家蚕の食道下神経節の神経ホルモン

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The Neurohormones from the Suboesophageal Ganglion of *Bombyx mori*: Separation of Melanization-and-Reddish-Coloration Hormone from Diapause Hormone

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The suboesophageal ganglion of the silkworm, *Bombyx mori*, is conceived to secrete two different hormones; diapause hormone (DH) for the silkworm and melanization and reddish coloration hormone (MRCH) for the common armyworm, *Leucania separata*. Whether or not these hormonal activities are derived from the same chemical entity was determined by comparing the activities of each hormone recovered from the sequential steps of purifications. By the solvent extraction of silkmoth heads, DH-A and MRCH were effectively partitioned into the same fraction. These two hormones behaved similarly on the column chromatographies with Sephadex LH-60, the first Merckogel OR 6000 and DEAE-Sepharose CL-6B. However, DH-A was clearly separated from MRCH in the subsequent purification steps such as the second Merckogel column chromatography or isoelectric forcusing. As for another kind of DH, DH-B (the smaller in molecular size) was quite different from MRCH as well as DH-A in extractability and chromatographic behaviors. The highly purified sample of MRCH exhibited linear dose-response relationship in cuticular melanization of common armyworms.

The hormonal activities of DH-A and MRCH dropped down by the proteolytic digestions with trypsin, α-chymotrypsin or pronase P and also by some physical or chemical treatments. Interesting selective decrement of the two activities occurred only on MRCH by alkaline treatment with 0.1N NaOH and dansylation with DH-A activity unchanged.

Thus, it is concluded that MRCH is a peptidal hormone, whose chemical properties and structure(s) are not identical but is likely to be similar to those of DH-A.

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The suboesophageal ganglion (SG) is well known as a principal neurosecretory organ responsible for the diapause induction in the eggs of the silkworm, ***Bombyx mori*** (HASEGAWA, 1952) as well as ***Orygia antiqua*** (KIND, 1972) and ***Adelphocoris lineolatus*** (EWEN, 1966). The SG's from ***Antheraea yamamai***, ***Antheraea pernyi*** (HASEGAWA, 1952), ***Phalenoides glycinae*** (ANDREWARTHA *et al.*, 1974) and the common armyworm, ***Leucania separata*** (OGURA and SAITO, 1973) which never need them for its diapause induction were demonstrated to have potent diapause-inducing activities in these SG's when implanted into the non-diapause type of silkworm pupae. These facts imply that the endocrine activity of SG would not be restricted to the diapause induction but also might contribute to the other physiological processes. In ***Carrausius morosus***, SG's were shown to be concerned with the regulation of physiological color changes (RAABE, 1966), and in ***Leucania separata***, melanization and coloration of the integument were also shown to be under the control of SG (OGURA *et al.*, 1971). Further, transplantation experiments figured out that SG's from silkworm exhibited the potency to induce the melanization in common armyworms (OGURA, 1975a). Accordingly, SG's of silkworms are concluded to have at least two different functions in endocrinological aspects.

Since diapause hormone (DH) was extracted from the SG's of silkworms by HASEGAWA (1957), our efforts have largely been paid for the effective extraction and purification of this hormone (see review by ISOBE and GOTO, 1980). At present, the hormone has been chromatographically purified and separated into two active principles, DH-A and DH-B. Both DH-A and DH-B are peptides with the respective molecular weights of 3300 and 2000 and the relative activity of the former is lower than that of the latter. Their chemical characters were determined to be labile to proteolytic digestion but stable in neutral or basic media, suggesting the hormones to be peptidal one (ISOBE *et al.*, 1973, 1975; KUBOTA *et al.*, 1976, 1979).

In the preliminary experiments with our DH samples, crude DH extracts were shown to induce cuticular melanization in common armyworms (private communication of Dr. OGURA, 1971). Further, the active substance was extracted from the silkworm moth heads with some modified methods developed for the prothoracicotropic hormone extraction and was named as melanization and reddish coloration hormone (MRCH) by SUZUKI *et al.* (1976). Progress of our chemical studies on DH in silkworms lead us to determine the question whether or not one chemical compound should be concerned with the dual functions exhibited by SG of silkworms; thus, with diapause induction in silkworms and with cuticular melanization in common armyworms.

This paper deals with the answer to this question. In this study, crude DH extracts (ISOBE *et al.*, 1973) was employed as the starting materials for the MRCH purification, and nothing was performed on extraction from the common armyworm. The activities of DH and MRCH were sequentially compared along with the

purification steps for DH, which involve the gel permeation chromatographies (ISOBE *et al.*, 1973) and a new method such as isoelectric focusing. Chemical properties of MRCH were estimated and compared with those of DH.

MATERIALS and METHODS

Bioassay of DH activity in silkworms and MRCH activity in common armyworms. A polyvoltine race (N_4) of ***Bombyx mori*** was employed for assay of DH as was described previously (HASEGAWA, 1964 ; ISOBE *et. al.*, 1973). In the cases of injecting highly purified samples, bovine serum albumin (Type V, Sigma Chem., Mo. USA: 200 μg/animal) was used as a hormone carrier to ensure and to enhance the sensitivity of the recipients to DH (YAMASHITA *et al.*, 1980). The hormonal activity was evaluated by counting the diapausing eggs after hatching of non-diapause eggs. The results are expressed as mean per cent of diapause eggs and S.E.M. from 9 individuals, but in some cases DH activity is conveniently shown in graded ranks as "−" or "卄" according to ISOBE *et al.* (1973). One DH unit was defined as the amounts of a given sample which was required to induce about half numbers of the laid eggs to be in diapause.

The larvae of common armyworms were reared at 25℃ under long day photoperiod on leaves of maize, ***Zea mays***, on Italian rygrass, ***Lolium italian*** or on an artificial diet (Oriental Feed Co., GC-4 ; SATO, 1965). They were grown in plastic cases (8×30×23 cm) under a crowded condition (300 to 500 larvae at 5th instar) to ensure sensitivity of cuticle to the hormone. The activity of MRCH in the different samples were examined on the 5th-instar larvae according to the method of OGURA (1975b) with slight modification. Around 20 hr before the 5th larval ecdysis, five to ten microlitters of test solution were injected into the hemocoal with an aid of a microsyringe attached with fine glass capillary. The injected larvae were immediately ligated between the 2nd and 3rd abdominal segment and the anterior parts were cut off to deprive the endogenous hormonal effects. In 24 hr later, new cuticle of the final 6th instar larvae could be clearly observed but the old cuticle did not shed. Thus, the animals were again ligated just behind the 3rd abdominal segment for successful scraping of the old cuticle. The hormonal activities were estimated 45 hr after the injection according to the scoring system of OGURA (1975b). Type I represents no melanization, while type V does the most intense melanization and dorsal region being velvety black. Type Ⅱ through type Ⅳ are the intermediates between them. The results are expressed on means ± S. E. M. from 8 to 10 individuals. For comparison of the resultant specific activity, MRCH unit was temporaly defined as the amount (μg/animal) necessary for the half maximum activity, which is obtained from the dose responce curve (see Fig. 6).

Purification of DH and MRCH from silkworm heads. The crude DH, DH-A (50g) and DH-B (5. 8g) were extracted from 2. 4 million heads of male silkworm

adults according to the methods reported previously (ISOBE *et al.*, 1973, 1976). DH activity of this crude sample was about 900 μg/DH unit for DH-A and 60 μg/DH unit for DH-B (see Fig. 1). For common armyworms, the crude DH-A sample exhibited an appreciable activity of MRCH at a dose of 100 μg/animal which is the maximum dose permitting the larvae to survive. Whereas, almost no MRCH activity was found in the crude DH-B sample. From the preliminary experiments, the fractions other than DH-A did not exhibit any MRCH activity, so the crude sample of DH-A was used for the purification of MRCH. Throughout the experiments, the substances from silkworms brought about a considerable melanization in cuticle but not any reddish coloration due to ommochrome pigments in the epidermis of common armyworms. MRCH was purified by the following steps effective for DH-A; thus, gel permeation chromatography in organic solvents, ion exchange chromatography and isoelectric focusing (See Fig. 1). These methods for each purification step were carried out at ca. 3℃ and the details of the experiments will be described in the ***Results***.

Enzymic and chemical treatments. For the purpose of detecting some characters of MRCH different from those in DH, potent MRCH samples were treated with enzymes or reagents; 200μg each of MRCH with an activity of 13μg/MRCH unit was subjected to the reaction condition, as shown in Table 2. Each reaction mixture was evacuated to dryness, dissolved in water, and then one tenth of the solution was injected into each of nine larvae of common armyworms. The resultant MRCH activities are depicted in the table together with the results in the case of DH for comparison.

The enzymic digestions were carried out with trypsin, α-chymotrypsin, protease V8 obtained from *S. Aureus*, pronase P and carboxypeptidase A in 5 mM phosphate buffer at pH 8. The digestion with a mixture of α- and β-glucosidase was done in 50mM sodium acetate buffer at pH 6.0. MRCH samples were dissolved or suspended in the buffer solution so as the final concentration became 1μg/μl, and they were incubated with each enzyme amounting 3.8 to 5.0 per cent (W/W) to the substrate at 37℃ for the indicated period in Table 2.

For the treatments under different physical or chemical conditions, MRCH samples were dissolved in the media shown in Table 2 (lower column) with or without the reagents cited therein. A sealed tube was used for heating. High pressure mercury lamp was placed 5cm away from the sample through Pyrex filter for light irradiation. N-Bromosuccinimide was recrystallized from hot water prior to use.

Enzymes and chemicals. The following gels, enzymes and reagents were purchased from the supplier in the parenthesis; Sephadex LH-60, DEAE-Sepharose CL-6B, Sephadex IEF and Pharmalite 3-10 (Pharmacia Fine Chemicals A B, Uppsala, Sweden); Merckogel OR 6000 (E. Merck, Darmstadt, Germany); trypsin, α-chymotrypsin, carboxypeptidase A, glucosidases and dansyl chloride (Sigma, St. Louis, Mo.

USA); Protease V8 from *S. Aureus* (Miles, Elkhard, Ind. USA); pronase P (Amano Chem., Aichi, Japan), N-bromosuccinimide (Nakarai Chem., Kyoto, Japan).

RESULTS

Purification of DH and MRCH from silkworm heads. DH and MRCH were sequentially purified with Sephadex LH-60, Merckogel OR 6000 (ISOBE *et al.* 1973) and DEAE Sepharose CL-6B column chromatographies and also with the flat bed isoelectric focusing (See Fig. 1). The chromatographic behaviors of DH-A and MRCH along the purification processes were compared to determine whether both hormones were the same or not. The following distribution pattern of the hormonal activities were obtained at each step of purification.

Step A-1 : Sephadex LH-60. Although Sephadex LH-20 had long been utilized for DH-separation (ISOBE *et al.*, 1973), the recent lots of this gel showed only a poor separatian for DH due to its extremely stronger polarity than before. While Sephadex LH-60, a similar gel, gave a quite satisfactory result for the first purification step of the crude DH-A for which we adopted Sephadex LH-60 and a mixture of methanol-dichloromethane (9:1) as eluent (ISOBE *et al.*, 1973). A resultant typical chromatogram is shown in Fig. 2, in which the applied materials were eluted

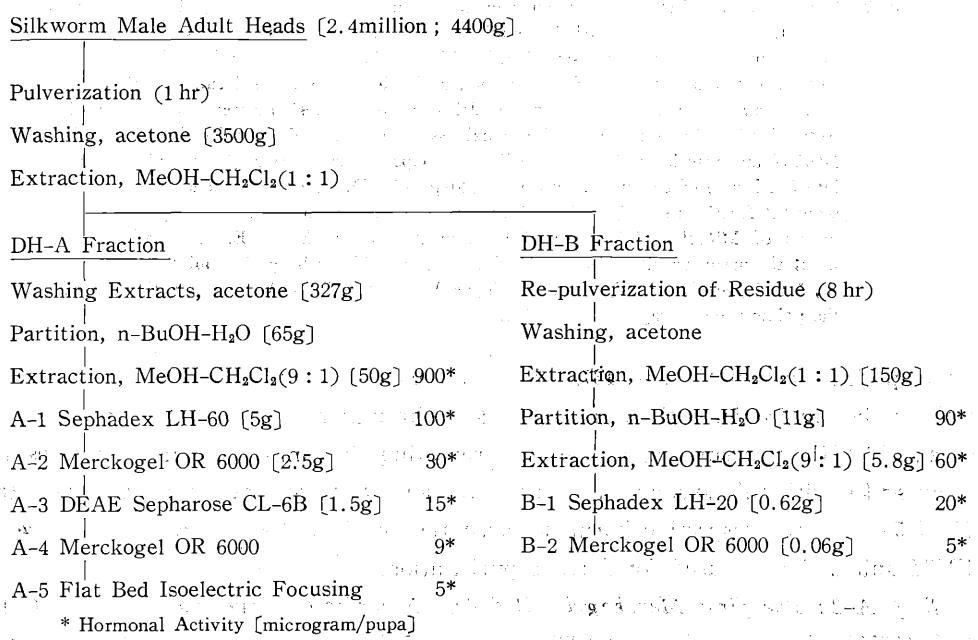

Fig. 1. Flow Sheet for selective extraction and purification of DH-A and DH-B preparation of DH from male adult heads of silkworms. Numbers in parentheses represent the recoveredweights of active substances at each steps and the numbers with asterisks are the hormonal amounts in microgram to exhibit an activity corresponding to one DH unit.

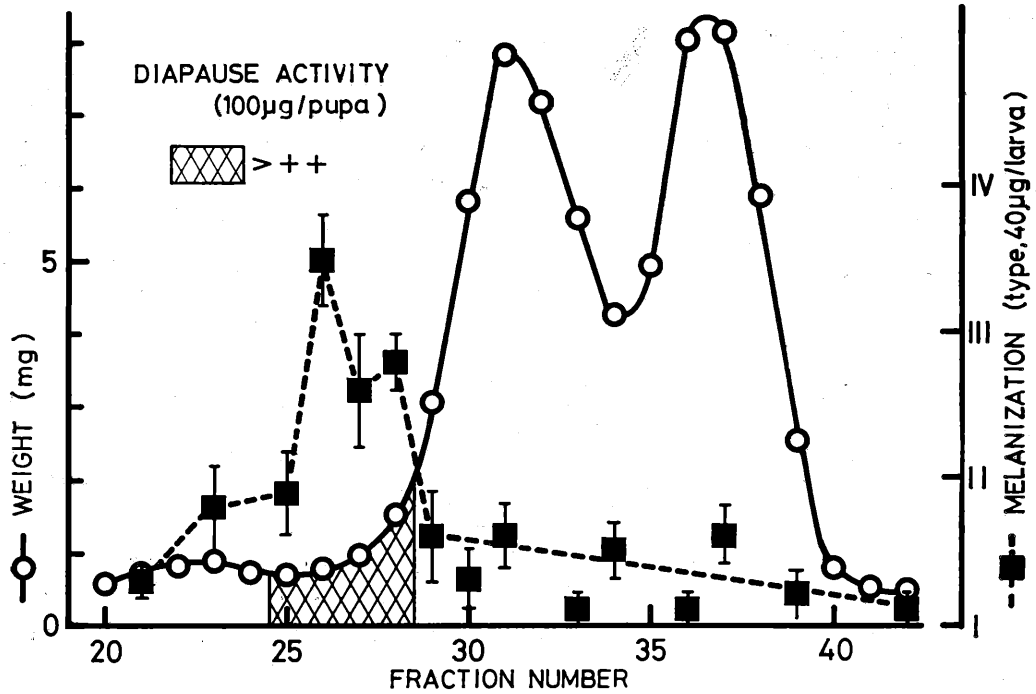

Fig. 2. Chromatogram of step A-1 on sephadex LH-60 and the hormonal activities of DH and MRCH. Crude extracts of DH-A (79mg) was applied onto the Sephadex LH-60 column (1×100cm) which was preconditioned with a mixture of methanol-dichloromethane (9 : 1), and was eluted at a flow rate of 2 ml/hr by the same solvent. Fraction size was 1.1 ml/tube and each fraction was dried up and the residues were weighed gravimetrically in millgram. For the hormonal assays, each sample was dissolved in 0.28% NH_4OH and was injected into non-diapause pupae of silkworms (100μg/animal) and into 5th larvae of common armyworms (40μg/animal). The cross hatched area represents the fractions showing DH activity above 50%. The black square represents the means of MRCH activity determined from 9 larvae with ± S.E. shown as vertical bars; the mean activity was calculated from 9 individuals by giving point-0 to Type-Ⅰ, 1 to Type-Ⅱ, 2 to Type-Ⅲ, 3 to Type-Ⅳ and 4 to Type-Ⅴ, and then the sum of the points were averaged with ± S.E.

as two peaks, fraction #31 and #37. The activities of DH and MRCH were recovered in the higher molecular weight fractions, #25 through #28; the elution patterns of DH and MRCH were superimposable. By repeating the similar chromatographies in larger scales with this system, the active fractions were collected and combined to afford 5g with the respective hormonal activities of about 100μg/DH unit and 40μg/MRCH unit and were used for further purification.

Step A-2: The first Merckogel OR 6000. According to the method of ISOBE *et al.* (1973), the active fraction (238mg out of 5g) prepared in the previous step was separated with the Merckogel OR 6000 using mixture of methanol-dichloromethane (1:1). Although the chromatogram is not shown herein, the elution pattern clearly resembled that described previously (ISOBE *et al.*, 1973). In this case, both

Table 1 DH and MRCH activities on the fractions separated by DEAE-Sepharose CL-6B column chromatography

Fraction No.	Weight recovered (mg)	MRCH activity (points)	DH activity
1	26.5	2.9±0.4	++
2	64.5	0.7±0.2	+
control		0.0±0.0	−

Samples (53mg with an activity of ca. 1800 DH units) were applied onto the DEAE-Sepharose CL-6B column (1.4×20cm) and eluted i) with a mixture of methanol-dichloromethane (1 : 1) (25m*l*) as Fraction No. 1; and ii) with the mixture containing 0.1M N-ethylmorpholinium acetate (40m*l*) as Fracton No. 2. After evaporating the solvent of the eluates, the residues were weighed and dissolved in 0.28% ammonia. Solution of the sample (15μg/animal) was each injected into 5th larvae of common armyworms and pupae of silkworms as described in the text. As the control, 10μl of 0.28% ammonia was injected. DH activity is expressed as ++ for over 50% induction of diapause eggs, + for below 10% and − for no diapause eggs. MRCH activity is depicted as the average of the points giving 0, 1, 2, 3 or 4 to the resultant individual Type −Ⅰ, Ⅱ, Ⅲ, Ⅳ or Ⅴ, respectively.

the activities for DH and MRCH were found in the same fractions which showed 30μg/DH unit and 20μg/MRCH unit, respectively. The corresponding active fractions with similar activities were collected by repeating larger scale chromatographies of Merckogel OR 6000 to give 2.5g in total.

Step A-3: DEAE-Sepharose CL-6B. The slurry of DEAE-Sepharose CL-6B was washed successively with water, 30%, 50%, 70% and 100% methanol, mixture of methanol-dichloromethane (7:3) and then (1:1). The sample (53mg) was dissolved in a mixture of methanol-dichloromethane (1:1) and then applied onto the column, which was first developed with the mixed solvent and then with the solvent containing 0.1M N-ethylmorpholinium acetate. In this ion-exchange chromatography, the active fractions from the preceding step was separated into two fractions. The first fraction (Fr. 1) was 26.5mg and the second one (fr. 2) was 64.5 mg contaminated by some of the salts. The results of bioassay on these fractions are summarized in Table 1 were no separations of DH and MRCH and both activities were recovered in Fr. 1. This separation was again repeated to give 1.5 g of the active material which was used for the following steps, A-4 and A-5.

Step A-4: The second Merckogel OR 6000. The active fraction from Step A-3 (44mg) was dissolved in 0.1m*l* of methanol-dichloromethane (1:1) and then rechromatographed with a small Merckogel OR 6000 column (0.8×150 cm) at a slow flow rate (1.8m*l*/hr). The chromatogram was monitored again by weight as well as by the hormonal activities (Fig. 3). MRCH activity assayed on 7.5 μg/larva of each fraction was found at the highest level in Fraction #21. The activity peaks for DH and MRCH were separated to show the latter being a little smaller in its molecular size than the former, but the peaks were quite close to each other. So we attempted to separate better the active fraction from Step A-3 by a different method.

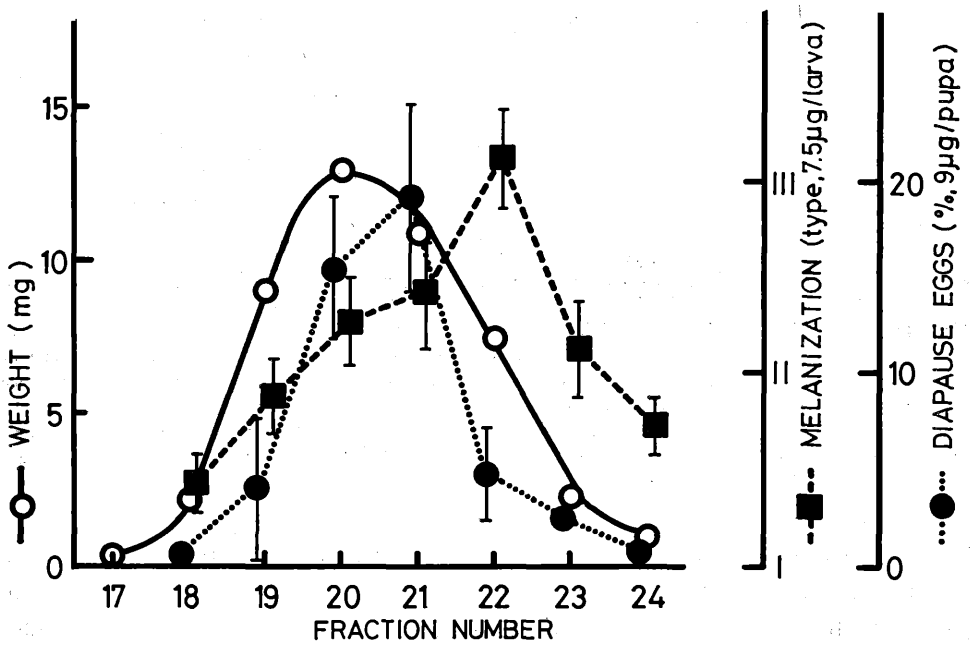

Fig. 3. Chromatogram of step A-4 on Merckogel OR 6000 and the hormonal activities of DH and MRCH. The sample (44mg) obtained from the DEAE-Sepharose CL-6B column chromatography (Step A-3; Fr. 1) were applied into the Merckogel OR 6000 column (0.8×150cm) which had been preconditioned with a mixture of methanol-dichloromethane (1 : 1) and eluted at a flow rate of 1.8ml/hr by the same solvent. Fraction size was 1.5 ml/tube and after evaporation of the solvent, the residues were weighed. The samples injected into silkworm pupae (9 μg/animal) and into armyworm larvae (7.5 μg /animal) for determination of the respective hormonal activity. Each point represents the means determined from 9 individuals and with ± S.E.M. shown as veritical bars.

Step A-5 : Isoelectric focusing. Flat bed gel isoelectric focusing was employed for the separation of Fr. 1 of Step A-3, by using a Sephadex as the support (FBE 3000 and IEF kit from Pharmacia Fine Chemicals). For this purification, about 350 mg of the material deprived from Step A-3 was applied onto 20×20×0.5cm flat bed gel of Sephadex IEF suspended with Pharmalite 3-10. The electrophoretic conditions were in constant two watts with a Power Supply (ECPS 2000/3000 type, Pharmacia Fine Chemicals) at 3℃ for 15 hr. After the electrophoresis, the gel was cut into 9 pieces corresponding to the fraction numbers in Fig. 4 (roughly judged by the brown lines) and then each piece was packed into a glass column. Samples were eluted with water and the eluates were extracted with butanol which was then evaporated to dryness. Rough values of pH and the hormonal activities of each fraction of DH and MRCH are illustrated in Fig. 4. The samples were separated into two peaks on Fraction #4 and Fraction #7 to #8 as determined gravimetrically. DH activity was also separated into two peaks, Fraction #2 and Fraction #5. Whereas, MRCH activity showed somewhat broad distribution with the maximum activity at

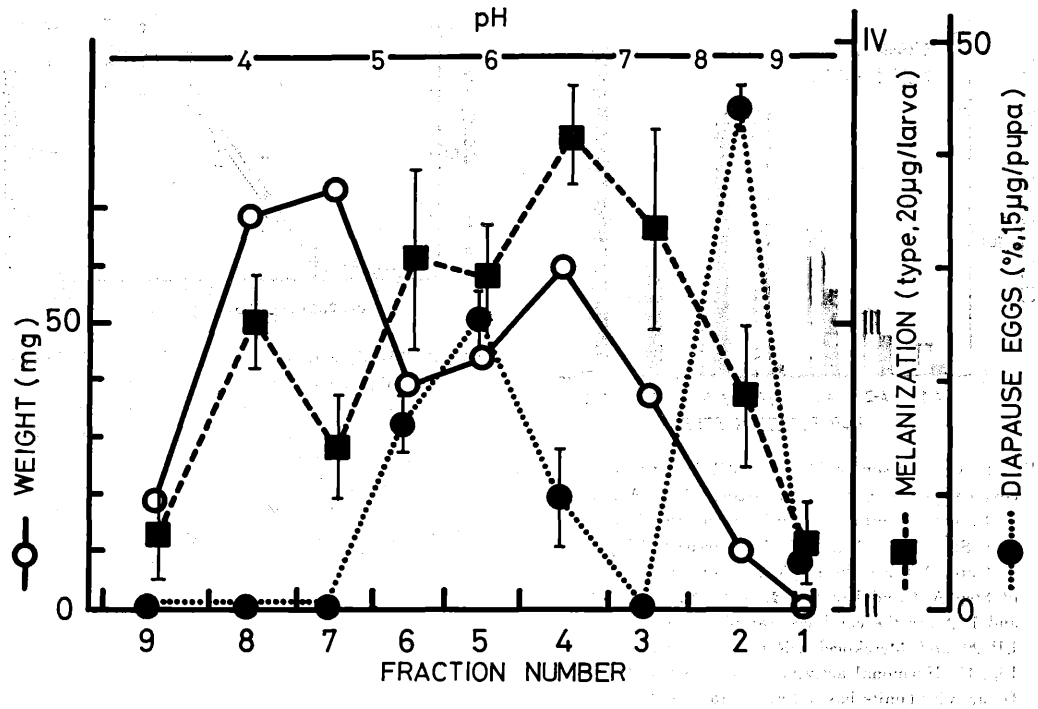

Fig. 4. Flat bed isoelectric focusing of step A-5 and hormonal activities of DH and MRCH. Sample (about 350mg) deprived from DEAE-Sepharose CL-6B column chromatography (Step A-3) were applied onto a flat bed gel (20×20×0.5cm) of Sephadex IEF suspended with Pharmalite 3-10. Electrophoresis was carried out at a constant watt of 2 at 3°C for 15 hr. The bed gel was cut off into 9 fractions, and each of them was eluted with water, extracted with n-butanol, and then evaporated to dryness. The residues were weighed, dissolved and then injected into the test animals for DH (15μg/pupa) and for MRCG (20μg/larva). Each point represents the means from 9 incividuals with ±S.E. M. shown as vertical bars.

Fraction #4 which did not exhibit any significant DH activlty.

Summary of the DH-MRCH activities. Fig. 5 illustrates the summary of the distribution of DH and MRCH through the purification steps. The specific activities (hormonal units/mg sample) were estimated on the combined samples in the steps A-1, A-2, A-3, B-1 and B-2. But a single fraction in Step A-4, #22 and Step A-5, #2 was taken for Fig. 5. As is clearly indicated, specific activity gradually increased according to the purification and DH-A and MRCH showed about 20 and 10 fold increment in these process. The activities of DH-A and MRCH did not separate either in the extraction and solvent partition process nor in the chromatographies Step A-1 through A-3. In Step A-4, however, the highest MRCH activity appeared in Fraction #22 in which only a weak DH activity was found. Fraction #20 and #21 in Step A-4, on the other hand, showed comparable activities for both of the two. The contrast between DH-A and MRCH was more striking in Step A-5; thus, very high DH activity was found in Fraction #2 and #5, while MRCH activity

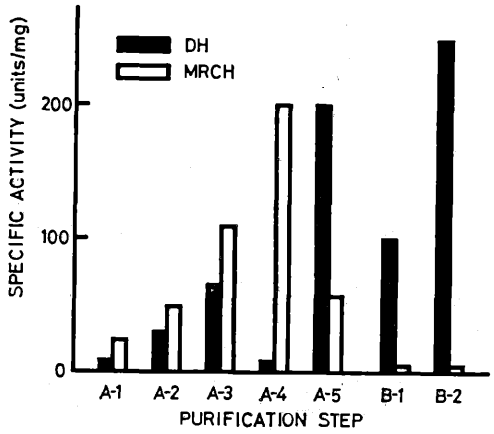

Fig. 5 Comparison of DH and MRCH activities recovered in the purification steps. A-1, A-2 and A-3 are the combined samples from the Purification Step A-1, A-2 and A-3, respectiviely, and A-4 and A-5 are the single fraction, Fraction #22 of Step A-4 and Fraction #2 from Step A-5. B-1 and B-2 are the combined samples after Sephadex LH-20 and Merckogel OR 6000, respectively (see Fig. 1). Hormonal activity is expressed as a specific activity (units based 1 mg of sample); the specific activity for DH was calculated from the amount of sample to induce 50% diapause eggs; for MRCH it was determined from each dose responce curve (see Fig. 6) by the amount of sample showing half activity of the maximum.

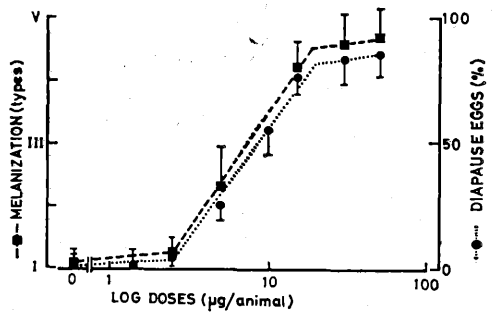

Fig. 6 The dose-Response curves for DH-A indiapause induction of silkworm EGGs and for MRCH in cuticular melanization of common armyworm larvae. Hormone samples from DEAE-Sepharose column chromatofraphy were dissolved in various concentrations. Each dose was injected into silkworm pupae and common armyworm as described in the text. Each point represents the means from 9 incividuals and with ± S. E. M. with vertical bars.

was widely distributed in most of the fractions, particularly in Fraction #4. Fraction #7 and #8 are of interest because they had considerable MRCH activity but had no DH activity at all.

Although the precise purification processes are not shown herein, the crude DH-B extracts (Fig. 1) were sequentially purified by column chromatographies with Sephadex LH-20 as Step B-1 and Merckogel OR 6000 as Step B-2, the hormonal activities on DH as well as MRCH were examined with the same procedure as in the experiments on DH-A, However, no appreciable activity of MRCH was found in any fraction throughout these chromatographies as expected by the absence of MRCH in the original crude extracts of DH-B.

Dose-response relationship of DH-A and MRCH. To presume the action prosess of MRCH isolated from silkworms, a dose-response relationship of MRCH was compared with that of DH-A. Fig. 6 shows the dose-response curves for DH-A and MRCH from Step A-3. Both curves represented a linear relationship between the hormonal activities and the logarithmic doses of samples injected in the certain ranges. These results gave the following doses for the half-maximum activity; 6μg

Table 2 Effects of the Enzymic and Chemical Treatments on the DH-MRCH Activity

Enzyme or Reagent	Solvent§	Reaction temp(℃)	Reaction time(hr)	Activity* MRCH	Activity* DH-A
trypsin	phosphate	37	1	−	−
α-chymotripsin	phosphate	37	18	−	−
protease V_8	phosphate	37	18	++	+
pronase P	phosphate	37	12	−	−
carboxypeptidase A	phosphate	37	12	++	++
α, β-glucosidases	acetate	37	12	++	++
heat	M/C (1 : 1)	60	6	±	±
light	M/C (1 : 1)	25	2	±	−
acid	0.1N HCl	37	4	±	±
alkali	28% NH_3	37	12	++	++
alkali	0.1N NaOH	37	12	−	++
N-bromosuccinimide	M/C(9 : 1)	22	1	++	++
dansylchloride	0.2M aq. $NaHCO_3$/acetone	45	1	±	++
control	M/C (1 : 1)	25	12	++	++

* Resultant activity is expressed as follows:
++ retained;
+ partly diminished;
± almost inactivated;
− completely inactivated.
§ phosphate=0.005*M* phosphate buffer at pH 8.
acetate=0.05*M* sodium acetate buffer at pH 6.
M/C=mixture of methanol and dichloromethane

/pupa for DH-A and 8 μg/larva for MRCH. Although data are not given here, there was a clear dose-response relation in crude samples of MRCH, but requiring much higher doses for the half-maximum activity depending on the purification steps. Thus, the active principle of MRCH exerts its function as hormonal action.

Stability of MRCH to enzymic and chemical treatments. Unseparability of MRCH from DH-A in the earlier purification steps through Step A-3 might indicate the active principles are in a similar class of chemical category. The characterization of DH-A and DH-B has revealed that both of them are peptidal hormones (ISOBE *et al.*, 1979). The similarity of the chromatographic behaviours of MRCH and DH-A is suggestive the MRCH being also (a) peptidal molecule(s). This was confirmed by applying the enzymic and chemical treatments for MRCH as in the case for DH. Thus, MRCH activity disappeared when incubated with some proteolytic enzymes, such as trypsin, α-chymotrypsin and pronase-P. Incubation with protease V8, a more selective enzyme, did not affect the MRCH activity (Table 2). Further, carboxypeptidase and some kinds of glucosidases did not inactivate the hormone. These profiles are similar to those of DH-A.

Some clear differences between DH-A and MRCH were also found in the stabili-

ty of some chemical treatments (Table 2). When MRCH was kept warm in 0.1 N NaOH solution for 12 hr, MRCH activity was completely lost, suggesting MRCH to be a depsi-peptide, while DH-A and DH-B were not affected under this condition (ISOBE *et al.*, 1975; KUBOTA *et al.*, 1979). Although the modification of DH into dansylated derivatives maintained the activity, the same modification of MRCH yielded almost no activity.

DISCUSSION

Although melanization in larval cuticle of common armyworms is a quite different process from the diapause induction in silkworm eggs, SG of the silkworm can control these two physiological processes. The question encountered in the present study concerns whether these functions of SG are derived from an identical and single chemical entity. The DH is highly purified by a combination of column chromatography with wide range of chromatographic media and isoelectric focusing, and also by an improvement of bioassay system; however, the chemical structure of DH still remains open. Then, the first experiment was directed at comparison of both DH and MRCH activities in accordance with the processes of isolation and purification steps (Fig. 1 to 5 and Table 1). Although no activitis of MRCH were recovered in DH-B fractions, DH-A and MRCH behaved similarly each other in the course of the extraction and early purification processes (Fig. 2 and Table 1). But the different behaviors of DH-A and MRCH on their further purification steps (Fig. 3 and 4) suggest that the both activities should derive from different chemical substances. This possibility is supported by the different specific activity (Fig. 5) and the different behaviors against 0.1N NaOH and dansylation (Table 2). However, it should be noticed that DH-A and MRCH are similar in responsibility to the various kinds of enzymatic digestions and to some physical and chemical treatments (Table 2). Molecular weight of MRCH is assumed to be slightly smaller than that of DH-A (3300, KUBOTA *et al.*, 1976) judging from the gel permeation column chromatography (Fig. 3). By referring the properties of DH-A, MRCH from the SG of silkworm is also a peptidal hormone but its chemical properties are not identical to those of DH-A. The active principles for MRCH and DH, thus, are concluded to be different to each other judging from all of the above aspects.

According to the electron microscopic observations, the two different types of the neurosecretory cells are identified from the view points of their neurosecretory granules in the SG of silkworm pupae (PARK, 1973). Thus, it is also possible to speculate that two different molecules are attributed to the different origins of the neurosecretory cells of SG. The answer to this question demands the chemical approach to get pure form of the two hormones, which is the scope of the present series of studies.

The SG of silkworms is supposed, only by a transplantation experiments, to have

an endocrine potency to induce cuticular melanization in common armyworms (OGURA, 1975a). The linear relationship between the activities and the logarithmic doses injected (Fig. 6) has supplied a direct evidence indicating that the MRCH act.ve substance isolated from silkworms function in common armyworms as a hormone for inducing cuticular melanization through mediations of several processes. This point will be supported by the similar dose-response curves of MRCH and DH-A whose action is mediated by enhancing a specific enzyme activities and the establishing the metabolic shift (see review by YAMASHITA *et al.*, 1981). However the control mechanisms of MRCH on melanization processes in common armyworms are unknown at all as yet.

Over recent years, SG of the silkworm is believed to be a neurosecretory organ only concerning DH production (see review by YAMASHITA *et al.*, 1981). The present results throw a new light on the endocrinological functios of the SG of silkworms, which secretes at least two different kinds of hormones; DH and MRCH. However, the MRCH activity is not manifested in silkworms throughout the life cycle. Melanization of larval cuticle is under the control of juvenile hormone which acts to suppress the deposition of melanine pigment in the larval markings (KIGUCHI, 1972). The same function of juvenile hormone in cuticular pigmentaion is also shown in larvae of tobacco hornworm, *Manduca sexta* (TRUMAN *et al.*, 1973). Whereas, melanization of pupal cuticle of the black mutant (bp) of silkworms is induced by a kind of peptide hormone which is secreted from the metathoracic ganglia not from SG (HASHIGUCHI *et al.*, 1965). However, in eggs, the pigmentation due to the ommochromes is shown to be controlled by SG or DH (YAMASHITA and HASEGAWA, 1964). Thus, MRCH is not a true hormone for silkworms in respect to the pigment metabolism. But it remains as a possibility that MRCH manifests another function in silkworms which is not elucidated as yet. The present chemical studies on the hormones secreted from SG of silkworms support such a possibility and lead us to the research into the new endocrinoligical functions of SG.

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Note added in proof; Quite recently, the highly purified MRCH fractions from adult heads of silkworms have been shown to exhibit no DH activity (MATSUMOTO, S., A. SUZUKI, N. OGURA and H. SONOBE (1981): Insect Biochem., 11, 725—733.).

今井邦夫・近藤直人・磯部 稔・後藤俊夫・山下興亜・長谷川金作：家蚕の食道下神経節の神経ホルモン：休眠ホルモンと皮膚黒化ホルモンの分離，**日蚕雑**，**51**，111—125，1982
家蚕の食道下神経節は家蚕卵の休眠を誘導する休眠ホルモン(DH)とアワヨトウ幼虫の皮膚を黒化するホルモン(MRCH)を分泌していることが知られている。しかしこれらのホルモン作用が同じ神経ホルモンによって発現されているか否かは不明のままである。そこで本研究においてはこの点に関して，ホルモン

活性物質の溶媒に対する溶解性，各種クロマトグラフィーによる分離パターンの比較ならびに等電点電気泳動による挙動から検討を行った。その結果，DH は溶媒抽出過程で2種 (DH-A, DH-B) に分離されたが，MRCH は DH-A と同一分画に回収された。DH-A と MRCH は Sephadex LH-60 および第1回目の Merckogel OR 6000 クロマトグラフィーでは同一画分に回収されたが，第2回目の Merckogel OR 6000 カラムクロマトグラフィーと等電点電気泳動によって明らかに分離された。したがって DH-A と MRCH は異なったホルモン物質であると結論した。

DH-A と MRCH を種々の物理化学的な処理ならびに数種の酵素による分解実験を行ったところ，両者ともトリプシン，α-キモトリプシン，プロナーゼ P によって失活した。しかし，DH-A は 0.1N NaOH およびダンシル化に対して安定であったが，MRCH はこれらの処理によって明らかに失活した。これらの結果は DH と MRCH は異なる神経ホルモンであることを示すものである。

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