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ISOLATION AND PURIFICATION OF JUVENILE HORMONE OF BOMBYX MORI

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ABSTRACT
The effect of vertebrate hormones, plant hormones, juvenile hormone mimics and pesticides were tried by several researchers, in increasing the feeding and larval weight of Insects. Hence, our study has focused on the purification, kinetics and the effect of Juvenile hormone, in increasing the silk production in *Bombyx mori.* The study was concentrated on the unrevealed mysterious of the Juvenile hormone biosynthesis and their indirect effect on the silk production. It throws light on the darker sides of the following aspects. The purification and isolation of Juvenile hormone from *Bombyx mori* upto 4000 fold by employing the methods of extraction such as methanol, saponification, silic acid chromatographyI, silic acid chromatographyII, succinoylation, crystallization and gas liquid chromatography. The GLC results show peaks at 12.9min in F and M model Instrument with hydrogen flame ionization detector.

KEYWORD: JH, *Bombyx mori*, purification, isolation, extraction, methanol, saponification, silic acid chromatographyI, silic acid chromatography etc.

INTRODUCTION

In silkworm the corpus allatum is gland tissue which has a function along with prothoracic gland to induce larval moulting. In hemimetabola that the corpus allatum was the secretory centre of an inhibitory hormone (Wigglesworth, 1936). This is named as "Juvenile hormone" when silk worm larvae were ligated with cotton thread between head and thorax around two thirds of through the fourth larval instar most larvae become precocious pupae skipping the fourth larval moulting. This suggests that an organ existing in the head is necessary for the induction of the fourth larval instar. Furthermore the corpus allatum from silkworm larvae resulted in the production of precocious pupae even in the second and third instars. The results clearly indicates that the corpus allatum as an important role in the induction of larval moulting. In the observation of Fukuda, the influence of implantation of corpora allata from pupae of various ages into fourth instar larva which had been depride of their own corpora allata. When corpora allata at the beginning of pupal stage were implanted, the recipients became early maturing larvae because of the secretion of the implanted corpora allata had not yet reached the action threshold contrasting to that the larvae which received corpora allata from middle age or older pupae never showed early maturity. Larvae implanted with corpora allata from more or individual at the end of the pupal stage moulted normally. The corpora allata was proved to be active in secretory function from the middle to the end of pupal stage (Morohoshi, 1959) reported that the corpus allatum was related to the non diapause egg in the silkworm. Ching *et al.*, 1972; Cymborowski, B and Stolarz, G., 1979 concluded that the moulting character, is predominantly determined by the brain corpora allata system and the voltinism is predominently determined by the brain sub-oesophagal ganglion system further Morohoshi, 1968 further proved that an extract of corpora allata could increase the pulse rate, promoting the decomposition of lipid and carbohydrate in the silkworm. These findings gives the idea of axonal transport of neurosecretary material through the brain corpus cordiacum that allatum system indicated not only that Juvenile hormone was secreted from incorporated corpora allata but also prothoracic gland of diapausing puape were activated by the implantation of corpora allata which presumably released neuro secretary material originating from neurosecretory cells in the brain. Further evidences are also available endocrine function in prothoracic gland at sub-oesophogal ganglion. The major role of the prothoracic gland in the induction of larval at pupal moultings was observed in silkworm (Muroga, 1939, 1940, Fukuda; 1940, 1944, Gangwar, S.K., 2009). It is also observed by Ogura and Saito, 1973, that some hormonal factors released from both the brain corpora cordiace-corpora allata complex and sub-oesophageal ganglion of the common army worm at directly on the developing ovaries of *Bombyx mori* pupae inducing embryonic diapause.

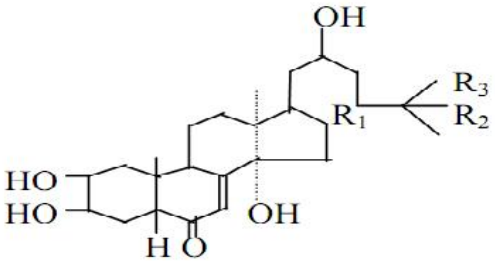
Chemical nature

The crude extract of the brain, when identified from its chemical properties as cholesterol and steroid hormones. It is also noted that the brain hormone purification is done by sephadex column, the molecular weight is ranges from 10,000 to 15,000. The hormone activity is resistance to

tripsia at chymotrypsin but destroyed by pronase. It is heat stable, the pH ranges from 8.55 to 8.65.

The juvenile hormone is separated by GLC. The main component was methyl-9-epoxy-hexa decanoate. Finally in 1970, Roller and Dahm, 1970, concluded that the juvenile hormone is methyl trans, *cis*-10-epoxy-7-ethyl-3, 11-dimethyl-2,6-tridecadienaate produced and secreted by corpora allata.

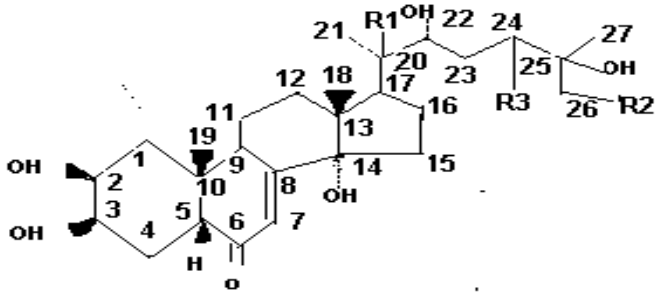
The moulting hormone is the ecdysone it is a derivative of stigma sterol. Sidal and co-workers, 1966 proved as 3-β-hydroxy-22, 23-Bisnorchol-5 anacorid finally four structures have been proved as shown below.

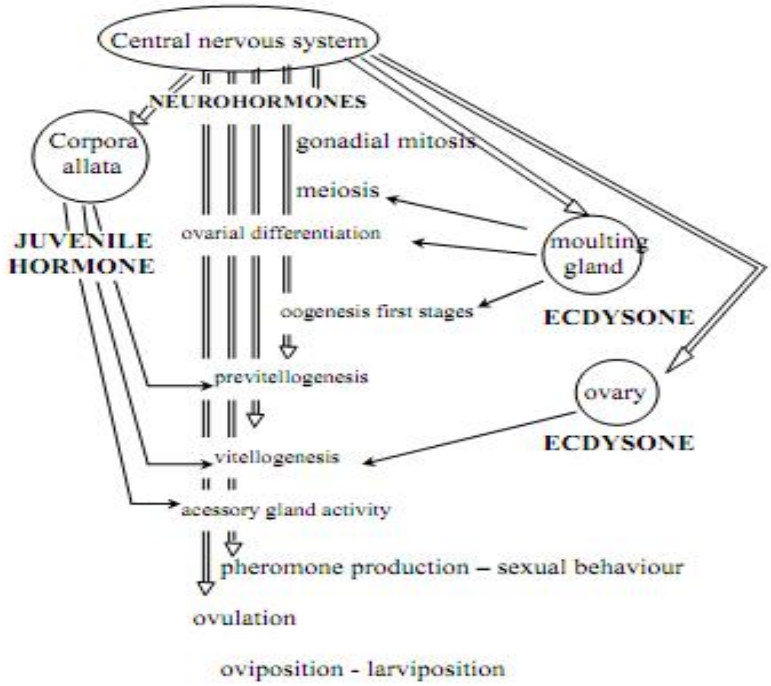


Structure of the Moulting Hormone (Ecdysone) and Related Compounds

Structure	R_1	R_2	R_3	
I	H	OH	CH_3	Ecdysone
II	OH	OH	CH_3	Ecdysterone(20-hydroxy ecdysone)
III	OH	H	CH_3	Ponasterone A
IV	OH	H	CH_2OH	Inokosterone

Structure of the moulting hormone (Ecdysone)





The diapause hormone in silkworm was extracted from sub-oesophagel ganglion (Hashigava, 1957; Trivedy *et al.,* 1993; Sehnal *et al*., 1986; Murakoshi *et al*., 1972). Now it is proved that it is synthesized in the brain and transfer to sub-oesophagal ganglion, where it is accumulated or released.

Mode of action of neurohormones

The neurohormones play a pivotal role in reproduction control by acting in two different way.

It directly acts upon the target organ itself or indirectly upon the endocrine glands which intern regulates various reproductive steps.

The successive processes involved in reproduction and their hormonal and neurohormonal regulation. The regulation of ecdysone synthesis is during growth, the brain of the reproductive insects controls the synthesis of ecdysone (Charlet *et al.,* 1979) and the factor involved may be identical larval brain hormone. But the ecdysone originates no longer in the moulting gland but in the follicular cells of the ovary.

The successive processes involved in reproduction and their hormonal and neurohormonal regulation.

The regulation of juvenile hormone helps in regulating the activity of corpora allata during growth, in the adult, in the reproductive period. Corpora allata regulation depends as both stimulatory inhibitory nervous at neuro hormonal inhibits. It regulates vitellogenesis the functionity of accessory glands. The production of pheromones, sexual behaviour etc. It is also noted that the hormone regulates milk glands the pheromone production and sexual behaviour also depends on the corpora allata, previtellogenesis was also depend on juvenile hormone (Mordue, 1965, Laverdue, 1972).

MATERIALS AND METHODS

Juvenile Hormone Bioassay

The Tenebrio test of Karlson and Nachtigall (1966) was modified in several respects. Tenebrio monitor larvae were maintained until pupation on wheat bran at 30°C and 40% humidity. Pupae, collected once daily at 8.00 AM., were 1-25 hours old at the time of injection. Pupae were narcotized with CO_2 and injected by means of a 10µl Hamilton syringe. They were punctured inter segmentally between the fourth and fifth abdominal sternites with a 26 gauge needle which was insected anteriorly to deposit the standard injection volume of 1.0µl between cuticle and epidermis of the third abdominal sternite. Materials for injection were diluted with olive oil (USP) to the desired concentration. Injected puape were maintained at 30°C and 40 percent humidity until the adults hatched (6-8 days).A minimum of 50 pupae were injected with each concentration. Hatched adults examined under a binocular microscope at 120 X magnification were graded as positive, negative, or dead (mortality approximately 10 %) to be considered a positive response, the presence of a spot of typical pupal cuticle (with characteristic pupal thickness and coloration and the absence of adult surface architecture, setation and pigmentation) over the area of sample deposition was required. Unsclerotized or otherwise abdominal adult cuticle was not considered to be a positive response. Controls injected with 1-4 µl of olive oil showed no response.

JH activity was quantified on the basis of the Tenebrio bioassay as follows:

One Tenebrio unit (TU) of JH activity was defined as the minimum amount of pure JH-active substance which induced a positive response in 40% of the total pupae injected. One Tenebrio Unit volume (TUV) was defined as the volume (in µl) of oil or fraction which contained one TU (In the case of pure substances, TU = TUV). Each of the various oils and precipitates were assayed at several concentrations. A dose response curve was constructed for each preparation and the characteristic TUV determined from the graph obtained. The specific activity, expressed as TU/µl, was calculated for each active preparation as the reciprocal of the TUV.

Zones from thin layer chromatography (TLC) and components trapped from gas liquid chromatography (GLC) were assayed at two or more theoretical activity levels which were determined as follows; for each TLC or GLC purification step, the total JH activity available (in TU) was calculated as the product of the volume of stacking material times its specific activity.

It was assumed that the activity would be discretely resolved and completely recoverable in one fraction. (for example, if 1000 µl of purified oil with a specific activity of 2.5×10^2 TU/µl were spotted in TLC system I, the total JH activity recoverable in one fraction assuming complete resolution would be $1000\ \mu l \times 2.5 \times 10^2\ TU/\mu l = 2.5 \times 10^5$ TU).

Bioassay solutions were prepared by detecting each fraction or a portion thereof with olive oil to a TU concentration theoretically present based on the assumption of total recovery in that fraction (t:1 to indicate theoretical concentration of one TU) For each TLC or GLC step, a bioassay response of 40% or more positive at t:1 TU for one fraction, together with negative results below T:10 TU-t:20 TU for other fractions, was considered to verify the assumption (i.e. to indicate that the activity had been discretely resolved and completely recovered in one fraction) and to identify the active fraction. From the nature of the bioassay results, it was also possible to determine if poor resolution or incomplete recovery of the activity had occurred.

Source of JH extracts

Abdomens of adult male *Bombyx mori* from several groups of animals raised in different years under various conditions were used. The animals were hatched and maintained 7-8 days at 25°C and 40-50 percent humidity.

During this time they were allowed to mate so that a maximum decrease in the lipid content would occur. Abdomens were clipped off, weighed, and stored in ether at -20°C until extracted.

Reagents

All solvents were analytical reagent grade. In some cases further purification by column chromatography and/or redistillation was necessary.

Solvent evaporation

Rotary evaporation under vacuo using a calab No.5000 model C Rotary Evaporator was performed in a +5°C cold room to concentrate large volumes of extracts or TLC effluents.

Micro evaporation was accomplished by blowing a stream of day nitrogen across the surface of small volumes of extract held in a water ice bath.

Storage

The extracts, TLC fractions, GLC trapped materials and bioassay solutions were kept at -20°C between procedures.

Extraction

Abdomin were homogenized in either at room temperature using a Vir Tis Model 23 Homogenizing Mill at 23,000 rev/min for 10 min. A volume of ether was added to establish the ratio of 10:1, solvent volume (ml); Fresh tissue weight (g). The suspension was extracted with magnetic stirring at room temperature for 24 hr, filtered, and the residue re-extracted under the same conditions.

The second filtrate was rotary evaporated to a residue which was added to the first filtrate. The volume of combined extracts was adjusted to maintain the 10:1 ratio. A small aliquot was removed from which the crude oil was recovered by rotary and micro evaporation for determination of its quantity and JH activity. The remaining major portion of the extract was treated by the low temperature precipitation procedure.

Low temperature precipitation

Extracts of the 10:1 ratio were immersed in a dry ice-ethanol both until thermal equilibrium at minus 75-80°C was reached. The cooled extract was filtered through S & S No 576 filter paper on a Buchner funnel pre-cooled to and maintained by a cooling jacket at the temperature of dry ice. The filtrate was reduced to purified oil by rotary and micro evaporation. A portion of the precipitate was micro evaporated to remove traces of solvent before bioassay.

Growth and development of larvae, extraction of hormone, pupal assay and purification of Juvenile Hormone

Abdomen of adult male *Bombyx mori* moths were used as the source of juvenile hormone cocoons were harvested in early July from netted wild cherry trees on which the larvae had been reared from fertile eggs. In certain years it was possible to purchase several thousand cocoons from collectors. Each cocoon was cut open with scissors, the male pupae were segregated at 6°C for atleast 10 weeks in order to activate their brains. To obtain adult moths, the previously chilled pupae were placed in cages at 25°C. The male moths which emerged after 4 or 5 weeks were stored for upto 1 week and then sacrified. With a single transverse cut with scissors, the abdomen was severed from the thorax, cut longitudinally into two pacts, and placed in a flask containing anhydrous ethyl ether. Over a period of weeks or months about 1000 male abdomens were accumulated in the stoppered flask at room temperature.

It may be noted that juvenile hormone can be extracted from dead male cecropia moths-even from museum specimens that have been dried and stored for as long as 8 years. However as one would anticipate, the yield is less satisfactory than in the case of fresh material.

Extraction of hormone

The yellow ethereal solution was decanted from the abdomen and filtered with suction through a coarse sintered filter into a separatory funnel. The abdomen was then homogenized in fresh ether by means of an explosion proof waring blender.

The brei was filtered and the solid material returned to the blender for further extraction with ether. The procedure was repeated several times until no further yellow pigment was extracted.

The filtrates were pooled in separatory funnels and extracted three times with equal volumes of distilled water, the aqueous hypophase being discarded. The extract was placed in a round bottom flask and the ether and residual traces of water were eliminated in vacuo by means of a rotary 'flash evaporator' with distillation temperatures gradually rising to 80°C. In this manner clear golden oil, approximately 0.2-0.3 ml per abdomen, was obtained.

The Bombyx oil was already highly active in the pupal assay for juvenile hormone. The crude extract can be stored indefinitely at 5°C in a stoppered brown bottle without loss of activity. Though similar golden oil is obtained from ether extracts of the abdomen of female Bombyx moths and from the male and females of most other species, highly active extracts, as previously mentioned, were obtained only from male cecropia and cynthia moths.

THE PUPAL ASSAY

1. by injection into previously chilled pupae

In one experience the most quantitative assay is provided by pupae of the polyhemus silkworm. After storage at 6°C for 4-10 months, polyhemes pupae initiate adult development within a few days after being placed at 25°C. The extract was injected as soon as the pupae were returned to room temperature and, in any event, prior to the initiation of adult development, as signalled by the detachment and refraction of the wing epidermis. Both male and female pupae were equally useful in the assay.

The crude extract was injected without dilution. We routineley used 1 ml tuberculin syringes equipped with 27 gauge needles, in conjunction with an Agla micrometer injection apparatus (Burough Welcome Co). The extract was drawn into the syringe, capped by the need,e and all bubbles expelled. Meanwhile, a group of previously chilled polyhemus pupae were removed from their cocoons and anaesthetized with carbon dioxide. The injection was performed into the thoracic tergum just lateral to the midline, the needle pointing posteriorly. The volume of the injection did not exceed 0.1 ml and not uncommonly was as little as 0.001 ml. Purified extracts, in weighed amounts, were dissolved in ether, diluted with a precise volume of peanut oil, sesame oil, or propylene glycol, and the ether evaporated by ventilation with prepurified nitrogen. All pupae, after injection, were placed at 25°C.

The presence of juvenile hormone was indicated by the failure of the pupae to metamorphosis into a normal adult

moth. The assay was based on the principles previously described for the assay of living corpora allata (Williams, C.M. and Kafatos, F.C., 1997 & Williams, C. M., Law, J. H., 1965). In brief, one measures the activity of the extract in terms of the preservation of pupal characteristics in the resulting moth.

Highly active extracts cause the formation of what is essentially a second pupa. The degree to which pupal characters are retained is a measure of the juvenile hormone activity of the injected material. We have made use of the same scoring system as previously described for assays of living corpora allata. Each assay was scored from zero (normal moth) to five (second pupae) (Akai et al.,1985). The effects of highly active extracts are first visible in the antennae about 4 or 5 days after the initiation of development by moistening the overlying cuticle with 70% ethanol, one can detect a failure of the underlying epidermis to subdivide into the backs and segments characteristics of the feather-like antennae of adult saturniids.

In our experience, the several grades in the assay provide only a semi-quantitative appraisal of the juvenile hormone content of the injected material. One source of difficulty was the extent to which the water insoluble oil was distributed sub divided and compartmented after injection into the test animal. An even more serious case was encountered when the test pupa failed to initiate development soon after the injection. The hormone is progressively broken down or inactivated by the pupae. Consequently, pupae which failed to undergo prompt development gave weaker tests for juvenile hormone than did pupae which underwent prompt development. For those several reasons, false negative assays were encountered from time to time in tests of extracts of known activity. But, as far as we can judge, false positive tests were not encountered.

2. Topical application of extracts

As pointed out previously extracts of juvenile hormone are able to penetrate the intact pupal cuticle. This observation formed the basis of a simple and successful assay which was usually performed on previously chilled pupae of the cynthia silkworm.

In brief, a drop of extract and two drops of peanut oil were placed in the bottom of a shell vial. The test pupa was then placed tail down in the upright tube so that the tip of the abdomen was continuously moistened by the extract. After 1-2 weeks of development at 25°C, the activity of the extract was scored in terms of the preservation of pupal characters in the head and thorax.

3. The wax-wound assay

Site of integumentary injury have long been known to show a greatly amplified sensitivity to juvenile hormone (Williams, 1997) have exploited this fact by dissolving extracts in paraffin and applying the melted wax to a small area of wounded integument.This is an incredibly sensitive and specific test for juvenile hormone. The assay is essentially qualitative in character in that it permits one to score extracts as active or inactive a serious disability in purification procedures where one must routinely discriminate between fractions having feeble and substantial activities.

Our version of the wax-wound test was simplifed. In brief, a zone of cuticle, about 1 mm square was excised from the thoracic tergum of an anaesthetized, previously chilled polyphenus pupa, the underlying epidermis being broken with forceps. A small drop of the oily extract was applied to the exposed epidermis and the wound then capped and sealed with melted wax. The oil wets the overlying wax and is thereby retained at the wounded site. If the extract contains juvenile hormone, a patch of pupal cuticle forms at this position during adult development. If the extract is highly active, enough partitions into the blood to provoke the formation of pupal characters in distant regions of head, thorax and abdomen.

PURIFICATION OF HORMONE

1. Extraction into methanol

The active golden oil was subdivided into several large centrifuge tubes and 3 volume of anhydrous methanol were added to each tube. The tubes were stoppered, vigorously shaken, and centrifuged briefly to break the emulsion. The alcoholic epiphases were aspirated into a round bottom flask and the latter placed on a flash evaporator to remove the methanol in vacuo at temperatures gradually rising to 60°C. Fresh methanol was added to the tubes and the extraction of the oil repeated six times, the alcoholic epiphases being pooled.

Evaporation of the methanol yields a golden oil of low viscosity which retains all the juvenile hormone of the initial extract in a volume reduced by about 75 percent purification of cecropia oil.

* 1 unit of activity is defined as the amount of extract which gives a 3+ assay when injected into a previously chilled *Bombyx* pupa.

2. Saponification

Vigorous saponification at high temperatures caused a large or complete loss of activity in all fractions. However, a large pact of the active substance survived very mild saponification. In a typical experiment, 32 g of the methanol-soluble oil was dissolved in 150 ml of methanol and to this solution was added 15 ml of 50% KOH. The flask was flushed with nitrogen and stoppered. The mixture was shaken for about 15 minute at room temperature until the oil dissolved. It was then allowed to stand for an additional 15 min. The solution was diluted with 2 vol of water and thoroughly extracted with ethyl ether to recover the unsaponified fraction. The combined ether extracts were washed with water and reduced to dryness wt 11 g. The aqueous alkaline solution was acidified and extracted with ether to recover the saponified fraction. The extract was washed with water and reduced to dryness, wt 16 g. Only the unsaponified fraction was active when assayed. Moreover, the saponified fraction was not activated after reduction by lithium aluminium hydride or after treatment with diazomethane to convert fatty acids to methyl esters.

3. Silic acid chromatography I

In a typical experiment, 9.33 g of the unsaponified fraction was dissolved in 200 ml of 1:1 mixture of benzene and petroleum ether (b.p. 30-60°C) and percolated onto a 2 cm (I.D) column composed of a slurry of 50 g silic acid (Unisil clarkson chemical co. Williamsport, Pa.) prepared in the same solvent mixture. The column was eluted successively with 500 ml volume of petroleum ether-benzene 1:1, pure benzene, two portions of 5% ethyl ether in benzene, two portions of 10% ethyl ether in benzene, and 25% ethyl ether in benzene. The two fractions eluted by 5% ether in benzene were highly active and were combined, wt 1.31g.

4. Silic acid Chromatography II

Silicic acid, 50 g, was packed on a 2 cm (I.D) column as slurry in benzene. The sample, 1.3 g was applied in 20 ml benzene and washed on with 80 ml of pure benzene. A linear gradient was established consisting of a mixing flask containing 500 ml benzene and a reservoir containing 500 ml of 10% ethyl ether in benzene. 14 ml fractions were collected by means of an automatic fraction collector. Aliquots were taken of every fifth tube for biological assays. Fractions 30 and 35 were shown to be active; tubes 27-37 were therefore pooled and reduced to dryness (373 mg). These tubes correspond to an ethyl ether concentration of 4-5%. This material was highly active in the biological assay.

5. Succinoylation

This step was not invariably included in the purification since it proved laborious and the results were not reproducible from one rich to another; it is described here because it provides useful information about the nature of the hormone 365 mg of material from silicic acid chromatography II were dissolved in 5 ml pyridine and 400 mg succinic anhydride were added to the solution (Butenandt *et al.,* 1961). The mixture was warmed to 40 for 2 hr and then allowed to stand 24 hr at room temperature. The solution was diluted with ethyl ether and washed three times with small portions of 2N HCl and twice with water. The ether layer was diluted with an equal volume of methanol and titrated to a phenolphthalein pink with 1N Na_2CO_3. The solution was then diluted with water and the ether phase separated. The latter was washed twice with small portions of water and the aqueous phases combined. The etherial non alcoholic fraction was taken to dryness, wt 240 mg. The aqueous phase was acidified and extracted with ether. The etherial succinate ester fraction was taken to dryness, wt 104 mg.

The non alcoholic fraction was highly active at the level of 5 µg per animal while 250-500 µg of the succinate ester fraction was required for biological activity. No increase in activity was obtained after mild saponification of the succinate ester fraction.

Infrared spectroscopy indicated that the non alcoholic fraction still contained hydroxylic compounds. Therefore, 18 mg of this material were subjected to a second succinoylation. The non-alcoholic fraction now showed no evidence of hydroxyl groups, while biological assay of their fraction demonstrated high juvenile hormone activity.

6. Crystallization of impurities

The active fractions obtained from silicic acid chromatography II contained in active alcoholic components which easily crystallized from warm methanol solution. Purification equal to or better than that obtained by the succinoylation procedure was obtained merely by removing this material by crystallization. The crystals appeared to be a mixture of sterols and were not examined further.

Crystallization of impurities resulted in an additional threefold concentration bringing the total purification to about 4000 fold. This material was active in amounts of less than 5 µg/5 g animal.

7. Gas Liquid Chromatography

Early exploratory experiments indicated that the hormone could be recovered inactive form from a gas liquid chromatographic column, although large losses in total activity were observed. This technique was exploited to bring about a further purification of the active material. An F and M instrument (Avondale Pa) with an 8 ft X ¼ in column packed with 5% SE-30 silicone rubber on chromosorb W was employed in conjunction with a hot wire detector. The column was operated at 190°C with a helium pressure of 20 lb/in^2

Several successive injections were made until a total of approximately 50 mg had been chromatographed. Fractions were collected at arbitrary time intervals, since most of the injected material never emerged from the column and since essentially no response was recorded by the detector. Two adjacent fractions were shown to have high activity.

These two fractions were examined by a more sensitive method using a hydrogen flame ionization detector in an F and M instrument or an argon detector in a Research specialities Co instrument (Richmond, California). The first of these fractions showed several peaks, of which the major one constituted about 30% of the total effluent. The second of the two fractions contained this same major peak, which in this case constituted about 90% of the total effluent. When this major peak was collected and assayed, it showed extremely high hormonal activity. On a non-polar column (SE 30) a relative retention time of 0.6 was evident compared to a standard sample of methyl eicosanoate. On a polar column (10% polydiethyleneglycol succinate) the peak showed a relative retention time of 1.8 compared to methyl cicosanoate. The amount of material present in the active peak was estimated as 200-300 µg by comparises of the peak area with that given by a standard solution of methyl hexadecanoate.

Approximately 1% of the fraction was dissolved in peanut oil and assayed in eight animals. The lowest dose tested (about 0.3-0.4 µg) gave a 3+ assay in the 5 g test animal, this corresponds to 1 part of extract per 10 million pacts insects active fraction obtained by gas-liquid chromatography of juvenile hormone concentrate. A column 8 ft X ¼ in packed with 5% SE-30 on chromo sob W was operated at 190°C. The F and M model 1609 instrument with hydrogen flame ionization detector was used the nitrogen cauier gas had a flow rate of about 170 ml/min. The major peak emerged at 12.9 min and had a relative retention time of 0.6 compared

with methyl eicosanoate. The active hormone emerged either with or slightly in advance of the major peak. The major peak was identified as the 9.10 epoxide of methyl hexa decanoate.

Bioassay objectives and choice of bioassay

Since all the effects that are known from endogenous JH may theoretically be used for bioassays, the possibilities are manifold. Good consideration should therefore be given to the objectives. Five types of assays may be distinguished.

1. Titer determinations in different physiological stages in insects.
2. Evaluation of purification and isolation of endogenous JH for the purpose of identification of new JH structures from extracts.
3. Determination of structure activity relationships for different compounds and insects.
4. Studies on specificity and activity.
5. Evaluation for possibility of practical application, ranging from laboratory to field assays.

The requirements for these five categories are very different. Titer determinations and JH isolation procedures require extremely sensitive assays with adequate reproducibility and good quantitation. Only one assay, the pupal wax test on certain species of Lepidoptera, has been fulfilling these requirements so far. Its use may be limited to insect hormones with a specificity similar to that of Cecropia JH. The isolation of new JH of different insects certainly needs exploration and comparison with more species specific assays. Successful purification of Cecropia JH has been completed through use of an assay technique on Tenebrio pupae. Although the identified materials are not the most potent JHA existing for beetles, they come close to that on Lepidoptera. The possibility that a different beetle JH exists cannot be excluded as yet.

Structure activity correlations for JHA are the most common objective and since usually greater amounts of synthetic compounds are available, these tests do not have to be so extremely sensitive. It nevertheless pays off to select the most sensitive stage of the prospective test insect for the test because it allows for greater reproducibility. Accurate synchronization of the animals in days and sometimes even hours after an easily observed process like for instance a behavior change, ecdysis, spinning, apolysis, oviposition etc. is always a basic requirement.

Since most evaluations are directly or indirectly aiming at ultimate application purposes, topical tests should be preferred. The inherent variance in all JH tests can usually be decreased by taking larger numbers of test animals when compound availability is no limiting factor.

Evaluation for practical purposes suggests the use of asynchronous colonies under conditions that approach those of the field situations. The use of plants, animals or stored commodities as the insect's substrate, may require an entirely different application approach. For stored food pests, the short cut of overlapping of objectives 3 to 5 exists. Actual bioassays for both can be done by mixing the compound into the commodity and infesting it with the insect-be it as a synchronous, or, as an asynchronous population. Results from work on asynchronous populations are very difficult to evaluate and to interpret and may require repeated observations after the actual treatment. We do see it as the ideal situation when this work can be backed up by a complete profile of activities on any given moment in the development cycle of the insect compiled from assays on synchronous batches.

Bioassay technology

There are many ways of administering JHA for evaluation purposes and only the most important ones can be mentioned. Many other papers mention specially adapted techniques. No clear picture has evolved explaining why certain JHA have a higher activity than others. With present knowledge we have to admit that we do not know whether a JHA is more active because it penetrates more rapidly or more slowly through the cuticle, binds easier or less easy to a carrier in the bloodstream, breaks down more or less rapidly when exposed to the insect's metabolism, etc. The different possible types of tests on one single insect species may differ tremendously in their sensitivity, depending probably on factors related to penetration, critical sensitivity periods, speed of breakdown at all levels, etc.

Pupal Wax Test

This highly sensitive test (in our laboratories, the Galleria unit corresponds to 5×10^{-6} micrograms of Cecropia JH) is laborious, but the only one sensitive enough for the +1 objectives. In young Galleria pupae, a small section of epidermis plus cuticle is removed and the wound is sealed with a molten mixture of solid paraffin wax and a dilution in mineral oil of the compound or extract to assay. The regenerating epidermis proves to be highly sensitive to exogenous JH and will secrete a local patch of pupal cuticle once again when JH is present. This pupal cuticle is conspicuously different from adult cuticle. The explanation of the high sensitivity may be in the sealing mixture that hardens to a paraffin skeleton in which the oil circulates continuously and contact with the regenerating integument is maximized. Serial logarithmic dilutions with a factor of 10 usually yield a distinct curve (even from as little as 5 to 10 animals per concentration) of which the intersection with the 50% response line indicates the active concentration or inhibition dose (ID 50). In our test procedure, only positive and negative responses are distinguished. No synthetic material equals the Cecropia JH activity in this test. The wax test values obtained with other JHA are not well correlated with values obtained by topical application on intact animals, and are therefore no reliable indicators for practical evaluation.

RESULTS

TABLE 1. Purification of Golden oil of *Bombyx mori* Juvenile hormone

Fraction	Initial Wt (g)	Final wt (g)	Wt of 1 unit (mg)	Specific activity (units/mg)
Crude extract	-	152	23	0.06
Methanol soluble	161	35	6	0.3
Non -Saponifiable	36	12	0.8	1.6
Silicic acid chromatography I	9.73	1.41	0.086	14
Silicic acid chromatography II	1.42	0.392	0.028	43
Non crystalline	0.46	0.162	0.006	226
Gas liquid chromatography	0.07	0.00092	0.0006	2600

*one unit of activity is defined as the amount of extract which gives a 3+ assay when injected into a previously chilled pupa
Values given are the mean value ($\bar{X}$) of 4 datas
d.f. = degrees of freedom = n-1
Significance ++ = $p < 0.001$
+ = $p < 0.05$
NS = Not significant

Purification of Juvevenile hormone from *Bombyx mori*

Bombyx mori larva was taken and was excised of 1 mm square from thoracic tergum of anaesthesized and previously chilled and broken with forceps below the epidermis. A small drop of oily extract containing juvenile hormones was taken outside.

The active golden yellow oil was divided into three portions and add three volume of anhydrous methanol was added to each tube, the tubes were stoppered, vigorously shaken and centrifuged briefly to break the emulsion. The alcoholic epiphases were aspirated, into a round bottom flask and later placed on a flask evaporator to remove the methanol in vacuo at temperatures gradually raising to 60°C. Fresh methanol was again added to tubes and repeated for 6 times. The evaporation of the methanol yields a golden oil of low viscosity which contains juvenile hormone.

Saponification

The vigorous saponification at a high temperature caused a large or complete loss of activity at all fraction.32 grams of methanol soluble oil was dissolved in 150 ml of methanol. To this solution 15ml of 50% KOH was added, The flask was flashed with nitrogen and stoppered. The mixture was shaken for 15 minutes at 32°C until the oil is dissolved. Then the solution was diluted with 2 volumes of water and thoroughly extracted with ethyl ether to recover the fraction. The combined ether extracts was washed with water and reduced to dryness. The weight was 11 g. Then the aqueous alkaline solution was acidified and extracted with ether to recover the saponified fraction.

The extract was washed with water and reduced dryness. That time the weight was measured as 16g.

Only the unsaponified fraction was active when assayed. The saponified fraction was not activated after reduction by lithium aluminium hydride.To convert fatty acids into methyl ester.

Silicic acid chromatography I

About 9.33g of the unsaponified fraction was dissolved in 200 ml of 1:1 mixture of benzene and petroleum ether and percolated on to a 2cm column composed of a slurry of 50g silicic acid prepared in the same solvent mixture.The column was eluted with 500ml of petroleum ether benzene 1:1 pure benzene. Two portions of 10% ethyl ether in benzene and 25% ethyl ether in benzene. The two fraction eluted by 5%ether in benzene well highly active and were combined the weight measured was 1.31g.

Silicic acid chromatography II

Silicic acid chromatography II was packed with 50 grams of silicic acid on 2cm column with benzene.1.3g sample was supplied in 20ml benzene and washed on with 80 ml of pure benzene. A linear gradient was established by mixing 5ml benzene and 500 ml of 10% ethyl ether in benzene.14ml fractions were collected by means of an automatic fraction collector. The 30^{th} and 35^{th} fraction were active in JH assays. When this is pooled and reduced to dryness 373 mg were obtained.

Succinoylation

The 365 mg of JH material obtained from silicic acid chromatography II was dissolved in 5ml pyridine and 400 mg succinic anhydride were added to the solution. The mixture was warmed to 40°C for 2 hours and allowed to stand at 32°C. The solution was diluted with ethyl ether and washed 3 times with 2N HCl and twice with water. The ether layer was diluted with equal volume of methanol and titrated with phenolpthalein pink with 1N Na2CO3.The solution was then diluted with water and ether face was separated.The non alcoholic fraction was dried and the weight was measured as 240mg.The aqueous phase extracted with ether was dried weighed as 104mg.The active fractions obtained from silicic acid chromatography II was crystallised. It was purified for about 4000 folds.

Gas Liquid Chromatography

The F and M instrument with 8 feet X ¼ inch column packed with 5%SE, silicon rubber was operated at 190 °C with a helium pressure of 20lb/in2 .Successive injections were made to reach 50mg had been chromatographed. Fraction were collected at arbitary time intervals since most of the injected material never emerged from the column and since essentially no response was recorded by the detector, the two adjacent fraction were active and examined by using a hydrogen flame ionisation detector in a F and M

instrument. This shows several peaks in which 2 fractions shows 90% of hormonal activity and this was assayed. The active peak shows the material estimated to 200 – 300 µg. When compared by a standard solution of methyl hexadeconate % of the fraction was dissolved and assayed in 8 animals. The lowest dose of about 0.3 to o.4 µg gave a 3+ assay in the 5g test of animal. This corresponds to 1 part of extract /10 million parts of insects.

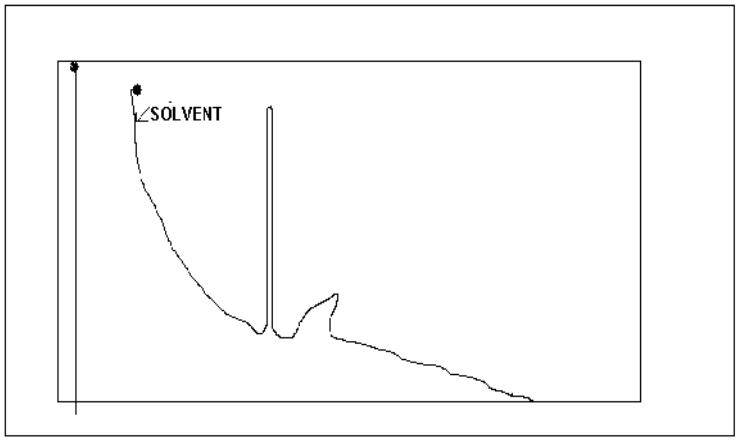


FIGURE 2. Active fractions obtained by Gas-liquid chromatography of juvenile hormone concentrate

A column 8ft X ¼ in packed with 5% SE-30 on chromosorb Kl was operated at 190°C. The F and M model 1609 instrument with hydrogen flame ionization detector was used. The nitrogen carrier gas had a flow rate of about 170 ml/min. The major peak emerged at 12.9 min and had a relative retention time of 0.6 compared with methyl ricosanoate. The active hormone emerged either with or slightly in advance of the major peak. The major peak was identified as the 9,10 epoxide of methyl hexadecanoate.

DISCUSSION

The interaction between effects of juvenile hormone and molting hormone at the molecular level poses a particular challenge in insect endocrinology. Each hormone does not necessarily have a single primary site of action, and may function by effects at several levels of organization. However, current evidence indicates that most steroid hormones apparently exert their main action by controlling transcription of specific genes, thus inducing protein synthesis. The mechanism has been explained by several models. The mechanism of controlling gene expression in eukaryotic organisms has proved recalcitrant, since recent findings have led to re-appraisal of the structural organization of chromatin itself. These findings will undoubtedly influence substantially future ideas regarding the molecular mechanism of gene switching by hormones.

Juvenile hormones and ecdysterrids play an important role in the regulation of growth and reproduction of Insects. The vertebrate hormones like prolactin (PRC), thyroxine (THY). Insulin (INS) and other pituitary entracts caused shortening of larval duration, increased the larval, silk gland weights and fecundity of the silk worm. Is reported that thyroxine works more efferinely when applied to 2nd instar larvae causing the enhancement of haemolymph pralines and ecdysteroid levels in the silkworm (Charlet *et al.,*1982, akai *et al*., 1981; Sashindran, 1999; Nagaraju, 2000) reported that vertebrate gonadotrophins such as follicular stimulating hormone (FSH), Leutinizing hormone (LH), and human chorionic gonadotrophic hormone (HCG) induce steroid biosynthesis in the gonads of insets. However, the precise mechanism of action of the vertebrate hormone in invertebrates remains to be understood. It may be a direct effect up on body cells or regulations of endocrine gland secretions or by both.

REFERENCES

Akai H., Kiguchi, K., Kobari, Y. and A. Shibukawa (1981) Practical utilization of juvenoids for increasing silk production. In: Regulation of Insect Development and Behavior, F. Schnal, A. Zabza, J. J. Menn, and B. Cymborowski, (Eds.), Wroclaw Technical University Press, Wroclaw. pp. 781-792.

Akai H., Kimura K., Kiuchi, M. and Shibukawa, A. (1985) Increase of silk production by repeated treatment with a juvenile hormone analoge. *J. Seric. Sci. Jpn.* 54(4): 297-299

Charlet, M. and Hoffmann, J.A. (1979) Prothoracic glands of *Locusta migratoria* secrete ecdysone together with proteins. *Gen. Comp. Endocrinol.* **46:** 374

Charlet, M., Goltzene, F., and Hoffman, A. (1982) Radioimmunological determinations of concentrations of six C_{21}, C_{19} and C_{18} steroids during the reproductive cycle of female *Artemia* S.P. (Crustacea: Anostraca),Comparative Biochemistry and Physiology Part A: Physiology, Volume 89, Issue 4, 1988, Pages 595–599.

Ching-Fun-Chang, Shigeo murakorshi and Saburo Tamura (1972) Giant cocoon formation in the silkworm *Bombyx mori* L,Topically treated with Methylenedioxy phenyl derivatives. *Agr. Biol. Chem.*, 36: 692-694.

Cymborowski, B. and Stolarz, G. (1979) The role of juvenile hormone during larval pupal transformation of spodoptera littoralis. Switch over in the sensitivity of the prothoracic gland to juvenile hormone. *J. insect. Physiol*, 25: 939-942.

Fukuda, S. (1940) Hormonal control of moulting and pupation in the silkworm. *Proc. Imp. Acad. Tokyo* 16: 417-420.

Fukuda, S. (1944) The hormonal mechanism of larval molting and metamorphosis in the si1kworm. *Anal. Rec.* 134, 569.

Gangwar, S. K. (2009) Effect of juvenile hormone mimic R394 on silkworm, *Bombyx mori* L., growth and development of silk gland. *ARPN Journal of Agricultural and Biological Science*. Vol. 4(6), pp .65-67.

Hashigava, K. (1957)The diapause hormone of the silkworm, *Bombyx mori*, Nature, 179: 1300–1301.

Karlson and Nachtigall, W. (1966) Die Kinematik der Schlagflugelbewegungen von Dipteren: Methodische und analytische Grundlagen zur Biophysik des Insektenflugs. Z. vergl. Physiol. 53: 155—211.

Laverdure, A. M. (1972) L'evolution de l'ovaire chez la femelle adulte de Tenebrio molitor - La vitellogenese. *J. Insect Physiol.* 18: 1369-1385.

Mordue, (1965) Neuro-endocrine factors in the control of oöcyte production in Tenebrio molitor L. J. Insect Physiol., 11 (1965), pp. 505–511

Morohoshi, S. (1959) Hormonal studies on the diapause and non diapause eggs of the silkworm, *Bombyx mori* L. *J. Ins. Physiol*. 3: 28-40.

Morohoshi, S. (1968) Effect of the brain-corpora allata complex on production of voltinism in *Bombyx mori*, *Proc. Japan Acad*. 45 (1969), pp. 308–313.

Murakoshi S., Chang, C. and Tamura, S. (1972) Increase in silk production by silkworm *Bombyx mori* L. due to oral administration of juvenile hormone analogue. Agric. Biol. Chem. 36(4): 672-674.

Muroga, H. (1939)Function of the ganglia in *the Bombyx morii* L., *Jour. Seric. Sci., Japan*,10:231.

Muroga, H. (1940) Radioimmunological determinations of concentrations of six C_{21}, C_{19} and C_{18} steroids during the reproductive cycle of female *Artemia* SP. (Crustacea: Anostraca) *J. Seric. Sci. Japan,* 11. P51.

Nagaraju, J. (2000) Recent advances in molecular genetics of the silk moth, *Bombyx mori* Current science,78:151-160.

Ogura and Saito (1973) Effect of corpora cardiaca in *Bombyx mori*,. *Appl. Ent. Zool*. 8: 46-48.

SashindranNair, K., Vijayan,V. A., Jula, S., Trivedi, K. (1999) Juvenilomimic compounds for enhanced productivity in silkworm, *Bombyx mori* L. *Indian J. Seric*, 38: 119-124.

Sehnal, F., Delbecque, J., Maroy, P. and Mala, J. (1986) Ecdysteroid titres during larval life and metamorphosis of Galleria mellonella. *Insect Biochem*, 16: 156.162.

Trivedy, K., Remadevi, O. K., Magadum, S. B. and Datta, R. K. (1993) Effect of a juvenile hormone analogue, Labomin on the growth and economic characters of silkworm, *Bombyx mori* L. *Indian J. Seric*. 32(2): 162168.

Wigglesworth, V. B. (1936) The function of the corpus allatum in the growth and reproduction of Rhodnius prolixus (Hemiptera). *Quart. J. Micro. Sci*., 79:91-121

Williams, C. M. and Kafatos, F. C. (1997) Theoretical aspects of the action of Juvenile Hormone, *Appl. Ent. Zool*. 8, 29-41.

Williams, C. M., Law, J. H. (1965)The Juvenile hormone IV its extraction,assay,and purification.J.Insect.Physiol,11,569-580.