one DH unit by 900 μg injection into non-diapause egg producers and consequently 30 g of the extracts from one million heads contain about 33,000 DH units. From the previous work (Hasegawa, 1964), we estimated the extracts from 35 male adult heads as active as to produce about 40 per cent diapause eggs. Hence, total DH units in one million male adult heads equal to about 28,500. Thus, the efficiency of the method presented in Fig. 2 is so high as to be comparable to that used for extraction from the brain-SG complexes.

2) Stability of the Diapause Hormone in Crude States

For the purification of DH its stability was examined by treating a crude hormonal fraction with acids, bases, heat and light such as those listed in Table 3. In this table are summarized the results performed on DHE, the DH activity of which was +++ in 400 μg, <+++ or ++ in 200 μg and + or > + in 100 μg injections (degree of DH activity, see bioassay).

As shown in this table, DH is stable to weak acids or weak bases but a little labile in the solutions containing high concentration of HCl or KOH. DH in crude states was also stable even when it was exposed to 60°C overnight or light for 6 hr; the former being tested on DHE dissolved in glacial acetic acid, ammonia or methanol-dichloromethane (1:1) and the latter tested in a methanol or methanol-dichloromethane (1:1) solution with a mercury lamp.

Details of its stability to heat and light are shown in Fig. 11, where its hormonal activity was little affected by exposure to heat or light.

Table 3. Stability of the diapause hormone in crude extracts.

Treatments	Degree of DH activities by injections of		
	400 µg	200 µg	100 µg
None	+++	<+++ or ++	+ or >+
Methanol:dichloromethane (1:1)*	+++	<+++ or ++	>+
Conc. ammonia:methanol (1:3)	+++	<+++ or ++	>+
Conc. ammonia*	+++	<+++	>++
Pyridine	+++	>++	+ or >+
2N KOH:methanol (1:3)	<++ or ++	>+	+ or ±
Glacial acetic acid*	+++	<+++	++ or <++
1N HCl:methanol-chloroform (1:3)	<++	+	+
Light†	+++	++	+ or >+

DH extracts, dissolved in test solvents indicated in this table in concentrations of 0.25 to 0.5% and left overnight except † for 6 hr exposure to mercury lamp in methanol or methanol:dichloromethane (1:1); * exposed to 60°C.

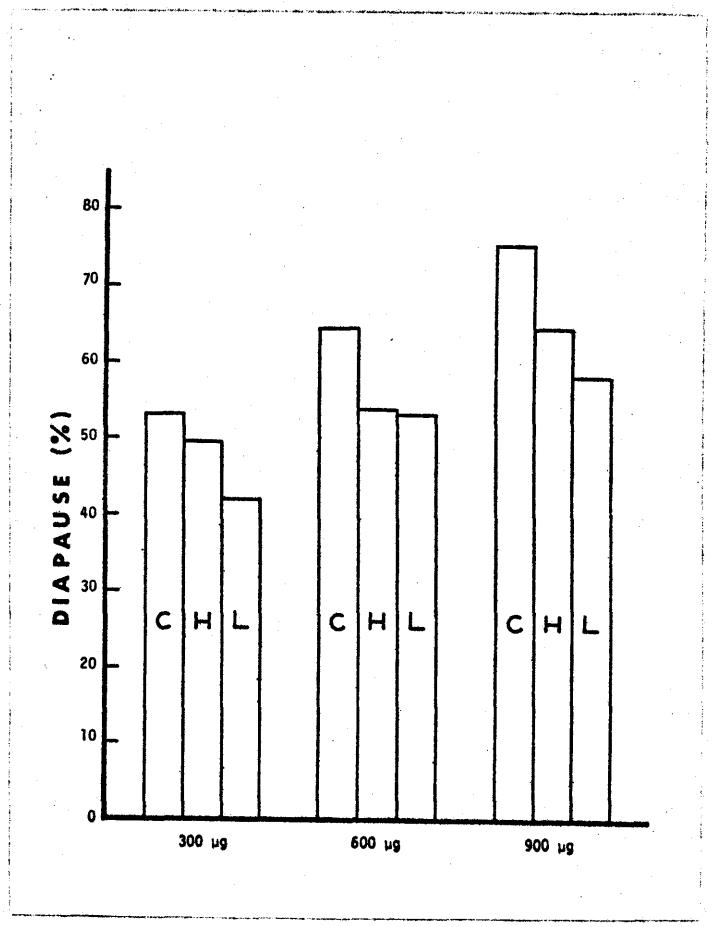

Fig. 11 Effects of heat and light on the crude diapause hormone.

Original sample, 0.8% solution of a crude diapause hormone (1 DH unit/300 µg) in methanol-dichloromethane (1:1); H, 8 hr heating at 60°C; L, 2 hr exposure to a mercury lamp; C, non-treated; bioassay, N_4 pupae; ordinate, average percent of the diapause eggs.

3) Chromatographic Characteristics

Stability of DH to those reagents in Table 3 seemed promising further purification of DH from DHE, especially for fractionation by column chromatographies with silicic acid (Mallinckrodt), silicic acid-Hyflo Super Cell (Wako), magnesium silicate (Woelm), Florisil (Woelm) or cellulose powder (Toyo Roshi). None of these chromatographies, however, were satisfactory to give high active fractions from DHE. Other chromatographies using polyamide (Woelm), DEAE cellulose (Brown) and bio-gel P-10 (Bio-Rad) were also ineffective.

Before Sephadex LH-20 became commercially available in 1968, we tried gel filtration chromatographies of DHE with Sephadex G-type by aqueous solvent system consisting two parts of water and one part of methanol with a little ammonia since DHE is hardly soluble in water. DHE was chromatographed on Sephadex G-25, G-50 and G-200, all of which passed active fraction without any partition. These gel filtration data suggested that DH in aqueous solution would form aggregates consisting of large molecular sizes. Even after purification, DH in an active fraction having 1 DH unit in 30 μg formed aggregates in aqueous solvent system.

It is already known that in polar solvents gangliosides form micelles having a molecular weight of about 200,000 to 250,000 (Wiegandt, 1968). In dimethylformamide or tetrahydrofuran, on the other hand, they form molecular solutions

characterized by a molecular weight of 1,000 to 3,000. Since extraction method of DHE is very similar to that of the gangliosides (Kuhn and Wiegandt, 1963; Wolfe and Lowden, 1964), an effective separation of DHE would be expected by a gel permeation chromatography using organic solvents where no aggregates would be formed. For such a gel permeation chromatography, Sephadex LH-20 was commercially produced in 1968. Then, we adopted this Sephadex and tested its separation efficiency using mixtures of methanol and chloroform as eluants. The results are summarised in Fig. 12, & Table 4.

Thus, the mixture of 9:1 showed the best separation of original DHE in about 9 per cent of fractions with 10-fold DH activity. This activity could be obtained quantitatively when dichloromethane was used instead of chloroform with the preparative column as also shown in Table 4. Since dichloromethane is more stable to light than chloroform, methanol-dichloromethane (9:1) was used as the best solvent system also in Sephadex LH-20 column for the first and second chromatographies.

As stated in Purification step I, repeating column chromatographies of Fraction A and B by Sephadex LH-20 gave finally respective single peak* but did not increase DH activity higher than that of the original fractions. The active principle in highly purified fractions is degraded during

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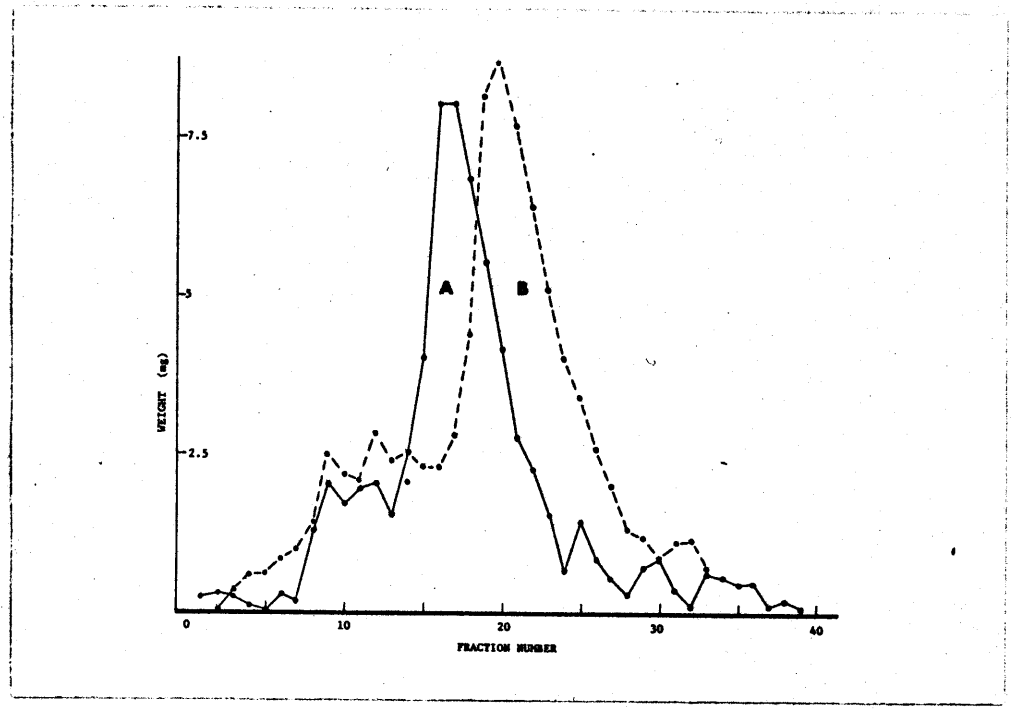
* The chromatograms are illustrated on the following page.



Further chromatographies of Fraction A and Fraction B of the diapause hormone with Sephadex LH-20 column chromatography.

Fraction A and Fraction B separated in the second chromatography (Fig. 6) were respectively re-chromatographed further three times with the same manner as that of the 2nd chromatography. Finally they afforded these chromatograms with only a slight DH activities (++ or >+ by 18 µg/pupa).

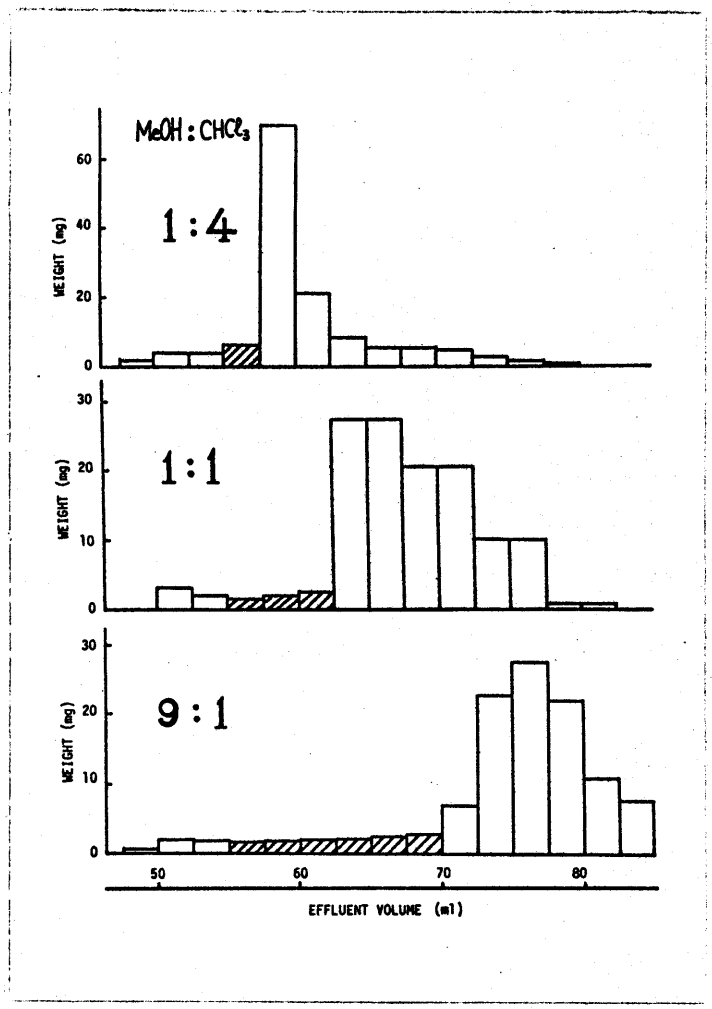


Fig. 12 Solvent effects of methanol and chloroform on Sephadex LH-20 column chromatography for separation of DHE.

Top, 1-4; Middle, 1-1; Bottom, 9-1 v/v portions of methanol and chloroform.

Table 4. Solvent effects of methanol and chloroform on Sephadex LH-20 column chromatography for separation of diapause hormone extracts (DHE).

Solvent system CH_3OH : $CHCl_3$	Sephadex LH-20	Column	DHE (1 DH unit/ 900 µg) used	Yield of fractions (1 DH unit/90 µg)	
(v/v)	(g)	(cm)	(mg)	(mg)	(%)
1 : 4	50	1.5 x 150	150	6.4	4.3
1 : 1	50	1.5 x 150	150	6.5	4.3
9 : 1	50	1.5 x 150	150	14.2	9.4
9 : 1	160	2.5 x 250	500	42.7	8.5
9 : 1*	160	2.5 x 250	500	51.6	10.3

* Dichloromethane was used instead of chloroform.

further chromatography at room temperature. Then stability of DH in highly purified fractions was re-examined. In this case, 0.01% of methanol-dichloromethane (1:1) solutions of purified DH fraction, which show 1 DH unit by 30 μg injection, were heated to 60° for 5 hr or exposed to a mercury lamp for 2 hr. These samples as well as non-treated samples were bioassayed, and the results are presented in Fig. 13. As shown in this figure, DH activity of these treated samples almost disappeared. It is in contrast with the case of DH in crude states (Fig. 11). This indicates that DH becomes very labile to heat and light as the purification proceeds.

Thus, further purification of the active Fraction A (1 DH unit/20 μg) had to be carried out in the dark at a low temperature. In this case, the best solvent system (9:1, methanol-dichloromethane) for Sephadex LH-20 chromatography could not be used because of low solubility of Fraction A at low temperatures but they showed poor separation. Therefore, we had to search another gel for permeation chromatography having high separation ability at low temperatures, and examined two Merckogels of Type OR (polyvinyl acetate), OR 6000 and OR 20000 (limited molecular weight to be permeated, 14,000), which have been commercially available since 1971. We found the former to exhibit better separating ability than the latter using methanol-dichloromethane (1:1) in the cold.

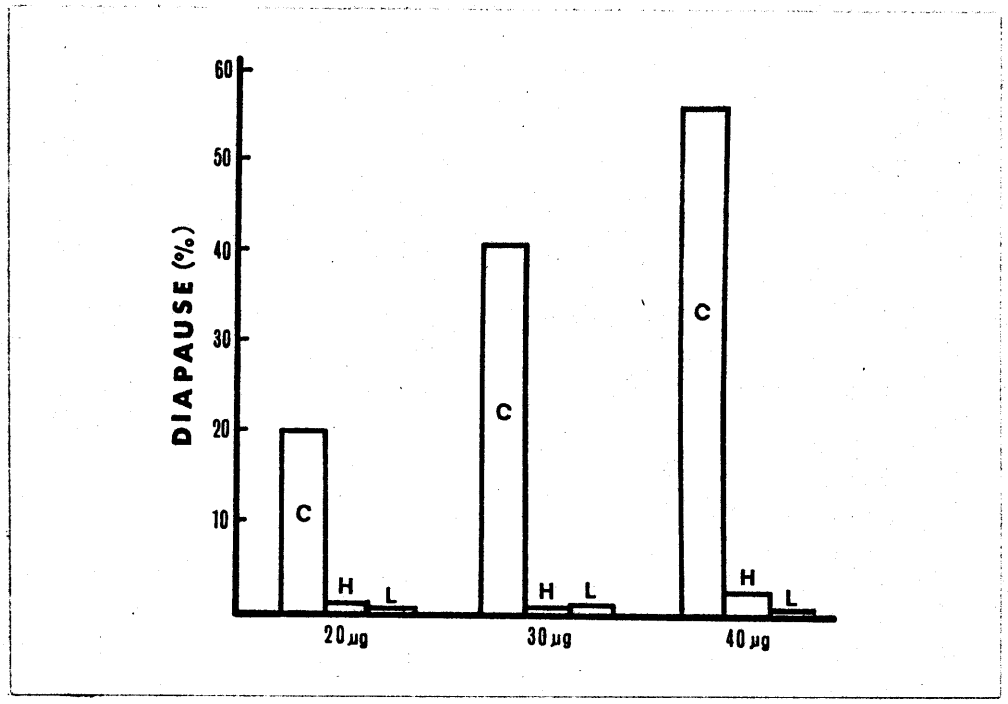


Fig. 13 Effects of heat and light on the diapause hormone.

Original sample, 0.01% solution of purified DH fractions (1 DH unit/30 μg) in methanol-dichloromethane (1:1); H, 5 hr heating at 60°C; L, 2 hr exposure to a mercury lamp; C, non-treated; bioassay, N_4 pupae; ordinate, average percent of the diapause eggs.

4) The existence of two species

As Fig. 6 shows, the peptide and the dihydrosphingomyelin are effectively separated in a narrow range, the half-height width being about 4 tubes, while fractions, which show high DH activity by 20 μg injections, widely located in 13 tubes. Hence we suggested the presence of two hormonally active fractions in the second chromatograms. This suggestion was further confirmed by the third chromatography (Fig. 7). Fraction A pooled from 2nd column chromatography was fractionated with the Merckogel^{R} Type OR 6000 column. The active fractions located in both sides of the peak; the right fraction corresponding to Fraction B and the left Fraction A, respectively. This inference was visualized by further chromatography of the peak fractions (tube numbers, 41 to 43) in an identical way as stated in the 3rd chromatography, and the result was monitored by weight together with DH activity measurements by 6 μg injection (Fig. 14).

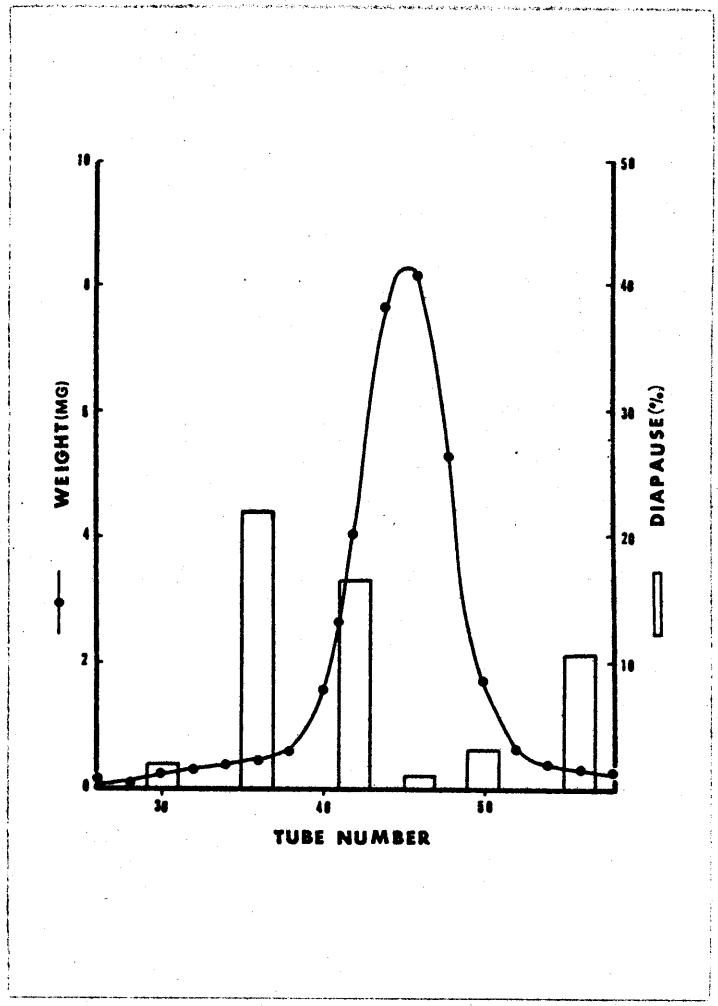


Fig. 14 Re-chromatography of the major fraction of the 3rd chromatography.

Original sample, 34 mg of fractions collected from tube numbers 41 to 43 of the 3rd chromatography; chromatographic conditions, identical to the 3rd chromatography; assayed fractions (bars), average percentages of the diapause eggs produced by 6 μg/pupa injections.

5) Molecular Weight

The molecular weight of DH in Fraction A was estimated to be between 2000 and 4000 from the following evidences; DH eluted after the peptide fraction from Sephadex LH-20 as well as Merckogel^{R} Type OR 6000 columns whose limited molecular weight to be permeated are both 4000 and hence the molecular weight of DH does not exceed 4000. On the other hand, it far exceeds that of dihydrosphingomyelin (molecular weight, ca 830) as shown in Fig. 5 and 6. The molecular weight of DH in Fraction B was suggestive also to be between 2000 and 4000 and slightly smaller than that of DH in Fraction A.

6) The Diapause Hormone

As Fig. 8 and Table 1 indicate, the fourth chromatogram of DH shows a single peak, and 6 μg of the peak was as active as one DH unit and yielded more than 100 diapause eggs on an average. For further confirmation, the active substance obtained from the fourth chromatography was chromatographed again (Fig. 10). The chromatogram (Fig. 10) shows only a single peak eluting symmetrically in a narrow range; 5 μg of the top-fraction having produced 74 diapause eggs.

We have compared the activity of our final DH preparation with that of other insect hormones. Brain hormone separated from silkworm brains is functional to induce pupal-adult development in brainless pupae of Bombyx mori in 0.02 μg injections (Yamazaki and Kobayashi, 1969) and of Samia cynthia recini in 0.002 μg injections (Ishizaki and Ichikawa, 1967). These activities should, however, not be compared to that of other hormones, for brain hormone acts in two-step endcrine mechanism; the hormones stimulates the prothoracic glands to release moulting hormone which, in turn, stimulates further the prothoracic glands themselves (Williams, 1952).

Fourth instar silkworm larvae deprived of their corpora allata at the beginning of the instar become mature precociously and transform into pupae. Using these allactectomized silkworm larvae weighing 0.25 to 0.3 g, Ohtaki et al. (1971) reported that 1 μg injections of dl-C_{18} JH are required to make 60 per cent of 4th instar larvae to develop normally. Another example was present by Williams (1968) in α-ecdysone.

In isolated pupal abdomens of diapausing male Samia cynthia pupae weighing 2 g, 20 μg injections of α-ecdysone are necessary for half of them to develop. Since the weights of our test animals are 0.7 to 0.8 g, our final DH preparations seem to be highly purified from biological point of view.

Recently Sonobe and Ohnishi (1971) reported that crude samples extracted from female adult heads with 80% methanol and fractionated by a Sephadex G columns exhibited the diapause factor (hormone) activity.* They used an indirect bioassay to assess its activity without testing whether their active preparations would actually produce diapause eggs or not. They traced the hormonal activity by measuring 3-hydroxykynurenine (3-OHK) content in the ovaries one day after the injections. According to their data, several

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* Their procedure for extraction (80% methanol) of the factor is the same as that reported by Hasegawa in 1957. And we have already failed to apply the procedures to the purification of DH using adult heads because the extracts interfere normal metabolism of the silkworm by its injection. Although experimental details are not present in their paper, we examined their data by following them using 17,000 adult heads. Finally, we have obtained a confidence that the diapause factor is similar to the very crude diapause hormone based on the Sephadex LH-20 chromatographic analysis and other experiments which are discussed herein.

thousand micrograms of the diapause factor fraction obtained by Sephadex G-50 chromatography were required for elevation of the 3-OHK content. Our final DH preparation is as active as to produce more than 100 diapause eggs (39 %) on an average by 6 μg injections. Although direct comparison of their hormonal activity with that of our preparation is extremely difficult, the degree of purification of the diapause factor seems to be far less (1/1,000) than that of ours, even if the factor could produce diapause eggs. They reported that the molecular weight of the factor lays between 5,000 and 10,000 using Sephadex G-50 column, and that there exists large molecule substances which might be aggregates of the factor or other high-molecular-weight substance(s) of the factor. If their factor be the same as our diapause hormone, the molecular weight suggested in their paper would be questionable because of aggregates formation of the factor in aqueous solvents.

During the course of DH purification before Sephadex LH-20 became available, we have tried to degrade impurities or proteins in DHE by enzymes, such as lipase, phospholipase D, papain, nagase and bromelain. The DH activity of DHE was little affected by such lipid hydrolases, while surprisingly the proteinases completely inactivated the active principle. Similar observation was also reported by Sonobe and Ohnishi. Although the extraction procedure that has been used by us was very similar to that for complex lipids, the enzyme experiments suggested the presence of peptide linkages in

the DH molecule. This discrepancy was recently reconciled by finding that the purified DH indeed contains several kinds of amino acids as well as some other components. These results on the chemical analyses of the DH are discussed in Chapter II.

Although we have not yet purified another species of DH in Fraction B, this species seems to be a similar type of compound to that in Fraction A.

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CHAPTER II

CHEMICAL NATURE OF THE SILKWORM DIAPAUSE HORMONE

ABSTRACT

In Chapter I is described the isolation of the diapause hormone (DH) which is responsible for the induction of the embryonic diapause of the silkworm, Bombyx mori, L. The stability of DH was re-examined to make it clear whether DH even after purification is fairly stable or not to some reagents such as weak acid, bases, sodium periodate, acyl halide or acid anhydride, although it is very labile to heat or light. Investigations on DH with various enzymatic digestion led us to conclude that our final preparation of DH contain fairly large portion of peptide residue in its molecule, and the peptide linkages play an important role for diapause induction in the silkworm. Amino acid analysis together with amino sugars' showed that DH, in fact, contains amino acids and amino sugars such as Lys, Arg, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Phe, Tyr, Glc-NH_2 and Gal-NH_2.

INTRODUCTION

In Chapter I we have reported on purification[1] and isolation[2] of the diapause hormone (DH) which is responsible for the induction of the embryonic diapause of the silkworm, Bombyx mori, L.[3] It was efficiently extracted into a complex lipid fraction; pulverized head powder of more than two millions of silkworm adults as the extraction source was washed with acetone and then extracted with methanol-chloroform (1:1). The extracts were partitioned by butanol and water, the active substance in the butanol layer having almost quantitative amount of DH in the brain used. The DH extracts were twice chromatographed with Sephadex LH-20 column chromatography by a mixture of methanol and dichloromethane (9:1). Two active principles were obtained and one of them was further three times purified by Merckogel^{R} Type OR 6000 column chromatography with a mixture of methanol and dichloromethane (1:1). Finally we obtained hormonally active sharp single chromatogram. Another DH fraction obtained during the course of its purification is not yet purified.

Biological comparison of this DH with other insect hormones, α-ecdysone[4] and C_{18}-juvenile hormone,[5] shows that the purified DH inducing about 40 percent of diapause eggs (40 DE%) by 6 μg/pupa injection, is to be regarded as an pure insect hormone. Chemical analyses discussed herein have also given further evidence of the final preparation of DH to be pure.

Although the extraction procedure of DH was very similar to that for complex lipids, our enzyme experiments surprisingly showed that DH activity was not lost by incubation with lipid hydrolases such as lipase and phospholipase D, but with proteolytic enzymes such as nagase, papain and bromelain immediately after incubation.

In this paper, we report further confirmation of the DH to have peptide linkages in its molecule by means of its degradation with chemical reagents or highly specific proteolytic enzymes such as trypsin, α-chymotrypsin and carboxypeptidase.

EXPERIMENTAL

1) Bioassay

i) Each bioassay consists of 9 pupae of non-diapause egg producers (N_4 race), (for details, see BIOASSAY in Chapter I or Reference No. 2).

ii) Degree of DH activity of a given sample was directly estimated by observing the eggs laid by the adults injected with test samples as reported. When diapause egg percentages are required as DH activity of a given sample, eggs laid by each adult were counted 2 weeks after oviposition and each diapause egg percent was averaged. The averaged values (DE%) were referred to as the DH activities of injected samples.

2) Materials

i) Highly purified DH with more than 80 DE% by 20 ug per pupa was used to test the stability of DH to chemical reagents or enzymes.

ii) For chemical analysis of DH, the finally obtained pure DH was employed together with partially purified fractions obtained during the course of purification.

3) Stability of Highly Purified DH to Chemical Reagents

We have already reported that DH is fairly stable to acids, bases, heat or light so long as it remains in a crude state and that it becomes more labile to heat and light when highly purified. Thus, comparatively pure DH fraction (40 DE% by 30 μg/pupa) immediately loses its activity into less than 2 DE% when exposed to heat (60°C, 5 hr) or to light (mercury lamp, 2 hr)(Fig. I-13), whilst a crude DH (75 DE% by 900 μg/pupa) retains its activity amounting to ca. 80% of the original one after the same treatments (Fig. I-11).

A purified DH fraction having high activity (more than 80 DE% by 20 μg/pupa) was tested on the stability of DH to acids or bases. Solutions containing 400 μg/tube of this DH dissolved in acid (0.1N or 1N HCl) or bases (0.1N or 1N NaOH, conc. aq-ammonia or pyridine) were allowed to stand overnight at room temperature and then evaporated under N_2 stream and dessiccated at ca. 10^{-2}mmHg with vacuum pump. These treated samples were dissolved in 0.2 ml of water and then every 0.02 ml of this solution was injected into 9 silkworm pupae, thus,40 μg/pupa. The results are summarized in Table 1.

Acylation of DH was carried out by dissolving the sample in 0.1 ml of pyridine and mixing with acetic anhydride (0.1 ml), benzoic anhydride (ca. 1 mg) or p-nitrobenzoyl chloride (ca. 1 mg) for 15 hr at room temperature. The reaction mixtures were evaporated and assayed in the same manner as described above, and the results are shown in Table 1.

Periodate oxidation was performed as follows[6]: the DH was dissolved in sodium acetate buffer (0.3 ml, 0.1M, pH 5.6) and to this solution was added a sodium periodate solution (0.03 ml, 0.1M) and allowed to stand at 5°C for 1, 10 or 20 hr. Ethylene glycol solution (0.01 ml, 0.5M) was added to stop the reaction into the mixtures and they were concentrated and subsequently extracted with methanol-dichloromethane (1:1) three times. The extracts, after evaporated, were assayed by injecting one tenth of the extracts per pupa, and the results are shown in Table 1 together with those by incubation in the acetate buffer without periodate as the control.

4) Stability of Highly Purified DH to Some Enzymes

As the DH even after purified is fairly stable to acids or bases, DH is considered to be stable under the conditions where DH is incubated in slightly basic or acidic buffered solutions for enzymic experiments. The enzyme experiments were performed to deduce the entity of DH using highly purified DH with more than 80 DE% by 20 μg/pupa as the substrates to the enzymes as follows.

i) Trypsin : Sigma Cat. No. T-8003, Type I from bovine pancreas, 2x crystallized, ethanol precipitate with 10,000 units/mg was used.. A solution of this enzyme (400 μg in 1 ml of 0.001N HCl) was prepared and then pre-incubated at 25°C

Table 1 STABILITY OF HIGHLY PURIFIED DIAPAUSE HORMONE TO SEVERAL REAGENTS.

Treatment*	Diapause activity remained (DE%)	Treatment*	Diapause activity remained (DE%)
non-treated#	82.7	28% aq-NH_3	80.1
0.1N-HCl	57.8	Pyridine	81.4
0.1N-NaOH	82.8	Ac_2O/Pyridine	75.2
1N-HCl	75.6	Bz_2O/Pyridine	68.4
1N-NaOH	18.6	PNBCl/Pyridine	64.2
NaOAc (20 hr)	74.7	IO_4^- (10 hr)	72.3
IO_4^- (1 hr)	74.3	IO_4^- (20 hr)	72.0

* Treatment : solutions of DH (400 µg/0.3 ml) were allowed to stand overnight at room temperature except those indicated in the parentheses at 5°C in the dark.

Original sample : 82.7 DE% by 40 µg/pupa, 80.7 DE% by 20 µg/pupa and 62.6 DE% by 10 µg/pupa; compared by 40 µg/pupa.

for several minutes.

Every 400 μg of the purified DH was placed in a tube and dissolved in Tris-HCl (0.05M, pH 7.2) buffered solution (0.2 to 0.28 ml) so as to become its final volume to be 0.3 ml after addition of the enzyme solution. Fifty micro liter (containing 20 μg of trypsin) of the enzyme solution was introduced into the DH solution which was pre-incubated beforehand for a few minutes, and the reaction mixture was allowed to stand at 25°C for a period of 10 min, 30 min, 1 hr or 3 hr. Five μl (2 μg), 20 μl (8 μg) and 100 μl (40 μg) were introduced into the DH solution and incubated for 30 min. Hydrochloric acid solution (100 μl, 0.001N) containing no enzyme was also incubated as the control.

After the incubation was stopped at the indicated period by addition of 0.5 ml of ethanol, each tube was stored at -20°C and then evaporated to dryness. The residue was extracted three times with methanol-dichloromethane (1:1) and evaporated in vacuo. The extract in each tube was dissolved in 0.2 ml of water, 0.02 ml of which was injected into each pupa. The results of assay summarized by relative activity to incubation time were illustrated in Fig. 1 and those to enzyme percentage (to sample in weight) in Fig. 2.

ii) α-Chymotrypsin : Boeringer Cat. No. 15139ECAA from pancrease, cryst, lyophilized, 10,000 units/mg was used. A solution of this enzyme (400 μg in 1 ml of 0.001N HCl) was prepared.

The DH solution were prepared as the same manner as

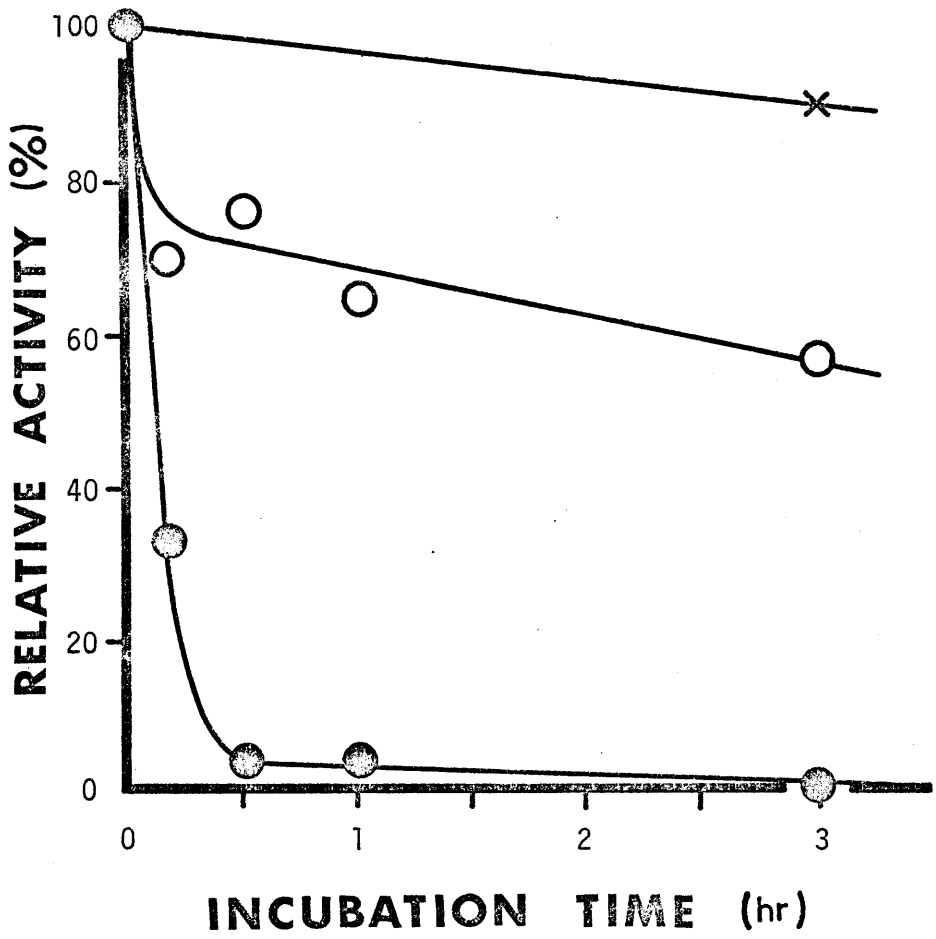

Fig. 1 Effect of incubation time on the diapause hormone hydrolyzed with trypsin and α-chymotrypsin.

The diapause hormone with 84.3 DE% by 40 μg/pupa was used as substrate; reaction mixture, 400 μg of the DH and 20 μg of each enzyme; enzymic hydrolysis was allowed to proceed at 25°C. Remained DH was assayed, and the activity was plotted relatively so as the original activity to be 100%.

—×— control; —○— α-chymotrypsin; —●— trypsin.

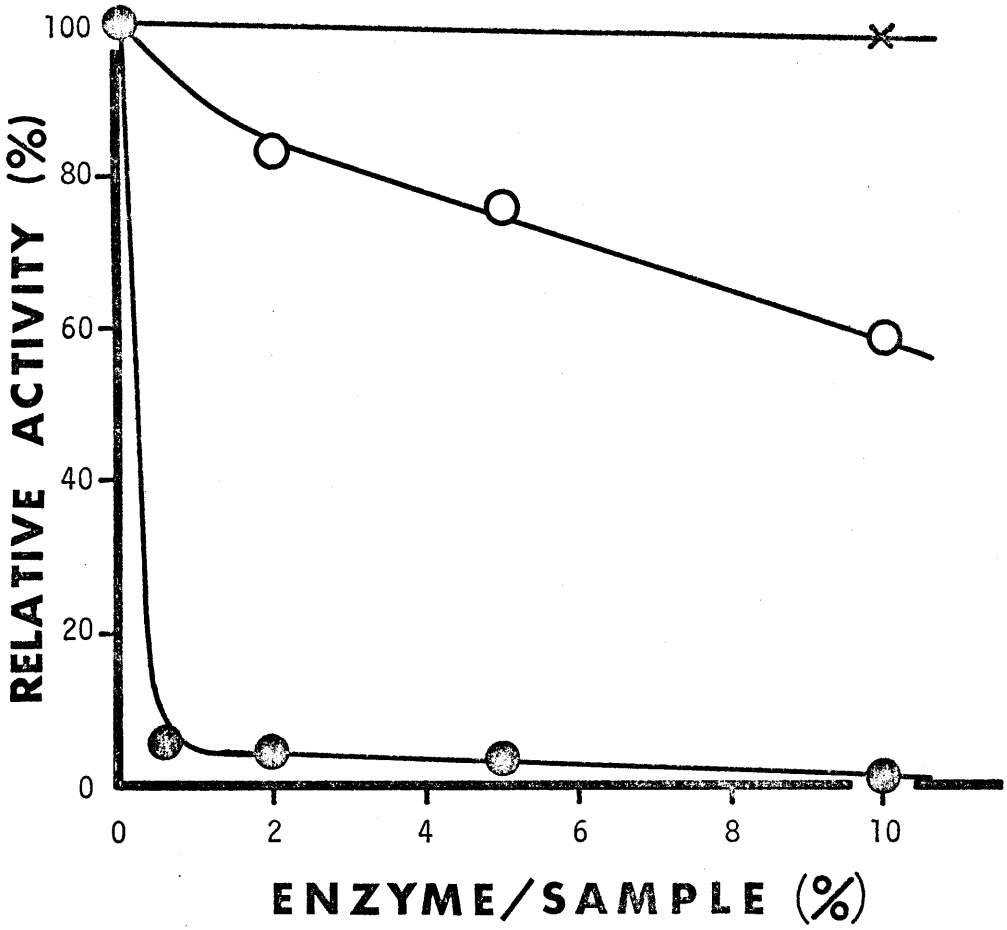

Fig. 2 Effect of enzyme concentration on the diapause hormone hydrolyzed with trypsin and α-chymotrypsin.

Incubation time is 30 min; other conditions are the same as stated in Fig. 1. (For detail, see the text)

—×— control; —○— α-chymotrypsin; —●— trypsin.

in the case of trypsin but for the phosphate buffer (0.05M, pH 7.0) for incubation.

All treatments of and after the incubation were carried out just the same as done in trypsin experiment, and the results of assay are summarized also in Fig. 1 and Fig. 2.

iii) α-Chymotrypsin : Sigma Cat. No. C-4129 from bovine pancreas, 3x cryst, lyophilized, salt free, Type II, 45 units/mg was also used to digest the DH in the same manner (but for long incubation up to 10 hr) as (ii), and the results are summarized in Table 2.

iv) Carboxy peptidase : DFP-carboxypeptidase A made by Worthington COADFP OIA (50 units/mg) was used for the incubation of the DH which is dissolved in 0.25 ml of N-ethyl-morpholine acetate (0.2M, pH 8.5) buffer. Enzyme solution was prepared by diluting the enzyme suspension (5 μl) with 10% aq-LiCl solution (50 μl) and the morpholine buffer (450 μl); 50 μl containing ca. 25 μg of the enzyme was introduced into the preliminarily incubated DH solution containing 400 μg/tube. To these reaction mixtures was added 10 μl of toluene and then they were incubated at 37°C for 0, 3, 7, 24, 36 and 48 hr.[7] All reaction mixtures were evaporated and assayed by 40 μg/pupa. Only 0.05 ml of 10% (10% aq-LiCl) of the morpholine buffer instead of the enzyme solution was incubated with the DH solution as the control. The results are shown in Table 3.

v) Glucosidases : α-Glucosidase (Boeringer, Cat. No.

Table 2. STABILITY OF THE DIAPAUSE HORMONE TO α-CHYMOTRYPSIN (Sigma).

Enzyme conc*	Incubation time				
	10 min	30 min	1 hr	3 hr	10 hr
(%)	(DE%)	(DE%)	(DE%)	(DE%)	(DE%)
0	76.5	74.1	79.9	74.6	81.0
2		50.1			
5	49.3	49.9	49.6	27.2	1.8
10		34.5			

Numbers in this table show average diapause egg percentages (DE%) by DH remaining after the incubation (40 µg/pupa).

* Percentage in weight of the enzyme to the substrate (DH).

Table 3 STABILITY OF THE DIAPAUSE HORMONE TO CARBOXYPEPTIDASE

	Incubation time (hr)					
	0	3	7	24	36	48
Control	80.5			80.1		
Digest. CPase-A	77.9	71.6	74.3	75.1	73.3	71.9

Numbers in this table show average diapause egg percentages by DH remaining after the incubation which was performed at 37°C on each solution of DH (400 µg with activity of 82.7 DE% by 40 µg/pupa) in N-ethylmorpholine acetate buffer (0.3 ml, 0.2M, pH 8.5) containing ca. 0.6 mg (0.2%) of LiCl with 25 µg (ca. 6% to the substrate) of carboxypeptidase A (CPase-A).

7150202)(20 μl) or β-glucosidase (Sigma, Cat. No. G 8625) (100 μg) was mixed with the DH dissolved in 0.3 ml of acetate buffer (0.1M, pH 5.6) was incubated at 25°C overnight and then evaporated to dryness. The residue were bioassayed, and the results are summarized in Table 4.

5) Amino Acid Analysis

The hydrolysis was performed by dissolving the sample in 6N HCl (once distilled at an atmospheric pressure at 108°C) (0.3 to 0.5 ml) and the solution was placed in a tube which was then degassed under reduced pressure (ca. 10^{-2}mmHg) by repetition of freezing and melting and finally sealed in vacuo.[8)]

The reaction time was determined firstly by hydrolyzing a fraction (tube numbers 43, of the third chromatography; Fig. I-7) by heating its 6N HCl solutions at 110°C for 12, 24, 48 or 96 hr. The resultant amino acids and amino sugars* are summarized in Table 5, where maximum values are underlined.

Further amino acid and amino sugar analyses were carried out by hydrolyzing the sample (80 to 600 μg) at 110°C for 48 hr.

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* Free amino acids and amino sugars thus obtained were measured with JEOL Model JLC-5AH Amino Acid Automatic Analyser, where amino sugars were detected by eluting them after tyrosine with citrate buffer (pH 5.28).

Table 4 STABILITY OF THE DIAPAUSE HORMONE TO GLUCOSIDASES

Enzyme	Specific activity of the enzyme used (10^{-3} μmol/min)	Diapause activity remained (DE%)
non-treated	-	73.0
control	0	73.4
α-glucosidase	500	76.6
α-glucosidase	1000	66.6
β-glucosidase	125 - 350	70.3
β-glucosidase	250 - 700	74.8

Each solution of DH (400 μg with its DH activity of 73.0 DE% by 40 μg/pupa, 69.9 DE% by 20 μg/pupa and 51.0 DE% by 10 μg per pupa) in sodium acetate buffer (0.3 ml, 0.1M, pH 5.6) was incubated at 25°C overnight. After incubation the mixture was evaporated with 0.5 ml of ethanol and then assayed by 40 μg/pupa.

Table 5 DETERMINATION OF THE HYDROLYSIS TIME OF THE DIAPAUSE HORMONE

	Liberated amino acids & amino sugars			
	12 hr*	24 hr*	48 hr*	96 hr*
Lys	14.9	18.5	24.4	27.3
His	trace	trace	4.8	6.9
NH_3	180	186	316	489
Arg	trace	trace	13.7	13.7
Asp	21.0	24.2	28.3	28.3
Thr	11.8	13.5	17.5	18.2
Ser	29.2	30.0	35.3	34.6
Glu	26.9	36.2	41.2	47.0
Pro	54.0	72.6	75.2	87.0
Gly	36.1	36.4	46.7	43.8
Ala	39.4	46.2	50.6	52.9
Cys	0	0	0	0
Val	23.2	21.5	31.9	33.3
Met	trace	0	trace	0
Ile	14.7	21.9	34.7	37.2
Leu	33.1	48.8	61.0	63.8
Tyr	9.5	9.6	11.1	11.3
Phe	8.7	10.5	14.5	13.8
Glc-NH_2	133	185	218	163
Gal-NH_2	300	254	210	180

* Hydrolyses were performed for these period with 6N HCl in vacuo at 110°C; the numbers, calcd. 10^{-3}µmol/1mg.

RESULTS AND DISCUSSION

1) Stability of DH to Chemical Reagents

Almost all treatments in the acid or bases in Table 1 gave no considerable effect on the DH to exhibit its hormonal activity remaining after the treatments but for the case with 1N NaOH.

Acylation of DH was intended to modify it into a chromophore-containing derivative which is detectable by UV absorption and would be available for high performance liquid chromatographic analysis of DH. The DH after these treatments retained its hormonal activity, and HPLC analysis would reveal whether the DH would be modified well or not.

Glucosamine and galactosamine were found in DH hydrolysate, which were simultaneously analyzed with amino acids. The confirmation of these amino sugars to be necessary for the diapause induction was failed by the fact that the DH was not affected by treatment with sodium periodate in acetate buffer (Table 1). This fact suggests the presence of the sugars to situate on the position where they do not affect the hormonal activity; or the fact suggests that the amino sugars have glucosidic linkage at 1 position and protective group (such as acetyl) on the 2nd and 4th position of the sugars and are innert to periodate oxidation. The question how they are in the DH molecule will be solved by further study on DH with reference to the amino acid sequence.

2) Stability of DH to Enzymes

Highly purified DH indeed degraded by proteolytic enzymes as illustrated in Fig. 1 and Fig. 2, where DH was incubated with and without trypsin or α-chymotrypsin. Both of the controls in Tris-HCl or phosphate buffer were slightly (10-11%) less active than the original which had an average of 84.3 DE% by 40 μg/pupa. All of the activities of DH treated with the enzymes were calculated relatively so as the original activity to be 100%. In Fig. 1 was plotted the relative activity against incubated time (hr), where the enzymes equivalent to 5% of the substrate (DH) in weight were used. In Fig. 2 was plotted the relative activity against enzyme amount (wt. percent to the substrate), where incubation was performed for 30 min. DH incubated with trypsin lost its activity immediately after (only less than ten minutes') incubation; or less than 1% of this enzyme is necessary to degrade half of the activity in 30 minutes' incubation. On the other hand, α-chymotrypsin shows relatively slower hydrolyzing ability of DH than trypsin. More than 3 hrs' incubation or more than 10% of this enzyme would be necessary to degrade half of the original activity.

Table 2 shows the stability of DH to another α-chymotrypsin produced by Sigma, where observation of almost similar tendency is illustrated.

Consequently, these enzymic experiments suggest that there exist peptide linkages in DH molecule responsible for the induction of the diapause hormonal activity.

Table 3 shows that no significant decrease of the activity is observed during the incubation with carboxypeptidase A. The fact that DH is not affected by carboxypeptidase suggests the C-terminus of DH molecule to be absent (cyclic peptide) or to be brocked by some substituent other than a neutral amino acid.

In Table 4 are summarized the results on DH treated with α- and β-glucosidase, where only minor effect of the enzymes to DH activity was observed. These experiments together with the failure affecting DH by acylation or periodate oxidation (Table 1) suggest that glucose, if any it is in DH molecule, might not be concerned with DH activity.

3) Constituents of DH

From the results of enzyme experiments, DH molecule contains some peptide linkages which are responsible for the hormonal activity in the silkworm diapause. Based on this fact the amino acids were systematically analyzed on several fractions concerned in order to determine them as accurately as possible. The analyses were performed on not only the fractions of the final chromatogram but also those in the chromatograms which have been obtained during the course of DH purification.

As already stated in the first chromatogram (Fig. I-5), we have obtained a peptide fraction (tube numbers 22-24) which precedes the DH fraction in the chromatogram. Results of

amino acid analysis of this fraction (tube number 23) is depicted in Table 6, A. In this table are also shown the results on B : DH fraction of the 4th chromatogram (Fig. I-8), tube numbers 53 to 61 and on C : DH-inactive fraction* of rechromatographed one in Fig. I-14, tube numbers 44 to 49. The relative composition of amino acids in B are very similar to those in C, and the total amounts in B are about five times of those in C. There is little relation between the components of B and A. On the other hand, the hormonal activity of C could be estimated from Fig. I-14, to be one fifth to one tenth of that of B. And hence these amino acids may be assigned to those originating from DH fraction:B. Whilst amino sugars are not found in A but in B and C, and the contents in C are higher than the ones in B by five to six times. Although contents of the amino sugars in B are comparable to those of other amino acids in their order, it is questionable whether the amino sugars originate from the DH molecule itself or not, since fraction:C, in which the amino sugar contents are very high, might contaminate in the fraction:B.

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* The major components of this hormonally inactive fraction:C are analyzed by acid hydrolysis to afford fatty acids and neutral sugars such as glucose, fucose, mannose and unknown sugars together with the amino sugars. This fraction may be a kind of glycolipids such as ganglioside. Slight amounts of amino acids found in this fraction are considered to be contaminated substances from the hormonal fraction.

For further confirmation of these amino acids and amino sugars in DH fraction to originate, in fact, from DH molecule four of the fractions (tube numbers 33, 40, 43 and 48) of the 3rd chromatogram (Fig. I-7) were analyzed (Table 7), where distribution of only neutral amino acids and amino sugars was determined. Tube number (t.n.) 40 and t.n. 48 have been estimated to be very active; t.n. 43 to be less active; and t.n. 33 to be inactive. From the values of amino sugar content analyzed, it is evident that t.n. 48 is not contaminated by the components in t.n. 43, indicating that when two fractions are separated by five or more tubes, the latter fraction is not affected by contamination of the former fraction. This fact applies to the case of t.n. 40 which is, therefore, not contaminated by t.n. 33, although the total amino acid contents of each other are very similar. Since the ganglioside-like fraction (C in Table 6) essentially contains no amino acids, the amino acids in t.n. 40 must originate from the DH active fractions (t.n. 36 to 40). Final chromatogram shown in Fig. I-10 gave the amino acids which are, thus, originate from DH molecule.

The relative mole-ratios of the amino acids and amino sugars on three of the fractions (t.n. 34, 38 and 42) in the final chromatogram are summarized in Table 8, where aspartic acid (Asp) is taken as standard value, 1.00. All constituents of the three fractions are almost identical. Slight deviation of the values in this table from integer numbers is considered to be due to incompleteness of the hydrolysis or decomposition of the products. It is not unreasonable since the hormone contains some non-amino acid components that would affect to

Table 6 CONSTITUENTS OF THE DIAPAUSE HORMONE (I)

	Liberated amino acids & amino sugars		
	A Fig.I-5 t.n.23	B Fig.I-8 t.n.$^{53-}_{61}$	C Fig.I-14 t.n.$^{44-}_{49}$
Lys	83	-	12
His	135	-	7
Arg	190	-	11
Asp	440	89	17
Thr	314	58	11
Ser	232	85	22
Glu	151	102	23
Pro	754	142	19
Gly	442	101	26
Ala	898	128	26
Cys	0	0	0
Val	270	73	21
Met	trace	trace	0
Ile	218	72	17
Leu	702	135	31
Tyr	283	47	8
Phe	92	43	8
Glc-NH_2	0	56	327
Gal-NH_2	0	51	261

Hydrolysis : 6N HCl, 110°C, 48 hr; calcd. 10^{-3}µmol/1mg.

Table 7 CONSTITUENTS OF THE DIAPAUSE HORMONE (II)

Distribution of amino acids and amino sugars in the 3rd chromatogram (Fig. I-7).

	Liberated amino acids & amino sugars			
	t.n. 33	t.n. 40	t.n. 43	t.n. 48
Asp	80	69	20	19
Thr	59	50	17	19
Ser	60	69	30	25
Glu	54	86	34	52
Pro	-	-	-	-
Gly	120	105	44	58
Ala	165	131	46	41
Cys	0	0	0	0
Val	89	91	44	51
Met	0	0	0	0
Ile	89	90	41	48
Leu	186	156	63	75
Tyr	55	37	12	6
Phe	47	47	15	16
A.A. total	1004	932	366	410
Glc-NH_2	trace	147	316	trace
Gal-NH_2	trace	124	228	trace
A.S. total	trace	271	544	trace

Hydrolysis : 6N HCl, 110°C, 21 hr; calcd. 10^{-3}µmol/mg.

Table 8 CONSTITUENTS OF THE DIAPAUSE HORMONE (III)

Relative mole-ratios of amino acids and amino sugars of the three fractions in the final chromatogram (Fig. I-10).

	Relative mole-ratios of the tube no. of		
	34	38	42
Lys	0.59	0.58	0.75
His	trace	trace	trace
Arg	0.70	0.61	1.08
Asp*	1.00	1.00	1.00
Thr	0.97	0.77	0.77
Ser	1.02	0.74	0.90
Glu	1.08	1.56	1.26
Pro	1.73	2.24	2.55
Gly	1.29	1.56	1.28
Ala	1.88	1.67	1.68
Cys	0	0	0
Val	0.99	1.23	0.98
Met	trace	trace	trace
Ile	1.35	1.37	1.34
Leu	2.10	2.21	2.11
Tyr	0.75	0.63	0.60
Phe	0.68	0.68	0.71
Glc-NH_2	1.00	0.67	1.03
Gal-NH_2	1.09	0.63	0.85

* Taken as standard value.

the yields of amino acids in the hydrolysate.

Infrared spectrum of DH is shown in Fig. 3, the characteristic absorptions being 3350 cm^{-1} (OH, NH), 2900 (methylene), 1720 (shoulder, ester), 1220 (ester), 1650, 1530 (amide) and 1060 (C-O or O-H). This spectrum supports that DH molecule consists mainly of amino acids in the form of peptide linkages, but there are indications that is also contains some other components. Tryptic and α-chymotryptic digestions of DH have shown the presence of peptide linkages in DH molecule as a part responsible for the diapause induction. Consequently, the silkworm diapause hormone (in Fraction A; pp 51) contains the 14 amino acids and 2 amino sugars as depicted in the final table. Analysis of other components of the hormone are now under investigation using several micro analytical techniques.

Further, isolation and the chemical studies on the diapause hormone in Fraction B are now in progress, and correlation of the constituents of the hormones in Fractions A and B will make the whole molecular structure of the hormone evident in near future.

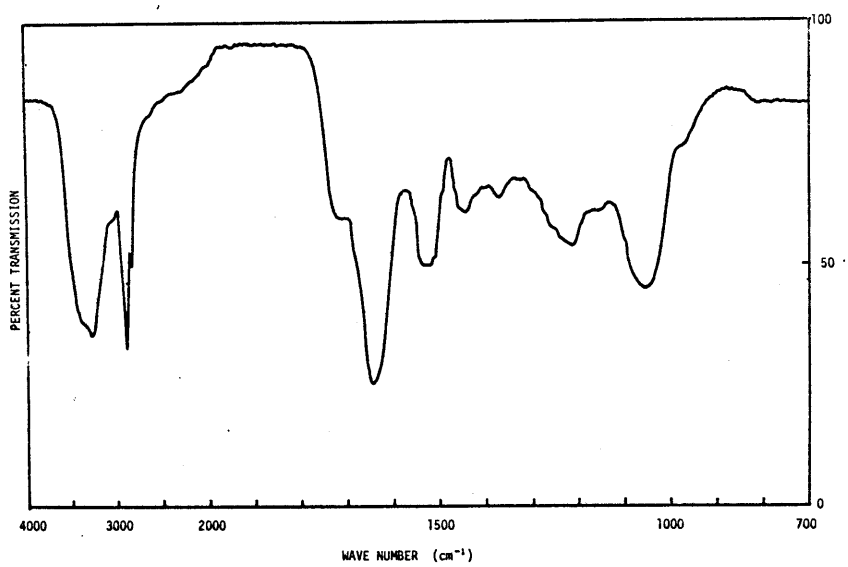

Fig. 3 Infrared spectrum of the diapause hormone.

Sample, cross-hatched area of the 4th chromatogram in Fig. I-8; measured in micro KBr disk.

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