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Prothoracicotropic hormone has been partially purified
from whole heads of tobacco hornworm larvae, Manduca sexta.
Activity was followed with a larval bioassay. Activity was
purified 4400 fold from crude extracts. The hormone was
found to be a heat-stable polypeptide with an approximate
molecular weight of 25,000 Daltons. The hormone has an iso-
electric point in the range pH 5.25-5.50. The most purified
preparations were shown to possess activity in vitro with a
prothoracic gland culture system.

Cyclic AMP and cyclic GMP were quantitated in the central
nervous system of Manduca sexta during the pre-metamorphic
endocrine activity that leads to a change from feeding to
wandering behavior and to the commitment to pupal development.

Cyclic AMP and cyclic GMP accumulated in the brain in tandem during this period. Cyclic nucleotide levels remained unchanged in abdominal and thoracic ganglia and in the sub-esophageal ganglion.

Proctolin was quantitated by radioimmunoassay in the central nervous system of the Maderae cockroach, Leucophaea maderae. Proctolin RIA activity was found throughout the CNS, with the sixth abdominal and thoracic ganglia being the richest sources. RIA activity was also found in the cockroach hindgut.

Prothoracicotropic Hormone, Cyclic Nucleotides,
and Proctolin in Insect Central Nervous System

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS USED IN THE TEXT

ACTH, adrenocorticotropic hormone

ATP, adenosine 5'-triphosphate

A.Z.T., arbitrary 'zeitgeber' time

BSA, bovine serum albumin

cAMP, adenosine 3',5'-cyclic-monophosphoric acid

cGMP, guanosine 3',5'-cyclic-monophosphoric acid

CMA, carboxylmethoxyamine (or (aminooxy)-acetic acid)

CNS, central nervous system

DCC, dextran coated charcoal

DEAE, diethylaminoethyl

EDC, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide-HCl

-ecdysone, 20-hydroxyecdysone

GTP, guanosine 5'-triphosphate

HPLC, high pressure (performance) liquid chromatography

IEF, isoelectric focusing

JH, juvenile hormone

PDE, phosphodiesterase

PTTH, prothoracicotropic hormone

RIA, radioimmunoassay

S.D., standard deviation

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electro-
        phoresis

ScNMPTME, succinyl cyclic nucleotide tyrosine methyl ester

S.E.M., standard error of the mean

TCA, trichloroacetic acid

TEA, triethylamine

PROTHORACICOTROPIC HORMONE, CYCLIC NUCLEOTIDES, AND PROCTOLIN IN INSECT CENTRAL NERVOUS SYSTEM

BACKGROUND FOR THE STUDIES DESCRIBED IN THIS THESIS

The concept of biologic homeostasis as originally form-
ulated by Claude Bernard oriented most early research on the
nervous system - endocrine interface toward the study of
steady state and stimulus response phenomena. Information
gained in the last few years, however, makes it clear that
many biological functions, including those driven by hormonal
secretions are under the control of endogenous oscillators
that are entrained to environmental periodicities. Particu-
larly striking are the circadian rhythms in mammals of ACTH,
cortisol, and prolactin secretion for which elevated levels
occur during the rest period of an activity cycle (Weitzman,
1980). It has been shown, too, that the secretions of the
peptides, eclosion hormone and prothoracicotropic hormone
in insects occurs with specific phases to photoperiodic cues
(Truman, 1970; Truman, 1972; Truman and Riddiford, 1974).

The basis for the biological clock(s) regulating hormonal
and behavioral rhythms is an area of intense interest to the
physiologist and endocrinologist. For instance, recent
progress has implicated retinohypothalamic projections to
the suprachiasmatic nucleus as being essential for circadian
expression of wheel-running and water-drinking activity in
hamsters (Zucker, 1980). In addition, the pineal gland

appears essential for activity rhythms in the house sparrow (Takahashi and Menaker, 1979), and it may be that vertebrate circadian organization is based on differentially weighted interactions between the suprachiasmatic nuclei, the pineal gland, and perhaps other brain regions. Less attention has been devoted to mechanisms underlying invertebrate circadian rhythms. The most notable exception to this statement is work done with Aplysia identifying the eye as a center of a circadian pacemaker (Eskin, 1979; Page and Hudson, 1979). Insects are notable in that many rhythms appear to be mediated through extraretinal photoreception (Truman, 1976). In an interesting preliminary study, Williams (1969) noted that a photoreceptive mechanism for the termination of diapause by PTTH release in Anthereae pernyi may be located in the region of the brain occupied by the lateral neurosecretory cells. Characterization of the neural and hormonal substrates of behavioral and physiological functions should provide a clearer understanding of biologic clocks. I have carried out a partial purification of prothoracicotropic hormone from Manduca sexta. The hormone was found to be a heat-stable polypeptide with an approximate molecular weight of 25,000 Daltons; its isoelectric point is in the range 5.25-5.50.

Much attention has been devoted to the role of steroids in regulating reproductive behavior (Davidson, 1980; Michael, 1980); however, behavioral influences of hormones extend beyond the steroids to include peptides. For example, feeding behavior in rats is inhibited by cholecystokinin (Smith, et

al., 1974).   It is also clear that peptides that may function
as neurotransmitters can have profound behavioral effects;
a case in point is the wet-dog shakes evoked by intracerebro-
spinal fluid injection of met-enkepalin, and α-,β-, and
γ-endorphin in the rat (Bloom, et al., 1976).   Invertebrate
behaviors known to be elicited by peptide neurohormones
include egg-laying in Aplysia (Arch and Smock, 1977) and
the eclosion behavior in silkmoths (Truman, et al., 1976;
Truman, et al., 1979).   In addition, proctolin (arg-tyr-
leu-pro-thr), a putative neurotransmitter in cockroach hind-
gut (Brown, 1975), elicits a rhythmic activity in the locust
extensor tibiae (Piek and Mantel, 1977; Piek, et al., 1979).
Ecdysone, the steroid molting hormone in arthropods, apparently
mediates quiescent behavior during molting in locusts (Haskell
and Moorhouse, 1963) and onset of wandering behavior in the
tobacco hornworm (Dominck and Truman, unpublished, cited in
Truman, 1978).

It is now known that biochemical changes occuring in
the neural substrates of behavior may include cAMP eleva-
tions.   These behaviors include the sensitization of the
gill-withdrawing reflex in Aplysia which may be mediated by
serotonin (Brunelli, et al., 1976) and the emergence behavior
in silkmoths mediated by eclosion hormone (Truman, et al.,
1976; Truman, et al., 1979).   The molecular mechanisms by
which steroids evoke behavioral changes is unclear, and
reports of steroid hormone - cAMP interactions in neural
tissue are scarce, indeed (see Nathanson, 1977).   A report

has appeared on the ability of ecdysterone to activate

a fluoride dependent adenyl cyclase in the tobacco horn-

worm brain (Vedeckis and Gilbert, 1973), but the signifi-

cance of this finding is presently unknown.

Invertebrates are excellent subjects for the study of

behavioral processes. Their nervous systems are composed

of thousands, rather than billions of nerve cells as in

mammals. The segmented nature of the central nervous system

and the relative hardiness of these organisms to surgical

manipulations allows accessibility to identifiable neurons

not thus far possible in higher organisms, including mammals.

One approach to the study of behavior would begin by quanti-

tating the distribution of the putative molecular substrates

of behavior. The next step would be the identification of

the individual neural elements containing these molecules

and the physiological characterization of the behavior medi-

ated by these elements.

I have carried out studies on the distribution of cyclic

nucleotides in the central nervous system of Manduca sexta

during endocrine activity leading to a change from feeding

to 'wandering' behavior. Cyclic AMP and cyclic GMP were

found to accumulate exclusively in the brain beginning with

the onset of endocrine activity that preceeds expression of

'wandering'. The distribution of proctolin in the central

nervous system of Leucophaea maderae has also been studied.

Radioimmunoassay activity was found throughout the central

nervous system with the sixth abdominal and thoracic ganglia

being the richest sources.

GENERAL METHODS

Insect Rearing

M. sexta eggs were obtained twice weekly from Mr. A.H. Baumhover, U.S.D.A. Res. Stn., Oxford, North Carolina. Larvae were mass reared essentially according to the procedures of Yamamoto (1969). Two diets were used: 1) Yamamoto's diet (1969) and 2) the CSM (cornmeal, soy flour, nonfat dried milk, and soy oil) diet formulated by Baumhover, et al. (1977), which was used exclusively for fifth instar larvae reared as source material for PTTH purification. The latter diet can be made at less than one-third the cost of the Yamamoto wheat germ based diet. Larvae were reared in constant temperature rooms at 25°C under a 12L:12D photoperiod cycle. Larvae for the in vitro PTTH assay were reared under a 16L:8D photoperiod. Lights-off is designated at midnight (2400), and times are given as arbitrary "zeitgeber" times (A.Z.T.) according to convention (Pittendrigh, 1965).

Studies requiring developmentally synchronous 4th and 5th-instar larvae were carried out on larvae selected from mass rearing trays and placed in individual rearing cells. Newly ecdysed 4th-instar larvae were selected from trays containing seven and eight day old larvae and placed in 50 cell plastic rearing trays (Bio-Serv, Inc., Frenchtown, N.J.) sandwiched between acrylic sheets; in this way 150-600 new fourth instar larvae could be set aside for use in the PTTH bioassay. Cyclic nucleotide studies utilized fifth instar

larvae selected from mass rearing trays the day of ecdysis
to the fifth instar (the tenth or eleventh day of development)
and placed in individual 4 oz. plastic cups.

Developmental Staging

According to convention (Truman, 1972), a new instar
"begins" with the dropping of the old head capsule. The
first day of the instar is designated day 0, the second day
is day 1, and so on. For the fifth (final) larval instar,
the abbreviations 5d0, 5d1, etc. will be used.

Newly ecdysed fourth and fifth instar larvae could be
reliably identified by the relation of body to head capsule
size, and by the presence of newly shed cuticle.

"Gate II" fourth instar larvae (Truman, 1972) were
selected for use in the PTTH bioassay. Larvae set aside two
days earlier were screened to eliminate the more rapidly
developing Gate I larvae (identified by apolysis of cuticle
around the spiracles) and, as suggested by Gibbs and Riddiford
(1977), those Gate II larvae under 0.9 g in weight at the time
the bioassay was performed (details given in PTTH chapter).

Fifth-instar larvae selected for cyclic nucleotide
studies were staged by a method combining weight and age
based upon a report by Nijhout and Williams (1974) (see
cyclic nucleotide chapter). Larvae were collected at specific
times during the photoperiodic cycle for dissection of nervous
tissue.

Dissections

Dissection of nervous tissue was done on animals lightly anesthetized with CO_2 and then placed on ice for approximately ten minutes. Manduca tissues were excised, rinsed in Ephrussi and Beadle ringer (1936) followed by distilled water and frozen in shallow plastic analyzer cups (Sherwood Medical) on dry ice. Time from onset of dissection to tissue freezing was 60-90 seconds. Dissection of Leucophaea tissues is described in the proctolin chapter.

Protein determination

Protein concentrations were determined by the method of Lowry, et al. (1951). TCA and methanol precipitates were solubilized in 1 N NaOH, clarified by centrifugation, and assayed. Proteins from PTTH separation procedures were assayed directly. Bovine serum albumin (RIA grade, Sigma) was used as a standard. When samples contained base, standards were made up to contain equal concentrations of NaOH. Folin's phenol reagent was added in amounts necessary to neutralize base.

PURIFICATION STUDIES OF THE PROTHORACICOTROPIC HORMONE IN <u>MANDUCA</u> <u>SEXTA</u>

<u>Introduction</u>

The role of the brain in insect development has been recognized since Kopec demonstrated its necessity for metamorphosis in the gypsy moth <u>Lymantria</u> <u>dispar</u> (Kopec, 1922). Similar observations by a number of other workers in the 30's and 40's (see Sakranek and Williams, 1980, for review) led to the theory that a brain hormone stimulated molting and metamorphosis.

Subsequent experiments by Fukuda (1940) showed the prothoracic glands to be the source of a molting factor, which was eventually isolated (Butenandt and Karlson, 1954) and characterized as the polyhydroxylated steroid 2β, 3β, 14α,-22R, 25 pentahydroxy-5β-cholest-7-en-6-one (Huber and Hoppe, 1965) and named ecdysone (Karlson, 1956). Work by Williams (1947) provided evidence that the molting factor was released by prothoracic glands in response to a blood-borne substance from the brain (brain hormone, activation hormone, prothoracicotropic hormone = PTTH). These results then became the basis for the classical theory of insect development and metamorphosis.

Wigglesworth demonstrated earlier (1934) that the head was the source of a factor that prevented metamorphosis in <u>Rhodnius</u> <u>prolixus</u> penultimate instar larvae. By parabiotically joining decapitated penultimate stage larvae to last stage larvae, metamorphosis was prevented in the latter,

demonstrating the circulatory nature of this "inhibitory hormone." In transplantation studies the source of this hormone was later identified as the corpora allata (Wigglesworth, 1936). In the early 1950's Williams found (see Riddiford and Truman, 1978) that the abdomens of male cecropia moths parabiotically joined to previously chilled cecropia pupae would exert a 'status-quo' effect on development, causing a molt to a second pupa. An active 'oily substance' was later extracted from the abdomens of cecropia male moths (Williams, 1956) and called juvenile hormone.

With the characterization of the 'status-quo' or 'juvenile hormone' from abdomens of male H. cecropia and Samia cynthia moths as the sesquiterpenoid methyl-10,11-epoxy-7-ethyl-3,11-dimethyl-2-trans-6-trans-tridecadienoate (Roller, et al, 1967) the chemical as well as the biological basis of insect molting and metamorphosis took form (Wigglesworth, 1970; Gilbert, 1974; Riddiford and Truman, 1978). The molting and metamorphic cycles are initiated by the release of PTTH from the brain or a remote neural hemal organ (corpus cardiacum or corpus allatum). The prothoracic gland responds by increasing synthesis and release of ecdysone which elicits cuticular apolysis and the subsequent synthesis of new cuticle. The nature of the molt is directed by juvenile hormone (JH) titers: high juvenile hormone ensures propagation of juvenile features in larvae and nymphs, while declining JH titers allow the metamorphic molts. Hemimetabolous insects, then, undergo an incomplete metamorphosis, while holometabolous insects go through a complete metamorphosis.

The purifications and subsequent availabilities have aided in the advancement of our knowledge of physiological aspects of ecdysone and juvenile hormone. Comparable understanding of the biology of PTTH will require its purification and development of sensitive and specific assays . Several questions regarding PTTH important to insect physiologists have arisen from application of the time-honored surgical methods of ligation, parabiosis, and implanation. These include the precise timing of the initial hormonal events leading to molting and metamorphosis. In tobacco hornworms Truman has demonstrated that completion of a larval molt requires an influence from the head (PTTH) for approximately four hours after the onset of the 'critical period' of PTTH secretion (1972). Direct measurements of PTTH in the hemolymph to identify bouts of secretory activity have apparently not been attempted with present assay methods. Simultaneous determinations of hemolymph titers of PTTH and ecdysone would clarify the temporal relationship between these hormones during larval and metamorphic molts and could distinguish secretory activity from the effects of that activity.

These endocrine events appear to depend on a circadian oscillator as well as on developmental factors (Truman 1972; Truman and Riddiford, 1974). Ligation studies indicate that PTTH is secreted during the early hours of the scotophase during larval molts (Truman, 1972), while PTTH release leading to wandering or pupation occurs during mid- or late-photophase, respectively (Truman and Riddiford, 1974). The

control of PTTH release in Manduca fifth instar larvae may
be more complex than in fourth instar larvae, involving
declining JH titers (Nijhout and Williams, 1974b). From these
results it is difficult to conclude whether the oscillator
for PTTH release changes for each developmental event or the
specific requirements for the developmental event itself
change. Again, knowledge of the circulating titers of PTTH
would be expected to shed light on this question.

How nutritional or developmental factors actually
influence the release of PTTH is little understood. The most
significant exception comes from Wigglesworth's studies with
Rhodnius prolixus (1934). He was able to show that an intact
ventral nerve cord was necessary for the molt following a
blood meal; he suggested that it was nervous activity result-
ing from abdominal stretching that provided the stimulus.
These observations have not been repeated in other insects,
and it appears quite probable that other phenomena come into
play even in Rhodnius, because five days are required to reach
the 'critical period' of hormone releases (Wigglesworth, 1934).
Understanding of these events may of necessity come from
developmental studies of rates of synthesis, transport, and
accumulation at release sites. Technical considerations
dictate the use of more sensitive immunochemical methods to
measure the minute amounts of hormone involved.

Another interesting biological aspect of PTTH is its
role in the induction of pupal diapause. It was shown from
Williams' work (1946) that the giant silkmoth Hyalophora
cecropia, which passes through an obligatory diapause,

requires a period of chilling for the 'brain activation'
that breaks pupal diapause by an activation of prothoracic
glands (Williams, 1952). In the oak silkworm <u>Antheraea</u>
<u>pernyi</u> pupal diapause results from exposure of larvae to
short-days which blocks PTTH secretion, (Williams and Adkisson,
1964). It appears that the block is at the level of release
of PTTH (Williams, 1967), because PTTH can be extracted from
diapausing pupal brains. Similar conditions of photoperiod
are responsible for pupal diapause in <u>Manduca</u> <u>sexta</u>. While
it has been determined that substantial PTTH activity is
present in the diapausing pupal brain (Gibbs and Riddiford,
1977), such brain implants are unable to evoke development in
either short or long-day reared debrained pupae (Safranek and
Williams, 1980). It would be of interest to examine PTTH
transport and storage in neuralhemal organs in long-day versus
short-day <u>Manduca</u>. <u>Antheraea</u> <u>pernyi</u> may be the preferred
subject for the physiology of PTTH in diapause termination,
since development can be synchronously initiated with a
change in photoperiod to 17L:17D (Williams and Adkisson, 1964).

The possibility that PTTH serves functions other than
in molting and metamorphosis has received support from the
finding of activity in the thorax-abdomen of virgin <u>Bombyx</u>
<u>mori</u> female moth (Ishizaki, 1969; Nishiitsutsuji-Uwo and
Nishimura, 1975) and the apparent consumption of PTTH by
ovaries (Nishiitsutsuji-Uwo and Nishimura, 1975). The presence
of bioassayable ecdysones in <u>Bombyx</u> developing ovaries (Hanaoka
Ohnishi, 1974) and unfertilized eggs (Mizuno and Ohnishi,
1975) indicate a role for ecdysones in egg maturation. Indeed,

it appears that ovaries from _Aedes aegypti_ synthesize the ecdysone (Hagedorn, _et al_. 1975) necessary for vitellogenesis in fat body in response to a hormone from the brain (EDNH = egg development neurosecretory hormone) (Hagedorn, _et al_., 1979). The additional fascinating observation was made by Hagedorn, _et al_. (1979) that EDNH activity was found in _Aedes_ larval brains, _Periplaneta americana_ larval and adult corpora cardiaca and larval brain of _Papilio polyxenes_.

PTTH activity has been found in pharate adult brain of _Manduca sexta_ (Gibbs and Riddiford, 1977 and my observations). It is generally held that prothoracic glands degenerate by early adult life (Herman and Gilbert, 1966); thus, it is possible that post-metamorphic PTTH affects target tissue directly, or that its effects are mediated through extra-ecdysial gland sources of ecdysone, such as ovarian tissue, as suggested for _Aedes aegypti_. As yet, no evidence exists to indicate a role for ecdysone in reproductive processes in _Manduca_. Post-metamorphic functions of PTTH might be uncovered by a survey of tissues for specific PTTH receptors, requiring, of course, its purification.

Earnest attempts to characterize PTTH can be traced to the demonstration of its proteinaceous nature in 1963 (Ichikawa and Ishizaki, 1963). In the next six years three publications appeared on its partial purification from pupal brains of _Bombyx mori_ (Kobayashi and Yamazaki, 1966; Ishizaki and Ichikawa,1967; Yamazaki and Kobayashi, 1969). One group (Kobayashi and Yamazaki, 1966), however, suggested that one component of the 'brain hormone' was cholesterol (Kirimura,

et al, 1962), although dose dependence of its activity had not been adequately demonstrated and its ubiquity in insect tissues made its hormonal activity seem implausible. Nevertheless, substantial progress was made and purifications of 2000-fold (Yamazaki and Kobayashi, 1969) and 8400-fold (Ishizaki and Ichikawa, 1967) were reported. However, homogeneity could not be demonstrated, and disagreements remained on the size and charge characteristics of the hormone, notwithstanding the use by both groups of Bombyx mori pupal brains as source material for purification. Later work using whole heads of Bombyx male adults (Nagasawa, et al., 1979) and Bombyx adults and pharate adults (Nishiitsutsuji-Uwo, 1972) indicated a small peptide (4400-5000 Daltons) was the active hormone. One group (Nagasawa, et al., 1979), using improved purification methodology, reported a 28,500-fold purification; however, tests of purity were not reported.

Activity in the above described work was followed with a 'dauer' pupal bioassay, that is, the newly ecdysed pupae were placed in a state of permanent diapause by surgical debraining. When injected with active substances, development would result in the emergence of a brainless moth in approximately three weeks. Although it is unclear whether anything but emergence was scored, it can be presumed that activity could be assessed in a shorter period of time by scoring apolysis, the onset of development. In either event, this assay suffers from a long turn around time, which must have hampered progress.

The present work was begun after the description of a larval bioassay using _Manduca sexta_ (Gibbs and Riddiford, 1977). It was felt that the rapidity with which activity could be assessed (24 hours) and the minimal expenditure of time in performing the assay (see Methods, below) would make it possible to progress at a reasonable rate. With these hopes, purification studies were undertaken.

Materials and Methods

Materials

Ammonium sulfate (Ultra Pure) was obtained from Schwarz/Mann. Acetone (reagent grade) was obtained from Mallinckrodt.

Sephadex gel filtration resins G-75, G-100, and G-200-SF used in chromatography and isoelectric focusing were obtained from Pharmacia or Sigma. Ion-exchange resin DEAE-Sephadex A-50 was purchased from Pharmacia. Hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad.

Electrophoresis grade reagents obtained from Bio-Rad included: acrylamide monomer, methylene bisacrylamide, ammonium persulfate, and N; N,N', N'-tetramethyethylenediame (TEMED). Riboflavin was obtained from Sigma. Sodium lauryl sulfate (SDS) was obtained from BDH Chemicals, LTD. Coomassie Brilliant Blue G-250 was from Schwarz/Mann.

Ampholytes for isoelectric focusing were obtained from LKB (Ampholine 3-10), Pharmacia (Pharmalyte 5-8) and Bio-Rad (Bio-Lyte 3-10 and Bio-Lyte 5-7).

All buffers and salts were of analytical grade and were obtained from Mallinckrodt, J.T. Baker, and Sigma.

PTTH: Bioassay

PTTH activity was assayed essentially following the method of Gibbs and Riddiford (1977). This assay is based on the observations that 1) PTTH release in fourth instar larvae occurs during a well-defined interval during the light:dark cycle (release is "gated" to the photoperiod) on the second or third night after the molt (Truman, 1972) and 2) larvae neck-ligatured just before the opening of this gate will complete a larval molt within two days if they receive ecdysone injections (Fain and Riddiford, 1976). Such larvae are sensitive to injected brain extract, responding with the ecdysone mediated molt in a dose dependent manner, with 50% showing the molting response when injected with 0.6 brain equivalents (Gibbs and Riddiford, 1977).

Larvae were isolated for use in the bioassay on the day of ecdysis to the fourth instar (see General Methods). Two days later larvae were examined under a dissecting microscope for spiracle apolysis, the first morphological sign of molting (Truman, et. al., 1973; Gibbs and Riddiford, 1977). Animals showing apolysis were discarded, since they had initiated the molt with hormone release the previous night. Of those not showing apolysis, larvae $\geq$ 0.9g in weight were selected for use. Generally about 25-30% of those isolated two days earlier could be used in the bioassay.

Larvae were then briefly anesthetized by immersion in water and neck-ligated with unwaxed dental floss. The ends of the floss were trimmed and a portion of the head cut away to insure removal of the brain. In preliminary studies ligations were done between 2300-2330 A.Z.T.; however, the "gate" for hormone release eventually shifted (for unknown reasons), opening two to three hours earlier. For the work reported here, ligations were done between 2100-2200 A.Z.T. Delay by as little as one hour resulted in a molting response in up to 50% of the control animals.

After ligations were completed, larvae were again anesthetized with water prior to injection with test or control substances. Injections were administered with a Hamilton syringe (50 µl, SN 30 gauge, 1/4" style #4) through the base of the first abdominal proleg. Volumes were 5-10 µl. Injected larvae were kept in a water-jacketed incubator at 30°C and scored for a response at 24 and 48 hr. At 30°C, 95% of the larvae showing spiracle apolysis at 48 hours had done so within 24 hours. This minimum response was sometimes followed within 48 hours after injection by synthesis of new cuticle with the black coloration that results from juvenile hormone deficiency (Truman, et al., 1973). This maximal response was qualitative for the potency of injected sample but was not incorporated into the scoring.

Activity was located qualitatively by injecting seven to ten larvae with aliquots from column fractions. Specific activities were estimated in crude extracts and in chromato-

graphy samples after pooling active fractions by injecting
25-35 larvae with aliquots of each preparation. The responses
were then converted to units of PTTH activity by interpolation
from a dose response curve constructed with "crude PTTH"
(30-65% acetone precipitate; see below). One unit of activity
was defined as that amount sufficient to elicit a larval
response in 35% of the injected animals.

PTTH: in vitro assay

In the latter stages of this work an in vitro PTTH assay
was described (Bollenbacher, et. al., 1979). The basis for the
assay is the synthesis of ecdysone by prothoracic glands from
newly pupated hornworms reared under long day(16 hrs. light:8
hrs. dark) conditions. The right and left glands are cultured
separately for six hours, one serving as a control, the other
as a test gland. Activity is expressed as a ratio of ecdysone
synthesized by test gland over control gland; this activation
ratio, "A_r", is typically four to five for maximally active
glands (Bollenbacher, et al., 1979).

This assay was used to confirm the presence of PTTH
activity in the final stages of its purification. Pupae were
selected within three hours after ecdysis. Glands were
dissected in Weever's Lepidopteran saline (4 mM NaCl; 40 mM
KCl; 18 mM $MgCl_2$. 6 H_2O; 3 mM $CaCl_2$), rinsed in saline,
and preincubated in pairs in 50 µl Grace's Insect Culture
Medium for 30 minutes. Glands were divided and cultured for
six hours in 25 µl standing drops of Grace's Insect Culture

Medium in multi-well tissue culture plates (Falcon Micro

Test II, No. 3008). The medium included one to five μl of

test or control substance. Ten μl culture medium was then

extracted with 0.3 ml methanol and aliquots assayed for

ecdysone by radioimmunoassay (see Appendix).

Tissue preparations

In the initial stages of this work brains and retrocere-

bral complexes (Nijhout, 1975) were dissected free of other

tissue from day 0 wandering larvae (Wd0). It eventually

became clear, however, that accumulating adequate amounts of

tissue would require a faster dissection to handle the large

numbers of animals required. Hence, the more rapid excision

of whole head was substituted. Excised heads were frozen

immediately in liquid nitrogen and stored at -80°C for

subsequent processing.

Frozen heads were powdered in liquid nitrogen in a

Tekmar A-10S analytical mill (with cryogenic attachment)

or, for large preps, in a one-gallon Waring Blendor. The

frozen powder was then blended with a nine-fold excess (volume:

weight) of acetone, previously chilled to -80°C. The result-

ing slurry was then filtered in a fume hood on Whatman No. 1

paper and subsequently washed with cold acetone three to four

times by filtration. The acetone cake was then placed on

paper towels and the large chunks broken up with a spatula.

After drying, the powder was stored in the presence of des-

sicant at -20°C. For immediate processing, the frozen powder

could be allowed to warm slightly, but not to thaw, and extracted directly. This modification became feasible because of the time saved by powdering large batches in the Waring Blendor. It was then possible to process a batch of tissue from whole heads through ammonium sulfate precipitate (see below) in one day.

Tissue extraction

The acetone or frozen powder was extracted with a ten-fold excess (volume:weight dry powder) cold 2% NaCl/0.1 mM phenylthiourea solution. The insoluble pellet was extracted twice more to yield a total volume of supernatant approximately 20-fold (volume:weight) that of the original dry powder. Insoluble material was removed by centrifugation in a GS-3 rotor at 6000 RPM for 10 min. It was found convenient for this crude extract and for the heated extract (see below) to filter the supernatant after centrifugation through Mira-cloth to remove small amounts of insoluble debris that would not pellet. The supernatants were pooled to give a clear dark-green "crude extract" (C.E.).

Heated fraction

The crude extract was heated in 300-400 ml batches on a boiling water bath with constant swirling. Coagulation was evident at 70°C; heating was continued until the extract reached and maintained a temperature of 85°C for two minutes. The mixture was then cooled rapidly on ice and centrifuged at 6000 RPM for 10 min. The pellet was rinsed once with 2%

NaCl/0.1 mM phenylthiourea, centrifuged and the clear super-
natants pooled. This "heated fraction" was deep amber in
color.

Ammonium sulfate precipitation

The volume of the heated fraction was measured, and
solid ammonium sulfate was added over a 30 minute period
to 30% saturation and stirred on ice for an additional 30
minutes. The precipitate was removed by centrifugation at
12,000 g for 15 min. The supernatant was then saturated
to 65% by the slow addition of solid ammonium sulfate followed
by stirring on ice for 30 min. Centrifugation gave a grayish-
white precipitate, designated 30/65% ammonium sulfate precipi-
tate (30/65 AS). This material was frequently stored over-
night at 4°C before proceeding to the acetone fractionation
step.

Acetone fractional precipitation

The 30/65 AS supernatant was decanted and discarded. The
precipitate was resuspended in glass distilled water (50 µl/
head equivalent) and dissolved with the aid of a rubber spatula,
resulting in a clear amber solution. The solution was stirred
at 0°C during the addition of acetone (previously chilled to
-80°C) to 30% (volume:volume) over a period of 10-15 seconds
to avoid local high concentrations of acetone.

The precipitate was removed by centrifugation at 8000 g
for 10 minutes. The supernatant was decanted, the volume
measured, and cold acetone again added slowly to 65% (volume:

volume). The precipitate was pelleted by centrifugation at 6000 g for 10 minutes and then resuspended in cold acetone followed by centrifugation three more times to remove the last traces of water. The precipitate was then dried under a stream of nitrogen with continuous stirring to speed drying. The dry, slightly gray powder (30/65% acetone precipitate, or 30/65 AP) was stored at -20°C with dessicant until the first chromatographic procedure.

Sephadex G-100 chromatography

The initial attempt at gel filtration of PTTH activity utilized Sephadex G-75 (2 x 44 cm); this column proved inadequate in resolving activity from high molecular weight contaminants. In subsequent columns Sephadex G-100-medium was used first in a 2.5 x 65 cm column, then in a 2.5 x 95 cm for the majority of the work reported. A 5 x 80 cm column was prepared for a single large prep.

Chromatography was done in 59 mM ammonium acetate, pH 7.2. In early preps active fractions were pooled and lyophilized. In later preps samples were concentrated either by pressure dialysis in an Amicon Model 52 stirred cell (with an Amicon UM10 membrane), by dialysis against powdered polyethylene glycol 6000, or by ammonium sulfate precipitation (70% saturation). Though the need for a volatile buffer no longer existed, its use was continued for reproducibility. In a single run in 50 mM sodium phosphate - 0.1M NaCl, pH 7.2, activity chromatographed with the same elution volume as with ammonium acetate, indicating that a high ionic strength

buffer would not be needed to minimize aggregation artifacts.

The 30-65% acetone precipitate was taken up in a volume of 59 mM ammonium acetate, pH 7.2 representing 2-3% of the column volume. The small amount of insoluble material remaining was removed by centrifugation and the sample loaded under a 5 cm head of buffer. Five ml fractions were collected and the activity located by bioassaying every fraction or alternate fractions. When SDS-PAGE was carried out on aliquots of fractions from the column, it was possible to correlate the appearance of certain bands on the gel with biological activity, obviating the need to bioassay every run. Typically, a column could be started at 11-12 PM and the flow rate adjusted to collect the void volume in a graduated cylinder by 8 AM. The fraction collector was then started, and an additional 200 ml was collected. The run was completed in time to bioassay collected fractions (injections done 2200-2300 hrs, that is 1-2 PM) and/or to run SDS gels on these fractions. Profiles of optical density at 280 nm were recorded, and fractions were concentrated later in the day when the gel pattern could be visualize, or they were stored at 4°C overnight when bioassays could be scored. After concentrating, samples were equilibrated with starting buffer for ion exchange chromatography.

DEAE-Sephadex chromatography

Samples from the G-100 column were taken up in .01 M Tris-HCl, pH 7.9 (ammonium sulfate precipitates), and dialyzed against the same buffer; concentrated (but not precipitated)

samples were dialyzed directly against Tris buffer. Samples

were judged to be equilibrated when the pH had risen to that

of the buffer (18-24 hours with two buffer changes).

DEAE-Sephadex was swollen in 0.01 M Tris, pH 7.9 (at

4°C) and a 1 x 15 cm column poured at 4°C (the amount of resin

was calculated to have a capacity for at least ten-fold

the amount of protein to be applied). The column was

washed with six to ten column volumes of starting buffer

(0.01 M Tris, pH 7.9); equilibration was monitored by

checking the pH of the outlet buffer. The sample was applied

to the column and allowed to drain into the bed. Elution

with starting buffer was begun and approximately two column

volumes collected. The column was next washed with 0.01 M

Tris containing 0.1 M NaCl, pH 7.9; alternatively a gradient

of NaCl (0-0.2 M NaCl) in Tris buffer was started. The

total volume of the gradient was generally eight to ten

column volumes.

When the column was eluted with a salt gradient frac-

tions were monitored by bioassay, by SDS gel electrophoresis,

and by conductivity measurements. As with the gel filtration

column, after initial characterization with the bioassay,

it was possible to judge location of activity from gel

patterns and conductivity measurements. After elution of

the column, fractions were pooled, an aliquot was reserved

for bioassay and Lowry protein determination and the frac-

tions were applied directly to the next column.

Hydroxylapatite chromatography

Material from the DEAE column was initially dialyzed against 0.01 M sodium phosphate, pH 6.8 prior to hydroxylapatite chromatography. However, it was soon learned that activity was bound by hydroxylapatite in the presence of 0.125 M NaCl and eluted with low concentrations of phosphate buffer. Hence, pooled fractions from the DEAE-Sephadex step could be applied directly to the hydroxylapatite column without concentration or buffer exchange.

Hydroxylapatite (Bio-Gel HTP) was swollen in 0.01 M sodium phosphate, pH 6.8 (starting buffer) and the "fines" were aspirated twice according to the manufacturer's recommendations. A 1 x 5 cm column was poured and washed with three column volumes of starting buffer. The buffer head was removed to the top of the column bed, and the pooled DEAE material was applied. After all material had drained into the matrix, the column was washed with two column volumes of 0.01 M Tris-HCl, pH 7.9. Column flow was then stopped, the Tris buffer head removed and replaced with 0.01 M sodium phosphate, pH 6.8. The column was eluted with a linear 0.01-0.15 M sodium phosphate gradient; total effluent volume was 20-25 column volumes. Fractions were collected and monitored for biological activity, concentration of phosphate by conductivity measurements, and for protein banding patterns on SDS polyacrylamide gels correlating with biological activity. Fractions were pooled and aliquots set aside for bioassay and protein determination. If protein

was less than 0.5 mg/ml, pooled material was concentrated
by dialysis against polyethylene glycol 6000 or by pressure
dialysis with an Amicon UM-10 ultrafiltration membrane,
followed by precipitation with the addition of solid ammonium
sulfate to 70% saturation. The ammonium sulfate solution
was kept at 0°C for one hour and then centrifuged at 12,000
x g for 15 minutes. The pellet could be stored at 4°C
without removal of the supernatant; if further purification
was to be carried out, the pellet was redissolved in glass
distilled water to a protein concentration of one to ten
mg/ml and dialyzed against glass distilled water for 24-28
hours with water changes every eight hours.

Isoelectric focusing

Isoelectric focusing (IEF) was carried out in a Desaga/
Brinkmann TLE Double Chamber apparatus. The chamber is
equipped with a metal cooling block in which cooling water at
4°C was circulated. The electrofocusing procedures used
were essentially as described by Radola (1973).

The support medium was Sephadex G-200 SF. Ampholytes
were obtained commercially from Bio-Rad (Biolyte 3-10,
Biolyte 5-7). Five g. Sephadex G-200 SF were swollen in 500
ml glass distilled water. The resin was washed repeatedly
by decanting water from the settled gel in a graduated
cylinder; finally, water was removed to yield a 3.5% suspen-
sion of resin. Ampholytes (60% Bio-Lyte 5-7, 40% Bio-Lyte
3-10) were added to 17 ml of the G-200 SF suspension to 2%
(weight:volume) total ampholyte. The mixture was poured

onto a 10 x 20 cm glass plate and spread to cover the entire

plate; the gel surface was brought to uniform thickness by

pushing the plate back and forth a few times on a level

wood surface. The gel was dried under an infra-red lamp to

a 40% weight loss, then placed in the focusing chamber.

Electrode strips ("print" paper, Desaga) were saturated

with .1 N NaOH (cathode) and .1 N H_3PO_4 (anode) and placed

on the plate. Electrodes were applied and the plate was

prefocused at 400V for one hour. After prefocusing, power

was turned off and the dialyzed sample was applied in a two

cm band across the width of the plate, leaving one cm at

either edge; sample volume was 200-500 µl. Focusing was

carried out at 600 volts for three hours, followed by 1000

volts for an additional three hours. Protein was recovered

by scraping the Sephadex in 0.5 cm zones from the plate and

eluting with distilled water in a disposable syringe with a

glass wool support bed. Samples were bioassayed and the pH

determined with stirring at 4°C with a Corning Model 119 pH

meter. Aliquots were removed for electrophoresis and

the samples were either dialyzed against distilled water

and lyophilized or precipitated with ammonium sulfate, washed

with 70% ammonium sulfate (to remove the last traces of

ampholytes) and taken up in a small volume of distilled

water for dialysis and subsequent lyophilization.

Electrophoresis

Two electrophoresis systems were used for analysis of

protein patterns in the PTTH purification. These were

based upon the non-denaturing system of Davis (Davis, 1964),
and the SDS system described by Laemmli (Laemmli, 1970).

One modification was made in the Davis buffer system:
anode and cathode buffer were 12.5 mM Tris 96 mM glycine,
pH 8.3. Three monomer:bis acrylamide formulations in the
separating gel were used: 1) 7.5% acrylamide (total),
37.5:1, monomer:bis, 2) 15% acrylamide, 200:1, monomer:bis,
and 3) 10% acrylamide, 200:1, monomer:bis. The third
formulation was found to give an adequate resolution in a
minimum running time.

SDS gels used included formulations with 1) 15% acryl-
amide, 37.5:1, monomer:bis, and 2) 18% acrylamide, 200:1,
monomer:bis in 0.75 M Tris-HCl, pH 8.9 (Thomas and Kornberg,
1975).

Electrophoresis was carried out in a microslab appara-
tus very similar to that described by Matsudaiira and Burgess
(1978) and constructed by a local plexi-glass fabricator.
Both SDS and non-denaturing gels were run in a 6°C cold-box.
SDS gels were run at 200V for one hour; non-denaturing gels
were electrophoresed at 90V until the bromophenol blue
tracking dye entered the separating gel and then at 150-200V
for one and one-half hours.

Both SDS and non-denaturing gels were stained in 0.04%
Coomassie Brilliant Blue G250 in 3.5% perchloric acid (Hol-
brook and Leaver, 1976) for 30 minutes. The stain was
intensified in 5% acetic acid for 30-60 minutes and then
destained in methanol:water:acetic acid, 25:65:10. For
exceptional sensitivity needed with highly purified prepara-

tions, a silver stain (Switzer, et al., 1979) modified for
simplicity and economy (Oakley, et al., in press) was used.

Enzymatic digestion

Proteinase K (32 Anson units/g) was obtained from
Beckman. α-chymotrypsin (65 U/mg) was obtained from Mil-
lipore. Both enzymes were tested in the same manner: 45
μg partially purified hormone (purified through hydroxyl-
apatite chromatography) was digested with 20 μg of either
α-chymotrypsin or proteinase K in 90 μl 0.04 M Tris-HCl,
pH 7.8 containing .056 M $CaCl_2$ at 37°C for one hour with
shaking; controls lacked enzyme. The tubes were immersed
in boiling water for three minutes and precipitated protein
removed by centrifugation. Five μl aliquots, containing
2.5 μg protein were bioassayed.

Results

PTTH Assays

Early in the investigation an attempt was made to
improve the sensitivity of the Gibbs-Riddiford bioassay
(1977) by looking at the primary endocrine event of molting,
ecdysone release. It was reasoned that endocrine events
preceeding molting might be detected as increases in hemo-
lymph ecdysone. Accordingly, larvae were selected using
the same criteria established for the bioassay (see above).
In addition to the neck ligature, a second ligature was

placed between the third and fourth abdominal segments. The
tip of the dorsal horn was cut away and 10 μl hemolymph
collected for ecdysone determination. After the remaining
abdomen was cut away, the isolated thorax (with three abdom-
inal segments) was injected with boiled 2% NaCl extracts of
WdO larval brains. After three hours hemolymph was collected
from a cut in a proleg for ecdysone determination. Ecdysone
in the thorax (t=3 hr) and abdomen (t=0 hr) was determined
by a radioimmunoassay procedure (see Appendix) and the ratio
of thorax to abdomen ecdysone expressed as a percent; a dose
response curve is shown in Fig. 1. Significant increases
over controls (zero brain equivalents) occur with 0.2 brain
equivalents. Despite the validity of this bioassay, it was
clear that it was not significantly more sensitive than the
bioassay scoring a morphological response (Gibbs and Riddi-
ford, 1977, and see below). Therefore, because of the addi-
tional time required to do the radioimmunoassays, this assay
was abandoned.

A photograph demonstrating the morphological response
scored at 24 hours post injection (spiracle apolysis) is
shown in Fig. 2.

The validity of the larval bioassay scoring spiracle
apolysis was confirmed with a sample of the 30-65% acetone
precipitate (see Methods). The powder was dissolved in a
volume of 0.15 M sodium phosphate, pH 6.8 to give a solu-
tion containing one head equivalent in 10 μl buffer.
Serial dilutions were injected into test larvae and scored
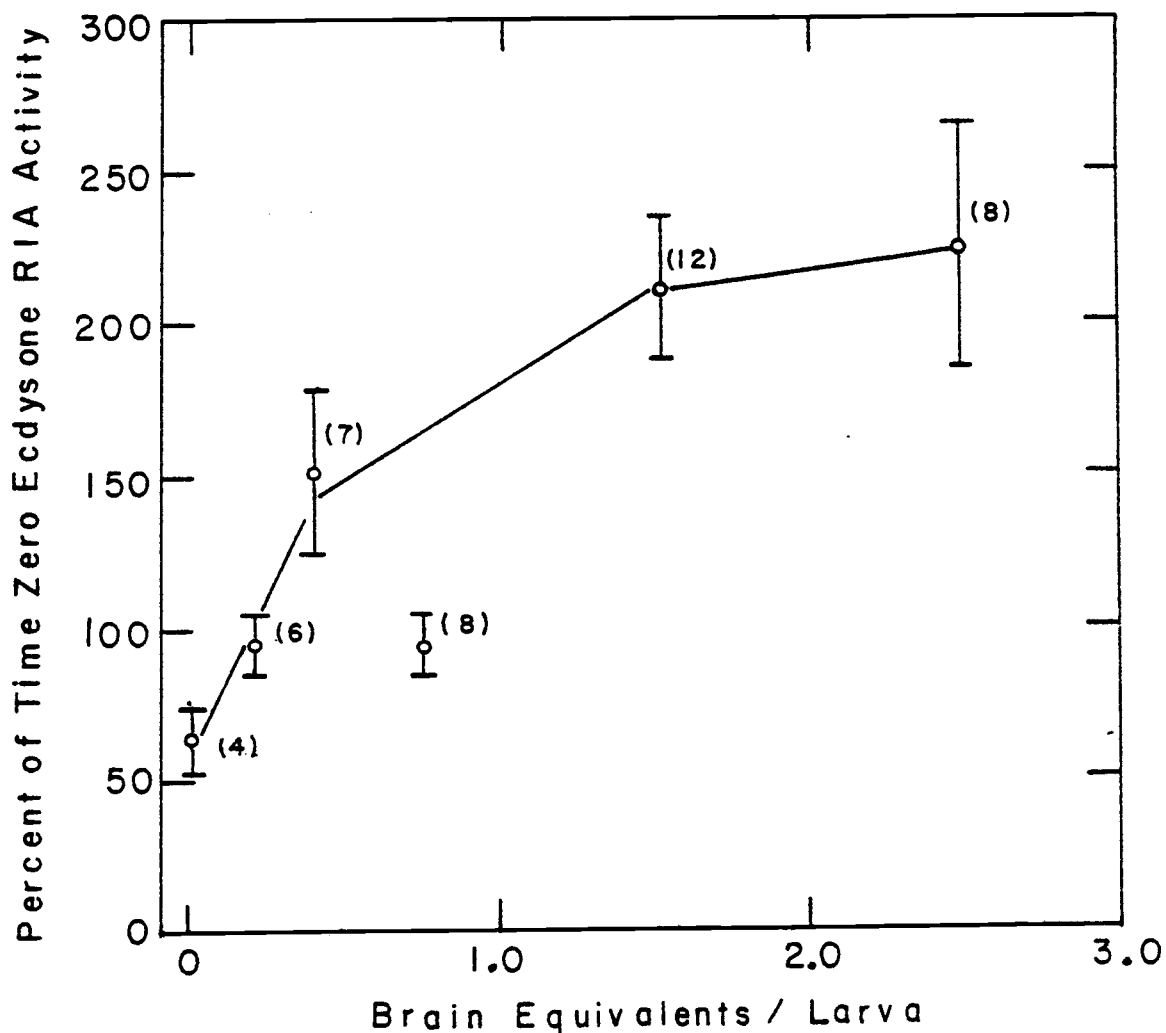
Fig. 1 Ecdysone titers in brain-extract injected isolated
thoraces. Response is t=3 hr thorax titer as a
percent of t = 0 hr abdomen titer. Number of assay
animals for each dose is shown in parentheses.
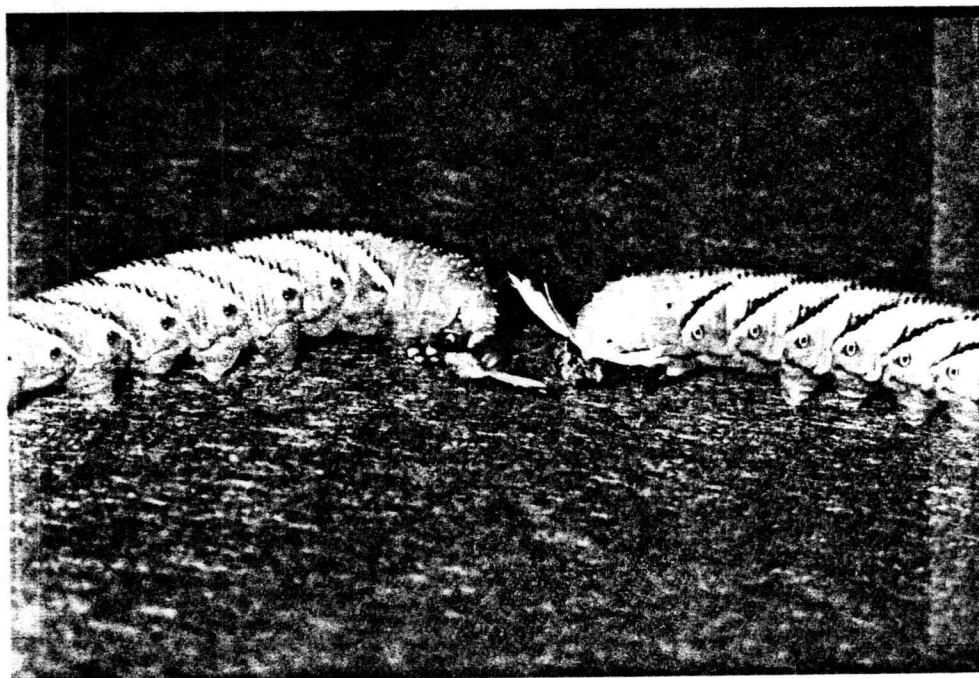
Fig. 2 Fourth instar control (left) and test (right)
larvae at 24 hours post injection. Note
spiracular apolysis in test larva.

at 24 hours. A dose response curve is shown in Fig. 3.

Comments on the larval bioassay

The importance of selecting larvae of $\geq$0.9g weight apparently lies in their "competence" to respond. When 0.7-0.9g larvae were used responses were invariably lower. In addition, inter-assay variation in response is reduced by narrowing the acceptable size range for assay larvae. As alluded to in Methods, some difficulty was encountered with a shifting gate for PTTH release, resulting in up to 50% false positives. It was essential that controls be kept below a 10% response to allow unambiguous identification of activity, whether the assay was being used quantitatively or qualitatively.

Crude Extract and Heated fraction

Because the crude extract and heated fraction were very dilute (with respect to activity) only a single dose was bioassayed, 0.125 head equivalents in 16 μl 2% NaCl/.1 mM phenylthiourea as shown below:

sample	no. test larvae	percent response	PTTH units/head equivalent
control	20	0	-
crude extract	33	6	1.3
heated fraction	32	13	2.0

Since 4.3 PTTH units are contained in one head equivalent 30-65% acetone precipitate (see Fig. 3), there are apparent-
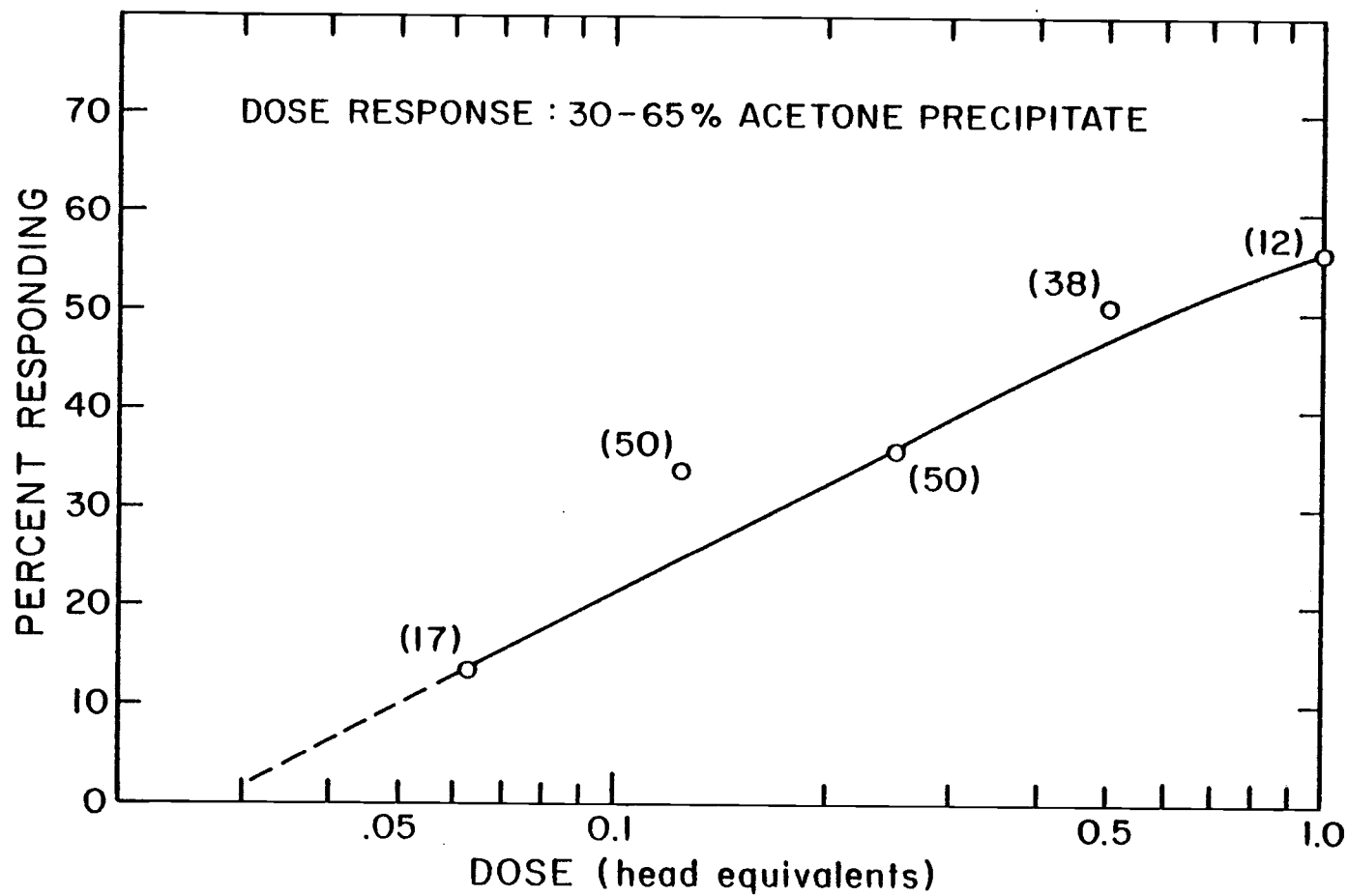
Fig. 3   Larval bioassay dose response curve: 30-65% acetone precipitate. Percent of controls responding was subtracted from test response. Number of assay animals for each dose is shown in parentheses.

ly substances present in the crude and heated fractions
that partially block expression of activity.

Ammonium sulfate fractionation

The solubility of PTTH activity in ammonium sulfate
solutions of varying saturations was investigated. The
heated fraction was aliquoted, saturated to three different
concentrations of ammonium sulfate (25,30,35%), and the
resulting precipitates bioassayed. The supernatants were
then saturated to 60%, 65%, and 70% ammonium sulfate, respect-
ively and the resulting supernatants dialyzed, lyophilized,
and bioassayed. The results are shown in Table 1. Detect-
able activity does not start to precipitate until 35% satur-
ation is reached. The apparent inconsistency in the pattern
of activities in the supernatants in all likelihood illus-
trates a difficulty occasionally encountered when the control
responses exceed 10% (here 17%); the control response becomes
more variable, resulting in unequal contributions to the
response of test animals. However, taken as a whole, the
three test groups indicate that substantially all activity
is precipitated by 60% ammonium sulfate. For routine use,
65% ammonium sulfate was adopted as an upper limit to pro-
vide some margin of safety. It was eventually appreciated
that a more precise determination of this upper limit would
have been useful, because of the substantial amount of
proteinaceous material precipitated in this range of ammon-
ium sulfate saturation (see comments on the purification).

Table 1.   Ammonium sulfate solubility of PTTH activity

sample	N	dose	%response	PTTH units/H.E.
Control	24	0	17	-
25% A.S. ppt	24	1.4 H.E.	0	0
30% A.S. ppt	24	1.4 H.E.	0	0
35% A.S. ppt	24	1.4 H.E.	17	.21
*pooled 2nd ppt	24	0.41 H.E.	46	4.77
C	23	0	17	-
60% A.S. sup	25	1.43	0	0
65% A.S. sup	24	1.43	25	.38
70% A.S. sup	24	1.43	4	.11

A.S. = ammonium sulfate; H.E. = head equivalent. The control
response has been subtracted from test response to arrive at
units of activity.

*The 60%, 65% and 70% A.S. precipitates were pooled and bio-
assayed. All samples were dissolved in .01 M Tris HCl - .1 M
NaCl, pH 7.1 for bioassay. The precipitates and supernatants
were assayed separately and are shown with their respective
controls.

The lower limit was less important as a much smaller amount of protein precipitates at 35% saturation.

Fractional precipitation with acetone

The pooled second precipitates from the ammonium sulfate series above were used to investigate the solubility of PTTH activity in acetone. The redissolved precipitates were diluted with distilled water to 50 µl/head equivalent; protein concentration was 1.7 mg/ml. The sample was aliquoted and cold acetone (previously chilled to -80°C) added with stirring. The 30% and 35% acetone supernatants were then further fractionated by the addition of cold acetone to 60% and 65%, respectively. In this series all precipitates were washed once with 95% ethanol, twice with diethyl ether, and dried under reduced pressure. Bioassay results are shown in Table 2. From these results, the lower and upper limits for fractionation were set at 30% and 65% acetone. A flow chart of procedures through the acetone fractionation is shown in Fig. 4.

Sephadex G-100 chromatography

The first gel filtration column, a 2 x 44 cm G-75 Sephadex column, was inadequate in resolving activity from the void volume. The second column, 2.5 x 65 cm Sephadex G-100 had improved resolution from the void volume but lacked sufficient capacity; in addition increased resolution from a longer column was desirable. These initial attempts did however, reveal that activity chromatographed

PREPARATION OF "CRUDE PTTH"

LARVAE WHOLE HEADS

　　　Pulverize in liquid N_2
　　　Wash in excess cold acetone

ACETONE POWDER

　　　Extract with 20× (vol:wt) 2% NaCl / 0.1 mM
　　　phenylthiourea

CRUDE EXTRACT

　　　Heat to 85°C for 5 min.; discard precipitate

HEATED FRACTION

　　　Saturate to 30% A.S.; discard precipitate
　　　Saturate supernatant to 65% A.S.

$(NH_4)_2SO_4$ PRECIPITATE

　　　Redissolve in H_2O
　　　Add cold acetone to 30% (vol:vol); discard
　　　precipitate
　　　Add cold acetone to 65%; wash precipitate
　　　with 100% acetone

ACETONE PRECIPITATE ("CRUDE PTTH")

Fig. 4　　Flow chart for preparation of "crude PTTH".

Table 2.   Acetone solubility of PTTH activity

sample	N	dose	%response	PTTH units/H.E.
control	21	0	0	-
30% AP	23	1.0 H.E.	17	.33
35% AP	22	1.0 H.E.	36	1.1
40% AP	23	1.0 H.E.	57	4.63
60% APsup	22	1.0 H.E.	32	.83
65% APsup	23	1.0 H.E.	0	0

Samples were dissolved in .01 M Tris HCl - .1 M NaCl for
bioassay. AP = acetone precipitate; H.E. = head equivalents.

as a single peak and gave an estimate of molecular weight range, 25,000-40,000 Daltons.

The third column, 2.5 x 95 cm Sephaex G-100, was calibrated with BSA, ovalbumin, chymotrysinogen A, and ribonuclease A (Fig. 5). This column separated PTTH activity from a large peak of UV absorbing material running near the void volume and a second smaller peak which co-elutes with BSA (Fig. 6). Because the column was heavily loaded (30-65 AP dissolved in 59 mM ammonium acetate, pH 7.2 to 135 mg/ml) it was felt that a second run of the same material could further remove inactive substances. Hence, active material was pooled, concentrated by pressure dialysis and rechromatographed on the same column with the result shown in Fig. 7. It can be seen that activity has been significantly enriched with respect to UV absorving material. Though the precise center of the activity elution profile cannot be determined because of the qualitative nature of the bioassay when seven to ten animals per fraction are injected, the peak appears to be in the 340-370 ml range for both chromatograms. This approximates the elution volume of chymotrypsinogen A (355 ml), a 25,000 Dalton protein.

DEAE-Sephadex chromatography

Preliminary studies indicated that all activity was bound by DEAE-Sephadex in 0.01 M Tris HCl, pH 7.9. When the column was eluted with 0.01 M Tris HCl containing NaCl in steps of 0.1M, 0.2M, and 0.5M NaCl, activity appeared
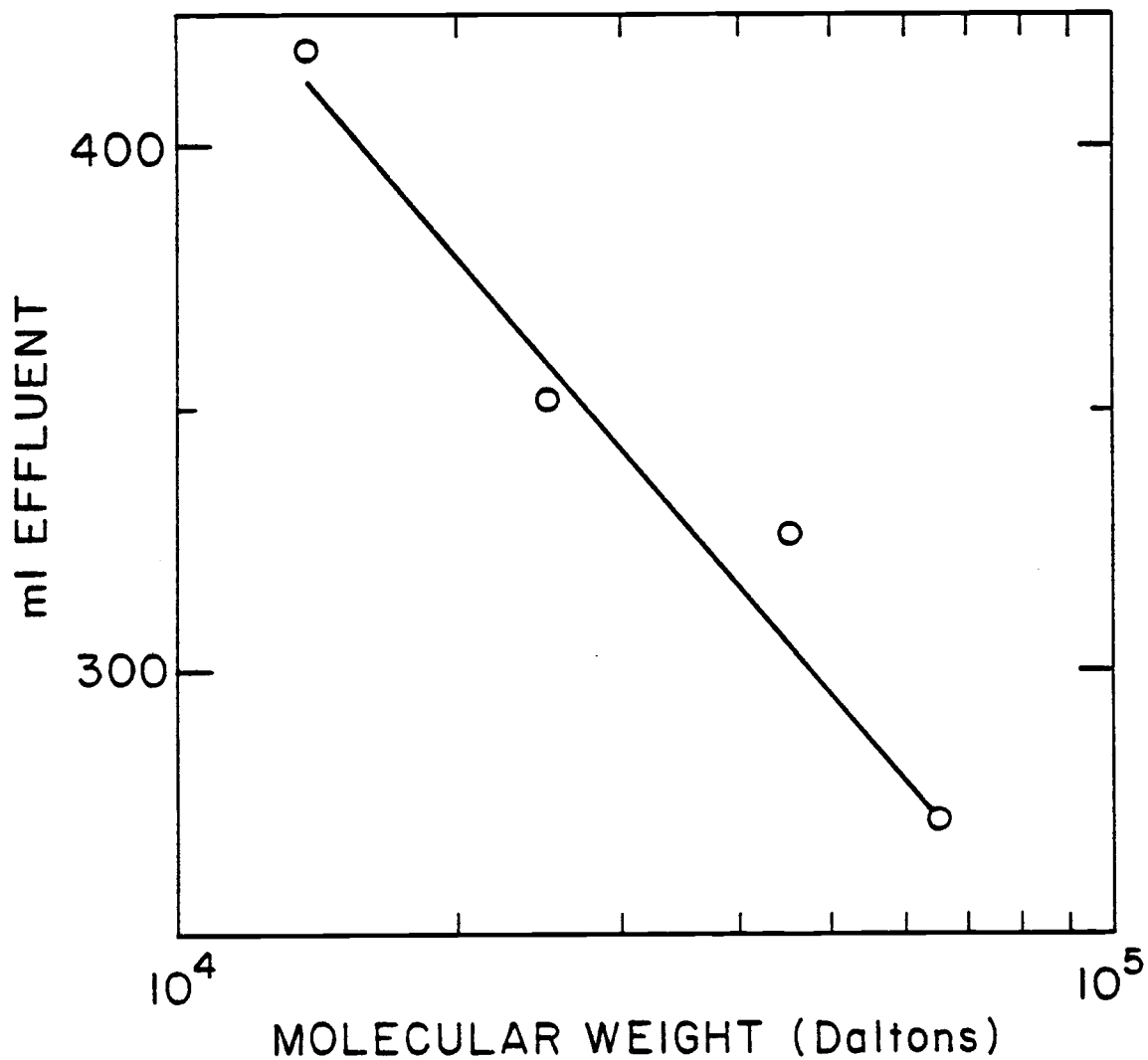
Fig. 5 Sephadex G-100 column calibration. Column dimensions, 2.5 x 95 cm; flow rate, 40 ml/hr. Standards: BSA (65,000), ovalbumin (45,000), chymotrypsinogen A(25,000), and ribonuclease A(13,700).
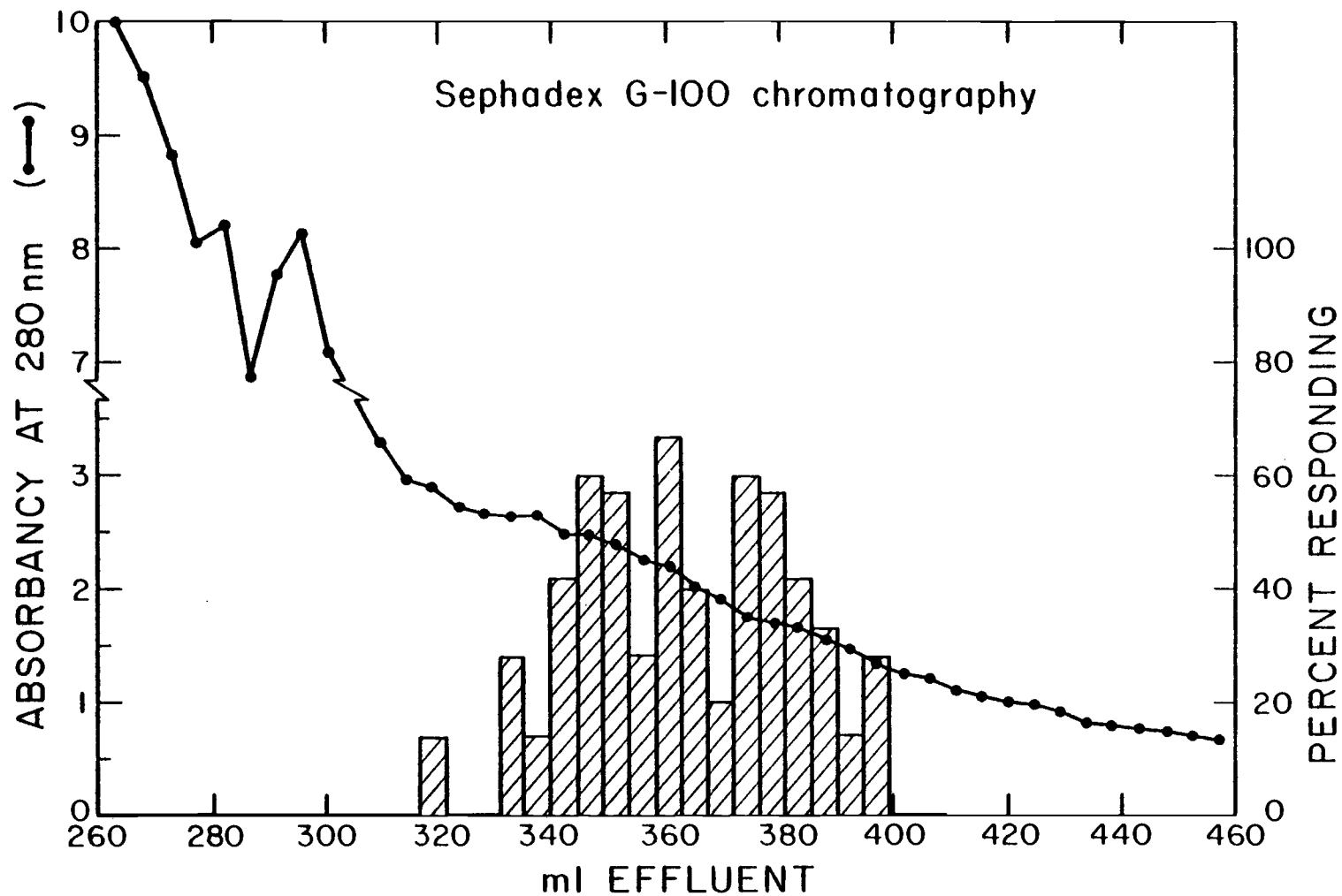
Fig. 6   Sephadex G-100 chromatography of 30-65% acetone precipitate.
Flow rate, 40 ml/hr; fraction size, 4.6 ml.
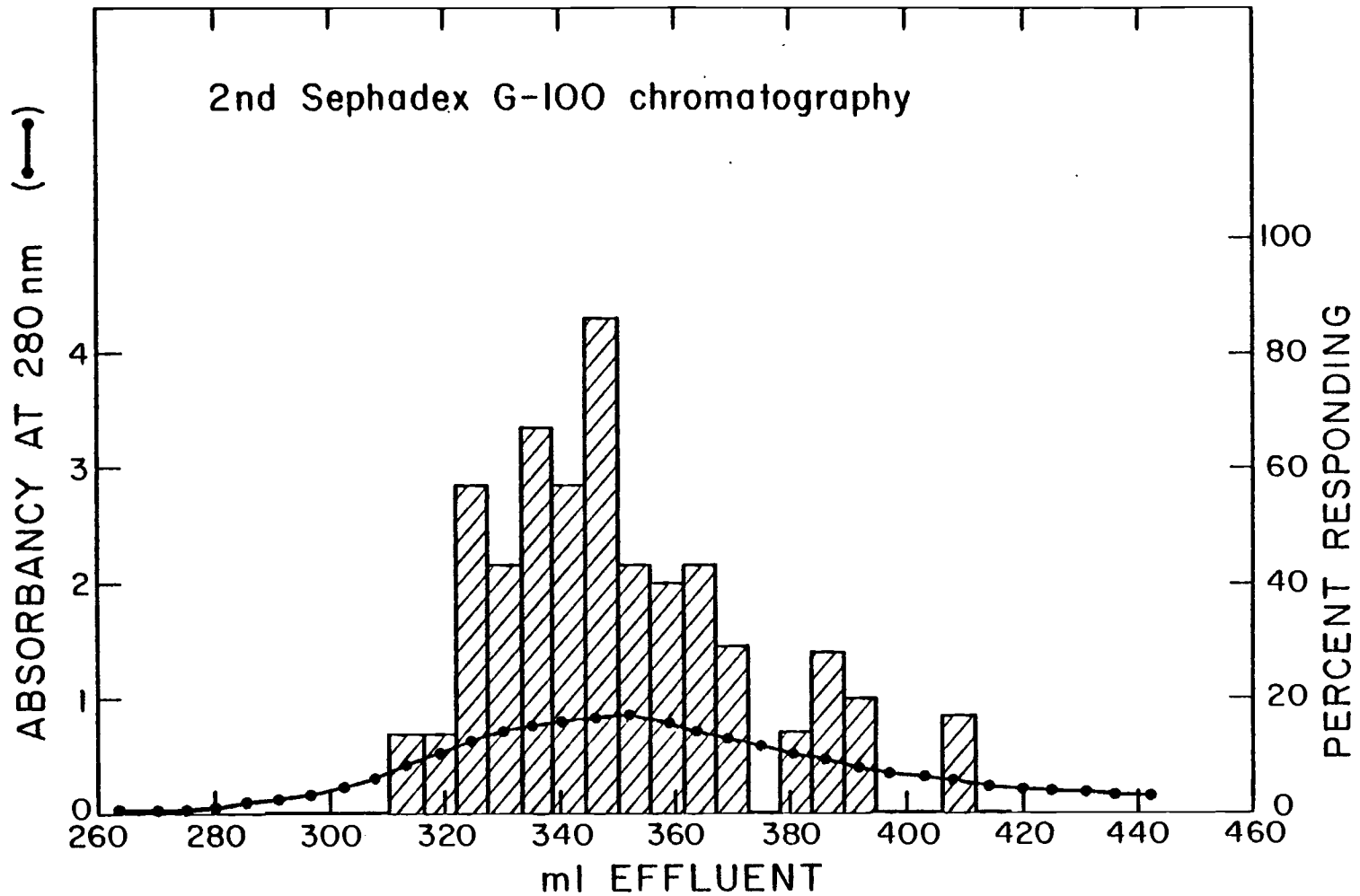
Fig. 7   2nd Sephadex G-100 chromatography.   Concentrated fractions
         from chromatography of "crude PTTH" (Fig. 5) rechromatographed
         on the same column; fraction size = 5.7 ml.

only in the 0.1M NaCl wash (Fig. 8); furthermore, it was
revealed that a substantial amount of inactive material
remained bound to the column.

Subsequent columns were developed with linear gradients
of NaCl in 0.01 M Tris-HCl, pH 7.9. One such activity elu-
tion profile is shown in Fig. 9. Although insufficient
numbers of bioassay animals precluded assaying every fraction
in the second half of the gradient, it can be seen that
nearly all activity is eluted below 0.11 M NaCl. Again,
because of the qualitative nature of the bioassay when
injecting seven to ten animals per fraction, the apparent
peaks of activity cannot safely be interpreted as evidence
of heterogeneity of PTTH activity.

Hydroxylapatite chromatography

As outlined in Methods it was possible to apply material
directly to Bio-Gel HTP without prior removal of salt.
Under these conditions approximately 40% of the total protein
was not bound by the resin, while all the PTTH activity was
bound. Activity could then be eluted with low concentrations
of phosphate in linear gradients, as shown in Fig. 10.
Material eluting in 5 mM phosphate contained PTTH activity
and was "loosely" bound. The remaining PTTH activity was
eluted between 0.02M and 0.08M sodium phosphate. As deter-
mined by co-migration on SDS gels and on Davis gels, the
material eluting with low phosphate and with 0.02 to 0.08 M
sodium phosphate contained several common proteins, includ-
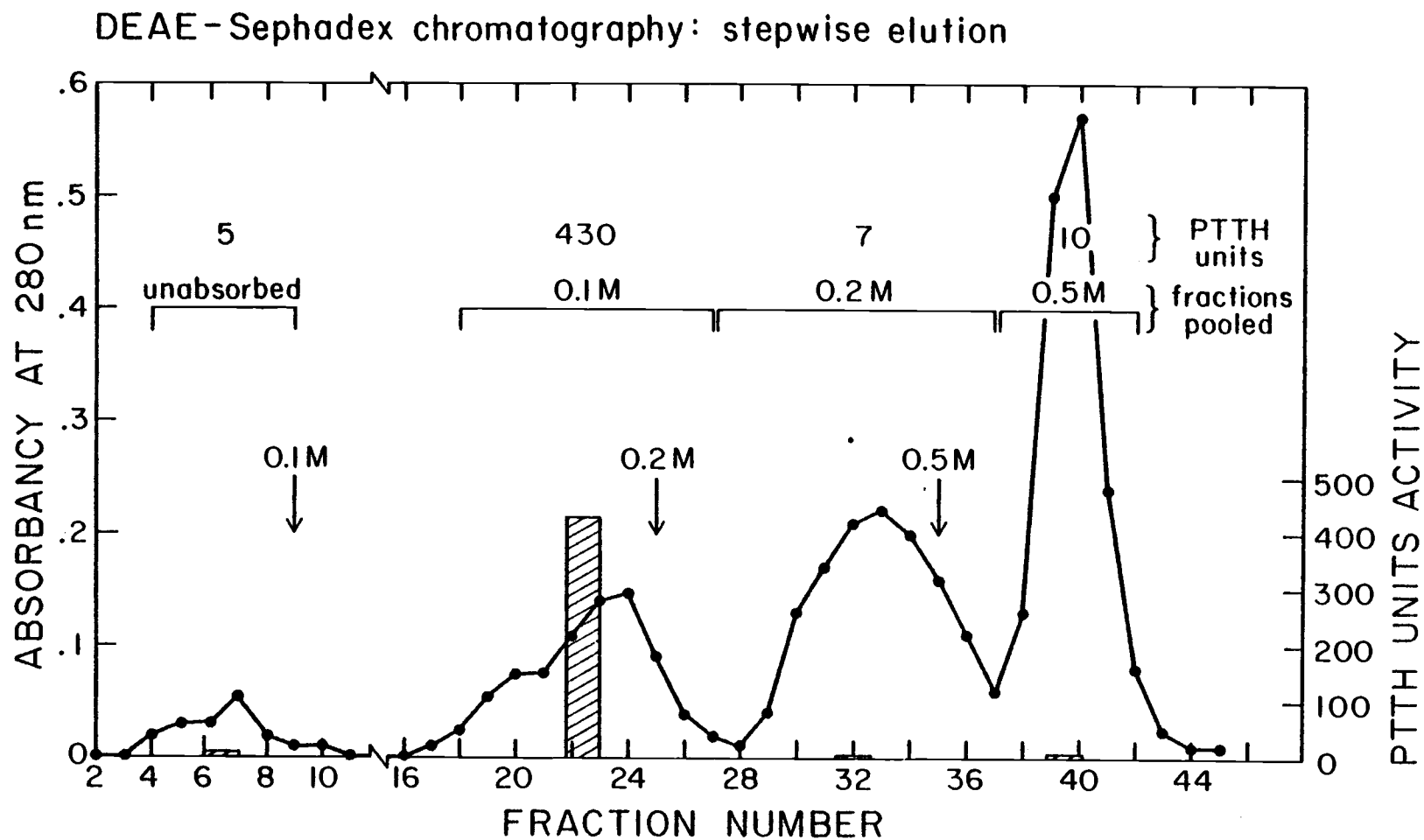
Fig. 8 DEAE-Sephadex chromatography: stepwise elution. Active fractions from
G-100 column adsorbed to DEAE-Sephadex in 0.01 M Tris-HCl, pH 7.9 and
eluted with steps of 0.1 M, 0.2 M, and 0.5 M NaCl in 0.01 M Tris-HCl,
pH 7.9.
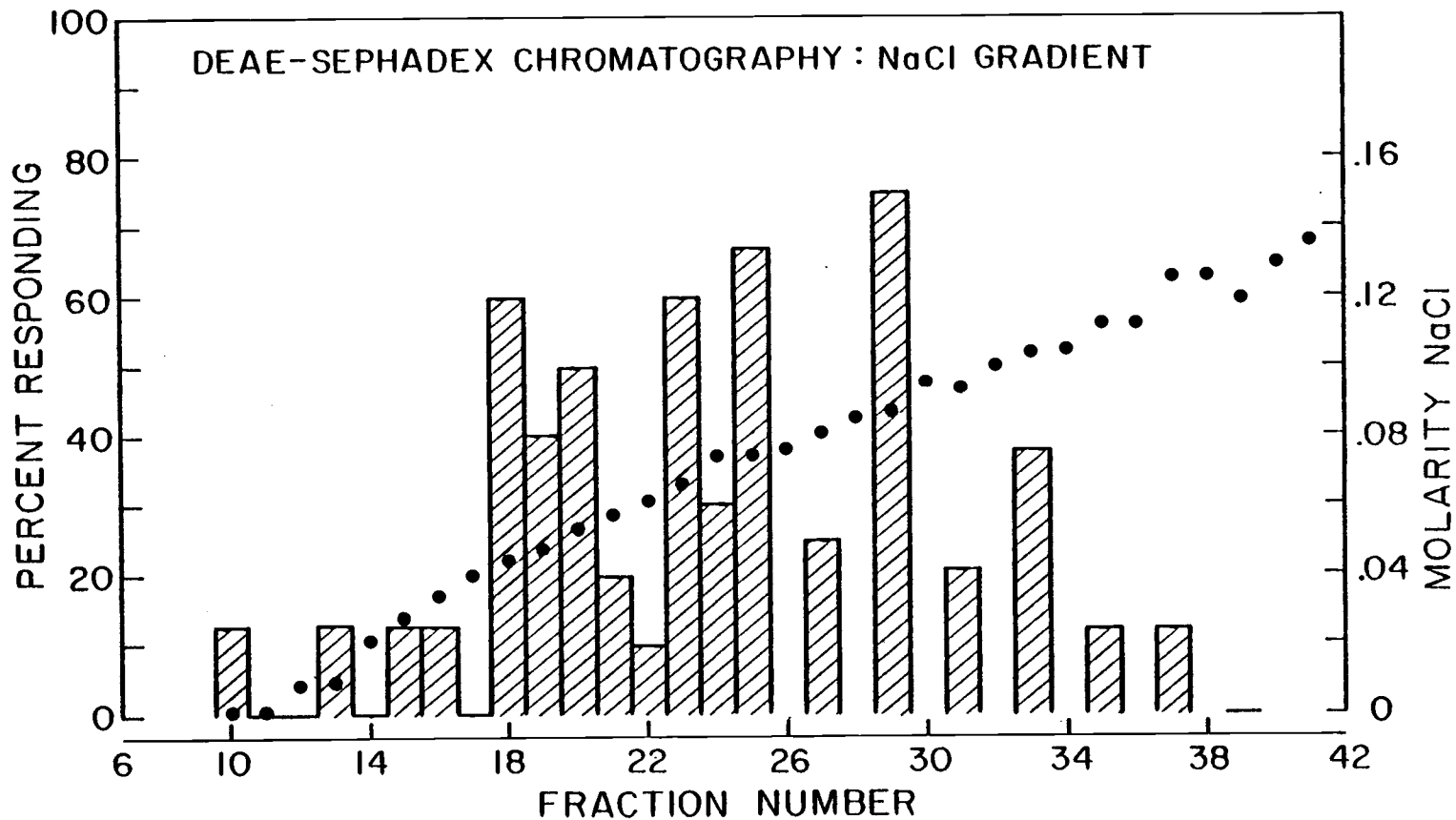
Fig. 9 DEAE-Sephadex chromatography: NaCl gradient elution. Column was
eluted with a linear gradient of 0 - 0.2 M NaCl in 0.01 M Tris-
HCl, pH 7.9.
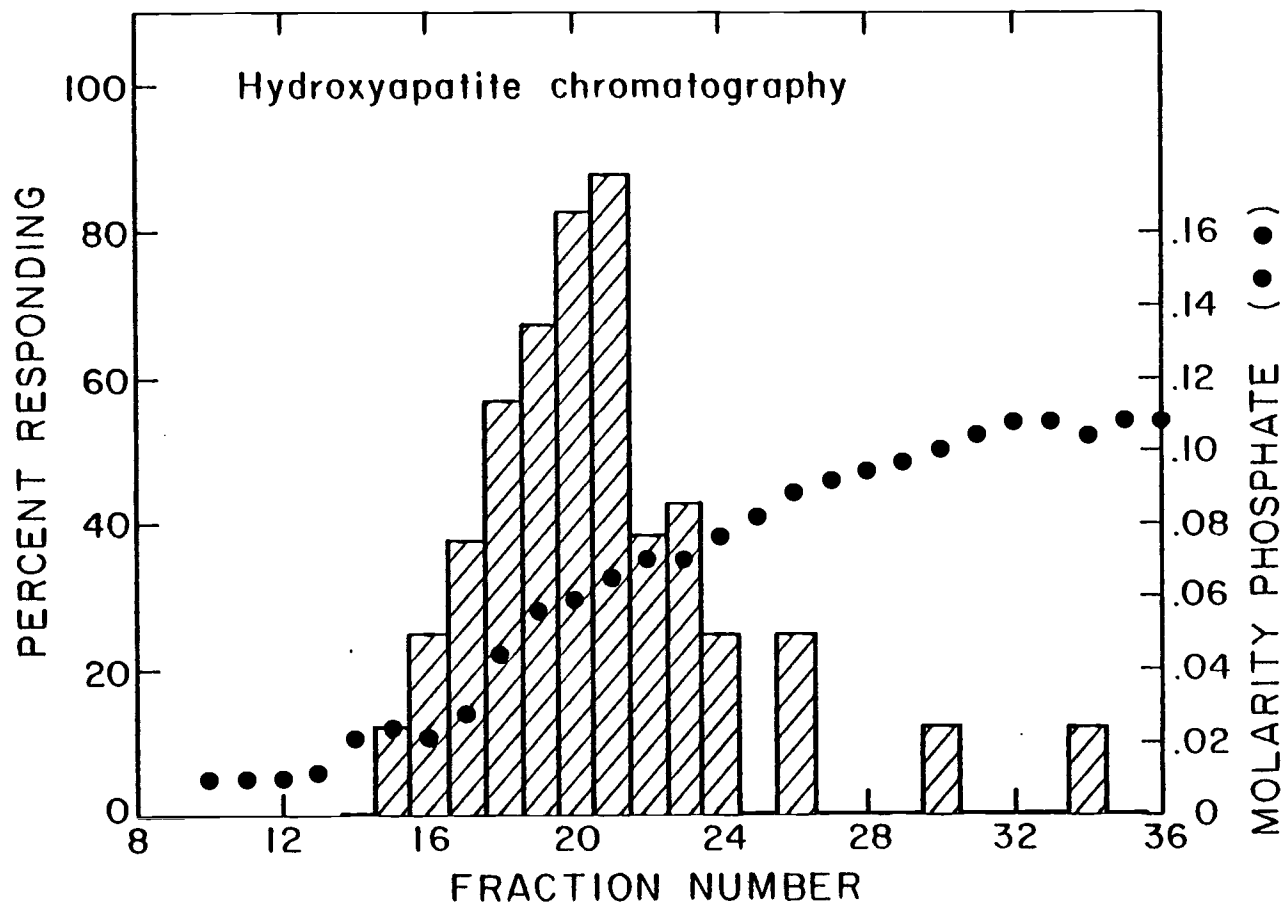
Fig. 10   Hydroxylapatite chromatography of active DEAE-Sephadex fractions. Active material directly adsorbed from DEAE column, eluted with a linear gradient of 0 - 0.15 M sodium phosphate, pH 6.8.

ing a major band at 25,000 Daltons on SDS gels. A summary
of the purification achieved through the hydroxylapatite
step is shown in Table 3. It will be noted that estimates
of activity recovery include averages when more than one
determination was made. In addition, percent recoveries
are normalized to activity in the 30-65% acetone precipitate
which was used to construct the dose response curve (Fig. 3).

Isoelectric focusing

A preliminary isoelectric focusing (IEF) experiment
with LKB 3-10 Ampholines of a preparation carried through
the Sephadex G-100 step showed PTTH activity occurring in
two adjacent fractions (one cm zones on a 20 cm plate) in
the pH range 5.7-6.5. No activity could be detected outside
of this zone. Hence, further uses of IEF were done with
narrow range ampholytes alone (Pharmalyte 5-8, Bio-Lyte
5/7) or in 60:40 or 80:20 combinations with broad range
ampholytes (Bio-Lyte 3/10).

Active material from hydroxylapatite was pooled and
subjected to IEF on flat beds of Sephadex G-200 SF; the
resulting activity and pH profiles are shown in Fig. 11. A
consistent observation was the separation of activity into
two zones. When focusing times were extended to eight
hours the approximate centers of the two zones were pH 5.25
and pH 5.50.

An unfortunate but equally consistent observation
was the spreading of activity across several fractions in

Table 3.   Purification of PTTH

sample	N^a	percent response (H.E.)	PTTH units[b] / H.E.	μg protein / H.E.	PTTH units / μg protein	percent[c] recovery
crude extract	33	6 (.125)	1.36	2230	.0006	42
heated fraction	32	12.5 (.125)	2.02	645	.0031	63
30-65% ammonium sulfate	24	42 (.45)	3.49	157	.0222	109
30-65%						
acetone - 1	22	33 (.46)	1.93			
acetone - 2	24	25 (.12)	4.70			
acetone - 3	31	39 (.42)	3.00			
			ave. 3.21	84.5	.0378	100
G-100-1	33	33 (.42)	2.17			
G-100-2	32	44 (1.0)	1.74			
			ave. 1.96	24.7	.0784	61
DEAE-Sephadex-1	32	56 (1.0)	4.35			
DEAE-Sephadex-2	35	40 (1.0)	1.35			
			ave. 2.85	5.7	.4914	89
hydroxylapatite	33	61 (1.0)	6.3	2.4	2.63	196

[a]N = number of larvae injected per test.

[b]Units of activity were interpolated from the 30-65% acetone precipitate dose response curve; H.E. = head equivalents.

[c]Percent recoveries are normalized to activity in the 30-65% acetone precipitate.
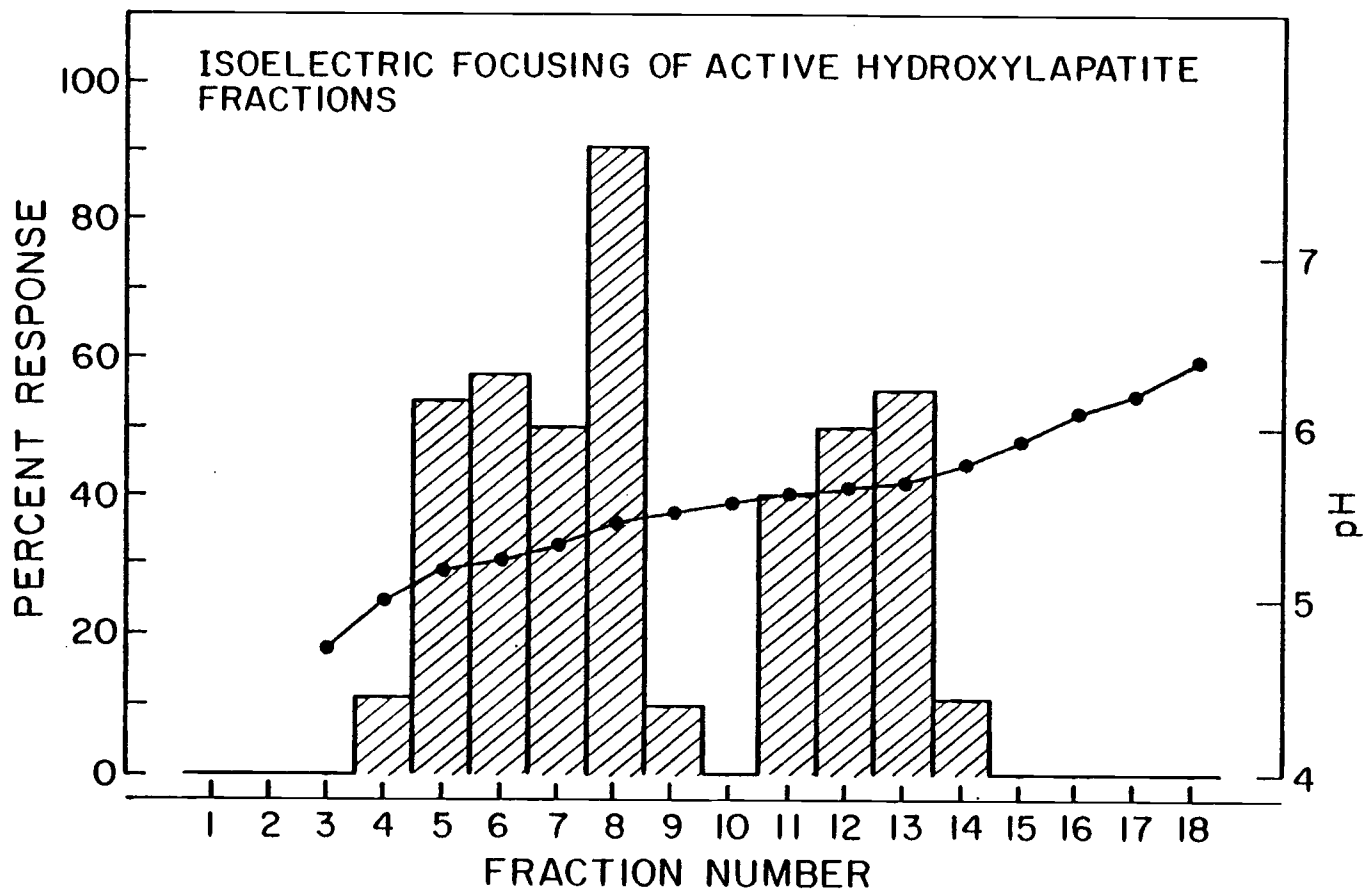
Fig. 11 Isoelectric focusing (IEF) of active hydroxylapatite fractions. IEF
done on thin layers of Sephadex G-200 SF with Biolyte 5-7 : Biolyte
3-10 (60 : 40). Fraction size = 0.5 cm on a 10 cm(w) x 20 cm(l) plate.

each zone which prevented resolution of individual compo-
nents in the mixture. When aliquots of fractions are elec-
trophoresed in SDS gels and non-denaturing gels, the in-
complete resolution of polypeptides is additionally apparent
(Fig. 12). It is clear from the SDS gel that a 25,000
Dalton peptide is the major component. However, it is
difficult to conclude that individual species are being
resolved in this molecular weight zone; a continuum from
acidic to basic fractions is broken only by a depletion
of staining material in fractions nine and ten. The non-
denaturing gel does reveal heterogeneity, and the basis
(though not the cause) of the heterogeneity becomes apparent.
"Band 1" of the three major components appears to be the
most basic, while "Band 2" and "Band 3" appear to be of
roughly equal acidity, both running closer to the anode
than "Band 1". The presence of unexpectedly large amounts
of "Band 2" in the richest "Band 1" fractions suggests that
individual species are not focusing completely independently
of one·another. This interpretation is reinforced by the
pattern of staining polypeptides in an acrylamide IEF (Fig.
13). Here "Band 1" is nearly completely resolved from the
more acidic fractions; however, it still contains signifi-
cant amounts of "Bands 2" and "3".

    A second problem not yet adequately addressed has been
the effect of ampholytes on bioassayable activity. A persis-
tent though qualitative observation has been the loss of
substantial amounts of activity upon electrofocusing; samples
are generally assayed without prior removal of ampholyte.

(a)　　　　　　　　　　　　　　　　(b)
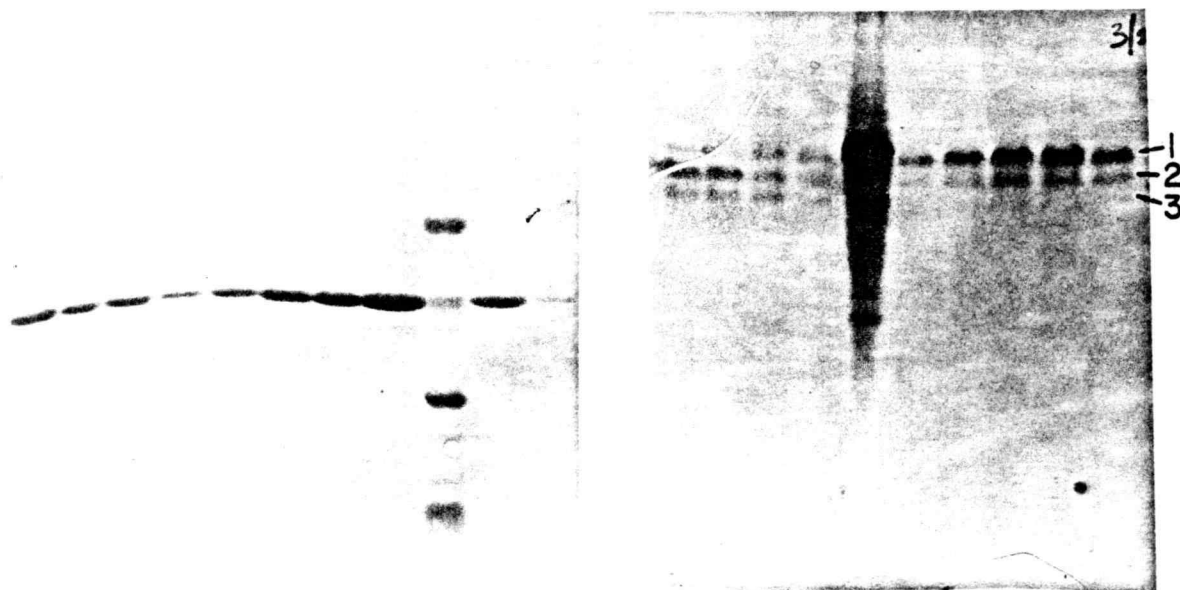
Fig. 12　Electrophoresis of proteins eluted following
IEF in thin layers of Sephadex G200 SF.　(a) SDS gel,
(b) nondenaturing gel.　IEF conditions:　see legend in
Fig. 13. Staining, CBB G-250.

(a)　　　　　　　　　　　　　　　　(b)

5.23　5.32　5.38　5.50　　　　　5.23　5.32　5.38　5.50
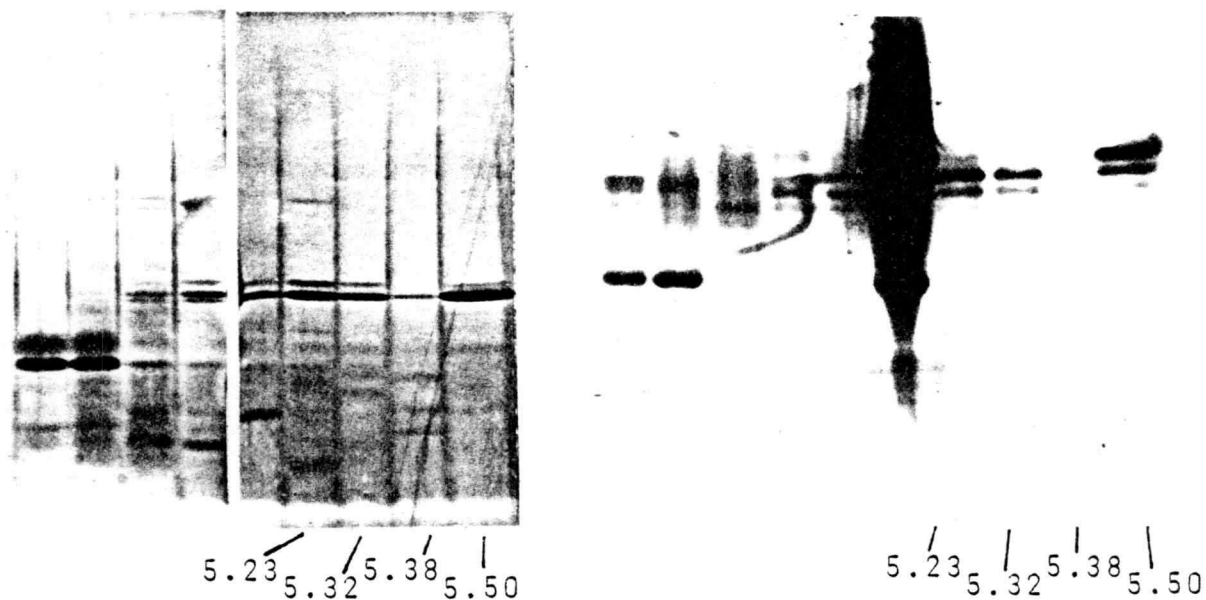
Fig. 13　Electrophoresis of proteins from polyacrylamide
IEF (according to Graesslin, et al., 1976). 2% total
ampholyte: Biolyte 5-7:Biolyte 3-10, 60:40 (a) SDS gel,
(b) non-denaturing gel.　Staining, silver stain.

That Pharmalyte 5-8 ampholytes bind avidly to focusing
material was demonstrated by their failure to dialyze or
to desalt on Sephadex G-50 F in 0.01 M Tris/1 M NaCl, pH
7.2 (bed dimensions: 1 x 90 cm); only ammonium sulfate
precipitation (70% saturation) was effective in separating
protein from Pharmalyte (protein precipitates while Pharma-
lyte remains soluble). This separation could be followed
because Pharmalytes are readily visualized in Coomassie
Brilliant Blue G-250 stained SDS gels. Bio-Lytes did not
comparably stain and hence their association with protein
was less readily assessed. If ampholytes do associate with
PTTH, they would be expected to alter the focusing of activ-
ity, perhaps accounting for the observed incomplete resolu-
tion (Figs. 12 and 13). In such an event, the addition of
non-ionic detergents or urea to the focusing system might
be necessary. In a preliminary experiment is has been deter-
mined that biological activity could be recovered from PTTH
samples containing 6M urea by extensive dialysis.

Preparative electrophoresis

Further attempts to identify PTTH activity involved
excising individual bands from non-denaturing polyacrylamide
gels and bioassaying the eluted material directly.

A preliminary experiment in which five adjacent strips
(numbers 1-5, from least mobile to most mobile stainable
band) were cut from the gel revealed activity only in strip
1; upon electrophoresis of aliquots of material eluted from
the five strips, strip 1 was shown to be homogeneous in the

least mobile band. However, a small amount of this band
contaminated the next more mobile band eluted from strip 2,
and yet no activity was present. This experiment was
repeated with a longer run time (3-1/2 hours/150V), and a
one mm strip cut between strip 1 and strip 2 was discarded.
Activity was tested in material eluted from no. 1 and no. 2,
and the strip cut just above no. 1 (no. 0). The results of
activity measurements and photographs of gels identifying
eluted polypeptides are shown in Fig. 14. Bands no. 1 and
no. 2 are now cleanly separated from each other and appear
to be homogeneous. Activity was recovered only in Band no.
1. On the SDS gel, this band comigrates with α-chymo-
trypsinogen (25,000 Daltons). In Fig. 13, it can be seen
that the majority of this peptide focuses at pH 5.5. While
these results appear to identify PTTH it must be cautioned
that this result is preliminary, and that its confirmation
awaits _in vivo_ and _in vitro_ specific activity measurements
with material freed of contaminants eluting from the poly-
acrylamide gel (e.g., unpolymerized acrylamide monomer). In
addition, it will be necessary to confirm the pI of purified
material by isoelectric focusing in a high resolution analy-
tical IEF system.

In vitro activation of prothoracic glands

    Preliminary experiments have been carried out in an
attempt to confirm the specificity of the substance puri-
fied in these studies as a prothoraciatropic hormone. A

band number	percent response	N
0	0	27
1	21	28
2	0	27

Band → number

HA 0 1 2   HA 0 1 2
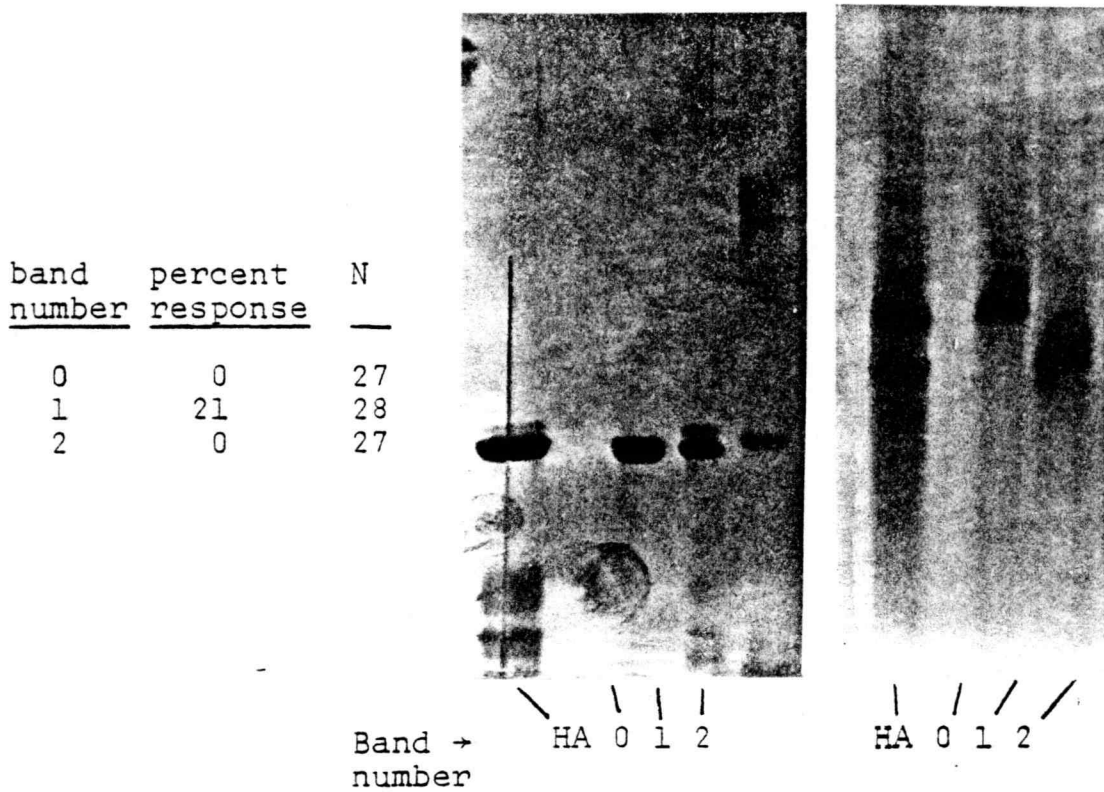
Fig. 14 Electrophoresis and activities of proteins eluted from preparative polyacrylamide gel of hydroxyl-apatite (HA) purified material. Bands 0,1, and 2 obtained by eluting strips cut from preparative gels; band 0 strip cut just above band 1 (see text).

simplified prothoracic gland culture system (Bollenbacher, et al., 1979) was used to check the activity in vitro of polypeptides purified by isoelectric focusing and preparative polyacrylamide gel electrophoresis that had previously demonstrated activity in vivo. The synthesis of right and left glands was compared and found to be 9.00 ± .82 ng/ gland/ 6 hr (left) and 10.25 ± 2.06 ng/gland/6 hr (right); thus synthesis by right and left glands should differ by no more than 12%, confirming Bollenbacher's findings (Bollenbacher, et al., 1979).

Prothoracic glands activated with long day reared (16L:8D) pupal brain homogenates demonstrate an increase in ecdysone synthesis. A_R's are, however, approximately 50-60% those reported by Bollenbacher, et al. (1979). Only two preparations from purified material demonstrate comparable A_R's, IEF 5.25 and PrepPAGE "Band 1." When both are compared to PrepPAGE "Band 3" only IEF 5.25 is significantly different (P < .1). These results are shown in Table 4. It is clear that with the present application of this culture system, many more determinations would be necessary to demonstrate statistical significance for increased rates of ecdysone synthesis. Nevertheless, because purified substances were shown to directly affect prothoracic gland activity, the present results strongly suggest that the material purified in these studies is a physiological prothoraciotropic hormone.

Table 4.   In vitro activity of IEF and PAGE purified
            polypeptides

sample	N	A_R
pupal brain		
.5 B.E.	5	2.65 ± .47
.1 B.E.	5	1.91 ± .47
IEF		
pH 5.25	10	2.34 ± .41
pH 5.5	5	1.61 ± .24
Prep PAGE		
"Band 1"	7	2.11 ± .67
"Band 2"	6	1.24 ± .19
"Band 3"	8	1.46 ± .36
"Band 4"	3	1.36 ± .30
"Band 5"	4	1.43 ± .40

A_R = activation ratio, synthesis of activated/control
glands (±SEM). One µl aliquots were added to test gland
cultures from material eluted from IEF plates or PAGE
bands. Day 0 non-diapausing pupal brains were extracted
with .1M Tris-HCl, pH 6.8, heated in a boiling water bath
for two minutes, and centrifuged to remove insoluble
material.

Enzymatic digestion

The susceptibility of PTTH activity to proteolytic enzymes was tested with α-chymotrypsin and proteinase K. Both enzymes appear to completely destroy activity: while 50% of the controls (enzyme absent) responded (N=14) when injected with 2.5 μg protein purified through hydroxylapatite chromatography, none of the test animals showed spiracle apolysis at 24 hours (N=14, α-chymotrypsin; N=12, proteinase K). This test demonstrates that PTTH contains peptide bonds necessary for activity.

Comments on the purification

As new methods for the purification were developed, possible refinements in methods already in use became apparent. These were not always tested because of limited time and/or synchronous insects for use in the bioassay. These can be mentioned here as "prime" areas for improving the purification.

One possible improvement has already been mentioned: narrowing the ammonium sulfate precipitation zone. Most of the purification associated with this step results from material left soluble at 65% saturation (the 30% ammonium sulfate pellet was small). It is conceivable that this could be reduced to 60% or lower without loss of activity.

It is possible that improvement can also be made in the acetone fractionation step. The 40% acetone precipitate contains 4.63 PTTH units/H.E (Table 2). This is nearly all (within the quantitative limitations of the bioassay) of

the activity present in the starting material (4.77 units/ H.E. in "pooled 2nd ppt.", Table 1). Thus, if the acetone upper limits can be brought down from 65% some additional improvement in the specific activity (on a protein basis) of "crude PTTH" can be expected.

Reducing the dry weight of "crude PTTH" to be loaded on the G-100 column might improve the first gel filtration and perhaps make the second column unnecessary. The loading of pooled G-100 material onto the DEAE-Sephadex column could perhaps be done directly or after dilution with water or low concentration Tris buffer, thus simplifying the procedure and eliminating any losses associated with the manipulations required for concentrating the sample.

The isoelectric focusing protocol can almost certainly be improved as evidenced by the apparent failure of polypeptides to focus independently of one another (Figs. 12 and 13). This deficiency may result from association of proteins with each other or with ampholyte. In either event it might be possible to eliminate such interactions by the inclusion of a non-ionic detergent and/or urea. This course was not originally pursued because of possible difficulties in recovering biological activity associated with subsequent renaturation procedures.

Very promising is the use of preparative polyacrylamide gel electrophoresis. Preliminary experiments indicate resolution superior to that obtained with IEF as used in the present studies. The very similar mobilities of the polypeptides to be resolved (Figs. 12-14) requires long

gels and extended running times. It was found that limita-
tions in resolution were partially associated with "trailing"
of bands, contaminating those of lesser mobilities. This
situation was improved by using low strength electrode
buffer (12.5 mM Tris - 96 mM glycine, pH 8.3); whether addi-
tional improvement could be obtained by having more buffer-
ing capacity in the anode chamber (Albin, 1974) was not
investigated.

For completeness it can be mentioned here that a cation-
exchange resin (SP-Sephadex) was tested and found not to
improve significantly the purification of the most highly
purified preps. In addition, an affinity resin for glyco-
proteins, concanavalin A-Sepharose (Pharmacia), was tested
because of a report that Bombyx PTTH contained 15% glucose
equivalent (Yamazaki and Kobayashi, 1969) but was found not
to bind Manduca PTTH activity.

Discussion

Previous studies on the purification of the insect
prothoracicotropic hormone (PTTH) have been carried out
on the commercial silkworm, Bombyx mori (Kobayashi and
Yamazaki, 1966; Ishizaki and Ichikawa, 1967; Yamazaki and
Kobayashi, 1969; Nishiitsutsuji-Uwo, 1972; Nagasawa, et
al., 1979). Comparisons of the silkworm hormone and the
tobacco hornworm PTTH await their purifications to homo-
geneity in sufficient quantity to allow chemical character-

ization. However, information obtained in the present study allows unambiguous comparison of selected properties of the two hormones.

Most notable amongst similarities is the heat stability of the two hormones. Heat treatment as the initial step in the purification of Manduca PTTH allowed a 3.5 fold purification (but, see below) and almost certainly removes many proteases that could be troublesome later in the purification.

PTTH activity can be salted out of solution between 30 and 65% ammonium sulfate (Table 1) which is similar to that used by Suzuki (Suzuki, et al., 1975). These same workers found PTTH activity to be soluble in 50% acetone, while Manduca PTTH is nearly completely precipitated by 40% acetone (Table 2).

Sephadex G-100 chromatography indicates an approximate molecular weight of 25,000 Daltons for Manduca PTTH. While this estimate is based on the behavior of "crude PTTH" (Figs. 6 and 7) corroborative evidence comes from the SDS gel electrophoretic patterns of material purified by IEF and preparative electrophoresis (Figs. 12-14). In addition, in a single experiment, PTTH activity was recovered from an SDS gel in a region co-migrating with α-chymotrypsinogen (25,000 Daltons) by a method of removal of SDS from protein by ion-pair extraction (Henderson, et al., 1979; data not shown). Molecular weight estimates of Bombyx mori PTTH vary and include 4,400 (Nagasawa, et al., 1979), 9,000-31,000 (three activities; Ishizaki and Ishikawa, 1967), and 20,000 (Yama-

zaki and Kobayashi, 1969). Ishizaki and Ishikawa's measure-
ments seem to have been done under suboptimal chromatograph-
ic conditions, and unfortunately, no activity profiles are
included with the UV absorbance profiles of their highly
purified material. Yamazaki and Kobayashi's estimate agrees
quite closely with that obtained in the present studies;
however, other chromatographic properties indicate that it
is a different molecule (see below). Nagasawa's estimate
(4,400) is in agreement with Nishiitsutsuji-Nuo's conclusion
(1972) that some active species of 5,000 Daltons exists.
Lack of chromatographic data make evaluation of the latter
conclusion difficult; however, she does report that approxi-
mately one-half the activity is dialyzable. In experiments
reported here, activity was not dialyzable, and it was
completely retained by an Amicon UM-10 ultrafiltration
membrane. Results from a Sephadex G-75 column show that
activity cannot be found outside of the region shown in
Figs. 6 and 7. The combined observations indicate that a
25,000 Dalton polypeptide is responsible for the majority
of PTTH activity in Manduca sexta.

Isoelectric focusing demonstrates PTTH to be an acidic
polypeptide, with a pI of 5.25-5.50. In this regard the
Bombyx hormone appears to be distinct (pI = 8.35-8.65;
Yamazaki and Kobayashi, 1969); however, Ishikawa and Ishi-
zaki's (1967) and Nagasawa's (Nagasawa, et al., 1979)
ion exchange chromatography results would seem to indi-
cate a more acidic pI for the Bombyx hormone.

The use of whole heads as starting material apparently
results in the extraction of substances masking PTTH activ-
ity that are removed throughout the purification. This is
manifested by the apparent increase in recovery of activity
at certain steps in the purification (Table 3). Because of
the very low levels of activity detected in the crude
extract and the heated fraction, recoveries were normalized
to the 30-65% AP, with which dose response curves were
constructed.

Measurements of interspecific activity indicate a bio-
logical relatedness between Bombyx (Lepidoptera:Bombycidae)
and Samia cynthia ricini (Lepidoptera:Saturniidae) (Ishizaki
and Ishikawa, 1967). In addition Gersch and Sturzebecher
(1968) found water soluble substances in the brain of Peri-
planeta americana (Orthoptera:Blattidae) that were active
in eliciting development in the 'dauer' pupae of Antheraea
pernyi (Lepidoptera:Saturniidae). However, Safranek and
Williams (1980) found no activity in highly purified Bombyx
mori PTTH with Manduca bioassays. Although some functional
homology in Lepidopteran PTTH's exists, the extent is still
an open question that will probably not be answered without
their purification and chemical characterization.

CYCLIC NUCLEOTIDES IN THE CENTRAL NERVOUS
SYSTEM DURING PRE-METAMORPHIC
ENDOCRINE ACTIVITY IN MANDUCA SEXTA

Introduction

The discovery and characterization of adenosine-3':
5'-mono phosphoric acid (cyclic AMP or cAMP) by Rall and
Sutherland (1958), the demonstrations of its norepinephrine
and glucagon responsiveness in liver and its mediation of
active phosphorylase accumulation in liver and heart (Suther-
land and Rall, 1958) heralded the central role that cyclic
nucleotides would take in research on the mechanisms of
action of neurotransmitters and hormones. This research
led to the formulation of the second messenger hypothesis
(Robison, et al., 1971), now well established for a number of
circulating hormones. Research continues unabated to the
present day, requiring annual series[1] just for the publi-
cation of review articles. Recent reviews emphasizing the
roles of cyclic nucleotides in nervous system function have
appeared (Nathanson, 1977; Standaert, 1979). Additionally,
Taylor has reviewed the evidence for participation by cyclic
nucleotides in invertebrate neurotransmitter actions (Taylor,
1977).

The studies reported here were undertaken as an attempt
to identify neurochemical correlates of behavioral processes.
Late in the fifth instar larvae Manduca undergoes a profound
change in behavior: feeding ceases, the gut is purged, the

[1]Advances in Cyclic Nucleotide Research, Raven Press.

dorsal vessel is exposed, and wandering activity is initiated.
It was reasoned that since the change from feeding to wandering
behavior came at the time of PTTH and ecdysone secretion
(Truman and Riddiford, 1974; Bollenbacher, et al., 1975), it
might be possible to correlate this change in behavior with
changes in CNS cyclic nucleotide content. Effects of ecdysone
on CNS interneuron and motor neuron activity have been reported
in the desert locust (Haskell and Moorhouse, 1963). Also, the
pre-eclosion behavior in silkmoths has been reported to be
hormonally triggered and mediated through an increase in cyclic
GMP (Truman et al., 1976; Truman et al., 1979). If an adequate
number of neural elements responded to this releaser effect of
hormones (Truman and Riddiford, 1974b) during the change in
behavior, it might be that the molecular substrates of this
change could be detected above the background of non-partici-
pating elements. If this approach was successful, it was
felt that it could aid in identifying the neural substrates
of this behavioral change, a very exciting prospect. Hence,
a survey of cyclic nucleotide content in the central nervous
system during the feeding to wandering transition was under-
taken.

Materials and Methods

Materials

Reagents obtained from Sigma for antisera preparation included $O^{2'}$-monosuccinyl cAMP, $O^{2'}$-monosuccinyl cGMP (sodium salt), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC), and human serum albumin. Nucleotides for testing the specificity of antisera included cyclic AMP, 3'AMP, 5'AMP, ADP, ATP, cyclic GMP, 3'GMP, GDP, and GTP were obtained from Sigma. Tyrosine methyl ester and ethylchloroformate for RIA tracer preparation were obtained from Sigma and Aldrich, respectively. ^{3}H cAMP and ^{3}H cGMP were obtained from New England Nuclear. ^{125}I-was obtained from Amersham-Searle and New England Nuclear. Chloramine T was from Sigma. Salts and buffers were reagent grade obtained from Mallinckrodt and J.T. Baker.

Preparation of Cyclic Nucleotide Antisera

Cyclic AMP and cyclic GMP antigens were prepared in identical manners, essentially according to Steiner's methods (Steiner, et al., 1969; Steiner, 1974). The succinyl-cyclic-nucleotides (ScNMP) were coupled to human serum albumin (HSA) by a two-fold molar excess of EDC in aqueous solution at pH 5.5. After exhaustive dialysis against a phosphosaline buffer (0.01M sodium phosphate, 0.15M sodium chloride, pH 7.4) the haptene/carrier multiplicity was calculated from the increases in the optical density at 258 nm assuming

that covalently bound ScNMP's are responsible

for any such increase. Using extinction coefficients of

14.65×10^3 l/m-cm (cAMP) and 13.7×10^3 l/m-cm (cGMP)

(Sober, 1968) and the measured optical density of a 1 mg/ml

solution of HSA, the multiplicities were calculated at 4.1

haptenes/carrier for the HSA-ScAMP and 11.2 for the HSA-

ScGMP conjugates.

Immunizations and bleedings were carried out by Dr. John

Patt in our laboratory. Three New Zealand White rabbits were

immunized, two with the HSA-ScAMP conjugate and one with the

HSA-ScGMP conjugate. One mg of the lyophilized conjugate was

suspended in 0.5 ml phosphosaline buffer, pH 7.40. This

solution was emulsified with 0.5 ml Freund's complete adjuvant

by vigorous shaking for three-five minutes, and 0.25 ml of the

emulsified antigen was injected into each footpad. Booster

injections of one mg conjugate in buffered Freund's incomplete

adjuvant were administered one month later. Bleeding was done

by venipuncture of the lateral ear vein and was continued at

one or two week intervals for four months. The collected blood

(up to 30 ml/bleeding) was allowed to clot, then the serum was

separated from cells by centrifugation. The serum was made

1:5000 in a 0.1% tincture of merthiolate and stored frozen

at -15°C.

Antisera Characterization

Cyclic nucleotide antisera were titered by measuring the

binding of a fixed amount of ^{3}H or ^{125}I cyclic nucleotide

by a series of different dilutions of the appropriate anti-

serum. The titer is expressed as the reciprocal of the dilution necessary to result in bound/free (B/F) = 1 when labeled antigen is present in trace amounts. When a stringent set of assumptions are met, the "least detectable dose" (Feldman and Rodbard, 1971) is minimized by setting B/F = 1. The functional significance of the mathematical theory for the present purpose is that the assay is most useful when the antiserum dilution binds 50% of labeled antigen ($*A$) and yet remains in the linear portion of the $*A$ titration curve. In the experiments reported here the titration curves for different antisera became non-linear at various percent bound $*A$ ranging from 40-80%. The basis for this phenomenon is not known, but the behavior is as if sera contain varying concentrations of binding inhibitors. Hence the titers listed will include the percent bound $*A$ at that titer.

Five bleedings from each of three rabbits resulted in ten cAMP and five cGMP antisera. These antisera were given code designations according to the cyclic nucleotide (A or G), the rabbit number for cAMP antisera (39 or 40), and the number in a sequence of bleedings. Hence A40-1 designates cyclic AMP antiserum, rabbit no. 40, first bleeding; G-2 designates cyclic GMP antiserum, second bleeding. Initially titered against the tritiated cyclic nucleotides were the five cGMP and one of the cAMP antisera.

Subsequent characterizations and radioimmunoassays were done with the G-2 and A40-1 antisera. Standard nucleotide

solutions of cAMP, cGMP, and homologous non-cyclic nucleotide mono-, di-, and triphosphates were made up using the extinction coefficients given above for cAMP and cGMP. The specificities of the antisera were then checked by the ability of different nucleotides to compete with the ^{3}H or ^{125}I tracer for its appropriate antiserum. The degree of specificity or "cross-reactivity" is expressed as the ratio (cNMP)/(competitor nucleotide) in percent when cNMP and competitor nucleotide concentrations are those necessary to inhibit tracer binding by 50%.

Cyclic Nucleotide Assays

Tissues were extracted for cyclic nucleotides essentially as described by Steiner, et al. (1969). Whole CNS were homogenized with 150 µl 10% trichloroacetic acid (TCA) in an all-glass tissue homogenizer. The homogenate and a 50 µl wash of the homogenizer pestle were pooled in a 6 x 50 mm test tube and centrifuged at 6000 RPM for 10 min in an SS-34 rotor. The pellet was washed once with 50 µl 10% TCA and the pooled supernatant material was extracted 5X with 500 µl cold, water-saturated diethylether to remove TCA. The remaining water was evaporated under a stream of nitrogen and the residues stored dry at -15°C or taken up in 0.05M sodium acetate, pH 6.2 for immediate assay.

Cyclic nucleotide assays were initially those described for ecdysone radioimmunoassays (Borst and O'Connor, 1974), except that the incubation buffer (0.05M sodium acetate, pH 6.2) was as described by Steiner (1974). Duplicate samples

or standards were pipetted into 6 x 50 mm test tubes. To these were added 50 µl ^{3}HcAMP or ^{3}HcGMP (approximately 4000 CPM) in RIA buffer followed by 100 µl cyclic nucleotide antiserum in RIA buffer containing 20% normal rabbit serum. Assay tubes were incubated for 15 hours at 4°C. Separation of bound from free nucleotide was accomplished by adding 200 µl saturated ammonium sulfate with immediate mixing with a vortex homogenizer. Tubes were allowed to sit at 4°C for 20-30 minutes and then centrifuged at 9000 RPM for 10 minutes in an SS-34 rotor. The supernatant was aspirated from the pellet which then was washed once with 400 µl 50% saturated ammonium sulfate in RIA buffer, followed by an additional 15 minutes at 4°C prior to centrifugation. The washed pellet was resuspended in 25 µl distilled water, and 600 µl of a scintillation solution (Handifluor, Mallincknodt) was added directly to the assay tube. The tubes were mixed, placed in 20 ml glass scintillation counting vials, and equilibrated for 24 hours prior to counting to allow the protein to settle out. During this time counting efficiency rose to a constant level of 36%.

The radioactivity was determined using a Packard Model 3330 Liquid Scintillation Spectrometer and analyzed using a Hewlett-Packard model 9821A desk-top calculator interfaced to an H-P model 2748B Tape Reader and a Packard Model 546 teletypewriter. The program utilized the "logit transformation" (Rodbard et al., 1969) for linearizing sigmoidal dose response curves and contained statistical analysis of data including

determinations of coefficient of variation (of duplicates)
and variances of constants determined by linear regression
analysis.

Recovery of extracted cyclic nucleotides was checked by
adding ^{3}H-cAMP immediately before extraction and found to be
95%. Reliability of the assay was checked by assaying multiple
volumes of the same extract and found to be ±6% when referenced
to the smallest volume assayed.

Protein pellets from TCA extractions were solubilized in
1N NaOH by incubation overnight at room temperature of the
suspended pellet. After removing insoluble material by centri-
fugation, protein was determined by the method of Lowry et al.
(1951) using bovine serum albumin, fraction V as a standard.
NaOH was added to standard curve tubes in equivalent amounts.

Cyclic Nucleotide Assays By Tissue Extract Acetylation

To allow the assay of small pieces of nervous tissue
for cyclic nucleotides, high specific activity ^{125}I cyclic
nucleotides were prepared, and extracts were acetylated to
enhance their ability to compete for antibody binding (Harper
and Brooker, 1975).

Tyrosine methyl esters of the succinyl cyclic nucleotides
(ScNMPTME) were prepared by the formation of a mixed carboxylic-
carbonic acid anhydride (Greenstein and Winitz, 1961) as
described by Steiner, et al. (1969), using commercially avail-
able succinyl cyclic nucleotides as starting materials. The
ScNMPTME's were purified by preparative layer chromatography
(PLC) on cellulose plates with butanol:acetic acid: water
(12:3:5) as a developing solvent. They were detected as the

new 1-nitroso-2-naphthol positive, ninhydrin negative spots.

The ScNMPTME derivatives were subjected to a second PLC and

found to run ahead of the ScNMP's from which they were incom-

pletely purified on the first PLC. The R_f's were: ScAMP

(.34), ScAMPTME (.47), ScGMP (.24), and ScGMPTME (.41). The

overall yields were 8% (ScAMPTME) and 10% (ScGMPTME).

Cyclic NMPTME iodination was by the method of successive

additions of chloramine T described by Cailla, et al. (1976).

The subsequent purification was by column chromatography on

Sephadex G-10 as described by Miyachi, et al. (1977). One

ml fractions were collected and the column monitored for

radioactivity and for immunoreactivity (see below). Immuno-

reactive material is retarded on the highly cross-linked

resin and emerges after unreacted ^{125}I; the product was

stored at -20°C.

Cyclic nucleotide standards and tissue extracts were

acetylated as described by Harper and Brooker (1975) except

that the reaction volume was 77 µl which included 1 µl

TEA and 1 µl acetic anhydride. Of this volume, 25 µl

was used for cGMP assays, and 10 µl was used for cAMP

assays, each in duplicate.

Assays were done as described above for conventional

RIAs with the following exceptions: 1) 0.1% human γ-glob-

ulin was used as the carrier protein in place of 10% normal

rabbit serum and 2) bound and free cyclic nucleotides were

separated by dextran coated charcoal precipitation of free

material (for charcoal preparation see proctolin chapter).

Radioactivity was determined using a Packard Model 3250 Auto

Gamma Scintillation Spectrometer. Data was processed by

the computer program as described above.

The specificity of the A40-1 and G-2 antisera was

determined by the ability of standard series' of acetylated

nucleotides to compete with the ^{125}I tracer for its homol-

ogous antiserum (see Results).

Results

Antisera Characterization

The titers of cyclic nucleotide antisera as measured

by dilution series of antisera against ^{3}H tracers are shown

in Table 5. Subsequent characterizations were done with

the A40-1 and G-2 antisera with final antisera concentrations

of 0.076% and 0.06%, respectively. The nominal sensitivities

of the cyclic nucleotide assays are 0.25 pmole (cAMP) and

0.1 pmole (cGMP) in which a 10% reduction in the response

variable (CPM) from the value at 0 pmole cNMP is arbitrarily

set; coefficients of variation of duplicate values were ±3%.

The specificities of these antisera were measured against

a limited number of cross-reacting nucleotides (data provided

by Dr. John Patt) and are shown in Table 6. Though K_a's

were not calculated it is clear that G-2 shows slightly

greater avidity and specificity for its homologous ligand,

cGMP, than does A40-1 for cAMP.

After the initial series of cyclic nucleotide assays on

whole CNS's was completed a more sensitive method of cyclic

Table 5.   Titers of Cyclic nucleotide Antisera: ^{3}H tracers

Date collected	Antiserum*	Titer	%CPM bound
9-16-77	A40-1	1316	49%
9-16-77	G-1	415	40%
9-22-77	G-2	1667	40%
12-17-77	G-3	428	40%
1-19-78	G-4	333	35%

*"A" designates a cAMP and "G" a cGMP antiserum. Titer
is the reciprocal of the percent antiserum required to
bind the portion of ^{3}H tracer shown.

Table 6.   Competition by Related Nucleotides for A40-1,
            G-2 Antisera

Antiserum	Nucleotide-pmoles	% cross rx.
A40-1	3'AMP - 10,000	.02
	5'AMP - 10,000	.02
	2'-3' cAMP - 10,000	.01
	3'-5' cGMP - 100	2.4
G-2	3'GMP - 10,000	.01
	5'GMP - 10,000	.01
	2'-3' cGMP - 10,000	.01
	3'-5' cAMP - 100	1.0

Cross reactivity is expressed as homologous cyclicnucleo-
tide as a percent of tested nucleotide when each depresses
binding of ^{3}H-tracer to an equivalent extent.
The ratio cNMP/tested nucleotide as a percent when compe-
tition by cNMP is equivalent to that of the quantity of
tested nucleotide.

nucleotide RIA was sought to facilitate their detection in smaller amounts of nervous tissue. The _in vitro_ succinyl- ation method of Cailla, _et al_. (1973), was tried and found to be quite sensitive, requiring less than 45 fm cAMP for detection. As reported (Cailla, _et al_., 1973) the binding of ^{125}I-tracer was found to suffer from competition by suc- cinate resulting in "flattened" dose-response curves when compared to those generated with exogenously added succinyl cyclic nucleotides. Whether or not this competition required sample dilution prior to RIA for reliability was not vigor- ously tested; instead, the _in vitro_ acetylation method of Harper and Brooker (1975) was tried and found to have compar- able sensitivity with no interference by acetate (the assay is done in acetate buffer). In addition, manipulation of reagents is lessened, since catalyst and acetylating agent are pipetted directly into the reaction tube.

^{125}I-ScAMPTME and ^{125}I-ScGMPTME were iodinated and then purified on Sephadex G-10; column functions were tested for immunoreactivity against the A40-1 (0.004%) and G-2 (0.008%) antisera, respectively. Immunoreactive material is retarded by partial adsorption on the Sephadex resin and is well separated from the salt volume. The minimum specific activity for the iodinated cyclic nucleotides was estimated to be 25 Ci/mmole.

Titrations of iodinated cyclic nucleotides with their homologous antisera revealed the dilutions necessary for radioimmunoassay. These are shown in Table 7.

Table 7. Antisera titers using ^{125}I ScNMPTME

Antiserum	Titer	Percent ^{125}I Bound
A40-1	125,000	44%
G-2	33,000	48%

See legend to Table 5 for explanation.

The specificities of the A40-1 and G-2 antisera exhibited in the acetylation assay were tested with dilution series of nucleotides. These data are shown in Table 8. It can be seen that the only possible troublesome spot is the cross-reactivity of cAMP with the cGMP antiserum. This will be discussed below as it relates to data interpretation.

Staging

Manduca larvae entering the fifth instar as a synchronous population (±7 hr) become wandering larvae on the fourth and fifth night after ecdysis (Truman and Riddiford, 1974a). Because hormone release initiating the change from feeding to wandering behavior occurs well before the actual change in behavior (Truman and Riddiford, 1974a), some reliable method

Table 8. Cross reactivities of related nucleotides
          with cAMP and cGMP antisera

	nucleotide	pmole [1]	cross reactivity,%
A40-1	cAMP	.14	-
	cGMP	80	.1750
	3'AMP	450	.0311
	5'AMP	3000	.0047
	ADP	32000	.0004
	ATP	60000	.0002
G-2	cGMP	.03	-
	cAMP	.45	5.34±1.28 [2]
	3'GMP	250	.0120
	5'GMP	4200	.0007
	GDP	19000	.0002
	GTP	4000	.0008

[1]Amount nucleotide required to reduce binding of homologous ^{125}I-ScNMPTME by one-half. Cross reactivity is homologous cyclic nucleotide as a percent of cross reacting nucleotide at 50% binding of ^{125}I-ScNMPTME.

[2]Average and range of two determinations.

of predicting the day of change over is needed if one is to
optimize chances of observing subtle biochemical changes
associated with this event. It had previously been observed
(Nijhout and Williams, 1974a) that larvae initiating wandering
on successive days show clear differences in their growth
rates during the first two days of the fifth instar. In the
present study larvae initiated wandering on day 5 and day 6
of the fifth instar, one day later than observed by Nijhout
& Williams (1974a). This was in part a consequence of the
method of selecting day 0 larvae, in which newly ecdysed
larvae were removed to individual cups late (2200-2300) on
day 0. The growth curves of these two groups is shown in
Fig. 15. Of 111 larvae followed, only one initiated wander-
ing on day 4 with the remaining initiating on day 5 (72
larvae) and day 6 (38 larvae). In this series 82% of the
larvae weighing $\geq$ 6.5g on day 3 (2200 hrs) begin wandering
on day 5 (N=67). The range of weights of these larvae is
6.5-8.1g; this range was adopted for the selection of larvae
for day 3 and day 4 CNS cyclic nucleotide content. Develop-
ment is resynchronized with the onset of wandering (Truman
and Riddiford, 1974a), however, only day 5 wanderers were
used.

Cyclic Nucleotides in Whole CNS

    Preliminary experiments on whole CNS cyclic nucleotide
content revealed approximately 5 pmole cAMP and 1.7 pmole
cGMP per mg protein with little change throughout the fifth
instar (data not shown). However, an apparent 20% increase
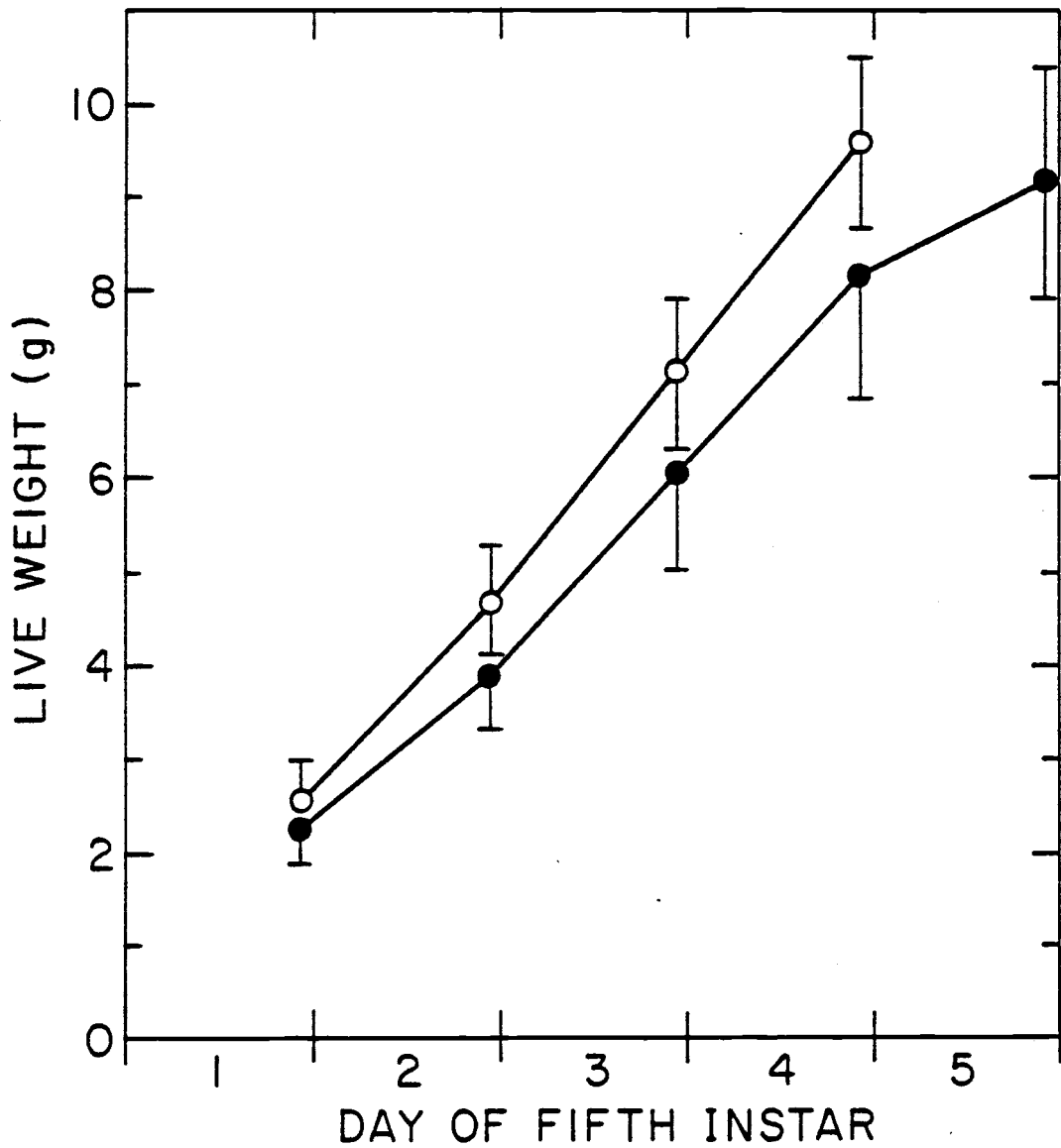
Fig. 15 Growth curves for 5d5 and 5d6 wandering larvae.
Open circles: 5d5 wanderers; filled circles:
5d6 wanderers. Live weights recorded 2100-2300
A.Z.T.

on day 4 (one day before wandering) in cAMP was found init-
ially, but insufficient replicates and time points precluded
any conclusions.

This experiment was repeated with four determinations
per time point; the results are shown in Fig. 16. The cyclic
AMP level is between six and seven pmoles per mg protein
prior to and during the presumed period of hormone release.
No change in content is apparent. From these data it is
clear that if endocrine, behavioral, or metamorphic events
mediated by cyclic nucleotides take place in the CNS, they
were not of sufficient magnitude or were not widespread
enough to be detected with this approach. Future efforts
in this area require the use of more sensitive methods to
look for changes in cyclic nucleotides in different parts
of the CNS.

Cyclic Nucleotides in CNS Ganglia

In order to facilitate the detection of these changes
in cyclic nucleotides, the femtomole sensitive method of
Harper and Brooker (1975, and see methods section, above)
was adopted. It was soon found that both cAMP and cGMP
could be quantitated in a single ganglion, thus making the
task of collecting and dissecting synchronous insects consi-
derably less formidable.

Larvae were collected at intervals over a sixty hour
period spanning the cessation of feeding and the onset of
wandering behavior. Cyclic nucleotides were assayed sepa-
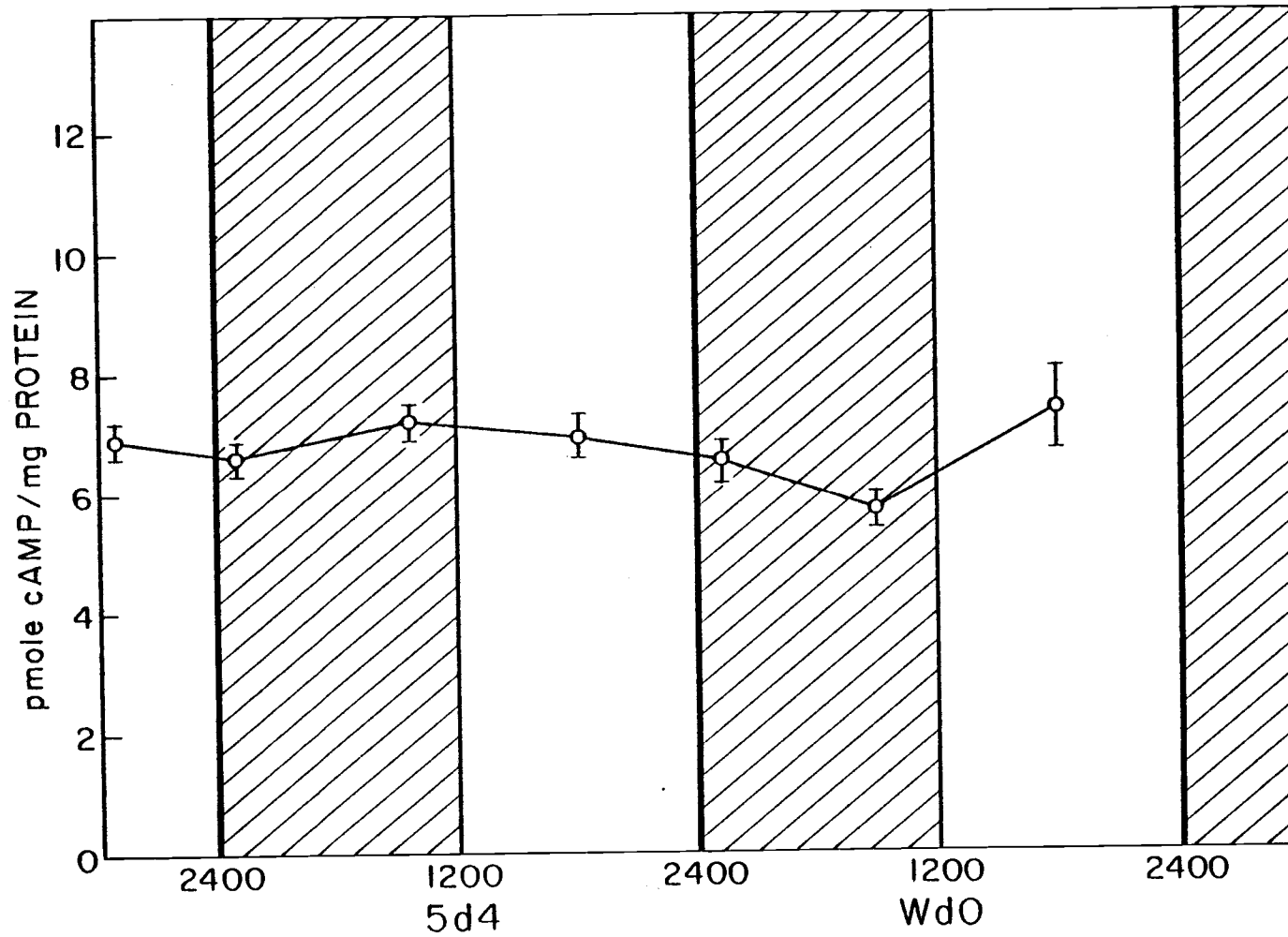rately in pooled abdominal ganglia, pooled thoracic ganglia,

Fig. 16   Whole CNS cAMP in larvae initiating wandering on 5d5.   Each point
represents the mean ± S.E.M. of four determinations.

subesophageal ganglia, and the brain. The results of these determinations are shown in Figs. 17-20. Cyclic AMP content shows a gradual decline in abdominal and thoracic ganglia from approximately 3 pmole/mg protein to 1 pmole/mg protein; abdominal ganglia contain a slightly greater cAMP content than do thoracic ganglia (Fig. 17). Additional determinations to verify the significance of the sharp decline in thoracic ganglia cAMP in the early hours of 5d4 were not done. Cyclic GMP profiles are virtually flat in abdominal and thoracic ganglia at 1 pmole/mg protein (Fig. 18). Similarly, cAMP and cGMP content in the subesophageal ganglion remains little changed during the transition in behavior (Fig. 19). The brain, on the other hand, accumulates cAMP and cGMP over this same time period (Fig. 20); cAMP levels increase from 90 fmole to 165 fmole/brain, while cGMP levels rise from 13 fmole to about 50 fmole/brain.

These increases begin early on 5d4 and are significant for cAMP by 0800 (P < .001) and for cGMP by 1700 hours (P < .05). This experiment was repeated yielding qualitatively similar results. Again, cAMP and cGMP increases were shown to begin approximately one day before the onset of wandering. Additional time points confirmed the constant levels during early feeding and show a slight decline 36 hours after the onset of wandering (data not shown). When the data from Fig. 20 are plotted on the basis of pmole cyclic nucleotide/mg protein, the same upward trend is seen; however, small increases in brain total protein mitigate the effect seen in the simple content plot.
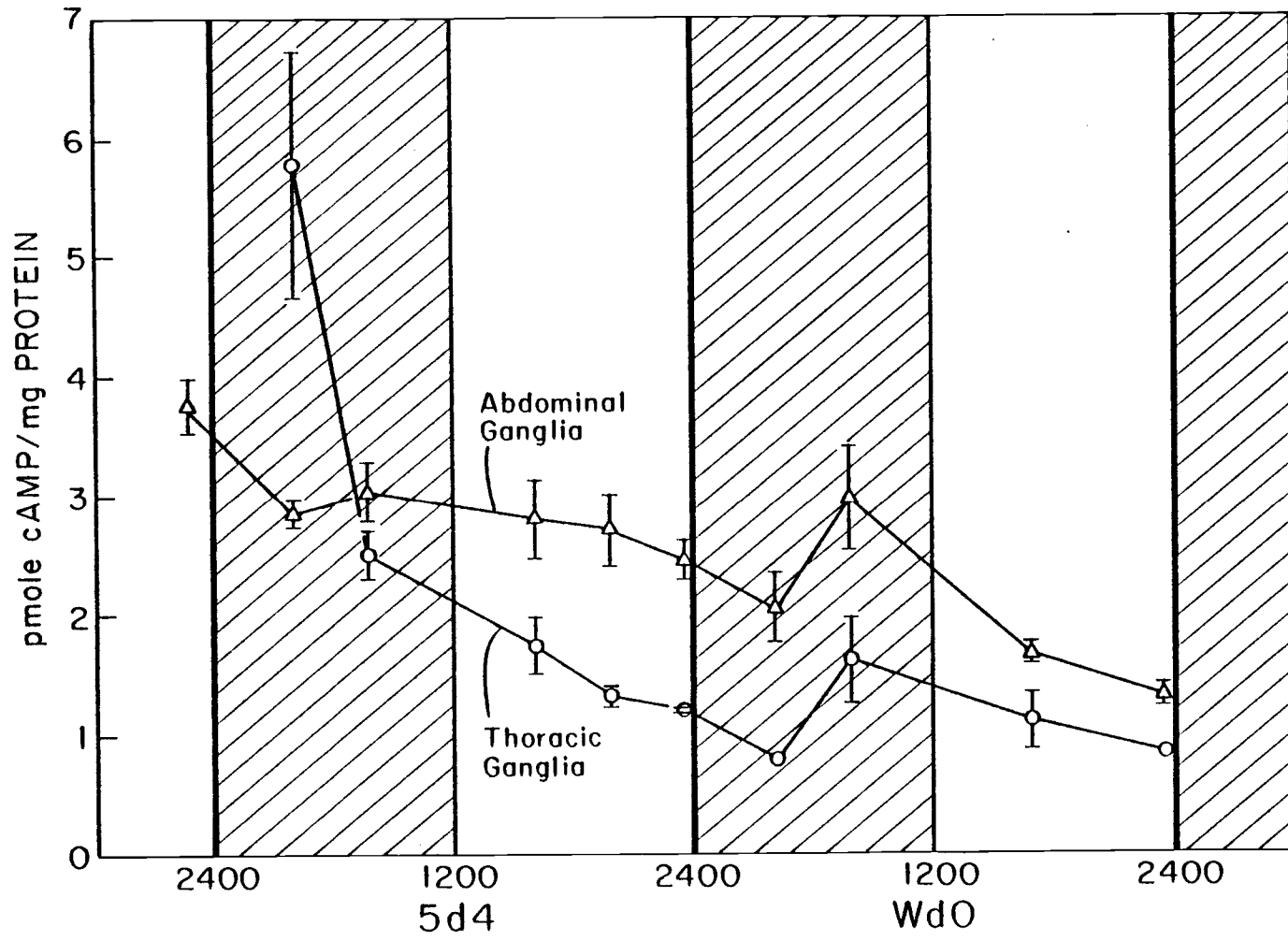
Fig. 17 Abdominal nerve cord and thoracic nerve cord cAMP in larvae initiating
wandering on 5d5. Each point represents the mean ± S.D. of three
determinations.
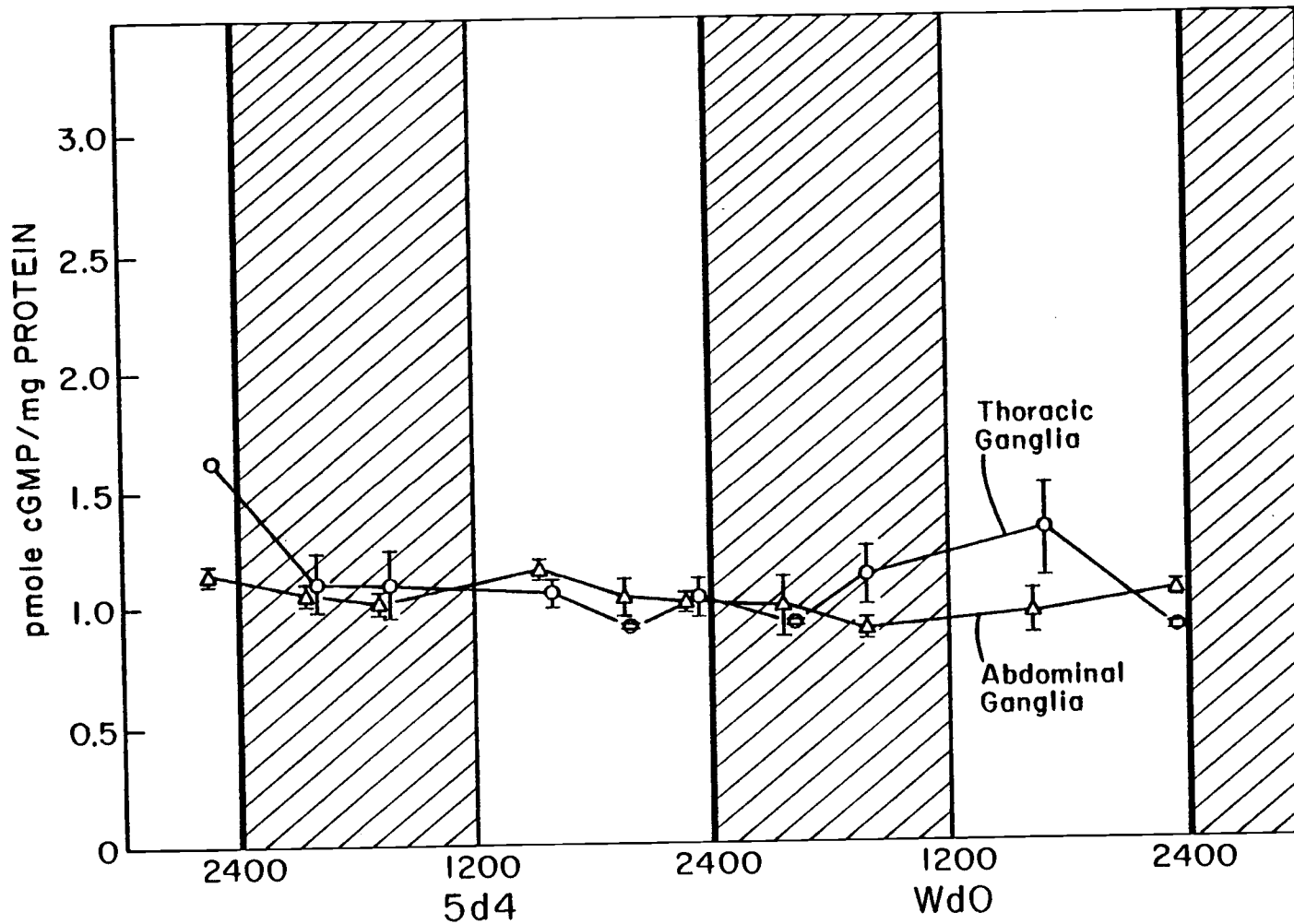
Fig. 18 Abdominal nerve cord and thoracic nerve cord cGMP in larvae initiating wandering on 5d5. Each point represents the mean ± S.D. of three determinations.
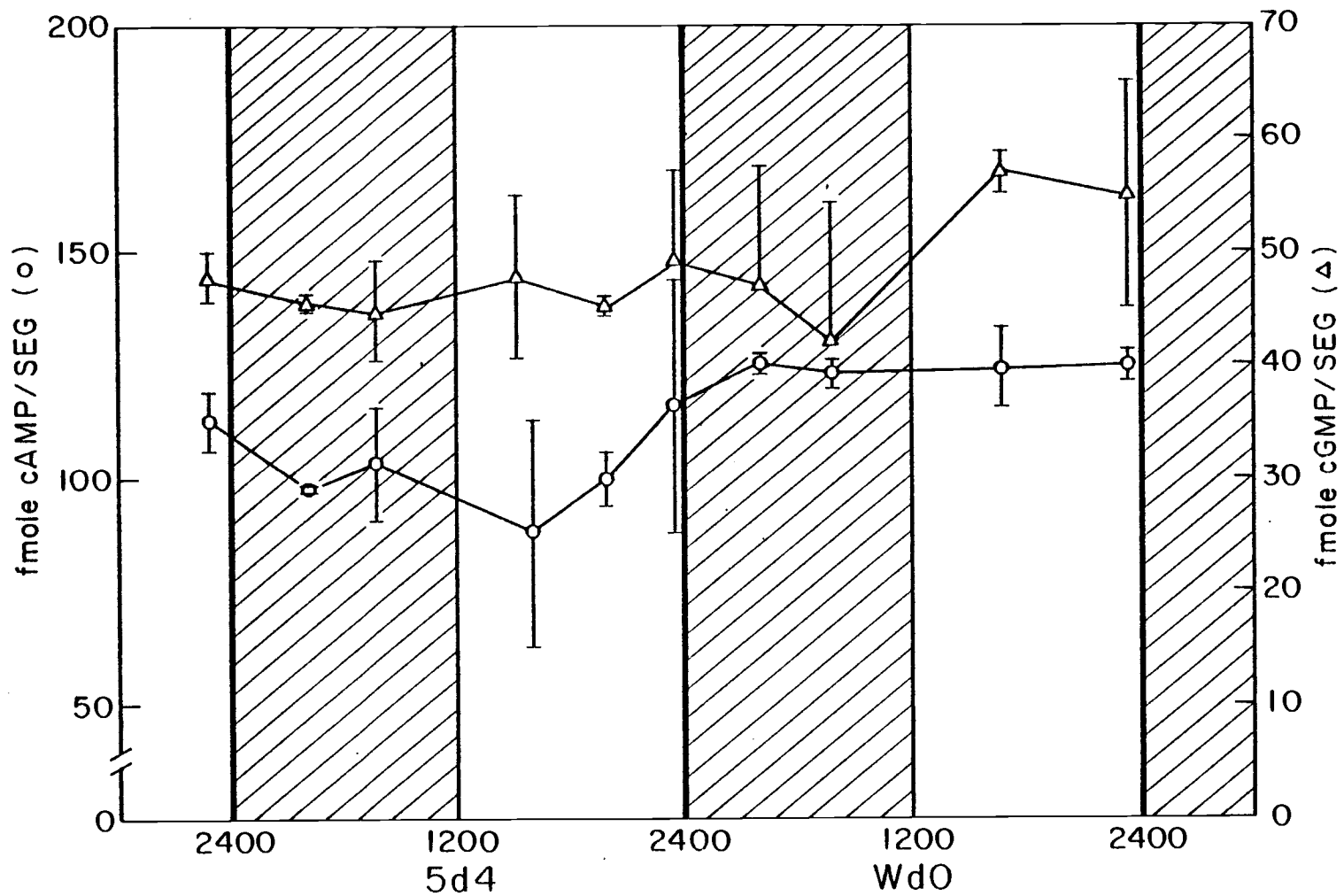
Fig. 19 Subesophageal ganglion cAMP and cGMP in larvae initiating wandering
on 5d5. Each point represents mean ± range (two determinations) or
±S.D. (three determinations).
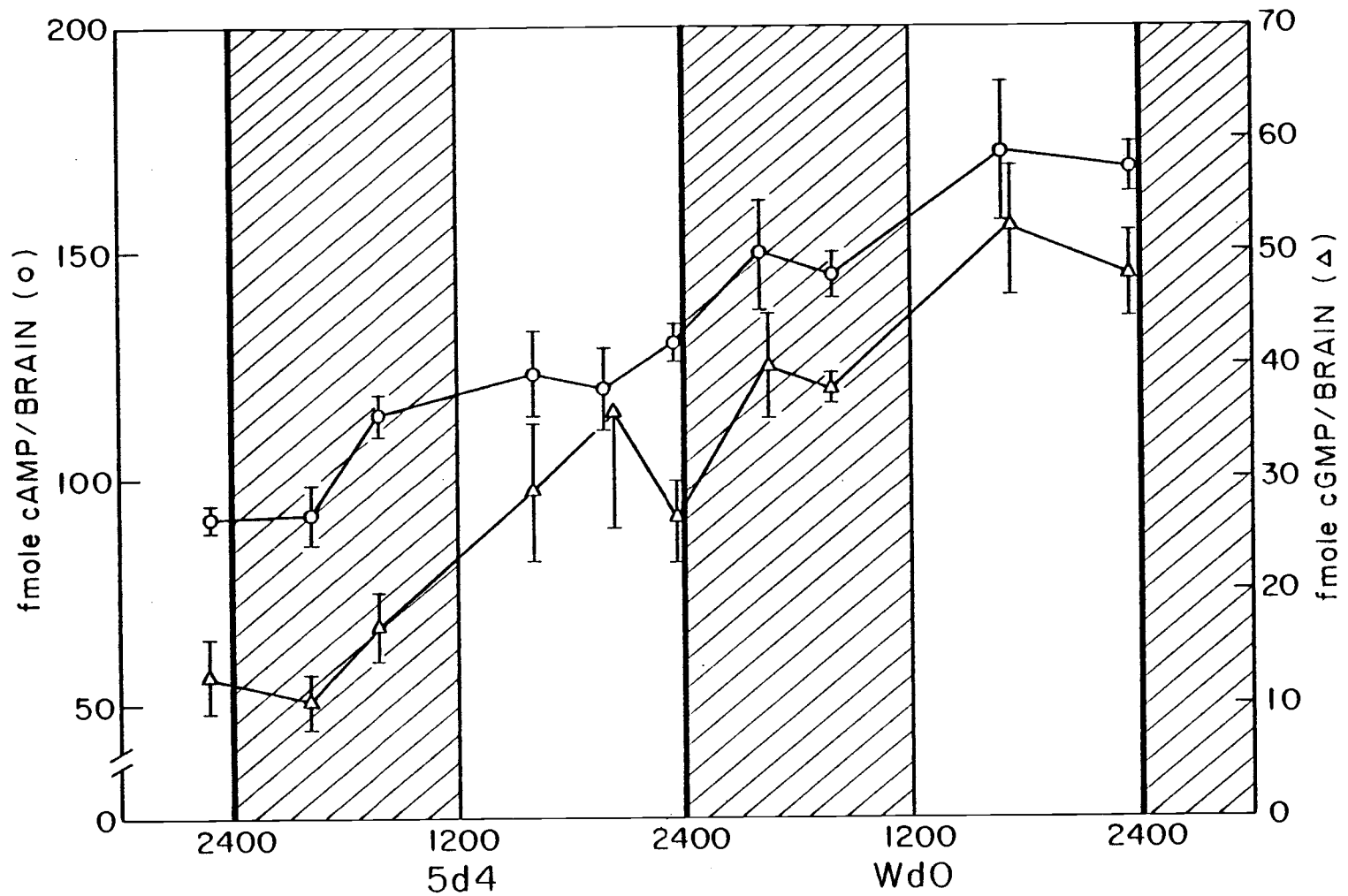
Fig. 20 Brain cAMP and cGMP in larvae initiating wandering on 5d5. Each point represents the mean ± S.E.M. of four to six determinations.

It can be seen in Fig. 20 that cAMP and cGMP profiles
follow one another. At least three explanations could be
put forth: 1) the antisera are not nucleotide specific. As
mentioned above, cAMP competes with about 5% the efficiency
of cGMP for the G-2 antiserum. However, cAMP goes nearly
undetected by the G-2 antiserum at the levels present (25-125
fmole cAMP) in the cGMP RIA. This is in part a consequence
of the sigmoidal character of these dose response curves:
a threshold cAMP must be reached to be detected in the cGMP
RIA. This conclusion was confirmed experimentally by showing
that cAMP internal standards in amounts encountered in cGMP
assays were not detectable by the G-2 antiserum (data not
shown). 2) The same enzyme is responsible for the synthesis
of both cyclic nucleotides; the activation by a single
substance (neurotransmitter or hormone) leads to the accumu-
lation of both cyclic nucleotides. It is clear from a
variety of mammalian studies that distinct adenylate and
guanylate cyclases catalyze the formation of cAMP and cGMP,
respectively. A number of studies have been done with
insect adenylate cyclases in nervous tissue (Rojakovick and
March, 1972; Harmar and Horn, 1977; Taylor and Newburgh,
1978). Guanylate cyclase has apparently not yet been examined
in insect nervous tissue; however, one study on a fat body
enzyme has appeared (Filburn and Wyatt, 1976). Whether the
same enzyme can catalyze the formation of both cyclic nucleo-
tides is not generally addressed; however, recent studies
with purified guanylate cyclases from various rat tissues

(Mittal, et al., 1979) have shown that a nitric oxide, sodium nitroprusside, sodium nitrite, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), and sodium arachidonate activated enzyme catalyzes formation of cyclic AMP. Although the physiological significance of the studies of Mittal, et al. (1979) is not yet known, single enzyme evoked accumulation of cAMP and cGMP in insect brain cannot be ruled out. 3) Adenylate and guanylate cyclases are both present and are independently activated. Since cAMP and cGMP accumulate in tandem with time, cyclase activations occur in response to agents released simultaneously, or to a single agent, perhaps acting through a receptor common to both cyclases.

Discussion

These studies have shown that in the central nervous system, cAMP and cGMP accumulate in the brain during the transition from feeding to wandering behavior. Cyclic AMP and cyclic GMP accumulation occurs in tandem beginning in the early hours of 5d4, approximately 24 hours before these animals would begin wandering on 5d5. Similar changes in cyclic nucleotide levels were not detectable in subesophageal, thoracic, or abdominal ganglia.

The pattern of changes in cyclic nucleotide levels in the brain cannot at this time be shown to be directly associated with a specific developmental or behavioral event. However, accumulation almost certainly occurs in response to release of a hormone or neurotransmitter. Ecdysone

released by the prothoracic glands (Bollenbacher, et al.,
1975) on the third or fourth day of the fifth instar is
affecting a changeover in developmental program preparatory
to pupation (Riddiford, 1976a) and apparently mediates the
cessation of feeding, gut purge, and onset of wandering
behavior required for locating a suitable pupation site
(Nijhout, 1976; O. Dominick, pers. comm.). In a single
attempt to establish a role for cyclic nucleotides in the
change over in behavior, theophylline injections into 5d3
larvae (6.4-8.7g live weight at injection) to concentra-
tions in the animal of 0.5 mm were ineffective in promoting
premature onset of wandering. Zero of twelve insects had
initiated wandering by 5d4-2330. In fact, because 5 of
15 controls initiated wandering on 5d4, there may be some
basis for inhibitory involvement of cyclic nucleotides.
That these concentrations of theophylline are effective in
inhibiting Manduca CNS PDE is suggested by Albin's work
(Albin, 1974) showing half-maximal inhibition in vitro at
one mm theophylline. The participation of hormones in
invertebrate behavior has been recently reviewed (Truman,
1978), and at least one example exists for the probable
mediation by cyclic nucleotides of a stereotyped pattern of
behavior (Truman, et al., 1976; Truman, et al., 1979).

The effects of hormones on developmental programs have
been studied in some detail in recent years, but such studies
have centered on epidermis (Riddiford, 1976a, 1976b) and
imaginal discs (Chiara and Fristrom, 1973). Much less
is known about the response of internal structures to hor-

mones, although the mediation by ecdysone of neurometamorphic shortening of nerve cord in Lepidopterans has been established (Robertson and Pipa, 1973). It can be argued that the cyclic nucleotide phenomena observed are not likely to be directly associated with events leading to reorganization of the nervous system since only the brain in the central nervous system accumulates cyclic nucleotides.

Another possibility is the involvement of cyclic nucleotides in neurosecretory phenomena. The insect brain (see Nijhout, 1975 for a study of Manduca brain) contains several discrete islets of neurosecretory cells. Recent reports have established increases in brain cyclic nucleotides during onset of adult development of previously chilled Cecropia (Rasenick, et al., 1976) or bertha armyworm (Bodnaryk, 1978) pupae. These workers postulate that cyclic AMP and/or cyclic GMP may be involved in activation of neurosecretory cells (Rasenick, et al., 1976) or in migration of secretory vesicles from site of synthesis to site of release (Bodnaryk, 1978). Unfortunately, changes in control tissue and under-chilled pupal brain cAMP obscure the significance of the cecropia work, and the bertha armyworm work suffers from lack of any data on control tissue. In addition, an earlier report (McDaniel and Berry, 1974) noted the potent inhibition of adult development in cecropia by the phosphodiesterase inhibitors caffeine and aminophylline, casting doubt on the involvement of cyclic nucleotides as positive effectors.

Nevertheless, evidence has accumulated in recent years from work in mammalian systems for the involvement of cyclic

nucleotides in hypothalamic regulation of adenohypophyseal function (reviewed in Jutisz and Kraicer, 1975). Evidence includes elevation of rat pituitary explant cGMP and cAMP in response to rat hypothalamic extract and increased growth hormone (GH) release into the culture medium with 10 mm cGMP (Peake, et al., 1972). More recently (Jutisz and Kraicer, 1975) 6 mM dibutyrl cAMP (see however Rosenfeld and Barrieux, 1979) was also shown to potentiate GH release from purified somatotrophs, and leutenizing hormone/follicle stimulating hormone releasing factor (LH/FSH-RF) could elicit theophylline potentiated LH release from rat pituitary explants.

The hormonal control of protein synthesis (including pituitary hormone synthesis under control of releasing hormones) is now well documented (Rosenfeld and Barrieux, 1979). If, as has been suggested (Bodnaryk, 1978), cyclic nucleotides play a role in transport and release of secretory factors, the diversity of possible cyclic nucleotide loci in secretory processes becomes apparent.

Fundamental to the present studies is the question of the temporal relationship between brain cyclic nucleotides and hemolymph hormone titers. Previous ligation studies (Truman and Riddiford, 1974a) have indicated the ecdysone "critical" period occurs some 7 to 11 hr. before onset of wandering. Such indirect methods of measuring hormone release only delimit independence of response from the secreting structure; more recent biochemical studies (Bollenbacher, et al., 1975) indicate that ecdysone release

may begin a full 24 hr. prior to onset of wandering. Such timing gives ecdysone release and brain cyclic nucleotide accumulation as observed in the present studies overlapping profiles; however, separate studies precludes precise comparison of the two events. If cyclic nucleotides accumulate in response to ecdysone release, physiological significance for an earlier observation on the ability of β-ecdysone to elicit cAMP synthesis in Manduca larval brains (Vedeckis and Gilbert, 1973) becomes possible. Ecdysones may serve regulatory functions in brain neurosecretion, acting in positive (Marks et al., 1972) and/or negative (Steel, 1975) feedback loops.

Just as likely, considering the available evidence, is the possibility that cyclic nucleotides signal an impending bout of neurosecretory hormone synthesis and release. As mentioned above, information on cause and effect relationships may be gained by simultaneous measurements of ecdysones and brain cyclic nucleotides. Immunocytochemical and conventional histological studies on the cyclic nucleotide accumulating cells and their processes would provide an additional bridge between observation and physiological significance. Finally, biochemical studies on the ecdysone stimulation of brain adenyl and guanyl cyclases and the effects of ecdysone in vitro on specific hormone (e.g., PTTH) content in the brain will aid in understanding the reported phenomena. The latter studies will be facilitated by purifying and obtaining antisera to PTTH.

PROCTOLIN IN ARTHROPOD CENTRAL NERVOUS SYSTEM

The roles of peptides as neurohormones, neurotransmitters, and neuromodulators has gained considerable interest in the last ten years long after von Euler and Gaddum (1931) first found a substance in brain and intestine that caused transient hypotension and elicited contraction of the isolated rabbit intestine. This substance was later termed substance P and found to be a peptide (Gaddum and Shild, 1934). The developing picture of a multiplicity of functions for substance P serves to illustrate the course of findings for many other peptides (see Barker, 1976; Stewart and Channabasavaiah, 1979).

Invertebrate studies in peptide neurotransmitters was aided by the isolation (Brown and Starratt, 1975) and characterization (Starratt and Brown, 1975) of proctolin, a pentapeptide (arg-tyr-leu-pro-thr) with potent excitatory effects on the slow, striated muscles of the protodaeum of the cockroach Periplaneta americana (Brown, 1975). When proctolin became commercially available in 1976 we (these studies were conceived in collaboration with Dr. Margaret Titmus, University of Oregon) set out to obtain an antiserum to proctolin. The intent was to screen a number of insect tissues to regionally locate proctolin. This was expanded after reports by Lingle (1979) of potent (5 x 10^{-8} M threshold) enhancement by proctolin of neurally evoked contractures of the GM6B nerve-muscle preparation in the spiny lobster foregut; selected tissues from three crustacea

were included in the screening. As of this writing, procto-
lin has been quantitated in whole insects (Brown, 1977) by
bioassay and HPLC (Holman and Cook, 1979b) and in hindgut
and foregut (Holman and Cook, 1979a) by bioassay and analyti-
cal HPLC. No reports of radioimmunoassay for proctolin have
yet appeared. The reported results are the first part of a
study in which proctolin-containing cells will be sought
immunocytochemically with follow-up on the neurophysiology
of proctolinergic cells (to be carried out by Dr. Margaret
Titmus).

Materials and Methods

Materials

Synthetic proctolin was obtained from Peninsula Labor-
atories (San Carlos, CA) and National Biochemical Corp.
RIA grade bovine serum albumin (B.S.A.) and carbodiimide
(EDC) for preparation of the antigen were obtained from
Sigma Chemical Co. Gamma globulin and dextran (ave. MW =
40,000) for the radioimmunoassays were also obtained from
Sigma. Norite "A" activated charcoal was from Matheson,
Coleman & Bell. Fetal calf serum and dialyzed horse serum
were obtained from GIBCO. Vials for gamma counting were
obtained from Evergreen Scientific (Los Angeles). $Na^{125}I$
was obtained from Amersham-Searle and New England Nuclear.
"Chlorox" for proctolin iodination was obtained from a
local supermarket. Synthetic peptides for testing anti-
serum specificity were generously provided by Dr. Terry

Moody and Dr. Robert E. Sullivan.

Animals

Mixed male and female adult Madera cockroaches (Leuco-
phaea maderae) were provided by Dr. Victor Brookes from
stocks maintained on Purina Dog Chow with water ad libitum.
Crustaceans were species collected locally (crayfish:
Pacifastacus leniusculus; crab: Cancer magister) or obtained
from suppliers (lobster: Panulirus interruptus).

Methods

Coupling of proctolin to carrier B.S.A. was accomplish-
ed with a seven-fold molar excess of EDC to proctolin.
Two mg each of proctolin and B.S.A. were dissolved in two
ml glass distilled water; 15.2 mg EDC was then added and
the solution stirred for 30 minutes at room temperature.
The reaction mixture was then diluted to six ml with distil-
led water, dialyzed 24 hrs., and lyophilized.

Immunizations and bleeding schedules, processing and
storage of antiserum were as described for ecdysone antisera
(see Appendix).

Iodination of proctolin was carried out by using dilute
Chlorox (hypochlorite) as the oxidizing agent (Dr. Edward
Herbert, personal communication). One µl of proctolin
(0.22 nmole) in water was diluted with 10 µl 0.1 M sodium
borate, pH 10.0. 0.5 mCi ^{125}I in 0.1 N NaOH was added
and the reaction initiated with 5 µl dilute hypochloroite
(20 µl Chlorox in 13.3 ml borate buffer). After 15 seconds

the reaction was stopped with 20 μl sodium metabisulfite
(10 mg/ml in borate buffer). In two minutes the reduction
was quenched with 20 μl NaI (100 mg/ml in 60% acetic acid),
and in an additional two minutes 50 μl 0.25% B.S.A. in 0.05
N sodium phosphate, pH 7.6 was added. The entire mixture
was then chromatographed on Sephadex G-10 in a Pasteur
pipet with 10% acetic acid as eluent. Aliquots of seven-
drop fractions were counted and checked for immunoreactivity
by assay procedures described below. Active fractions with
low backgrounds were diluted with one ml 50% aqueous n-propa-
nol and stored at 4°C.

Tissues for proctolin determination were excised in a
cockroach physiological saline (190 mM NaCl, 21 mM KCl, 7.7
mM $CaCl_2$, 1.8 mM $MgCl_2$) or artifical seawater (for lob-
ster, crab, and crayfish), and either frozen immediately on
dry ice or kept on ice for up to one hour for subsequent
homogenization. Tissues were extracted for proctolin by
homogenizing in minimally twelve-fold excess (vol:wt)
methanol:water:acetic acid, 90:9:1 (Holman and Cook, 1979a).
Insoluble material was removed by centrifugation and the
supernatant dried under a stream of nitrogen. Residues were
taken up in RIA buffer (0.1% γ-globulin in .05 M Na phos-
phate, pH 7.6, containing .02% sodium azide), clarified by
centrifugation, and assayed immediately.

The radioimmunoassay incubations were done in 200
μl 0.1% bovine γ-globulin in 0.05 M sodium phosphate,

pH 7.6 overnight at 4°C. Separation of bound from free
^{125}I-proctolin was accomplished with the addition of 100

μl 2% dextran-coated Norite "A" charcoal (DCC) followed

by centrifugation. (DCC was prepared by a method used in

Dr. Herbert's lab: 0.5g charcoal in 10 ml distilled water

was coated with 125 mg dextran for 1 hr. at room temperature.

2.5 ml fetal calf serum or dialyzed horse serum and 3.3 ml

.05 M Na phosphate, pH 7.6 were added and the mixture brought

to 25 ml with distilled water. This mixture was made fresh

weekly and stored at 4°C). 200 μl of clear supernatant

was counted and data analyzed as described in the cyclic

nucleotides chapter.

Solutions of proctolin were standardized for the assay

by amino acid analysis (kindly performed by Thomas Bailey

in this Department). Standards were stored in RIA buffer

including 0.02% sodium azide at 4°C and were stable for at

least four months.

Results

Antiserum characterization and radioimmunoassay

Two proctolin antisera were obtained after the second

booster injection, each with a titer of approximately 1:1000.

One of these, designated "7A/1018", was selected for more

extensive characterization and for use in radioimmunoassays.

A standard curve, showing competition of proctolin with

^{125}I-proctolin for antiserum is shown in Fig. 21. Non-

precipitable counts on newly prepared label was typically
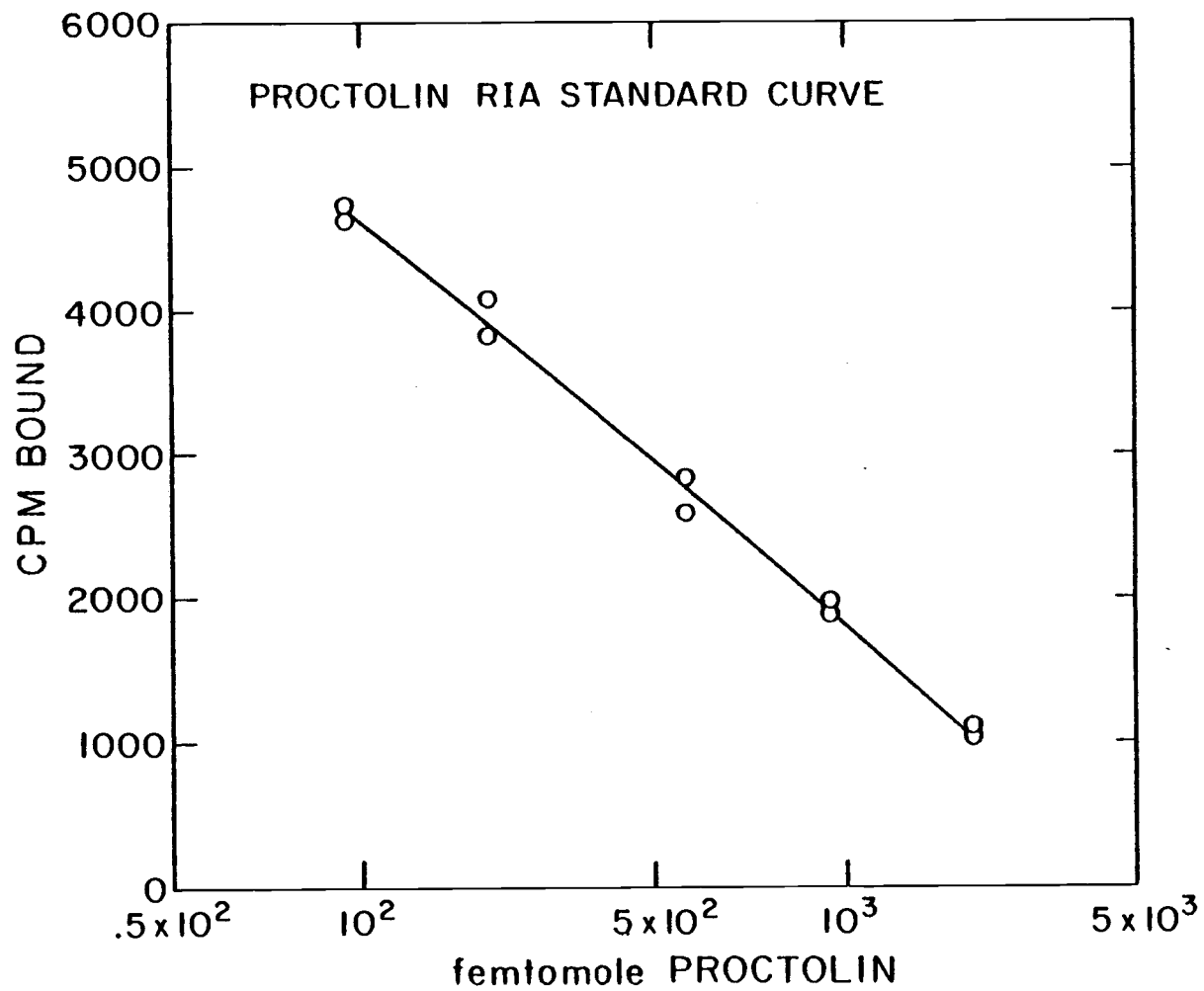
Fig. 21   Proctolin radioimmunoassay standard curve.

3% of the total. The specificity of the antiserum was checked against a series of peptides unrelated to proctolin and with the synthetic amino terminus dipeptide, arg-tyr. Only the latter showed detectable ability to compete with ^{125}I-proctolin, this activity being a very low 0.00064 %. These results are shown in Table 9.

Recovery of tissue proctolin was checked in two stages: 1) 95% ^{125}I-proctolin included during homogenization was recovered in the homogenate just prior to centrifugation. 2) Recovery of internal standards added to homogenate aliquots were 99 ± 8% (Manduca sexta whole CNS) and 89 ± 11% (Leucophaea madera hindgut). Recoveries were not changed by partitioning the water-soluble extract against ethyl acetate prior to assay, indicating a lack of interference by non-polar contaminants; hence, tissue extracts were assayed without further purification.

Leucophaea Proctolin RIA Activity

Proctolin RIA-activity was found throughout the central nervous system and in several non-neural tissues as well. These results are presented in Table 10. Values for insect tissue were obtained with freshly dissected material. It was determined that a dry ice freeze and subsequent thaw resulted in 33-50% lower RIA activity (n=4). The reason for these losses is not known; however, it is conceivable that damage to cell structure on thawing releases proteolytic enzymes.

The most abundant source of RIA-activity is the hind-

Table 9. Peptides not cross-reacting with proctolin anti-
serum

peptide	highest amount tested	% cross reactivity
Somatostatin	10 nm	0
Substance P	10 nm	0
Bombesin	5 nm	0
Neurotensin	10 nm	0
α-MSH	5 nm	0
met-enkephalin	10 nm	0
VIP	25 nm	0
Bradykinin	10 nm	0
Δ-sleep inducing factor	10 nm	0
TRF	10 nm	0
β-endorphin	10 nm	0
arg-tyr	50 nm	.00064

[*] The 7A/1018 proctolin antiserum was used in these tests.

Table 10. Leucophaea proctolin RIA activity

structure (n*)	pmole/structure (±SEM†)	pmole/mg protein (±SEM)
6th abdominal ganglion (3)	.68 ± .02	26.3 ± 2.2
1st-5th abdominal ganglia (4) (with connectives)	.11 ± .03	4.9 ± 0.7
Metathoracic ganglion (4)	.55 ± .02	13.8 ± 1.6
Mesothoracic ganglion (4)	.54 ± .02	11.3 ± 1.1
Prothoracic ganglion (4)	.45 ± .06	13.3 ± 1.3
Brain (excluding optic and antennal lobes (4)	.20 ± .02	3.7 ± 0.2
Optic lobe (1)	<.01/pair	-
Antennal lobe (2)	<.01/pair	-
Hindgut (4)	2.95 ± .22	1.85 ± .2
Skeletal muscle (1)	-	0.48
Tracheae (1)	-	6.9
Fat body (1)	-	0.18
Hemolymph (1)	-	0.29

*n = number of determinations. †SEM = standard error of the mean. All assays done with freshly dissected tissue.

gut, and the 6th abdominal ganglion is the richest source on a protein basis. The relative amounts are similar to those originally described by Brown (Brown, 1965) for the "gut-factor" now identified as proctolin (Starratt and Brown, 1975). The finding of proctolin RIA activity in thoracic ganglia parallels Brown's results from bioassay in Periplaneta americana (Brown, 1965). Lesser amounts were found in the brain and abdominal chain of ganglia. The existence of activity in non-neural tissues is an apparent anomaly, given its presumed role in neurotransmission, this will be discussed below.

Crustacea Proctolin RIA Activity

A number of crustacea tissues were found to contain measurable proctolin RIA-activity. These values are shown in Table 11. Tissues containing no measurable proctolin are shown in Table 12. Proctolin was found in the brain of all three crustacea and in the pericardial organ of the two species examined (Panulirus and Pacifastacus). Only one other report has appeared on proctolin-like peptides in crustacean tissues (Sullivan, 1980); in this report 500 pm proctolin bioassayable equivalents were found in the peri-cardial organ of the Gilbertese land crab, Cardisoma carnifex. Extracts from Cardisoma purified by silica gel TLC and provided by Dr. Sullivan contained 32 ± 8.5 pmole (n=3; ±S.D.) proctolin RIA activity. This represented just 6% of the biological activity as measured on the cockroach hindgut (R.E. Sullivan, personal communication). The

Table 11. Crustacea proctolin RIA-activity

Structure	pm/structure	pm/mg protein
Panulirus brain (1)	.728	.125
Panulirus heart-pericardial organ (1)	.299	.013
Panulirus thoracic ganglion (1)	.365	.027
Pacifasticus brain (2)	.351	1.20
Cancer brain (3)	.309	.672
Cancer pericardial organ (4)	.061	.296
Cancer eyestalk (6)	.093	.030

All values represent the amount in a single determination on the number of structures shown in parenthesis. Tissues were dissected in artificial seawater, frozen on dry ice, and stored at -20°C until being assayed.

Table 12. Tissues with no measurable proctolin

Panulirus abdominal nerve cord (1)

Pacifasticus abdominal nerve cord (2)

Pacifasticus thoracic ganglia chain (2)

Pacifasticus eyestalks (4)

Pacifasticus commissural ganglion (3)

Pacifasticus pericardial sac, heart (2)

Pacifasticus stomatogastric ganglion and accessory heart (2)

Cancer thoracic mass (3)

Cancer commissural ganglion (4)

Cancer stomatogastric ganglion (3)

Number of structures assayed in a single determination is
shown in the parentheses. Tissues treated as in Table 11.

discrepancy may have resulted from an undetermined source of error in the bioassay or in the co-purification of biologically active peptides not recognized by the proctolin antiserum.

Discussion

Since the purification (Brown and Starratt, 1975) and characterization (Starratt and Brown, 1975) of proctolin the question of its possible neurotransmitter role in cockroach hindgut has been discussed by Brown (1975) and Holman and Cook (1979a). Brown's studies (1975) have shown that proctolin and neurally-evoked responses have similar pharmacological properties (specific threshold of inhibition by tyramine at 3-5 x 10^{-8}M) and that a substance is released by a neurally-stimulated donor hindgut that potentiates a neurally-evoked response in a second hindgut; authentic proctolin but not glutamate has similar potentiating effects. While suggestive of neurotransmitter status, these studies do not rule out neuromodulatory functions (Holman and Cook, 1979a); iontophoretic techniques that could establish a common site of action at the postsynaptic receptor for proctolin, the natural neurotransmitter, and tyramine have not yet been reported. In addition, with the availability of proctolin antiserum reported here, neuromuscular junctions can be examined for proctolin immunocytochemically.

The present finding of 1.9 ng proctolin/ hindgut in L. Maderae is very similar to that reported by Holman and Cook

(1979a) as determined by bioassay after partial purification (3.3 ng/hindgut); furthermore, the high specific activity present in the 6th abdominal ganglion (26.3 pm/mg protein) is consistent with its known innervation of cockroach hindgut and supports Brown's contention that proctolin is the hindgut neurotransmitter.

Proctolin has previously been assayed only in partially purified whole insect homogenates (Brown, 1977; Holman and Cook, 1979b) and in partially purified extracts of Maderae cockroach hindgut, foregut, and whole head (Holman and Cook, 1979a). It is, however, likely that Brown's chymotrypsin resistant "gut-factor" (1967) from thoracic ganglia, thoracic nerve, and proctodeal nerve contained or was identical to proctolin. The finding of proctolin RIA activity in thoracic ganglia strongly suggests that proctolin serves functions other than its role in the hindgut. Of interest in this regard are reports by Piek and Mantel (1977) and Mantel, et al. (1979) that proctolin induces (10^{-9}M) the small myogenic rhythmic contractions of the locust metathoracic extensor tibiae originally reported by Hoyle and O'Shea (1974). That this is not the only function served by thoracic proctolin is made likely by the failure to demonstrate any such rhythm in pro- or mesothoracic extensor tibiae of three species of locusts examined and the complete absence of this intrinsic rhythm in three species of cockroaches and three of crickets (Hoyle and O'Shea, 1974). Important in this regard are reports that proctolin (10^{-9} M threshold) increases heartrate in a

semi-isolated prep of _Periplaneta_ heart (Miller, 1979).
Also of significance is a recent finding (Dr. Michael
O'Shea, personal communication) of a pair of large cell
bodies in each thoracic and abdominal ganglion of locust
that when stimulated causes increased heartrates. Extracts
from these ganglia show a peak co-migrating with proctolin
on HPLC. It will be interesting to test such ganglion
extracts for proctolin RIA activity.

At this time it is difficult to speculate on the
significance of proctolin RIA activity in the brain. The
accessibility of the insect CNS may lend itself to immuno-
cytochemical and neurophysiological identification of procto-
lin containing cells. A recent report (Holman and Cook,
1979a), however, denies the existence of proctolin in whole
head extracts of _Leucophaea_ _maderae_ cockroaches. This con-
clusion was based on rather difficult separations of phenyl-
thiocarbamyl derivatives of partially purified extracts on
HPLC. The authors state the minimum detectable derivative
to be 30-35 ng which cannot be seen in the extract of 100
heads. Assuming 0.20 pm/brain (Table 10) as the only source
of head proctolin, 100 heads = 13 ng, which is not enough
to be seen with their detector. These authors do report,
however, 1.5 ng proctolin equivalents/head in a quantitative
hindgut bioassay and propose that all activity is due to a
second, distinct peptide called hindgut stimulating neuro-
hormone or HSN (Holman and Cook, 1972). HSN is presumed to
have identical chromatographic properties (including on a
simple HPLC separation) and indistinguishable physiological

properties on <u>Periplaneta</u> hindgut. Confirmation awaits
purification of HSN, if in fact it is a distinct peptide.

The presence of proctolin RIA-activity in muscle, fat
body, and tracheae was initially a surprise (Table 10).
However, some activity might be expected in muscle if proc-
tolin modulates muscle tonus (see above). Smaller amounts
of RIA activity were present in hemolymph and fat body;
however, tracheae contain much greater amounts of RIA activ-
ity, 6.9 pmole/mg protein. The significance of this activ-
ity is not known. It should be pointed out, however, that
the question of antiserum specificity is dealt with in only
a limited way by looking at cross-reactivities of unrelated
peptides. The most controlled use of the antiserum would
dictate purifying tissue proctolin to determine the relation-
ship between authentic proctolin and proctolin RIA-activity.

The first report of proctolin-like peptides in crusta-
cean tissue has recently appeared (R.E. Sullivan, 1980).
Pericardial organs from the Gilbertese land crab <u>Cardisoma</u>
<u>carnifex</u> were found to contain a peptide with cockroach
hindgut stimulating activity that co-chromatographed with
proctolin in eight solvent systems and whose activity was
completely blocked by 30 μM tyramine. The enormous amount
of activity (500 pm proctolin equivalents/pericardial organ)
was not confirmed by radioimmunoassay (32 pm/pericardial
organ) suggesting the possibility of additional biologically
active peptides not recognized by proctolin antiserum. In
two other crustacea (<u>Pacifastacus</u> and <u>Cancer</u>) significant
but much less proctolin RIA-activity was found in pericardial

organs. The most interesting additional finding is the presence of RIA activity in the brains of all three species examined. The values presented must, however, be confirmed with fresh tissue; on the basis of findings with insect tissues (above), higher values would not be surprising. The significance of this activity may have to await identification of the proctolinergic cells.

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APPENDIX

APPENDIX

Ecdysone Antisera

A β-ecdysone-BSA conjugate was prepared essentially
as described (Borst and O'Connor, 1972, 1974). Briefly, 40
mg β-ecdysone was derivatized with 200 mg (Aminooxy)-
acetic acid (CMA) in 2.4 ml pyridine at 36°C overnight.
The entire mixture was streaked on two silica gel PLC plates
(500 μ) and chromatographed in methanol:chloroform (2:3).
Corresponding UV absorbing regions were scraped, pooled,
eluted with absolute ethanol, and evaporated to dryness
under reduced pressure. This material was then taken up in
methanol and rechromatographed in the same solvent system
alone or with the addition of 0.5% acetic acid (this resulted
in a more compact mobility of UV absorbing material).
After eluting, an aliquot was chromatographed on silica gel
PF-254 to yield a single spot at R_f=.12. The yield was
16.5 mg dry material = 34% of theoretical maximum.

A scan in the ultraviolet spectrum demonstrated that
E_{max} had shifted from 242 nm (β-ecdysone) to 259 nm
(presumptive β-ecdysone-CMA). IR spectroscopy showed a
loss of the absorbance at 1645 cm^{-1} (unsaturated ketone)
and a new peak at 1610 cm^{-1} (carboxylate anion). Initial
attempts to further characterize the derivative by nuclear
magnetic resonance and gas chromatography - mass spectroscopy
were inconclusive, possibly because of trace contaminants
in the preparation. Further sample clean-up and characteri-
zation was not undertaken. Free amines were quantitated

with 2,4,6-trinitrobenzenesulfonic acid (TNBS) as described (Fields, 1972); a contamination by CMA of 1.6% by weight was indicated.

Conjugation of β-ecdysone-CMA to BSA was done as described for a progesterone-CMA derivative (Erlanger, et al., 1958). The reaction proceeds through the formation of a mixed-acid anhydride (Greenstein and Winitz, 1961) with isobutylchloroformate and the subsequent displacement of acid by BSA to form the peptide bond of the BSA-β-ecdysone-CMA conjugate. Characterization of the conjugate by UV absorbance indicated three haptenes per carrier.

Three New Zealand white rabbits were immunized by multiple intradermal injections along the back (Vaitukaitis, et al., 1971), each with one mg conjugate in Freund's complete adjuvant. One booster was similarly administered one month later in Freund's incomplete adjuvant. 30 ml whole blood was taken weekly from the lateral ear veins and allowed to clot overnight at 4°C. Serum was separated from cells by centrifugation, brought to 1:5000 in a tincture of merthiolate and frozen at -20°C.

^{3}H α-ecdysone was a gift of Dr. David King (Zoecon Corporation) and had a specific activity of 68 Ci/mmole in 1975. The label was repurified on silica gel F-254 TLC sheets (EM Laboratories) in chloroform:95% ethanol (4:1) and stored in benzene:ethanol (9:1) in evacuated sealed ampules at 4°C.

Radioimmunoassays were done as previously described (Borst and O'Connor, 1972,1974).

Rabbits produced antiserum with titers and affinities shown in Table 13. Specificities and affinities were tested on antisera at peak titers, which occurred six and nine weeks after boosting for rabbits I and II, respectively. Curiously, rabbit I produced an antiserum with approximately ten-fold higher affinity for α-ecdysone than β-ecdysone, though the antigen was prepared with β-ecdysone. Neither antiserum recognized cholesterol.

Table 13.

Rabbit #	max-titer*	K_a**(1/m)		50% inhibition of ^{3}H-α-ecdysone binding (pmole).	
		α-ecd.	β-ecd.	α-ecd.	β-ecd.
I	1:43,000	6.6×10^9	7.2×10^8	.15	.61
II	1:650	7.5×10^8	7.3×10^8	.54	.58

*The titer representes the dilution necessary to bring binding of a high specific activity ^{3}H-α-ecdysone down to 50%.

**K_a's were calculated from Scatchard plots of dose-response series (Scatchard, 1949).