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TITLE: NEUROTRANSMITTERS, CYCLIC NUCLEOTIDES, AND ADENYL CYCLASE IN THE CENTRAL NERVOUS SYSTEM OF MANDUCA SEXTA

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Abstract Approved: R. W. Newburgh

The role of adenosine 3',5'-cyclic monophosphate (cyclic AMP) in neurotransmission in the ventral nerve cord of Manduca sexta has been investigated chemically. The findings of these studies are as follows: (1) Serotonin dramatically elevated cyclic AMP levels over controls in incubated cell suspensions of neurons bulk-isolated from intact nerve cords. (2) Cyclic AMP and cyclic GMP were present in intact nerve cords at levels comparable to those found in other insect nervous tissues. (3) In contrast to mammals and carnivorous insects, high potassium in the incubation medium decreased basal, serotonin (5-HT), and theophylline-stimulated accumulations of cyclic AMP in intact nerve cords. (4) In general, one mM ascorbate impaired cyclic AMP accumulation in basal, 5-HT- or theophylline-stimulated intact nerve cords, while a 100 μM concentration had no effect on basal accumulation at pH 5.3. (5) 5-HT stimulated cyclic AMP accumulation at pH 3.5 and 5.3; at pH 6.5 theophylline-stimulated accumulation is potentiated

by 5-HT which had no effect alone. (6) Aspartic acid, gamma-aminobutyric acid (GABA), norepinephrine (NE), and isoproterenol were effective in elevating cyclic AMP levels at only one of the tested pH's. (7) β-Ecdysone had no effect on cyclic AMP levels in intact nerve cords, but markedly increased cyclic GMP levels after ten and thirty minutes incubation. (8) Acetylcholine (ACh), aspartic and glutamic acids, GABA, glycine and theophylline stimulated cyclic GMP accumulation in intact nerve cords. (9) Synthesis of serotonin from radio-labeled tryptophan and ACh from labeled choline by intact isolated nerve cords in vitro was demonstrated. (10) Brain contained twice the level of free amino acids as did the nerve cord and was particularly enriched in glutamate. (11) The brain contained five times as much NE and 5-hydroxytryptophan as did the nerve cord, which contains an equivalent amount of 5-HT and was three-fold enriched in dopamine. (12) The adenosine triphosphate level in the nerve cord was determined and corresponded to an intracellular concentration of one mM. (13) A fluoride-stimulable adenyl cyclase was demonstrated in the nerve cord which was stimulated at μmolar concentrations by 5-HT but not by any other putative neurotransmitters tested.

Neurotransmitters, Cyclic Nucleotides, and Adenyl Cyclase in the Central Nervous System of *Manduca sexta*

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Duncan Paul Taylor

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Professor of Biochemistry
in charge of major
and
Dean of Graduate School

Redacted for Privacy

Head of Department of Biochemistry and Biophysics

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TO JEANNE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND TRIVIAL NAMES USED IN THE TEXT

ACh, acetylcholine

Adenyl cyclase, adenylate cyclase, adenylyl cyclase, (E.C.4.6.1.1), ATP: pyrophosphate-lyase (cyclizing)

5'-AMP, adenosine 5'-monophosphate

ATP, adenosine 5'-triphosphate

Bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino] propane

BOL, bromo-lysergic acid diethylamide

CNPase, cyclic nucleotide phosphohydrolase, (E.C. 3.1.4.1d), 2',3'-cyclic nucleotide 3'-phosphohydrolase

CNS, central nervous system

Cyclic AMP, adenosine 3',5'-cyclic monophosphate

2',3'-Cyclic AMP, adenosine 2',3'-cyclic monophosphate

Cyclic GMP, guanosine 3',5'-cyclic monophosphate

DA, dopamine, 3-hydroxytyramine

DOPA, dihydroxyphenylalanine

DTT, dithiothreitol, Cleland's reagent

EC_{50}, effective concentration at which 50% response is obtained

EDTA, ethylenediamine tetra-acetic acid

EGTA, ethylene glycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid

g, acceleration due to Earth's gravity

GABA, γ-aminobutyric acid

GTCM, Grace's (insect) tissue culture medium

HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

5-HT, 5-hydroxytryptamine, serotonin

5-HTP, 5-hydroxytryptophan

IgG, immunoglobin G, gamma-globulin

Isoproterenol, isopropylnorepinephrine

K_M, Michaelis constant

NE, norepinephrine

OA, octopamine, ρ-hydroxyphenyl ethanolamine

Phosphodiesterase, cyclic nucleotide phosphodiesterase, (E.C.3.1.4.1c), 3',5'-cyclic nucleotide 3'-phosphohydrolase

PVP, polyvinylpyrollidone

R_F, relative migration = (migration of compound)÷(migration of solvent front)

RIA, radioimmunoassay

rpm, revolutions per minute

V_{MAX}, maximum enzyme velocity

NEUROTRANSMITTERS, CYCLIC NUCLEOTIDES, AND ADENYL CYCLASE IN THE CENTRAL NERVOUS SYSTEM OF *MANDUCA SEXTA*

INTRODUCTION

Purpose of the Studies Described in this Thesis

A role for cyclic AMP as a second messenger in mammalian neurotransmission has permeated the literature for the past twenty years, since the discovery of the compound itself. In the last five years it has become apparent that cyclic AMP probably plays a similar role in the insect nervous system. More specifically, cyclic AMP may mediate stereotyped behavior in *Manduca sexta* and may act in this animal's developmental neuroethology. Thus, this insect with its relatively less complex nervous system may act as a neurochemical and behavioral model for higher animals.

The purpose of the studies presented in this thesis was to determine if the cyclic AMP system in the *M. sexta* CNS was stimulated by any neurotransmitter candidates. Initially, accumulation of cyclic AMP in intact tissues was studied, both in the whole nerve cord and in preparations of enriched cell fractions from the nerve cord. As exposure to the literature of neurotransmission increased and the list of potential neurotransmitter candidates expanded, it became advisable to determine which candidates were present in the system under investigation. Thus,

some of the physiological transmitter candidates were quantified. Because increases in cyclic AMP levels throughout the nerve cord or just within neurons may be below the sensitivity of detection by current methodology, a study of the characteristics of the adenyl cyclase activity became the approach of choice in this investigation. The background from other insects and mammalian studies on metabolism, physiology, and pharmacology of neurotransmitters and cyclic nucleotides will be reviewed prior to presenting details of the investigations with the M. sexta nerve cord.

Cyclic AMP

Since the discovery of adenosine 3',5'-cyclic monophosphate, more popularly referred to as cyclic AMP, the literature of this and other cyclic nucleotides has virtually exploded. Moreover, this explosion has now extended to reviews of the role played by cyclic AMP in a number of physiological situations. Cyclic AMP is essentially ubiquitous in Animalia, and has been found in bacteria and protists. Some debate surrounds the question of whether cyclic AMP occurs in plants.

Origin and Fate of Cyclic AMP

Adenyl cyclase[1] is the enzyme which catalyzes the cleavage of adenosine 5'-triphosphate to form cyclic AMP and pyrophosphate. The destruction of cyclic AMP is catalyzed by a phosphodiesterase which cleaves the 3'-phosphoester linkage to form adenosine 5'-monophosphate. In some animal tissues, particularly kidney, and most notably in slime molds, cyclic AMP is excreted intact from the cell. In most tissues and organisms the activity of the phosphodiesterase exceeds that of the adenyl cyclase to some degree; this degree reaches several orders of magnitude in insect tissues (Arch and Newsholme, 1976). A most detailed study of the cyclic nucleotide phosphodiesterase in the nervous system of M. sexta has been conducted previously in this laboratory (Albin, 1973).

The Second Messenger Hypothesis

Sutherland and his coworkers proposed that cyclic AMP functions as a second messenger (primarily in animals; Robison et al., 1971). Their hypothesis says that an extracellular messenger, such as a hormone, neurotransmitter, or ionic gradient, exerts its effect upon the

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[1]Adenyl cyclase is variably referred to as adenylate cyclase or adenylyl cyclase in the literature. No formal choice has been proposed, so the shortest trival name will be used in this thesis.

target cell's metabolism by increasing the intracellular concentration of some compound such as cyclic AMP. This intracellular compound is the second messenger and promotes the stereotyped response of the cell to hormonal stimulation. The first degree of specificity of hormonal stimulation is conferred by the possession of an appropriate hormone receptor coupled to adenyl cyclase in the target tissue. A number of elegant studies exploring the genetic independence of the β-adrenergic receptor and the adenyl cyclase of catecholamine-sensitive tissues have been recently published (Gilman and Minna, 1973; Insel et al., 1976). Cyclic GMP (Goldberg et al., 1973) and calcium (Rasmussen et al., 1972) have been proposed as additional second messengers.

The Protein Kinase Hypothesis

Greengard and his coworkers have proposed that, in animals, cyclic AMP exercises its second messenger role by the activation of a tissue-specific protein kinase (Kuo and Greengard, 1969). The activated kinase would catalyze the phosphorylation of other proteins which would lead to the cell's characteristic metabolic response: secretion or altered ion transport, enzyme activation, altered gene readout, or post-transcriptional modifications, for instance. Thus, a second or even third degree of specificity is implied in cyclic AMP actions, the activation of specific kinases and the

presence of specific substrates for them. Since the publication of this hypothesis, a number of papers have substantiated the correctness of this hypothesis, and none have provided data to alter it.

The Role of Cyclic AMP in Synaptic Transmission

An entire book, dedicated to Cyclic Nucleotides in the Nervous System[2], has just been published which summarizes the present findings on this topic. Greengard and his coworkers (Greengard, 1976; Nathanson and Greengard, 1976; 1977; Nathanson, 1977), as well as Daly (1976) and Bloom (1975), have recently published numerous extensive reviews of the evidence supporting a role for cyclic nucleotides and phosphorylated membrane proteins in synaptic transmission. Regulation of neurotransmitter synthesis in presynaptic neurons, alteration of microtubular function, and generation of postsynaptic membrane potentials are seen as roles for cyclic AMP and accompanying protein phosphorylation in neural function. Cyclic AMP would be elevated when a neurotransmitter activates a specifically sensitive adenyl cyclase present in the postsynaptic cell membrane. The cyclic AMP which is produced may remain near the membrane or diffuse into the cytosol. Microtubular

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[2]J. Daly, Plenum Press, New York, 1977, 401 pp.

function would be altered when an intrinsic protein kinase is activated by the diffusing cyclic AMP. However, it is unknown what parameters of microtubule function would be altered; possible parameters are rate of assembly or disassembly or axonal transport rate. Diffusing cyclic AMP may activate a protein kinase which is capable of increasing the synthesis of neurotransmitter-synthesizing enzymes such as tyrosine hydroxylase (Guidotti et al., 1975). Cyclic AMP which remains in the vicinity of the membrane could activate a membrane-bound cyclic AMP-dependent protein kinase which would lead to the phosphorylation of a membrane-bound substrate protein. In Greengard's model (1976), this substrate protein would be responsible for regulation of postsynaptic membrane permeability. A change in ion conductance or the rate of an electrogenic pump resulting from the phosphorylation would result in a change in the membrane potential (postsynaptic potential). A phosphodiesterase, to degrade cyclic AMP, and a phosphoprotein phosphatase, to return the substrate protein to its original form, would be necessary to ensure that the alteration of the membrane potential was a transient event. The membrane may become hyperpolarized, resulting in depression or inhibition, or it may become depolarized, resulting in facilitation

of neurotransmission. In the superior cervical sympathetic ganglion dopamine (DA) appears to be responsible for a cyclic AMP-mediated hyperpolarization of the membrane, the slow inhibitory postsynaptic potential, and a muscarinic cholinergic site (blocked by atropine) is responsible for a cyclic GMP-mediated membrane depolarization, the slow excitatory post-synaptic potential.

Some of the criteria for positing the cyclic nucleotide mediation of a postsynaptic potential in a specific tissue were given by Beam and Greengard (1975). There are six major criteria: (1) Synaptic activation should increase cyclic nucleotide levels, (2) the reputed neurotransmitter should elevate cyclic nucleotide levels, (3) the reputed neurotransmitter should stimulate a cyclic nucleotide cyclase in broken-cell preparations, (4) cyclic nucleotide levels in physiologically relevant cells, as detected cytochemically, should increase after electrical or chemical stimulation, (5) phosphodiesterase inhibitors should potentiate electrical or chemical stimulation of the postsynaptic potential and cyclic nucleotide levels, (6) application of cyclic nucleotides should mimic electrical and chemical stimulation. These criteria have been fulfilled for the mammalian superior cervical ganglion (Greengard, 1976; see above) and for the Purkinje cells of the cerebellum (Bloom, 1975), in

which a norepinephrine-sensitive adenyl cyclase mediates inhibition. Neurotransmitters which stimulate cyclic AMP levels in mammalian nervous tissue include dopamine, norepinephrine, serotonin, histamine, prostaglandins, morphine and the opioid peptides, substance P (Nathanson, 1977), aspartate and glutamate (Shimizu et al., 1974), and adenosine (Daly, 1976). Cyclic GMP levels are elevated by acetylcholine, norepinephrine, glycine, GABA, and glutamate (Ferrendelli, 1975).

Cyclic Nucleotides in Insect Nervous Tissues

The role of cyclic nucleotides in neuronal function of invertebrates has been much less explored than in mammals. In molluscs many of Beam and Greengard's (1974) criteria appear to be fulfilled, specifically for the abdominal ganglion of Aplysia (Levitan and Barondes, 1974; Treistman and Levitan, 1976) and Helix heart (Wolleman and S.-Rósza, 1975).

In insects it has been suggested that cyclic AMP mediates the octopamine-sensitive activation of cockroach nerve cord phosphorylase (Robertson and Steele, 1972). In their studies, Robertson and Steele (1972) demonstrated that OA decreased tissue glycogen by activating phosphorylase, and this effect was potentiated by caffeine. Exogenous cyclic AMP activated the phosphorylase as well (Hart and Steele, 1973).

Fluid secretion appears to be a cyclic AMP-mediated event in the serotonin-sensitive Malpighian tubules of Rhodnius and Carausius (Maddrell et al., 1971) and in the serotonin-sensitive salivary gland of Calliphora (Berridge and Patel, 1968; Berridge, 1970; Berridge and Prince, 1972). In the Malpighian tubule 5-HT and some of its analogs stimulated fluid secretion, and bromo-lysergic acid diethylamide (BOL) was a potent inhibitor, apparently acting competitively. Cyclic AMP and aminophylline (theophylline ethylene-diamine) separately evoked increased fluid secretion (Maddrell et al., 1971). In the Calliphora salivary gland 5-HT or cyclic AMP increased the rate of fluid secretion (Berridge and Patel, 1968). Theophylline alone stimulated fluid secretion and potentiated the response to 5-HT or cyclic AMP (Berridge, 1970). The cyclic AMP-mediated fluid secretion in the toad bladder has been used as a model for cyclic nucleotide-mediated neuronal permeability (Greengard, 1976).

Stimulus reception and chemosensory nerve function may be mediated by cyclic nucleotides in insects. Smith and Friend (1972) showed that cyclic AMP and theophylline potentiated the nucleoside phosphate-stimulated engorging behavior of Rhodnius. An adenyl cyclase has been observed in the compound eye of

Trichoplusia (Bitensky et al., 1972). The stimulus-induced spiking of the sucrose receptor, an identified neuron in Phormia, was inhibited by cyclic AMP, dibutyryl cyclic AMP, and aminophylline with or without cyclic AMP, and application of cyclic GMP caused increased neuronal firing in the presence of sucrose, while noncyclic nucleotides had no effect on firing frequency (Daley and Vande Berg, 1976). These authors have suggested that these results are consistent with a Yin-Yang mechanism (Goldberg et al., 1973) operating in the insect nervous system. Their work is supported by the cytochemical localization of adenyl cyclase (Felt and Vande Berg, 1977) and phosphodiesterase (Vande Berg, 1975) activities in the Phormia labellar chemoreceptor.

Another instance in which cyclic AMP may mediate a peripheral nervous function is in the luminescent response of larval photocytes in Photuris. It has been suggested that the firefly light organ is adrenergically innervated (Oertel and Case, 1976). The pharmacological characterization of the photocyte revealed that α-adrenergic agents depolarized photocytes and stimulated light production. Aminophylline and theophylline caused the light organ to glow (Oertel and Case, 1976).

In the insect CNS, adenosine, histamine, and norepinephrine produce significant increases in spontaneous electrical activity associated with neurohaemal tissue in Carausius. These increases were mimicked by cyclic AMP, dibutyryl cyclic AMP, caffeine, and theophylline (Finlayson and Osborne, 1977).

Several behavioral patterns of CNS origin in insects may result from cyclic nucleotide-mediated events. In the decapitated cockroach, the ability to learn to raise or extend a leg is mediated by various thoracic ganglia. The cockroach thoracic ganglia possess adenyl cyclases which are specifically sensitive to DA, OA, and 5-HT (Nathanson, 1973). In fascinating studies of this shock avoidance task, Nathanson (1973) demonstrated that learning and memory were facilitated by dibutyryl cyclic AMP and theophylline, while dibutyryl cyclic GMP antagonized the effect of dibutyryl cyclic AMP (Nathanson, 1973). The prepatterned behavior of eclosion in various moths (Truman and Riddiford, 1971) is triggered by the release of a neurohormone (Truman, 1971). This behavior is the result of a complex firing pattern which appears to be "hard-wired" in the nerve cord as electrical recordings from the deafferented tissue have demonstrated (Truman and Sokolove, 1972). This behavior may be elicited by injection of the "eclosion hormone" into isolated Cecropia abdomens

and enhanced by concommitant administration of theophylline (Truman et al., 1976). Similar results were obtained with injections of theophylline plus cyclic AMP, dibutyryl cyclic AMP, or cyclic GMP (Truman et al., 1976). Cyclic AMP and theophylline perfusion of deafferented nerve cords of Manduca sexta resulted in elicitation of eclosion behavior (Truman, personal communication).

This laboratory and others have investigated the cyclic nucleotide systems of insect nervous systems. The brain of Gromphadorhina contains an adenyl cyclase which was stimulated by fluoride, epinephrine, and norepinephrine (Rojakovick and March, 1972) and a corresponding phosphodiesterase (Rojakovick and March, 1974). Phosphodiesterase activity toward both cyclic AMP and cyclic GMP was highest in the head of Acheta versus other parts (Fallon and Wyatt, 1977). Periplaneta thoracic ganglia accumulated cyclic AMP in response to incubation in media containing norepinephrine, dopamine, octopamine, serotonin, and carbamoylcholine (Nathanson, 1973). The ganglia possessed an adenyl cyclase which was specifically stimulated by dopamine, octopamine, and serotonin (Nathanson, 1973). A cyclic AMP-dependent kinase was identified which catalyzed the phosphorylation of

specific ganglion proteins, and a phosphoprotein phosphatase activity capable of reversing this action was demonstrated (Nathanson, 1973). Specific adenyl cyclases sensitive to dopamine and octopamine have been found in cockroach brain as well (Harmar and Horn, 1977). Cyclic nucleotide phosphodiesterase activities and fluoride-stimulable adenyl cyclase activities were found in brains from Schistocerca, Blaberus, and Pachnoda (Arch and Newsholme, 1976). A cyclic nucleotide binding protein and a cyclic nucleotide-dependent protein kinase were found in the Galleria CNS (Tsuzuki and Newburgh, 1974). Sonicates of M. sexta brains synthesized cyclic AMP, and this synthesis was stimulated two-fold in the presence of 1 ug/ml β-ecdysone, but only if 12 mM fluoride were also present in the assay mixture. In the absence of fluoride, no production of cyclic AMP was observed with or without β-ecdysone despite the presence of aminophylline and cyclic AMP (Vedeckis and Gilbert, 1973). The nerve cord of M. sexta also synthesized cyclic AMP and cyclic GMP (Albin, 1973). Accumulation of cyclic GMP was stimulated by incubation in acetylcholine (Albin, 1973). A cyclic nucleotide phosphodiesterase, multiple cyclic nucleotide-stimulable protein kinases, and a phosphoprotein phosphatase have been studied in the M. sexta nerve cord (Albin, 1973). Endogenous protein substrates for the

protein kinases exist in the M. sexta nerve cord (Albin, 1973).

Thus, it appears that cyclic nucleotide systems probably play a role in insect neurotransmission which is analogous to that proposed by Greengard (1976) and Bloom (1975) for the mammalian superior cervical ganglion and the cerebellar Purkinje cell, respectively, as discussed above. It must be carefully noted that agents which affect the action and accumulation of cyclic nucleotides may be acting as neurotransmitters only if these agents are released as the result of presynaptic electrical activity. The possibility that putative neurotransmitters are acting as neurohormones (Hinks, 1967; Page and Carlsson, 1970) cannot be excluded solely on the basis of biochemical studies.

GENERAL MATERIALS AND METHODS

Materials

In general, specialty chemicals and enzymes were obtained from Sigma and stored according to their instructions unless otherwise noted. Standard laboratory chemicals were of reagent grade.

Insect Rearing

M. sexta eggs were obtained from Mr. A. H. Baumhover, U.S.D.A., Oxford, North Carolina, on a weekly basis. Larvae were raised essentially according to the procedures of Yamamoto (1969); modifications to the diet were made according to Riddiford (personal communication to R. W. Newburgh). As studies in this laboratory progressed, both "short day" and "long day" (12 hours light: 12 hours dark and 17 hours light: 7 hours dark, respectively) photoperiods were installed in separate growth regimes. Animals for dissection were removed from either regime indiscriminately. Initially, animals which had entered the fifth larval instar were chosen for dissection on the basis of body weight. As the expertise within the laboratory grew, it became feasible to choose a more precise developmental stage, the first day after the initiation of "wandering" (Truman and Riddiford, 1974).

Dissection of Insect Nervous Tissues

The morphology of the M. sexta nervous system and the means of dissecting brains and nerve cords has been explicitly described by Albin (1973). Originally, freshly-excised tissues were rinsed in Grace's (1962) insect tissue culture medium. However, following the work which indicated that high potassium and amino acid levels may affect cyclic nucleotide levels (see subsequent chapters), all tissues were rinsed in the high sodium ringer of Ephrussi and Beadle (1936). In most experiments, the nervous tissue was then immediately frozen by contact with the wall of a glass or plastic container held on powdered dry ice. Tissues were pooled, commonly in lots of ten, and stored at -80^{o}C until used.

Protein Determination

The method of Lowry et al. (1951) was routinely used to assess the levels of proteins in homogenates of nervous tissues. Bovine serum albumin, Fraction V, was used as a standard. Care was taken to ensure that standards contained the same concentration of sodium hydroxide as samples in order to avoid erroneous results. Absorbance at 750 nm was read on a Bausch and Lomb Spectronic 600 spectrophotmeter in one ml quartz cuvettes. Although this procedure can

accommodate sample volumes as high as 100 µl, usually only 2-10 µl were required to attain quantifiable levels of protein which remained within the linear portion of the standard curve.

CYCLIC NUCLEOTIDES IN NEURONAL- AND GLIAL-ENRICHED FRACTIONS FROM THE NERVE CORD OF *MANDUCA SEXTA*

Detailed biochemical study of neuronal function is often hampered by the heterogeneous population of cells present. A number of techniques have been described for the bulk-isolation of neuronal- and glial-fractions from mammalian brain tissues (Fewster *et al.*, 1967; Rose, 1967, 1969; Flangas and Bowman, 1970; Norton and Poduslo, 1970; Sellinger *et al.*, 1971; Sinha *et al.*, 1975). These methods rely on tissue dissociation by mechanical mincing with or without enzymatic treatment, followed by a meshing operation through various sizes of nylon bolting cloth. The various cell types and debris were then separated and enriched by one or more centrifugations on discontinuous sucrose gradients. The methods of Rose and his coworkers have received some criticism in the literature (Cremer *et al.*, 1968) for yielding heterogeneous and damaged populations of cells.

The purpose of the following study was to separate neuronal perikarya from glia in the fifth larval instar nerve cord and determine the effects of putative neurotransmitters on cyclic nucleotide levels in these fractions. The method of isolation used

in this study was patterned after the relatively simple, direct and successful procedure followed by Sellinger et al. (1971).

The degree of enrichment was monitored microscopically using the staining procedures of Flangas and Bowman (1970). A more quantitative measure of enrichment was obtained by assessing the activity of 2',3'-cyclic adenosine monophosphate 3'-phosphohydrolase (CNPase, E.C. 3.1.4.1d). In an effort to maintain a viable environment for the enriched fractions, suspension and incubation were carried out in Grace's (1962) insect tissue medium (GTCM) as suggested by Albin (1973). The use of absolute ethanol (Farmer et al., 1975) promised a means of easily and quantitatively extracting cyclic nucleotides while removing protein. Radioimmunoassay kits for the determination of cyclic nucleotides were chosen as the simplest and most specific means of assay. The relatively small yield of neuronal- and glial-enriched fractions constrained the investigation to pursue a few prototypical putative neurotransmitters, namely ACh, NE, and 5-HT, with theophylline as the classical phosphodiesterase inhibitor.

Materials and Methods

Materials

M. sexta larvae were raised and nerve cords dissected as described in General Materials and Methods. For cell separations, sucrose, ultracentrifuge grade, was obtained from Schwarz Mann, and Ficoll-400 from Pharmacia. Other specialty chemicals were obtained from Sigma. Nylon bolting cloth was a gift of Dr. J. M. Cardenas. Grace's insect tissue culture medium, without hemolymph, was acquired from Gibco, Grand Island, New York. Paragon Multiple Stain was from Paragon, Bronx, New York. Sodium deoxycholate used in the CNPase assay was a gift of Dr. D. J. Reed. *E. coli* alkaline phosphatase (E.C. 3.1.3.1), type III-S, containing in one ml activity to hydrolyze 144 μmoles p-nitrophenyl phosphate per minute at 37°C, was from Sigma. Absolute ethanol was purchased from IMC Chemical Group, Inc., Agnew, California. Kits for the assay of cyclic AMP and cyclic GMP were obtained from Collaborative Research, Waltham, Massachusetts, and contained standard solutions of cyclic nucleotides, ^{125}I-labelled antigens, cyclic nucleotide antisera, anti-rabbit IgG, and normal rabbit serum. Cellulose nitrate filters (HAWP 02500 0.25 μm) were obtained from Millipore Corp.

Aquasol was a xylene-based scintillation cocktail purchased from New England Nuclear. U-^{14}C-cyclic AMP, ammonium salt, specific activity 117 mCi per mmol, was purchased from Amersham Searle. Glass double-distilled water was used throughout.

Neural-Glial Enrichment

Preparation of neuronal and glial fractions was accomplished following the technique of Sellinger et al. (1971). Five hundred twenty-nine nerve cords weighing 2.1915 gm were minced with a razor blade on a petri dish, transferred in a wide-mouth Pasteur pipet by suspending in 15 ml 7.5% PVP, 1% BSA, 10 mM $CaCl_2$, and chopped at low speed for 1.5 minutes in a Virtis 45 minicup. The material was meshed twice through two layers of nylon screen, the first layer being 275 µm mesh, the second of 110 µm, by securing the mesh with rubber bands over plastic disposable syringes which had been altered by cutting away the tips. After each meshing, the screen was scraped with a razor blade to remove adhering material. The resulting filtrate was meshed once through layers of 110 µm and 70 µm cloth. This final filtrate was centrifuged on a discontinuous sucrose gradient consisting of 1.25 ml 1.75 M sucrose, 1% BSA, 1.25 ml 1.0 M sucrose, 1% BSA, 2.00 ml filtrate in ½" by 2"

nitrocellulose tubes in an SW 50.1 rotor at 23,000 rpm (50,000 x g) for 30 minutes at 0°C in a Sorvall OTD-2 centrifuge. The upper layer was aspirated and discarded. The middle layer (1.0 M sucrose) and its accompanying lower interface, containing glia and medium-density debris, were aspirated and diluted 2:1 with 1% BSA, 1% NaCl. The lowest layer was aspirated and discarded. The pellet consists of neurons and was washed once with one ml 1% NaCl. The suspended neurons and the glial fraction were pelleted at 23,000 rpm for 10 minutes in the SW 50.1. The washed neuronal pellet was resuspended in 1.5 ml GTCM. The glial fraction was resuspended in 7.5% PVP, 5% Ficoll, 1% BSA, and centrifuged on a second gradient at 23,000 rpm for 30 minutes in the SW 50.1 rotor as before. This second gradient was composed of 1.5 ml 1.65 M sucrose, 1% BSA, 1.8 ml 1.2 M sucrose, 1% BSA, 0.9 ml 30% Ficoll, 1% BSA, and 0.8 ml glial fraction. The lowest interface (1.65 M sucrose - 1.2 M sucrose) was enriched in glia; it was aspirated, washed with GTCM once, and resuspended in GTCM. The neuronal and glial fractions were refrigerated at 0-4°C until the various analyses could be completed.

Microscopic Inspection

A drop of the cell suspension to be viewed was smeared on a clean glass microscope slide and allowed

to air dry. The smear was stained by covering with several drops of Paragon Multiple Stain for two minutes. The excess stain was removed by rinsing the slide in a beaker of distilled water. The slide was then allowed to air dry.

CNPase Assay

The method of Prohaska et al. (1973) was followed for this assay: 20 µl of cell suspension were sonicated for ten seconds in 20 µl 0.32 M sucrose in a 12 x 75 mm culture tube using a Kontes Cell Disruptor at power setting 6. The CNPase was activated by incubating the sonicate with 20 µl 0.2 M Tris-HCl pH 7.5, and 40 µl 1% sodium deoxycholate on ice for ten minutes. The reaction was then initiated by the addition of 100 µl 100 mM Tris-maleate, pH 6.2, 15 mM 2',3'-cyclic AMP, and incubated ten minutes at 30°C in a shaking water bath. Tissue blanks were obtained by adding Tris-maleate without the 2',3'-cyclic AMP. The reaction was terminated by boiling for 30 seconds in a Tem-blok heater, and the tubes were held on ice for the next step. One hundred µl 0.3 M Tris-HCl, pH 9.0, 21 mM $MgCl_2$ and five µl E. coli alkaline phosphatase were added, and the reaction mixture incubated 20 minutes at 30°C on the shaking water bath. At this time, a standard curve consisting of 0, 100, 200, 500, 750, and 1000 µmoles of phosphate

(from a stock solution of 10 mM $NaH_2PO_4 \cdot H_20$) in 300 µl total volume was prepared. Inorganic phosphate liberated by the enzymatic reactions or present in the standards was determined by the formation of a phosphomolybdic acid complex by the addition of 1.2 ml 1.5% ammonium molybdate-tetrahydrate in 0.5 N H_2SO_4. The yellow chromophore was extracted by vigorously vortexing for ten seconds with 1.2 ml isobutanol:benzene (1:1,v/v). The upper layer was obtained after centrifuging five minutes at 2200 rpm (1000 x g) using the 50 ml buckets in a Sorvall GLC-1. After removal the organic phase was read in one ml cuvettes at 410 nm against isobutanol:benzene.

Incubation with Neurotransmitters

The test substances, in GTCM, and cell suspensions were preincubated at least ten minutes at 37°C on a shaking water bath. At time zero, 200 µl of test solution was added to 200 µl cell suspension in a three ml conical tube and incubation at 37°C continued for an additional ten minutes. At the end of this time, the reaction was terminated by placing the tubes in a test tube rack which was partially submerged in liquid nitrogen in a styrofoam box. Freezing was usually complete by 15 seconds after immersion. Samples thus prepared could be stored overnight at -20°C.

Cyclic Nucleotide Extraction and Deproteination

Incubates were individually thawed and sonicated for ten seconds. Two ml ice-cold absolute ethanol were used to rinse the sonicator probe and precipitate protein in the manner of Farmer et al. (1975). This method has also been described by Kebabian et al. (1975b). The ethanolic solutions were vortexed for ten seconds and centrifuged at 3,000 rpm (1800 x g) for ten minutes in the 50 ml buckets of the Sorvall GLC-1. The supernatant was removed and reserved, and the pellet was washed with an additional two ml absolute ethanol. The resulting supernatant was pooled with the original in 12 x 75 mm culture tubes and evaporated to dryness in a vacuum oven at 80^{o}C and 30+ inches of vacuum. The residues were suspended in 400 µl 50 mM sodium acetate, pH 6.2, and 100 µl was removed and placed in three other culture tubes.

Cyclic Nucleotide Assays

The protocol of Collaborative Research, issued with the purchased kits, followed the procedure of Steiner et al. (1972). This involved incubation of the buffered extracts with cyclic AMP or cyclic GMP antiserum and ^{125}I-succinyl cyclic AMP tetramethyl ester. After two hours at 4^{o}C anti-rabbit IgG was added as a second, precipitating antibody. After 16

hours incubation at 4°C the antigen-antibody-antibody complex was isolated by retention on Millipore filters as detailed by Albin (1973): The filter apparatus was chilled by filtering ten ml ice-cold 50 mM sodium acetate, pH 6.2; the assay solution was diluted with 1.5 ml ice-cold buffer and filtered within five seconds; the filter was rinsed with an additional 15 ml ice-cold buffer and allowed to air dry. The filter was placed in a glass scintillation vial and dissolved by heating at 60°C for 20 minutes in 0.5 ml ethylene glycol monomethyl ether. After addition of 15 ml Aquasol the sample was vortexed vigorously ten seconds and allowed to equilibrate overnight in the dark. The samples were counted in a Packard 3330 liquid scintillation spectrometer at a setting of 30% gain, windows 50 to 1000.

Comments

Deproteination is an important step. An early experiment deleted this step with a resultant requirement of nine hours to complete the "rapid" filtration and removal of antigen-antibody-antibody complex! After deproteination a sample filter could be washed in about 20 seconds normally.

It proved very difficult to visualize and retain the antigen-antibody-antibody complex after pelleting by centrifugation as suggested by the factory protocol.

The filter-retention technique seemed to side-step the difficulties inherent in the former procedure. It is interesting that the Collaborative Research protocols recently (since the completion of this work) now suggest charcoal-dextran precipitation or ammonium sulfate precipitation of the complex.

Results

Microscopic Inspection

Light microscopy of the fractions isolated from the CNS of M. sexta showed intact perikarya, which stained light blue, in the neuronal fraction (Fig. 1 and 2). It appears that axonal processes were sheared off in the enrichment process as noted by others (Flangas and Bowman, 1970). Cells found in the glial-enriched fraction were "gauzy"-appearing and dark magneta-stained (see Fig. 3), the color described by Flangas and Bowman (1970).

CNPase Activity

The neuronal-enriched fraction contained sufficient activity to hydrolyze 0.019 µmoles 2',3'-cyclic AMP per mg protein in ten minutes. The glial-enriched fraction contained 20 times as much activity, at the level of 0.366 µmoles 2',3'-cyclic AMP hydrolyzed per mg protein in ten minutes. Intact nerve cords, homogenized in 0.32 M sucrose, have an activity of 0.270 µmoles per minute per mg protein.

Figure 1. Cell body from neuronal-enriched fraction.

The cell suspension had been stained with Paragon Multiple Stain (see text). Micro-photograph taken at 400 X with phase optics.

Figure 1.

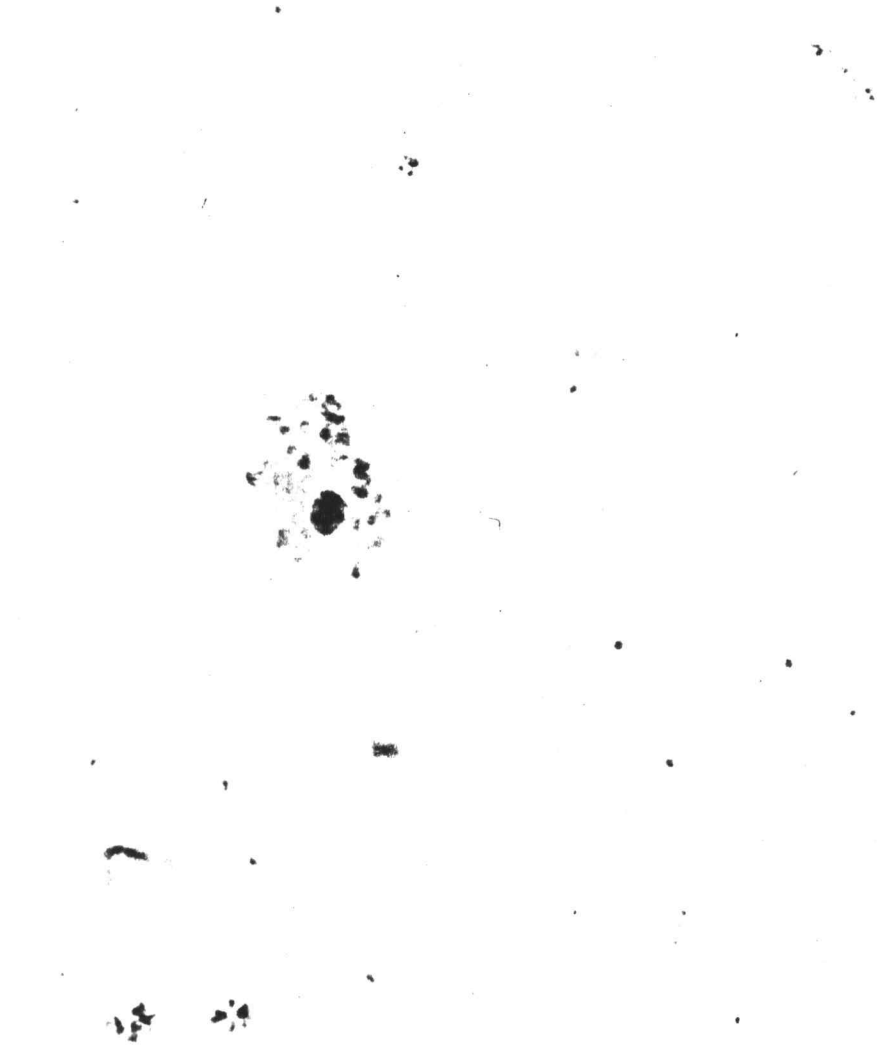

Figure 2. Cell body from neuronal-enriched fraction.

Details are given in legend to Figure 1, and text. 400 X, phase.

Figure 2.

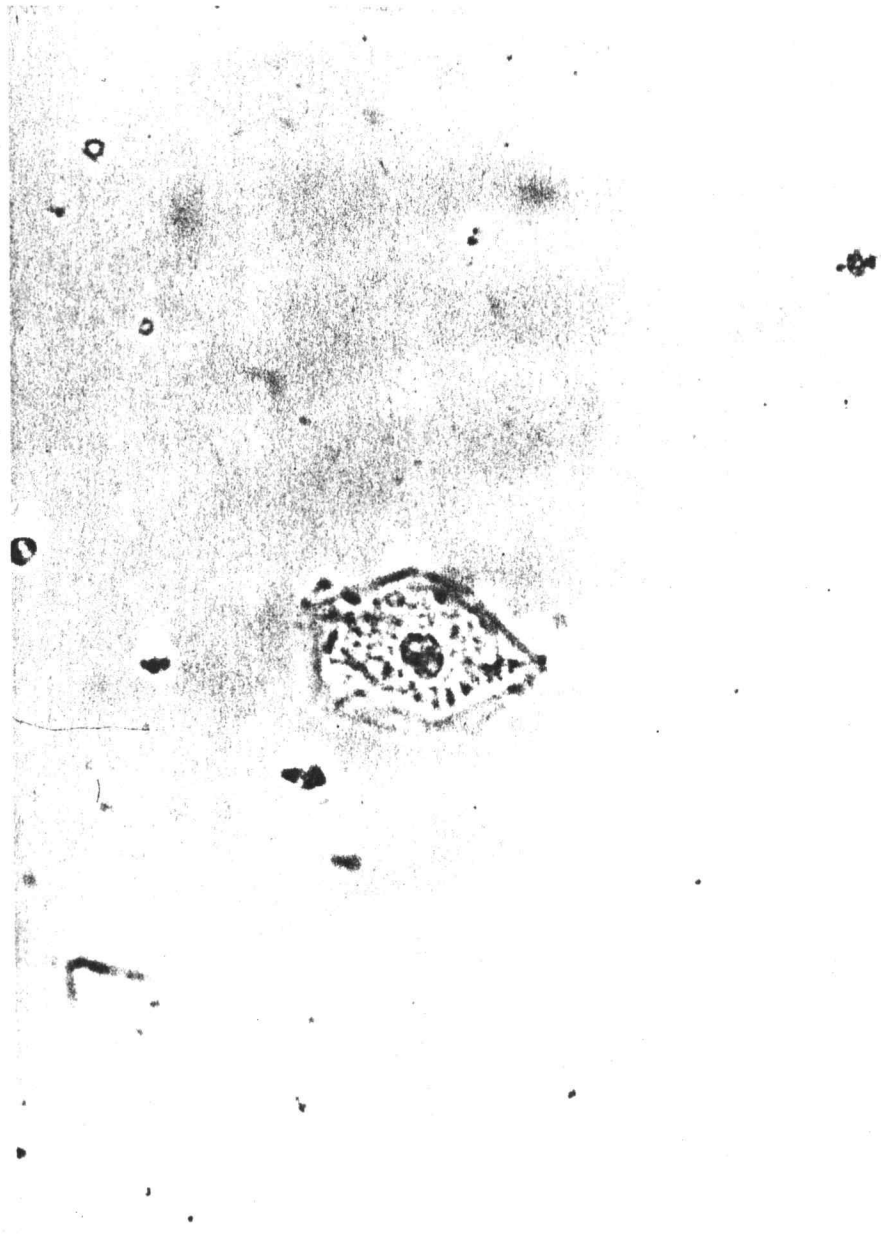

Figure 3. Cell body from glial-enriched fraction.

See legend of Figure 1, and text for details. 400 X, phase.

Figure 3.

Cyclic Nucleotide Levels

The recovery of cyclic nucleotides through the ethanol extraction procedure using equivalent amounts of BSA in place of cell protein and ^{14}C-cyclic AMP was 97.7% ± 0.9% (SEM) based on four replicate samples. Kebabian et al. (1975b) reported recoveries routinely in excess of 90%, and Farmer et al. (1975) reported theirs at 99-100%.

The results of the cyclic nucleotide radioimmunoassays are given in Table 1. In both cell fractions incubation in GTCM, which is similar to Lepidopteran hemolymph (Grace, 1962), resulted in cyclic GMP:cyclic AMP ratios in excess of ten. Exposure to 5-HT resulted in a dramatic 33,000-fold increase in cyclic AMP levels in neurons and a 430-fold increase in glia, about one percent of the neuronal increase. Theophylline was much less active than 5-HT in neurons and ineffective in glia in the elevation of cyclic AMP levels. NE had an even smaller effect and ACh essentially none on neuronal cyclic AMP levels. Cyclic GMP levels in both neuronal- and glial-enriched fractions were unaffected by any of the putative neurotransmitters.

Table 1. Effect of various test substances on cyclic nucleotide levels in neuronal- and glial-enriched fractions from nerve cords.

Results are given as multiples of control levels after ten minutes incubation. Control levels, as mean pmoles cyclic nucleotide per mg protein ± range for two determinations, were as follows: Neuronal fraction, cyclic AMP, 0.029 ± 0.015, cyclic GMP, 0.44 ± 0.09, glial fraction, cyclic AMP, 0.043 ± 0.020, cyclic GMP, 1.76 ± 0.13. Test substances were present in the final concentration indicated.

	Neuronal Fraction		Glial Fraction	
Test Substance	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
22.5mM Theophylline	344	3.2	0.67	0.56
0.1mM Norepinephrine	74	2.9	133	0.46
50mM Acetylcholine	6.4	2.5	0.77	0.29
20mM Serotonin	33,000	5.6	430	0.44

Discussion

Zanetta et al. (1972) have described CNPase activity as glial-specific in the nervous system, using C-6 clone of rat glioblastoma. Glial cells were higher in CNPase activity than neuronal cell bodies from adult rat brains prepared by Nagata et al. (1974). The activity of the M. sexta CNPase in the glial-enriched fraction is about two orders of magnitude less than that described by some workers (Zanetta et al., 1972; Nagata et al., 1974), but not by others (Poduslo and Norton, 1972) for normal mammalian tissues and one-tenth that of various human brain tumors (Kurihara et al., 1974). The glial fraction also has about one-tenth the CNPase activity of the intact nerve cord. It may be that the isolation/enrichment procedure is responsible for some inhibition of activity as PVP and sucrose inhibit acetylcholinesterase, succinate dehydrogenase and lactate dehydrogenase activities in mammalian brain tissue homogenates (Lisý et al., 1971). The degree of glial contamination of the neuronal-enriched fraction may be estimated by modifying the assumptions of Giacobini (as cited in Flangas and Bowman, 1970) to the effect that (a) all CNPase activity represented glial protein and (b) the glial fraction is not contaminated by neurons. If the second assumption were erroneous,

the amount of glial contamination calculated in the neuronal-enriched fraction would be too large. When the CNPase activity of the glial-enriched fraction is divided into that found in the neuronal-enriched fraction, the apparent glial contamination is less than 5.3%.

Cyclic AMP:cyclic GMP ratios on the order of five to ten were commonly observed in vertebrate nervous tissues while the reciprocal ratio is seen in some cricket nervous systems (Ishikawa et al., 1969). However, this latter finding has been shown to be misleading as all other tested cricket families (Fallon and Wyatt, 1975), as well as intact nerve cords of the lepidopterans Cecropia (Truman et al., 1976) and M. sexta (see next chapter), have cyclic AMP:cyclic GMP ratios near those for vertebrates. The value for the cyclic AMP:cyclic GMP ratio obtained here is probably erroneous as it will be shown in the next chapter that submillimolar concentrations of amino acids, present in GTCM at millimolar levels, are capable of elevating cyclic GMP levels over 20-fold in intact M. sexta nerve cords. This artificial elevation of cyclic GMP levels may actually mask any elevation caused by addition of the putative neurotransmitters.

CYCLIC NUCLEOTIDE ACCUMULATION IN THE CENTRAL NERVOUS SYSTEM OF *MANDUCA SEXTA*

A much larger number of putative neurotransmitters exist than could possibly be examined in the neural-glial preparation described in the previous chapter. It seemed most economical, in terms of time and material, to observe the effects of cyclic nucleotide levels of incubating intact ventral nerve cords in solutions containing various putative neurotransmitters as a screening technique for further investigations. As alluded to in the previous discussion the high amino acid content of GTCM caused (rightly) some concern about artifacts in the work. It was deemed desirable to investigate the effects of the high sodium ringer solutions of classical neurophysiology as incubation media versus a high potassium ringer patterned after the lepidopteran hemolymph. The RIA described in the previous chapter was shortened by removal of the incubation with the second precipitating antibody.

Materials and Methods

Materials

M. sexta larvae were raised and nerve cords dissected as described in General Materials and Methods.

Special chemicals and buffers were obtained from Sigma, except glutamic acid which came from Nutritional Biochemical Co. RIA kits, containing cyclic nucleotide standards. cyclic nucleotide antisera, normal rabbit serum, and ^{125}I-labelled antigens, were again obtained from Collaborative Research.

Tissue Incubations

Thawed nerve cords, usually in pools of ten, were transferred to three ml Kontes Duall all-glass tissue grinders and preincubated at least ten minutes at 30^{o}C in a shaking water bath followed by the addition of 200 µl preincubated saline solution with or without additional agents. The tissue and solution were incubated at 30^{o}C on the shaking water bath for ten minutes and frozen in liquid nitrogen as described in the previous chapter.

The saline solutions used were a high sodium ringer (Ephrussi and Beadle, 1936), consisting of 152 mM NaCl, 4.7 mM KCl, and 2.8 mM $CaCl_2$, or a high potassium solution, consisting of 150 mM KCl, 5 mM NaCl, and 2.8 mM $CaCl_2$. Solutions were buffered with 50 mM concentrations of formate (pH 3.5), citrate (pH 5.3), bis-tris propane (pH 6.5), or HEPES (pH 7.2). The pH at 30^{o}C of all incubation solutions was checked after constitution. Ascorbic acid was routinely added as an antioxidant at 100 µM.

Cyclic Nucleotide Extraction and Deproteination

These operations were essentially described in the previous chapter: The frozen incubates were homogenized in one ml ice-cold absolute ethanol using ten one-second passes of a motor-driven pestle at 500 rpm and the pestle washed with 0.5 ml ice-cold ethanol. The ethanol solutions were centrifuged five minutes at 2200 rpm (1000 x g) in the 50 ml buckets of a Sorvall GLC-1. The pellets were rehomogenized twice using five one-second passes of the pestle, one ml of ice-cold absolute ethanol for each homogenization and 0.5 ml ethanol for rinsing the pestle each time. The ethanolic supernatants were pooled in 15 ml conical glass tubes and evaporated to dryness in a vacuum oven in two stages to prevent bumping; the first being five inches of vacuum for five minutes, the second being 30+ inches until dryness was obtained, usually about four hours. Both stages were conducted at a temperature of $60^{o}C$. This residue could be held overnight at $-20^{o}C$. The ice-cold residue was resuspended in exactly 1000 µl ice-cold absolute ethanol, vortexed vigorously for ten seconds and aliquoted into 12 x 75 mm tubes as either duplicates of 400 µl or triplicates of 250 µl each. The ethanol was evaporated in the vacuum oven as described

above and the residues held at -20°C until a sufficient number for cyclic nucleotide RIA had accumulated.

Preparation for Protein Determination

The ethanol-washed precipitates resulting from the cyclic nucleotide extraction described above were dried for three to five minutes on a boiling water bath. Two hundred µl of ten N NaOH were added, and the tubes returned to the boiling water bath for an additional two hours. At the end of this period the tubes were sonicated with a Kontes Cell Disrupter at power setting six for ten seconds to evenly disperse any undissolved material remaining from the pellet. The probe was rinsed off with 200 µl of ten N NaOH, the basic, protein solution transferred to a volumetric two ml test tube and made up to volume with aqueous washes of the tissue grinder. This solution was then held at 4°C until assayed for protein as described in General Materials and Methods.

Cyclic Nucleotide Assays

Cyclic nucleotide residues were dissolved in 300 µl 50 mM sodium acetate, pH 6.2, and assayed using the RIA kits as described in the previous chapter, except that at the end of the first incubation with cyclic nucleotide antiserum the antigen-antibody complex was isolated by retention on Millipore filters as

described in the previous chapter. The filters were prepared and counted as detailed in the previous chapter.

Comments

At the time this work was being performed, a rash of papers appeared describing extremely sensitive, small volume (hence cheap) RIAs carried out by the microsuccinylation (Cailla et al., 1973) or microacetylation (Harper and Brooker, 1975) of cyclic nucleotides. Several attempts to duplicate the microacetylation technique (Rubin and Ferrendelli, J. Histochem. Cytochem., in press) gave monotonic, but nonlinear, standard curves using chemicals from Collaborative Research. Further efforts along these lines were curtailed in the interests of time.

Results

Freshly excised tissue which is frozen upon excision and assayed contained 3.22 $\pm$ 0.49 (SEM) pmoles cyclic AMP per mg protein (one pool of ten nerve cords assayed in triplicate). A similar pool was allowed to thaw after excision and freezing, incubated at 30^{o}C for 20 minutes, refrozen, and assayed. This latter pool contained 3.29 $\pm$ 0.12 (SEM) pmoles cyclic AMP per mg protein (assayed in triplicate).

The data in Table 2 indicate that the sodium: potassium ratio affects not only control levels of

Table 2. Specific accumulation of cyclic AMP in pooled nerve cords with various agents in different saline solutions.

Results are given as mean pmoles cyclic AMP per mg protein ± SEM or range (for two determinations). The number of pools of nerve cords assayed is indicated in parentheses. Additions were present in the final concentrations indicated at pH 5.3. All solutions contained 100μM ascorbate.

Addition	High Sodium Ringer (Na+/K+ = 32)	High Potassium Ringer (Na+/K+ = 0.033)
None	1.65 ± 0.89 (8)	0.440 ± 0.029 (2)
10mM Theophylline	7.88 ± 2.08 (4)	2.81 (1)
10mM Serotonin	5.15 ± 2.69 (4)	1.11 ± 0.09 (2)

cyclic AMP present but also the absolute responses of those levels to incubation with 5-HT or theophylline. Under the conditions employed, 5-HT and theophylline give 300% and 500% elevation of cyclic AMP levels in the high sodium ringer, whereas these agents are responsible for 250% and 600% respective increases in the high potassium solution. To maintain the possibility of observing the maximum degree of absolute accumulation, the high sodium ringer was used as a carrier in the remaining experiments.

The effects of incubation with various agents at several different pH values were recorded in Table 3. At pH 3.5 the values for cyclic AMP levels resulting from incubation with 5-HT, with or without theophylline, theophylline alone, and aspartic acid are markedly above the control levels. Only 5-HT or theophylline, alone or in combination, are effective at pH 5.3 in elevating cyclic AMP levels. Interestingly, 5-HT actually potentiated response to theophylline at pH 6.5, but was ineffective alone. In addition, GABA was active in stimulating cyclic AMP levels at this pH. Norepinephrine and the β-adrenergic agonist, isoproterenol, elevated cyclic AMP levels at pH 7.2 as did theophylline alone or in combination with 5-HT. As can be seen, a great deal of variation was present

Table 3. Specific Accumulation of Cyclic AMP in Pooled Nerve Cords Incubated at Different pH Values with Various Agents.

Results are given as mean pmoles cyclic AMP per mg protein ± SEM or range (for two determinations). The number of pools of nerve cords assayed is indicated in parentheses. Incubations were carried out in the high sodium ringer solution containing 100μM ascorbate, and test substances were present in the final concentrations indicated. N.D. = not determined.

Addition	pH: 3.5	5.3	6.5	7.2
None	0.235 ± 0.029 (5)	1.65 ± 0.89 (8)	0.546 ± 0.086 (4)	0.082 ± 0.003 (2)
10mM Serotonin	3.85 ± 3.40 (3)	5.15 ± 2.69 (4)	0.496 ± 0.297 (5)	0.119 ± 0.060 (2)
10mM Theophylline	8.36 ± 3.64 (4)	7.88 ± 2.08 (4)	7.71 ± 4.99 (2)	0.948 ± 0.613 (2)
10mM Theophylline + 10mM Serotonin	3.97 ± 2.43 (5)	7.19 ± 3.48 (5)	13.8 ± 5.5 (3)	0.887 ± 0.412 (3)
50mM Acetylcholine	0.113 (1)	1.12 (1)	N.D.	0.118 (1)
250μM Epinephrine	0.223 (1)	N.D.	0.260 (1)	0.086 (1)
250μM Norepinephrine	0.132 (1)	0.645 (1)	N.D.	0.178 (1)
250μM Dopamine	0.220 (1)	0.218 (1)	N.D.	0.067 (1)
250μM Octopamine	0.045 (1)	0.918 ± 0.431(5)	N.D.	0.089 (1)
250μM Isoproterenol	0.013 (1)	1.26 ± 0.45 (3)	N.D.	0.368 (1)
500μM Aspartic acid	1.81 ± 0.89 (3)	0.077 (1)	0.077 (1)	0.034 (1)
500μM Asparagine	N.D.	0.524 (1)	N.D.	N.D.
500μM Glutamic acid	0.760 ± 0.536(3)	0.106 (1)	0.194 (1)	0.040 (1)
500μM Glycine	0.037 (1)	0.482 ±0.183(3)	N.D.	0.043 (1)
500μM GABA	0.230 (1)	1.06 ± 0.93 (2)	1.27 ± 0.01(2)	0.087 (1)
2mM Glutamine	N.D.	0.537 ± 0.091(2)	N.D.	N.D.
10mM Sodium fluoride	N.D.	N.D.	0.239 (1)	N.D.

Table 4. Effect of Varying Ascorbate Concentration on Specific Cyclic AMP Accumulation in Pooled Nerve Cords With and Without Various Agents at Different pH Values.

Results are given as mean pmoles cyclic AMP per mg protein ± SEM or range (for two determinations). The number of pools of nerve cords assayed is indicated in parentheses. Incubations were carried out in the high sodium ringer solution, and test substances were present in the final concentrations indicated. N.D. = not determined.

	pH and Ascorbate Concentration:								
	3.5			5.3		6.5		7.2	
Addition	100µM	1mM	0µM	100µM	1mM	100µM	1mM	100µM	1mM
None	0.235±0.029(5)	0.214±0.132(2)	1.42±0.59(3)	1.65±0.89(8)	0.440±0.172(3)	0.546±0.086(4)	0.151±0.066(2)	0.082±0.003(2)	0.449±0.096(2)
10mM Theophylline	8.36 ±3.64 (4)	0.976±0.034(2)	N.D.	7.88±2.08(4)	2.55 ±2.22 (3)	N.D.	N.D.	0.948±0.613(2)	1.47 ±0.50 (2)
10mM Serotonin	N.D.	N.D.	N.D.	5.15±2.69(4)	0.778±0.358(3)	0.496±0.297(5)	0.108±0.047(2)	0.119±0.060(2)	0.394±0.031(4)
10mM Theophylline + 10mM Serotonin	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.887±0.412(3)	1.11 ±0.03 (2)

between replicate experiments. Such variation has been noted by others in similar experiments (Kebabian et al., 1975b).

An interesting sidelight was generated when a series of incubations was erroneously conducted with a ten-fold increase in ascorbate levels. A few experiments were performed with the ascorbate concentration varied as depicted in Table 4. At pH 5.3, the addition of 100 μM ascorbate did not seem to affect control accumulation of cyclic AMP. With the exception of the experiments at pH 7.2, one mM ascorbate levels reduced the amount of cyclic AMP accumulated in the presence or absence of 5-HT or theophylline. The relative magnitude of the response to theophylline was reduced from a ten-fold increase in cyclic AMP levels to about three-fold at pH 7.2 by the excess ascorbate.

Table 5 illustrates the effect of various putative neurotransmitters on cyclic GMP levels. Glutamate and GABA stimulated cyclic GMP accumulation over 20-fold over the control, even more than theophylline. Glycine, aspartate, and ACh were less potent. Cyclic GMP levels were not increased by ten mM 5-HT or fluoride, nor by 250 μM concentrations of the catecholamines or octopamine.

β-Ecdysone, the insect molting hormone, did not increase the level of cyclic AMP in the nerve cord,

Table 5. Specific accumulation of cyclic GMP in pooled nerve cords incubated with various agents.

Results are given as mean pmoles cyclic GMP per mg protein. The control value is the mean of four nerve cord pools ± SEM. Experiments with added agents, present at the final concentration indicated, each employed one pool of ten nerve cords assayed in triplicate. Incubations were performed in the high sodium solution at pH 6.5 containing 1mM ascorbate.

Addition	Specific Cyclic GMP Accumulation
None	0.098 ± 0.012
50mM Acetylcholine	0.191
500μM Aspartic acid	0.235
500μM Asparagine	0.702
500μM Glutamic acid	2.89
500μM Glutamine	0.488
500μM Glycine	0.751
500μM GABA	2.33
10mM Theophylline	1.35
250μM Epinephrine	0.107
250μM Norepinephrine	0.095
250μM Dopamine	0.119
250μM Octopamine	0.064
250μM Isoproterenol	0.069
10mM Serotonin	0.029
10mM Sodium fluoride	0.044

but did have a marked effect on cyclic GMP levels (see Table 6).

Discussion

The basal values of cyclic AMP in freshly excised tissue were approximately the same as those observed in insect tissues by other workers (Albin, 1973; De Reggi and Cailla, 1974, 1975; Catalán et al., 1975, 1976; Fallon and Wyatt, 1975; Arch and Newsholme, 1976; Bodnaryk, 1976; Truman et al., 1976). It appears that the incubation step resulted in basal values which were lower. It is likely that this decrease in cyclic AMP levels was brought about by the high activity of the M. sexta phosphodiesterase (Albin, 1973; Albin et al., 1975).

In the work described in the previous chapter, as well as other work on cyclic nucleotide accumulation in insect nervous systems (Albin, 1973) GTCM was used as the carrier for incubation solutions. Because this medium contains amino acids which could affect cyclic nucleotide levels, less complex media were investigated.

The M. sexta hemolymph has a low sodium:potassium ratio, 1:1, which will not support action potential production in naked axons; this implies a sodium-dependent action potential (Pichon et al., 1972). The ventral

Table 6. Specific Accumulation of Cyclic Nucleotides in Pooled Nerve Cords Incubated With or Without β-Ecdysone.

Results are given as mean pmoles cyclic nucleotide indicated per mg protein ± SEM or range (for two determinations). The number of pools of nerve cords assayed is indicated in parentheses. Incubations in high sodium ringer solutions were performed for the indicated times at the indicated pH values. β-Ecdysone, where added, was present at a final concentration of 50μg/ml. N.D. = not determined.

Addition, Length of Incubation	Cyclic AMP, pH 3,5	5.3	6.5	7.2	Cyclic GMP, pH 6.5
No addition:					
10 minutes	0.235 ± 0.029 (5)	1.65 ± 0.89 (8)	0.546 ± 0.086 (4)	0.082 ± 0.003 (2)	0.098 ± 0.012 (4)
30 minutes	0.102 (1)	N.D.	0.529 ± 0.492 (2)	0.027 (1)	N.D.
β-Ecdysone:					
10 minutes	0.029 (1)	N.D.	0.587 ± 0.274 (3)	0.108 (1)	0.49 (1)
30 minutes	0.079 (1)	0.799 ± 0.142 (2)	0.106 (1)	0.094 (1)	0.72 (1)

nerve cord is enveloped by a highly impermeable sheath of glia which allows production of action potentials even in sodium-free saline (Pichon et al., 1972). Because the freeze-thaw treatment necessitated to obtain sufficient pools of nerve cords for biochemical studies might have damaged this sheath, it was felt that an investigation of the effects of high sodium or high potassium ringer solutions, with or without additions, on cyclic AMP accumulation should be made.

The high potassium ringer markedly diminished the amount of cyclic AMP accumulated in the presence or absence of 5-HT or theophylline in comparison to the high sodium solution. Because the phosphodiesterase in this tissue is largely soluble (Albin et al., 1975), it would not be expected to be affected by extracellular ionic changes. The conclusion is that the activation of the adenyl cyclase was affected by the extracellular ions present. One appealing possible mechanism for the regulation of this activation is that the hormonal receptor conformation was altered by the ions present as was postulated for the opiate receptor (Pert and Snyder, 1974). This altered conformation would permit a certain level of basal interaction with the catalytic moiety of the cyclase, and the response to hormonal activators might be expected to be similarly altered.

Adenyl cyclase was variably affected by potassium in mammalian brain slices (Rall and Sattin, 1970), especially in the presence of theophylline (Shimizu et al., 1970; Roch and Kalix, 1975). The cockroach, a carnivorous insect with a high Na^+/K^+ hemolymph ratio, accumulated cyclic AMP over control levels in the presence of 50 mM K^+ with or without theophylline (Nathanson, 1973). This serves to illustrate some of the difficulties which may be encountered in the extrapolation of effects to phytophagous insects such as M. sexta.

In view of the dramatic effects of 5-HT on cyclic AMP levels in the neuronal-enriched fraction of the nerve cord described in the previous chapter, the effects of the indoleamine at various pH values were studied as was the possibility of potentiation of those effects by theophylline. A number of other putative neurotransmitters or drug agents were screened simultaneously. These efforts yielded mixed results. Serotonin had significant effects at pH 3.5 and 5.3 but not at the more physiologically relevant pH values, 6.5 and 7.2. However, 5-HT clearly potentiated the effect of theophylline at pH 6.5.

A number of factors may have contributed to these observations. First, it was pointed out by an anonymous reviewer of this data that freezing of nervous tissue

before incubation, necessary for the collection of sufficient quantities for assay, may disrupt the receptor-catalytic coupling, whatever its nature, required for increased synthesis of cyclic AMP. This did not appear to be a factor, however, in the hormonal response of the cyclases in mouse cerebellum (Schmidt and Nadi, 1977). Moreover, the work of the previous chapter was carried out using once-frozen tissue. Furthermore, the experiments carried out on the adenyl cyclase in a later chapter demonstrate that neither basal nor fluoride-stimulated activity are altered by freezing and holding nerve cords at -80^{o}C for up to five years! Second, as pointed out above, the intact nerve cord sheath is highly impermeable. The results of incubation with the high sodium and high potassium solutions indicated that these ions have an effect on cyclic AMP accumulation, but the blood-brain barrier may prevent the passive diffusion of larger neurotransmitter molecules (Twarog and Roeder, 1956; Lane, 1972; Treherne and Pichon, 1972; McLaughlin, 1974). Transport may be aided by the low pH values, 3.5 and 5.3, where 5-HT effects are seen. Third, it is actually surprising that any effects would be observed at all in view of the high phosphodiesterase activity in crude nerve cord homogenates (1000-2000 pmole cyclic AMP hydrolyzed per minute per mg protein, Albin et al., 1975)

which greatly exceeds the maximally-stimulated adenyl cyclase in the same homogenates (see later chapter). Fourth, these experiments were conducted without a clear view of the appropriate concentrations of test substances to be used. What constitutes a "physiological" concentration of a neurotransmitter may be an open question: Assuming a 1 μm diameter axon with a 100 Å synaptic cleft, one would have described a volume of less than 10^{-16} cm^3. A solute which was present at the level of 10 mM would make a contribution of 8×10^{-19} mole to a solution of this volume, or 5×10^5 molecules. The relationship of this number in this hypothetical system to a physiological concentration in a real system is uncertain, but it has been reported that 5×10^6 molecules of ACh per impulse per end plate have been recovered from perfusion experiments at the neuromuscular junction (Katz, 1966, p. 120). Twarog and Roeder (1957) used 10^{-3} M 5-HT to potentiate the giant fiber response to cercal nerve stimulation in cockroach. Because the aim of these studies was to obtain any significant stimulation, it was felt that larger doses were more appropriate in this screen. Treherne and Smith (1965 a,b) calculated that when an intact cockroach nerve cord was soaked in 10 mM ACh, the extracellular concentration was less than ten μM! These limitations should be borne in mind while reading the remainder of this discussion.

Aspartic acid, GABA, norepinephrine, and isoproterenol elevated cyclic AMP levels above those of controls at a single tested pH value, and no experiments were conducted to see if their effects could be potentiated by theophylline. The possibility that these results were of polysynaptic origin cannot be excluded in the case of these compounds as they were not tested or did not potently affect cyclic AMP levels in the monocellular neuronal-enriched fraction described in the last chapter (i.e., NE). Cox (1977) has reported on the lack of effect which glutamate has on cyclic AMP levels in rat brain slices.

Zemp and Thomas (1976, 1977) and Thomas and Zemp (1977) have reported on the ascorbate inhibition of the DA-sensitive adenyl cyclase in rat striatum. In these studies 10 μM ascorbate had no effect on the basal activity of the enzyme. In view of the link which these researchers noted between dietary and brain levels of ascorbate in guinea pigs, it is interesting to note that the M. sexta artificial diet contains approximately 0.25% ascorbic acid. If a "wanderer" fifth larval instar has consumed five grams of this diet daily during the previous five days (Nijhout and Williams, 1974), it will have been exposed to roughly 350 μmoles of ascorbate. Because the insect maintains its natural behavioral

patterns, this level of exposure must not be harmful to the function of its nervous system. However, it may well be that more appropriate antioxidants may be found for use in similar studies.

Levels of cyclic GMP in the M. sexta nerve cord were also studied, as well as the impact of incubation in the high sodium ringer containing various agents. The basal values for cyclic GMP levels are approximately one-fifth of those for cyclic AMP. This ratio compares favorably with cyclic AMP:cyclic GMP ratios found previously in Cecropia nerve cords (Truman et al., 1976), in most crickets (Fallon and Wyatt, 1975), in silkworm eggs (Takahashi et al., 1975), and in noninsect systems (Ishikawa et al., 1969). This value differs from that found in the neuronal- and glial-enriched fractions described in the previous chapter and from that previously found by Albin (1973) in the M. sexta nerve cord. As alluded to before, it is likely that this difference stems from the past employment of GTCM as carrier for incubations inasmuch as this medium contains millimolar concentrations of amino acids. From the results of this current work, it may be seen that incubation in half millimolar glutamate results in cyclic GMP levels fifteen times in excess of cyclic AMP levels, in agreement with the ratios observed for the

the neuronal and glial fractions in the previous chapter.

Glutamate potently elevates cyclic GMP levels and is thought to be an excitatory transmitter at the insect neuromuscular junction (Pichon, 1973; McDonald, 1975). While GABA is nearly as potent as glutamate in its effects, it has not been shown to be synthesized by isolated nerve cords from labeled glutamate (see next chapter). It seems likely that any or all of the amino acids which have an effect may be acting as analogues for one another or some as yet untested compound.

Acetylcholine appears to be a transmitter in the insect CNS (Pichon, 1973). ACh has already been proposed as a neurotransmitter in the sensory neurons of the antennae of the adult M. sexta (Sanes and Hildebrand, 1976). ACh synthesis from labeled choline by intact nerve cords has been observed (see next chapter), and Prescott et al. (1977) have characterized the synthetic and degradative enzymes in the M. sexta CNS.

Again, the possibility that ACh, glutamate or other amino acids are affecting cyclic GMP levels by a polysynaptic event cannot be excluded. However, it is noteworthy that 5-HT, catecholamines, and octopamine,

which might have a polysynaptic effect, do not elevate cyclic GMP levels above the control level.

These data do not establish a direct role for cyclic nucleotides in the action of ecdysone. However, our results are in agreement with the finding of Applebaum and Gilbert (1972) that ecdysone stimulated the guanyl cyclase in the pupal epidermis of Hyalophora. Cyclic GMP and dibutyryl cyclic GMP enhance the effectiveness of ecdysone in terminating diapause in Mamestra (Bodnaryk, 1975).

The elevations of cyclic AMP and cyclic GMP levels revealed by the work in this chapter do not supply us with a mechanism for the phenomena. Preliminary observations of the phosphodiesterase in crude homogenates indicate that 5-HT, but no other of the putative neurotransmitters tested in this work, may inhibit this enzyme's activity (Taylor et al., 1978). The effects of these putative neurotransmitters on adenyl cyclase activity will be detailed in a later chapter.

AMINO ACIDS, BIOGENIC AMINES AND THEIR SYNTHESES IN THE CENTRAL NERVOUS SYSTEM OF MANDUCA SEXTA

The value of indiscriminately dunking nerve cords in putative neurotransmitters may be as dubious as the "spritzing" of neurons seen in iontophoresis (Bloom, 1974). Therefore, a rapid assessment of some of the synthetic capabilities of the M. sexta nerve cord as well as a detailed analysis of putative neurotransmitter levels was undertaken.

The ready availability of a picomole-sensitive amino analyzer and the recent publication of simple techniques for the assay of putative neurotransmitter syntheses (Hildebrand et al., 1971) and storage (Smith, et al., 1975) placed all of the necessary techniques and equipment within the apparently easy grasp of this investigation. Of three flaws in the experimental scheme, GABA, octopamine, and histamine, the first two are being investigated in collaboration with two other laboratories.

Materials and Methods

Materials

(Methylene-^{14}C)-choline chloride, (U-^{14}C)-L-tyrosine, and 3-^{14}C-DL-tryptophan were obtained from New England Nuclear, and 2-^{14}C-glutamic acid was purchased from ICN Pharmaceuticals. Choline chloride was from

Mann Research Laboratories. Special chemicals were obtained from Sigma. Other chemicals were of reagent quantity, and double glass-distilled water was used throughout. Tissue culture slides (#4804) were obtained from Lab-Tek Products, Westmont, Illinois. Whatman 3 MM paper was graciously provided by Tony Auerbach, University of Oregon, Eugene. Opticlear dram vials were from Kimble, and Econofluor was a gift from New England Nuclear. Bio-Rex 70 and Dowex AG 1 x 4 ion exchange resins were obtained from Bio Red.

Insect Tissues

M. sexta larvae were raised and nerve cords and brains dissected as described in General Materials and Methods. Fresh tissues were used for studies of precursor uptake and utilization, while tissues for amino acid analyses and biogenic amine determinations were pooled and frozen and stored as described in General Materials and Methods. Hemolymph was obtained by bleeding insects from an incision in the terminal proleg and collecting the blood in culture tubes placed in ice.

Biogenic Amine Biosynthesis

Freshly-dissected tissues were placed in 50 µl of saline (Ephrussi and Beadle, 1936) per nerve cord containing 1 mM ascorbic acid, 5 mM trehalose, 50 mM

HEPES, pH 7.5 (at 30°C), and 20 µM radiolabeled precursor compound. The tissue was incubated in a tissue culture slide for 16 hours at 30°C in an atmosphere of 95% oxygen:5% carbon dioxide and agitated on a "Rocker Platform" (Bellco Glass, Inc.).

Following incubation, tissues were rinsed in the above incubation media less the radiolabeled precursor and then placed in 0.5 ml Kontes Duall tissue grinders in which they were promptly frozen by immersion in liquid nitrogen to prevent degradation while other tissues were processed. The frozen tissues were homogenized in 50 µl of a buffer consisting of 0.47 M formic acid, 1.4 M acetic acid (Hildebrand et al., 1971) and appropriate marker compounds[3] using a glass pestle. Aliquots of these homogenates were applied to Whatman 3 MM paper and dried under a stream of warm air. The entire paper was wetted by spraying with the formic acid/acetic acid buffer and carefully placed in a Savant Instruments electrophoresis tank. A potential difference of 4 kV was applied for 2.5 hours as described by Hildebrand et al. (1971). The radioactive medium was treated in a like manner as a control and to examine for possible product excretion.

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[3]For example, if the radioactive substrate were choline, unlabeled choline and acetylcholine were added.

After drying at 30°C for about an hour, electropherograms were examined under ultraviolet light for indole and phenol derivatives or sprayed with chemical stains, described by Hildebrand et al. (1971), to determine the location of the marker compounds. A spray consisting of 0.2 g of ninhydrin in 100 ml of absolute ethanol plus 0.5 ml of one N NaOH (Smith, 1958) was used to visualize GABA and glutamate. A diazotized sulfanilic acid reagent was used for OA (Dawson et al., 1969). The iodine spray suggested by Hildebrand et al. (1971) was supplemented with two two-step spray-on reactions using potassium ferricyanide and cobalt chloride for choline and hydroxylamine and ferric chloride for ACh (Dawson et al., 1969).

The location of the radioactivity was obtained by cutting the paper into strips from anode to cathode with the origin for each sample and its products centered on the strip. A crude profile of the location of radioactive compounds was obtained from a Packard radiochromatogram scanner. More accurate determination of radioactivity was obtained by cutting the strips into one cm segments, placing the segments in glass dram vials from five ml of Econoflour, and counting in a Packard 3330 Tri-Carb liquid scintillation spectrometer at 20% gain with window settings of 50 and 1000.

Free Amino Acid Analyses

Samples for amino acid analyses were obtained by homogenizing previously frozen tissues in ice-cold five percent trichloroacetic acid in three ml Kontes Duall all-glass tissue grinders, incubating 45 minutes at $4^{o}C$, and centrifuging at 2200 rpm (1000 x g) for 15 minutes in the Sorvall GLC-1 with 50 ml buckets. The supernatant was decanted and analyzed for free amino acid content. The pellet was dissolved in ten N NaOH as described in the previous chapter and assayed for protein. Preparations from whole larvae were obtained by homogenization in five percent trichloroacetic acid in a Waring blender, and the supernatant, obtained as described above, was used for the analysis. Hemolymph from three to five insects was pooled and treated with a three-fold excess of five percent trichloroacetic acid and processed as above.

Amino acid analyses were performed on an automated analyzer employing a single 0.3 x 30 cm column of DC-4A resin (Durrum). After 20 µl sample injection, amino acids were eluted using the Durrum Pico-Buffer System, reacted with an o-phthalaldehyde fluorometric reagent (Roth and Hampai, 1973), and detected by the system described by Ayres et al. (1974). Fluorescence peak areas were integrated and correlation factors were

calculated using an Autolab System IVb integrator.

Biogenic Amine Determinations

Tryptophan and other biogenic amines were extracted and separated on tandem columns as described by Smith et al. (1975, and personal communication). This involved homogenization and extraction of frozen tissue in formic acid/acetone followed by an heptane/chloroform wash. The residue was solubilized and analyzed for protein as described above. The extracts were dried, dissolved in water and buffer, and applied to a tandem arrangement of mini-columns of Bio-Rex 70 and Dowex AG 1 x 4. Resins had been prepared by stirring in one N HCl and one N NaOH alternately three times each. The Bio-Rex 70 was left in the Na^+ form, while the AG 1 x 4 was stirred in one N formic acid and left in the $HCOO^-$ form. Resins were then washed five times with 0.5 M phosphate buffer, pH 6.1, and five times with 0.02 M phosphate buffer, pH 6.1, and left in a solution of the latter buffer until needed (Smith et al., personal communication). The biogenic amines in the extracts were chromatographed by washing the column arrangement with buffer. The two columns were separated, and the Bio-Rex 70 column was washed according to Smith et al. (1975, and personal communication) to obtain an eluate which contained DA, NE, and 5-HT. The AG 1 x 4 column

was washed with 60% methanol followed by 60% methanol/ 0.04 N HCl, and the latter eluate reserved for tryptophan and 5-HT analysis. The Dowex AG 3 x 4 resin described by Smith et al. (1975) was not required as five-hydroxyindoleacetic acid was not being assayed.

Tryptophan, NE, 5-HT, and 5-HTP were assayed by the microfluorometric techniques described by Smith et al. (1975). DA assays were carried out essentially according to the method of Barchas et al. (1972), except that 100 µl instead of 300 µl of the alkaline sulfate solution was used to halt the oxidation step, and citrate and phosphoric acid were premixed instead of being added sequentially. An Aminco-Bowman spectrophotofluorometer was used to measure fluorescence.

Comments

It was thought that a knowledge of the amount of amino acids present in samples for amino acid analysis would reduce the time required to find an ideal dilution of the samples to bring the levels within the sensitivity range of the machine. To this end, the ninhydrin assay described by Hirs (1967) and utilizing the ninhydrin reagent of Moore (1968) was employed. No graduate student in this department in recent times has obtained a good linear standard curve with this method. A much better means would be to adapt the o-phthalaldehyde

method described by Maickel and Miller (cited in Smith et al., 1975).

Several dogged attempts were made to visualize catecholamines or 5-HT in the M. sexta CNS using the straightforward formaldehyde condensation described by Čech and Knoz (1970). Unfortunately, the nerve cord is so highly invested with cuticularized tracheae that there is an insurmountable degree of autofluorescence. Attempts to attain success with the presumably less-heavily invested pupal nerve cord met with resounding failure (Taylor and Haywood, 1976, unpublished).

The descriptions of the experimental procedures for the assay of biogenic amines in the paper of Smith et al. (1975) are somewhat less than complete. It was invaluable to read the original articles of Anton and Sayre (1962) and Hess and Udenfriend (1959) and the review of Udenfriend (1962).

Results

A Miscellaneous Observation

In view of the lack of information on the pH of M. sexta hemolymph, some of the blood obtained for free amino acid analyses was used for a pH determination. Hemolymph from three to five animals was pooled, brought to room temperature, and the pH measured. The results thus obtained yielded an average pH of 7.10 $\pm$ 0.02 (SEM) for six different pools.

Biogenic Amine Biosynthesis

Initially a four hour incubation period without oxygenation was employed for precursor uptake and utilization. However, insufficient uptake and metabolism were observed. Hildebrand et al. (1971) suggested that such difficulties may arise as a result of (1) failure to sufficiently label precursor pools, (2) feedback inhibition of transmitter-synthesizing enzymes, (3) tissue necrosis. By prolonging the incubation and incubating in 95% O_2: 5% CO_2, it was possible to circumvent these problems.

Figure 4A shows the results obtained from an experiment in which labeled tryptophan was added to M. sexta nerve cords and an extract applied to a chromatogram for separation and analysis. In the tissue extract, a non-mobile component, presumably containing peptides and larger molecules, was noted near the origin. Radioactivity also was found in the same regions where the standards for tryptophan and 5-HT migrate. In the extract from the medium, 74% of the total radioactivity is found in the tryptophan region, essentially none in the 5-HT region, and 15% in the region where 5-HTP, an intermediate in 5-HT synthesis, would be expected to migrate.

The results of a similar experiment using labeled choline are depicted in Figure 4B. As found in the

Figure 4. A. Synthesis of serotonin by the larval nerve cord of Manduca sexta.

Tissues were incubated in (U-^{14}C)-DL-tryptophan and extracts prepared for high foltage electrophoresis at pH 1.9 as described by Hildebrand et al. (1971). The regions in which fluorescence attributed to exogeneous marker compounds occurred are denoted by horizontal bars. The graph indicates the cpm obtained from 1 cm segments of the electropherograms of the extracted nerve cords (solid line) or the incubation medium (broken line).

B. Synthesis of acetylcholine by the M. sexta larval nerve cord.

(Methyl-^{14}C)-choline was added to the incubation medium. Other procedures are mentioned above and detailed in the text. Exogeneous markers were visualized chemically and are represented by horizontal bars. Counts from the electropherograms of extracted nerve cords (broken line) or the incubation medium (solid line) are portrayed in the graph.

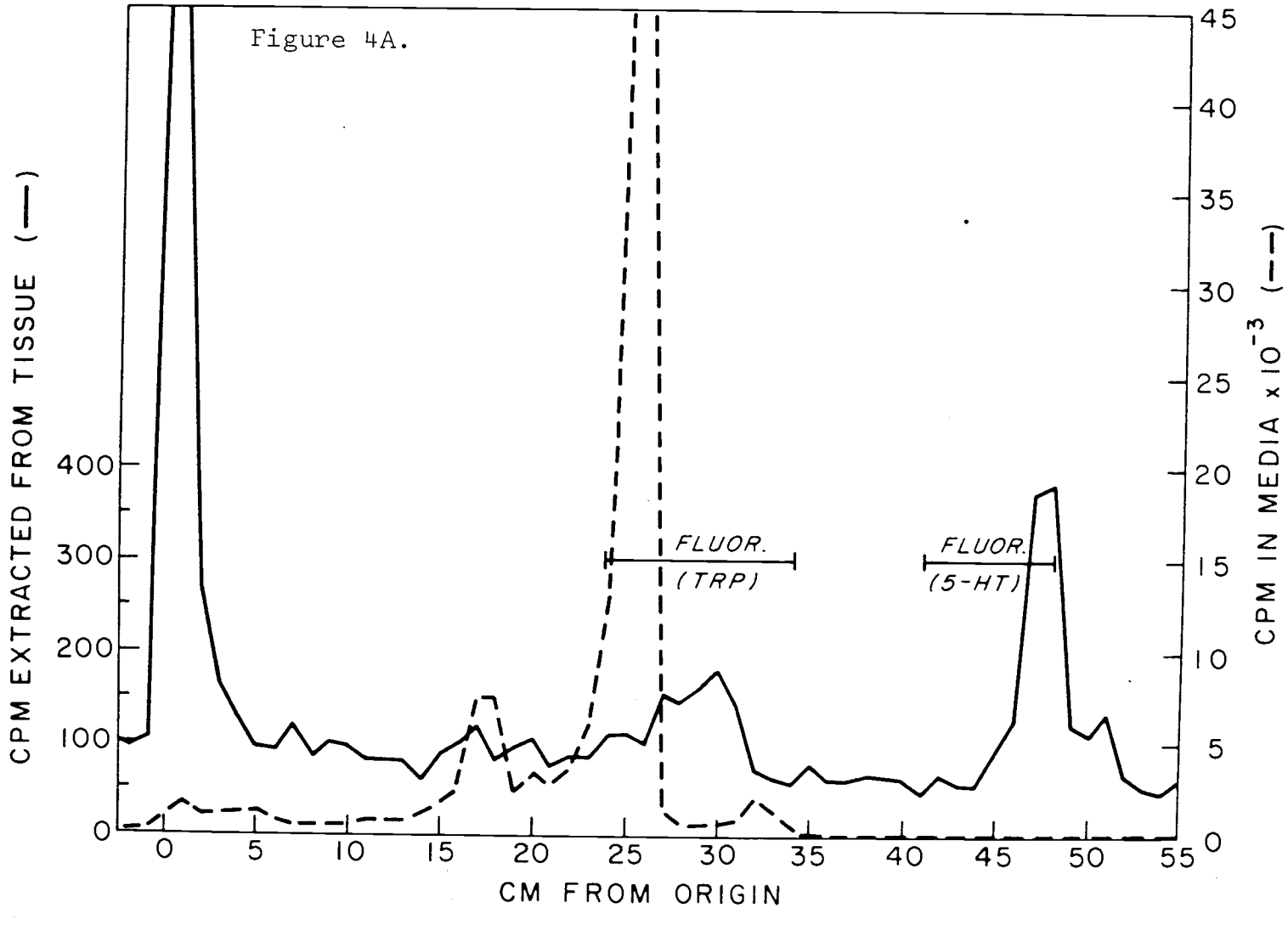


Figure 4A.
CPM EXTRACTED FROM TISSUE (—)
CPM IN MEDIA x10⁻³ (--)
CM FROM ORIGIN
FLUOR. (TRP)
FLUOR. (5-HT)
0
100
200
300
400
0
5
10
15
20
25
30
35
40
45
0
5
10
15
20
25
30
35
40
45
50
55

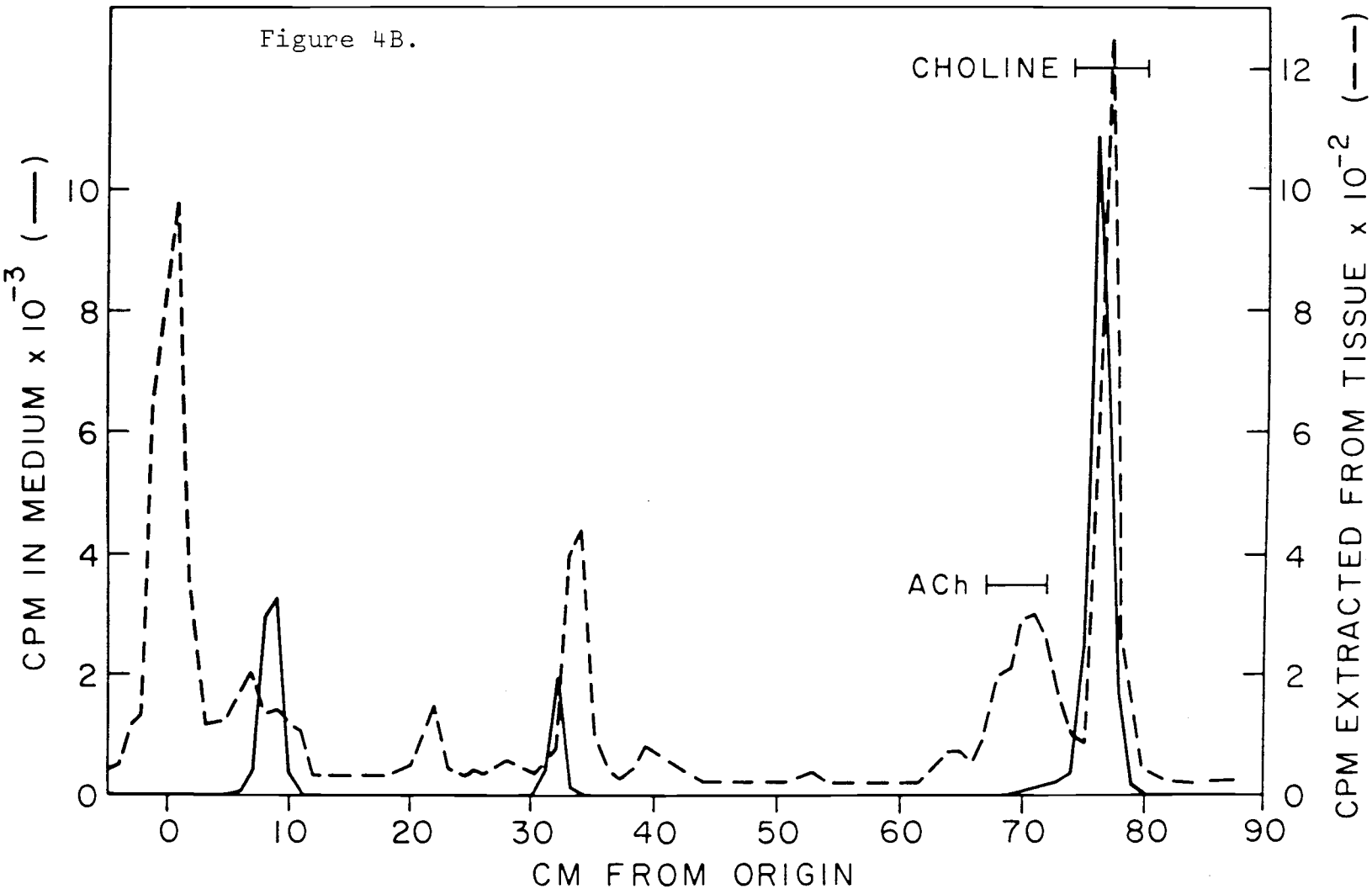


Figure 4B.
CHOLINE
ACh
CPM IN MEDIUM x 10^{-3} (——)
CPM EXTRACTED FROM TISSUE x 10^{-2} (— —)
CM FROM ORIGIN
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70
80
90

tryptophan experiment, a large non-mobile component occurs near the origin. In the extract from the external medium, 62% of the total radioactivity migrates in the choline region, and 2% migrates in the acetylcholine region. In the tissue extract 21% of the radioactivity is choline and 16% acetylcholine. A slower-migrating component (30-35 cm from the origin in this system) was observed in both tissue extracts and medium, and it comprised about eight percent of the total radioactivity.

Experiments with labeled glutamate and tyrosine gave profiles for both medium and tissue extracts similar to the above in that radioactivity was observed at the origin, where precursors were apparently incorporated into non-mobile components. However, in contrast to the previous experiments, radioactivity coincided only with the region where substrate migrated and not at the positions where markers for GABA, DA, NE, or OA migrated.

Amino Acid Analysis

The results of the amino acid analyses are given in Table 7. β-Alanine, DA, GABA, histamine, NE, OA or tyramine could not be resolved under the conditions employed, nor could proline or tryptophan levels be determined. Neither cysteine nor cyteic acid were

Table 7. Amino Acid Levels in Tissues and Hemolymph of M. sexta Larvae.

Hemolymph values represent the mean of the number of values given in parentheses obtained from several dilutions of two different pooled preparations ± SEM. Nerve cord and brain values represent the mean ± range of two pools: nerve cord pools contained ten nerve cords each; brain pools contained 41 and 48 brains. tr = trace.

Amino Acid	Whole Larvae Mole %	Hemolymph m molar	Mole %	Nerve cords µmol/mg protein	Mole %	Brain µmol/mg protein	Mole %
Alanine	12.1	3.61 ± 0.42 (6)	4.45	8.13 ± 0.84	15.4	17.7 ± 1.0	18.2
Arginine	6.2	1.43 ± 0.18 (5)	1.76	2.97 ± 0.52	5.57	5.83 ±0.42	6.02
Aspartic acid	1.1	1.46 ± 0.14 (5)	1.80	0.66 ± 0.05	1.25	1.95 ±0.28	2.04
Glutamic acid	7.8	2.89 ± 0.48 (6)	3.56	7.11 ± 0.65	13.5	24.8 ±13.9	23.1
Glycine	19.6	14.4 ± 1.5 (7)	17.7	10.5 ± 1.3	19.9	13.0 ± 2.4	12.9
Histidine	10.2	15.9 ± 0.8 (7)	19.6	3.90 ± 0.96	7.24	8.70 ± 0.72	8.77
Isoleucine	1.6	0.41 ± 0.13 (3)	0.51	1.01 ± 0.45	1.82	1.09 ± 0.07	1.10
Leucine	1.7	0.72 ± 0.09 (6)	0.88	1.11 ± 0.20	2.07	1.59 ± 0.05	1.61
Lysine	3.0	4.08 ± 0.93 (6)	5.02	1.21 ± 0.13	2.30	3.55 ± 0.32	3.68
Methionine	tr	1.23 ± 0.40 (2)	1.52	tr	tr	tr	tr
Phenylalanine	5.4	0.90 ± 0.05 (4)	1.11	0.23 ± 0.04	0.45	0.34*	0.15
Serine	24.8	24.2 ± 2.7 (7)	29.8	10.4 ± 1.0	19.8	12.7 ± 1.1	12.7
Taurine	tr	0.31 (1)	0.4	tr	tr	tr	tr
Threonine	1.5	4.43 ± 0.54 (3)	5.46	1.47 ± 0.14	2.79	2.16 ± 0.24	2.25
Tyrosine	tr	3.47 ± 0.18 (5)	4.28	1.16 ± 0.14	2.19	1.71 ± 0.20	1.78
Valine	5.1	1.79 ± 0.15 (5)	2.21	3.20 ± 0.73	5.95	5.62 ± 0.39	5.79

* Only one value obtained.

detected in any preparation. Protein values for the whole larval preparation were not determined, but the mole percent of amino acids present was calculated from the relative levels obtained in the amino acid analysis of an extract of 98 animals.

The total amount of free amino acids in the brain was approximately twice as great as the amount in nerve cords. In view of this difference, it is perhaps more meaningful to observe the differences in mole percent of amino acids present. The mole percents of alanine, arginine, glutamic acid, isoleucine, leucine, and valine were noticeably lower in the hemolymph than in the nervous tissues although histidine, threonine, and tyrosine were higher in the hemolymph than in other tissues. The mole percent of phenylalanine was highest in homogenates of whole animals. A higher mole percent of glutamic acid and a lower mole percent of glycine and serine were found in the brain than in the nerve cord.

An average nerve cord contains 5.4% protein based on an average value of 0.207 $\pm$ 0.003 (SEM) mg protein per nerve cord (from triplicate assays of 220 preparations of 2,373 nerve cords) and an average of 3.82 $\pm$ 0.74 (SEM) mg wet weight per nerve cord (from five preparations of 588 nerve cords). Thus, a level of one µmole of an amino

acid per mg protein represents a value of 54 μmoles per gm wet weight.

Biogenic Amine Levels

The characteristics of the assays used in determining the levels of biogenic amines are listed in Table 8. A preliminary experiment revealed that DA was present in concentrations at least ten times greater than NE, a not unexpected result in insects (Östlund, 1954; Brunet, 1963), but not usual for mammalian nervous tissue (Smith et al., 1975). It seemed prudent to investigate whether such a difference would have any effect on the assay for NE. The interference of DA in the NE assay is depicted in Figure 5. As a consequence of this finding, the NE content was calculated after first subtracting the fluorescence value due to the presence of DA.

The results of the assays for biogenic amines are given in Table 9. The brain contains five times as much NE and 5-HTP as the nerve cord, while the latter is almost three-fold enriched in DA relative to the brain. The levels of 5-HT and tryptophan do not differ significantly between the two tissues.

Table 8. Characteristics of Biogenic Amine Assays Employed.

References for the various assays are given in the text. Wavelengths for maximum excitation (λ_{ex}) and emission (λ_{em}) were uncorrected. Slits used for assays are given in the user's manual for the Aminco-Bowman Spectrophotofluorimeter as arrangement five, except that the tryptophan assay required slit #7 (photomultiplier turret) to be 1mm instead of 5mm. Sensitivity is defined as that level of amino which has twice the relative fluorescence of the blank.

Assay	$\lambda_{ex}/\lambda_{em}$	Sensitivity (pmoles)	Linearity (pmoles)	Recovery (%)
Dopamine	315/350	5	10 - 10,000	46.9
5-Hydroxytryptamine	365/465	10	5 - 1,000	109.1
5-Hydroxytryptophan	360/480	10	5 - 1,000	45.1
Norepinephrine	405/495	5	10 - 10,000	127.5
Tryptophan	370/440	25	10 - 10,000	66.5

Figure 5. Relative fluorescence of dopamine and norepinephrine in the fluorometric assay for norepinephrine.

Each point is the mean of two determinations. Standard curve for dopamine is indicated by triangles and broken line. Standard curve for norepinephrine is represented by circles and solid line.

Figure 5.

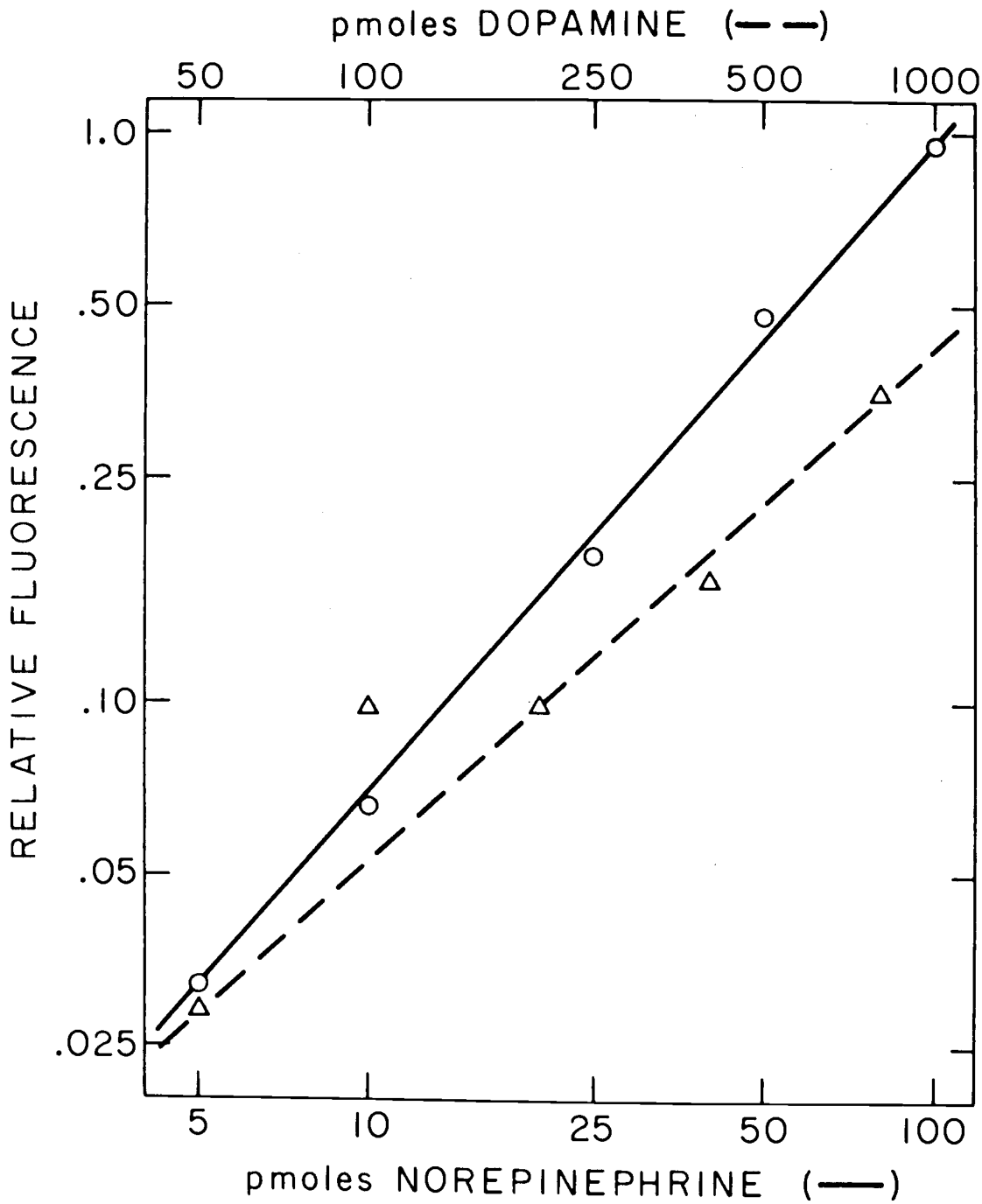

pmoles DOPAMINE (— —)
50
100
250
500
1000
1.0
.50
.25
.10
.05
.025
RELATIVE FLUORESCENCE
5
10
25
50
100
pmoles NOREPINEPHRINE (——)

Table 8. Tissue Levels of Biogenic Amines Found in M. sexta Larvae.

Values represent mean pmoles of amine present per mg protein ± SEM for four nerve cord preparations and ± range for two brain preparations. All preparations were assayed in duplicate. Values in parentheses represent the mean µg of amine present per gm wet weight, calculated from values given in the Results section.

Amine	Brain		Nerve Cord	
Dopamine	1410 ± 250	(11)	4840 ± 870	(40)
Norepinephrine	586 ± 543	(5.5)	99.5 ± 37.8	(0.9)
Tryptophan	3970 ± 385		3038 ± 550	
5-Hydroxytryptophan	541 ± 164		80.0 ± 26.7	
5-Hydroxytryptamine	93.1 ± 3.8	(0.8)	114 ± 20	(1.0)

Discussion

The demonstration of 5-HT synthesis from tryptophan by whole nerve cords and its presence in nerve cords and brains of M. sexta may be taken as further evidence that this substance plays a role in neurotransmission in the CNS of this insect. Other evidence for this suggestion is the increase of cyclic AMP levels in neuronal-enriched fractions of nerve cords after incubation in 5-HT-containing medium and the effects of 5-HT on cyclic AMP accumulation in intact nerve cords disclosed in previous chapters of this thesis.

The level of 5-HT observed is in agreement with that seen in the cockroach CNS (Kusch, 1975) and locust brain (Hiripi and S.-Rośza, 1973). 5-HT has been detected histochemically in the brain of Schistocerca (Klemm and Axelsson, 1973) and in the cricket corpora cardiaca (Lafon-Cazal, 1975). A decarboxylase capable of synthesizing 5-HT from 5-HTP has been found in cockroach brain and nerve cord (Colhoun, 1963), and in housefly tissues (Colhoun, as cited in Colhoun, 1968). Adenyl cyclase activity is stimulated by 5-HT in the thoracic ganglion of the cockroach (Nathanson, 1973; Nathanson and Greengard, 1974) and in the nerve cord of M. sexta (see following chapter).

ACh appears to play a role in neurotransmission in the M. sexta CNS as it does in other insects (Pichon, 1973). In addition to the work reported here showing ACh synthesis, it had been earlier reported that choline acetyltransferase and acetylcholinesterase exist in neuronal- and glial-enriched fractions of M. sexta nerve cord (Taylor et al., 1975); a more detailed study of these enzymes in this preparation is in progress. Others have demonstrated the presence of these enzymes in homogenates of whole nerve cords of M. sexta (Prescott et al., 1977), and Sanes and Hildebrand (1975) have proposed ACh as the transmitter between sensory neurons in the antennae of M. sexta.

Although no evidence was obtained for the synthesis of DA, NE, or OA from tyrosine, it has been shown that these compounds are present. It may be that the neurotransmitter precursor pools were not labeled sufficiently or that the large amount of DA present acted to inhibit tyrosine hydroxylase. The values of NE in the M. sexta nervous tissues are somewhat greater than those reported for cockroach (Frontali and Häggendal, 1969: 40 pmoles per mg protein) and locust brains (Hiripi and S.-Rósza, 1973: 60 pmoles per mg protein; Klemm and Axelsson, 1973: 15 pmoles per mg protein; Robertson, 1976: 25 pmoles per mg protein). It should be noted that these insects are hemimetabolous whereas

M. sexta is holometabolous.

The values for the DA content in the M. sexta brain is of the same order as values obtained from cockroach (Frontali and Häggendal, 1969; Kusch, 1975) and Locusta brains (Hiripi and S.-Rósza, 1973). The value for DA in the M. sexta nerve cord is much higher. It seems likely that this is due to its utilization in the cuticle sclerotization process (Brunet, 1963; Sekeris and Karlson, 1966) especially when the heavy tracheolar investment of the nerve cord is considered. DA has been proposed as the neurotransmitter which mediates fluid secretion in the salivary gland of the adult M. sexta (Robertson, 1975).

Octopamine is present at a level of 42 ± 4 (SEM) pmoles per mg protein in the M. sexta nerve cord (about 0.35 µg per gm wet weight; R. E. McCaman, personal communication), and it is probably synthesized from tyrosine as no phenylethanolamine can be detected in that tissue. This is somewhat lower than the levels seen in locust brain (Robertson, 1976) or cockroach nerve cord (Robertson and Steele, 1974), but typically higher than OA levels seen in mammals (Molinoff and Axelrod, 1972).

Glutamate is recognized as a neurotransmitter at the insect neuromuscular junction (McDonald, 1975), but

the evidence for a similar role in the CNS is not conclusive (Lunt, 1975). The levels of glutamate obtained in the M. sexta brain nerve cord are much higher than those seen for cockroach nerve cords (Ray, 1964) or the honeybee brain (Frontali, 1961). Glutamate is extremely potent in elevating cyclic GMP levels in intact nerve cords (see previous chapter).

GABA is presumed to be an inhibitory neurotransmitter in the insect CNS (Pichon, 1973). The inability to obtain GABA synthesis from labeled glutamate does not prove that the compound is not formed by and stored in the M. sexta CNS. Glutamic acid decarboxylase activity is very low in locust nerves (Bradford et al., 1969); if the activity of the M. sexta enzyme is also low this could explain the apparent lack of synthesis. A collaborative project investigating GABA levels and the possible presence of a GABA-binding protein in the M. sexta CNS has been undertaken.

These results provide further evidence for the suggested roles of 5-HT and ACh in M. sexta neurotransmission. None of these results denies the presence of any tested putative neurotransmitter in the insect's CNS, nor do the previous chapter's results exclude any of these compounds from a role in cyclic nucleotide metabolism or synaptic transmission.

ATP IN THE CENTRAL NERVOUS SYSTEM OF MANDUCA SEXTA

When confronted with the dramatic increases in cyclic AMP levels brought about in the neuronal-enriched fraction by 5-HT, it seemed that it would be most enlightening to determine just exactly how much ATP was present in the M. sexta CNS. Thus, one might calculate the degree of conversion under this stimulation. The method of Stanley and Williams (1969) had been previously used in this laboratory (T. Hinds and R. W. Newburgh, unpublished), so it was pressed into service for this investigation.

Materials and Methods

Materials

M. sexta larvae were reared and nerve cords dissected as described in General Materials and Methods. ATP and firefly lanterns were from Sigma. All other chemicals were reagent grade. Glass double-distilled water was used throughout.

Tissue Preparations

Two pools of nerve cords, containing five or six nerve cords, were frozen, sonicated with a Kontes Cell Disrupter at power setting 6 for one minute in 0.5 ml 0.6 N perchloric acid, homogenized for 1.5 minutes, sonicated again, and washed into a centrifuge tube with 1.0 ml 0.6 N perchloric acid. The homogenates were

centrifuged at 500 x g for 15 minutes at 4°C, the supernatant aspired for the ATP assay, and the protein pellet dissolved in sodium hydroxide and assayed for protein as described previously.

ATP Assay

The method of Stanley and Williams (1969) was followed. Standard curves were linear from 10^{-12} to 10^{-9} moles ATP.

Results

The average recovery of the assay procedure was 98.7%, and the mean ATP content for the two preparations, assayed in triplicate, was 14.7 $\pm$ 8.5 (range) nmoles ATP per mg protein.

Discussion

Assuming 80% of the wet weight to be intracellular water (Treherne, 1966; see also Albin, 1973), the value obtained here yields an intracellular concentration of the magnitude of one mM for ATP in the nervous system of M. sexta. This level is in agreement with levels obtained for various invertebrate and vertebrate nerve and muscle preparations (Beis and Newsholme, 1975). Formation of cyclic AMP at a rate of 15 pmoles per mg protein per unit time would degrade less than 0.1% of the ATP available in the M. sexta nerve cord.

ADENYL CYCLASE IN THE CENTRAL NERVOUS SYSTEM OF MANDUCA SEXTA

Having learned that essentially all putative neurotransmitters were probably present in the M. sexta CNS (see previous chapter), it appeared that the best way to screen these compounds for stimulatory effects on the cyclic AMP system would be to undertake an investigation of the appropriate synthetic and degradative enzymes.

Because the activity of the phosphodiesterase has already been the study of one doctoral dissertation from this laboratory (Albin, 1973), an investigation of the effects of neurotransmitters on this enzyme's activity was undertaken as an undergraduate research project in this laboratory (Taylor et al., 1978).

Cyclase activities had not been studied previously in this laboratory, so an appropriate methodology had to be evolved. A number of bad experiences with minicolumn chromatography and the previous success of paper chromatography in the separation of adenine compounds (Albin et al., 1974; Taylor et al., 1978) led to the adoption of the latter technique for this important procedural step. The substrate of choice was the α-^{32}P-form of ATP as this isotope emits highly energetic β particles (it can be counted on paper easily), and the label will not exchange as it does with the tritium compound (Schultz, 1974).

Materials and Methods

Materials

Dowex AG 50 x 8, 200-400 mesh, was obtained from Bio-Rad. Alumina, Woelm, activity grade I, was obtained from ICN Pharmaceuticals, Van Waters and Rogers, and Alupharm Chemicals, New Orleans, Louisiana. U-^{14}C-cyclic AMP, ammonium salt, specific activity 117 mCi per mmole, and α-^{32}P-ATP, sodium salt, specific activity 10-17 Ci per mmole were purchased from Amersham Searle. The following radiolabeled compounds were from New England Nuclear: 8-^{14}C-adenine, 50 mCi per mmole, 8-^{14}C-ADP, trisodium salt, 40 mCi per mmole, U-^{14}C-5'-AMP, diammonium salt, 500 mCi per mmole, U-^{14}C-ATP, tetrasodium salt, 500 mCi per mmole, and 2,8-^{3}H-ATP, tetrasodium salt, 30 Ci per mmole. A small quantity of ^{32}P-orthophosphate, from New England Nuclear, was a gift from G. D. Pearson. Whatman No. 1 paper was obtained from Scientific Products. Polyethylenemine (PEI) cellulose-F precoated thin layer chromatography sheets, 100 um thick, were from E. Merck, Darmstadt, Germany. Aquasol was from New England Nuclear, Handifluor from Mallinckrodt, and PPO (2,5-diphenyloxazole), scintillation grade, was from Allan Laboratories, Bellevue, Washington. Special chemicals were from Sigma, except glutamic acid which was from Nutritional Biochemical Co. Other chemicals were of reagent grade. Glass double-distilled water was used throughout.

Column Chromatography

Glass disposable Pasteur pipets (0.5 cm i.d.) were plugged with small pieces of glass wool. Four cm of Dowex or alumina were added. The alumina was added dry while the Dowex had been cycled three times with one N NaOH and one N HCl and exhaustively rinsed with H_2O. Samples were prepared from 25 µl of a solution containing 30 mM KCl, 9 mM $MgCl_2$, 9 mM theophylline, 0.1% BSA, 2 mM phosphoenol pyruvate, 0.01 mg per ml pyruvate kinase, 1 mM ATP (0.05 µCi 2,8-^{3}H-ATP), 6.7 mM NaF, after Neer (1973), plus 100 µl of a solution containing 12 mM ATP, 1.2 mM cyclic AMP (0.05 µCi U-^{14}C-cyclic AMP), and with or without 50 µl of one mg per ml BSA, and boiled for five minutes to simulate a terminated enzyme assay. The samples were applied to the columns and eluted with six one-ml washes of distilled water for the Dowex or three one ml washes of water or one mM KCl for the alumina. Samples were mixed with 15 ml of Aquasol and counted in a Packard Model 3330 Tri-Carb liquid scintillation spectrometer at settings of 75% gain, windows 20 to 1000 for ^{3}H (23.4% counting efficiency) and 12% gain windows 150 to 1000 for ^{14}C (37.5% counting efficiency for Dowex samples and 30.1% efficiency for alumina samples).

Substrate Purification

The α-^{32}P-ATP substrate was usually purified upon arrival by thin layer chromatography on PEI-cellulose (Böhme and Schultz, 1974). Plates were prechromatographed twice in distilled water before the substrate, usually one mCi, was applied as a streak on a 5 x 20 cm plate and dried under a stream of warm air. Chromatography in 0.2 M ammonium bicarbonate (suggested in the Amersham ATP specifications) required just over one hour. The purified ATP was visualized on the dried plate by UV absorbance, and the region was cut out of the chromatogram. The cellulose was scraped off the plastic backing and onto a large piece of glassine weighing paper from which it was carefully transferred into a three ml conical tube. The cellulose was washed six times with two ml each 0.2 M ammonium bicarbonate. Routine checks of the last wash showed that less than one percent of the radioactivity was present in the total volume. The washes were pooled in a 30 ml tube, shell-frozen in liquid nitrogen and lyophilized. The residue was washed into a 12 x 75 mm tube with three 500 µl volumes of water. This was frozen and lyophilized, and the residue dissolved in 1000 µl 50% ethanol and frozen at -80^{o}C until needed for use.

Enzyme Preparations

Insects were reared and nerve cords were dissected as detailed in General Materials and Methods. Nerve cords used were either fresh or frozen as detailed in results. In general, about ten nerve cords were rinsed in a buffer containing 50 mM HEPES, pH 7.5, one mM DTT, one mM EGTA, and homogenized in 100-200 µl buffer in a three ml Kontes Dual all-glass tissue grinder by 15 one-second passes of a motor-driven pestle.

Adenyl Cyclase Assays

Adenyl cyclase activity was monitored by determining the rate of conversion of α-^{32}P-ATP into ^{32}P-cyclic AMP. Assays were performed in a system similar to that described by Harmar and Horn (1977), containing 50 mM HEPES, pH 7.5, one mM DTT, one mM EGTA, 10 mM theophylline, two mM magnesium acetate, ATP as indicated in the results (0.2-0.3 µCi α-^{32}P-ATP per assay tube), and tissue homogenate (usually containing about 100 µg protein) plus test substances as indicated in a final volume of 50 µl. The assay system was preincubated at least five minutes on ice prior to the initiation of the reaction with the addition of the substrate. Following incubation at 30^{o}C in a shaking water bath, usually for five minutes, the reaction was halted by boiling for five minutes in a Tem-blok heater. Blanks

with heat-killed enzyme or without enzyme were identical, and the latter were used routinely. The condensate was concentrated by centrifuging at 2200 rpm (1000 x g) for five minutes in the omni-carriers in a Sorvall GLC-1, and 25 µl aliquots were removed for paper chromatography.

Paper Chromatography

Aliquots from adenyl cyclase assays were spotted on Whatman No. 1 filter paper along with a marker solution containing cyclic AMP, adenosine, and 5'-AMP (10 mg of each in ten ml water), and chromatograms developed for six to seven hours ascendingly with 1 M ammonium acetate - 95% ethanol (30:70, v/v) or isobutyric acid: water: concentrated ammonium hydroxide: EDTA (100:56:4.2:0.06, v/v/v/w, as suggested in the Amersham specifications) at room temperature as described by Albin et al. (1975). After drying at room temperature, marker compounds were located by their absorption under ultraviolet light. The chromatograms were cut into strips representing the origins plus products of the aliquots, and the cyclic AMP region of the strip was cut out, and all three segments of the strip were put in one to five ml of a fluor containing 5.5 gm/L PPO in toluene (H. W. Schaup, personal communication).

Samples counted with essentially 100% counting efficiency at 2% gain, windows at 20 to 1000, in a Packard 3330 Tri-carb liquid scintillation spectrometer. When ^{3}H or ^{14}C marker compounds were run for authentication purposes, they were extracted from the chromatogram by soaking overnight in two ml of 0.1 N HCl. One ml of the extract was mixed with three ml of Handifluor in a glass dram vial and counted at 12% (^{14}C) or 70% (^{3}H) gain, windows 50 to 1000.

Comments

Attempts to circumvent the long chromatography by a simple one- or two-step precipitation of ATP, ADP, and 5'-AMP by the coprecipitation techniques described by Chan and Lin (1974) met with failure. Well over 1% of the total counts was left in solution, an unacceptably high amount. An attempt to lower cyclic AMP background by P_i precipitation after the method of Sugino and Miyoshi (1964) was without effect. Purification of the α-^{32}P-ATP by the method given reduced cyclic AMP counts in the substrate from 0.6% to often less than 0.1% of total radioactivity. Because basal activity was usually of the order of the latter figure, it was excruciatingly important to have low backgrounds, otherwise experimental scatter would obliterate the activity. Descending paper

chromatography, extolled by Albin (1973), was time-consuming and no more sensitive than the technique employed here. Placing the paper sheet between glass plates for ascending chromatography (Tao, 1974) markedly decreased the time required for chromatography but absolutely destroyed resolution. While time-consuming, the method described is very inexpensive: not only are costly ion-exchange resins not required, but the scintillation cocktail is very inexpensive to begin with, and it may be reused. After removing the filter papers, the vials were recounted to eliminate those which had high backgrounds. These "hot" vials were set aside for two weeks and recounted. Thus the only requirement for scintillation fluor was to replace that which had soaked into the filter paper and was removed when the papers were discarded.

Methods and materials for the preparation of neuronal- and glial-enriched fractions and for the assay of CNPase activity were used as described in a previous chapter of this thesis.

I wish to thank Ms. C. Marra and Dr. J. A. Patt, Jr., for performing phosphodiesterase and acetyl-cholinesterase assays, respectively. Details of these assays are published (Albin, 1973) or in preparation along with further studies of this system.

Results

Column Chromatography

The results of column chromatography with Dowex and alumina are depicted in Table 10. Only one chromatography system excluded more than 90 percent of the ATP counts, and one alumina column (ICN) eluted only three percent of the cyclic AMP counts in three ml.

Consideration of the Assay

In the ammonium acetate-ethanol paper chromatography system cyclic AMP was well-separated from other nucleotides. The following R_f values were determined using visual markers and authenticated by chromatographing and counting various radiolabeled compounds: ATP, 0.08; ADP, 0.14; 5'-AMP, 0.20; cyclic AMP, 0.50; adenosine, adenine, and inosine, 0.66. Cyclic AMP was further authenticated by chromatographing identical labeled samples with markers in a two-dimensional system: Cyclic AMP represented the same percentage of counts in a total strip, run in one dimension with the ammonium acetate-ethanol system as it did when compared to other marker compounds after running in one dimension with ammonium acetate-ethanol and in the second dimension with isobutyric acid: water: concentrated ammonium hydroxide: EDTA. It should be noted that 5'-AMP and cyclic AMP

Table 10. ^{14}C-Cyclic AMP Retention and ^{3}H-ATP Recovery from an adenyl cyclase assay reaction mixture by mini-columns.

Results are given as the percent recovery or exclusion ± SEM (or range, for two samples) of the number of replicate preparations given in parentheses.

Chromatography	Cyclic AMP Recovery	ATP Recovery
Dowex:		
Fractions, 3,4,5*	83.6 ± 0.8 (3)	42.6 ± 5.2 (3)
All fractions	87.4 ± 0.8 (3)	67.8 ± 4.7 (3)
Alumina:		
Without BSA -		
VWR cationic	93.5 ± 1.5 (4)	11.0 ± 7.0 (4)
VWR neutral	88.1 ± 0.7 (2)	17.7 ± 5.2 (2)
VWR neutral (KCl)**	86.8 ± 0.7 (2)	7.3 ± 0.9 (2)
ICN neutral	2.99 ± 0.61 (5)	23.0 ± 2.4 (5)
With BSA -		
VWR neutral	89.2 ± 1.1 (3)	22.2 ± 4.3 (3)
Alupharm neutral	94.3 ± 0.3 (4)	41.7 ± 2.4 (4)

* These fractions account for 95.6% of all ^{14}C-cyclic AMP counts recovered.
** This set of columns was washed with 1 M KCl; all others were washed with water.

cochromatograph in the second system, so this method alone cannot be used for the separation of adenyl nucleotides. Less than 0.008 percent of total counts migrated in the cyclic AMP region when radioactive ^{32}P-orthophosphate was chromatographed in the ammonium acetate: ethanol system.

Freezing and thawing up to seven times had no effect on the chromatography patterns resulting from labeled adenyl nucleotides, nor did boiling at neutral pH. Addition of acid followed by boiling resulted in the hydrolysis of phosphoester bonds of either ATP or AMP to give predominately adenosine as the product (see Table 11).

The distribution of ^{32}P on the filter paper, the amount of folding, or the quantity of scintillation fluor present had no effect on the counting rate of the sample. It was sufficient for the filter paper to be wetted with fluor solution as the paper appeared to be transparent for the purposes of liquid scintillation counting in this procedure. The papers were routinely wedged in the bottom of a 20 ml counting vial and sufficient fluor added to a depth of about one cm (usually four to five ml). The amount of protein present, and whether or not it was applied to the chromatogram, had no effect on the distribution of radioactivity.

Table 11. Effect of Boiling at Neutral or Acid pH on Adenyl Nucleotide Stability.

Results are given as percent of total recovered counts ± range for two replicate determinations of each procedure. Ten µl of 50mM HEPES, pH 7.5, plus 5µl of ^{14}C-cyclic AMP or ^{14}C-ATP, with or without 10µl 1N HCl were treated as shown. Other experimental details are given in Methods and Materials.

Labeled Compound and Chromatogram Regions	No treatment	Boil, pH 7.5	Boil, pH 2
Cyclic AMP:			
Other nucleotides	4.4 ± 0.2	3.5 ± 0.1	3.4 ± 0.3
Cyclic AMP	90.1 ± 0.8	91.3 ± 0.1	70.1 ± 0.2
Adenosine	4.7 ± 0.6	4.3 ± 0.2	25.2 ± 0.1
Solvent front	1.0 ± 0.1	1.1 ± 0.0	1.5 ± 0.1
ATP:			
All nucleotides	94.6 ± 0.2	93.5 ± 0.2	46.9 ±12.2
Cyclic AMP	1.1 ± 0.0	1.6 ± 0.1	8.6 ± 2.7
Adenosine	2.1 ± 0.3	2.6 ± 0.1	42.3 ± 9.5
Solvent front	2.3 ± 0.1	2.4 ± 0.1	2.4 ± 0.1

Time Dependence of Substrate Disappearance and Product Formation

Figure 6 illustrates the time course of the degradation of the substrate, as measured by the difference in counts found in the ATP spot of the chromatogram compared to blanks. Almost all of the radioactivity which disappears was found in the 5'-AMP spot. The presence of ten mM fluoride reduced this degradation by a factor of two at all times. Double reciprocal analysis of this data revealed that fluoride did apparently prevent half of the basal degradation, from 20 to 10.5 nmol per mg protein, and doubled the apparent half-life of the substrate from 2.4 to 4.8 minutes, if hyperbolic kinetics are assumed. An investigation of the effect of time on cyclic AMP production revealed that the loss of ATP did not affect the linearity of production for at least five minutes (Figure 7). The correlation coefficient of a least-squares generated line for the production of cyclic AMP is 0.40 for seven minutes of basal activity and 0.93 for the first five minutes of fluoride-stimulated activity.

Effect of Protein Levels on Product Formation

Figure 8 illustrates the effect of increasing amounts of nerve cord homogenate on the production of

Figure 6. Loss of ATP with time.

The destruction of ATP, initial concentration 100 μM, is shown in the presence (filled circles, broken line) and absence (open circles, solid line) of 10 mM sodium fluoride. Each point represents the mean of four replicate experiments and bars indicate SEM. The enzyme preparation for the basal experiments was obtained from fresh, unfrozen nerve cords, and each experiment contained 102 μg enzyme protein. The enzyme preparation for the experiments exploring fluoride effects came from frozen tissues, and each experiment contained 239 μg enzyme protein.

Figure 6.

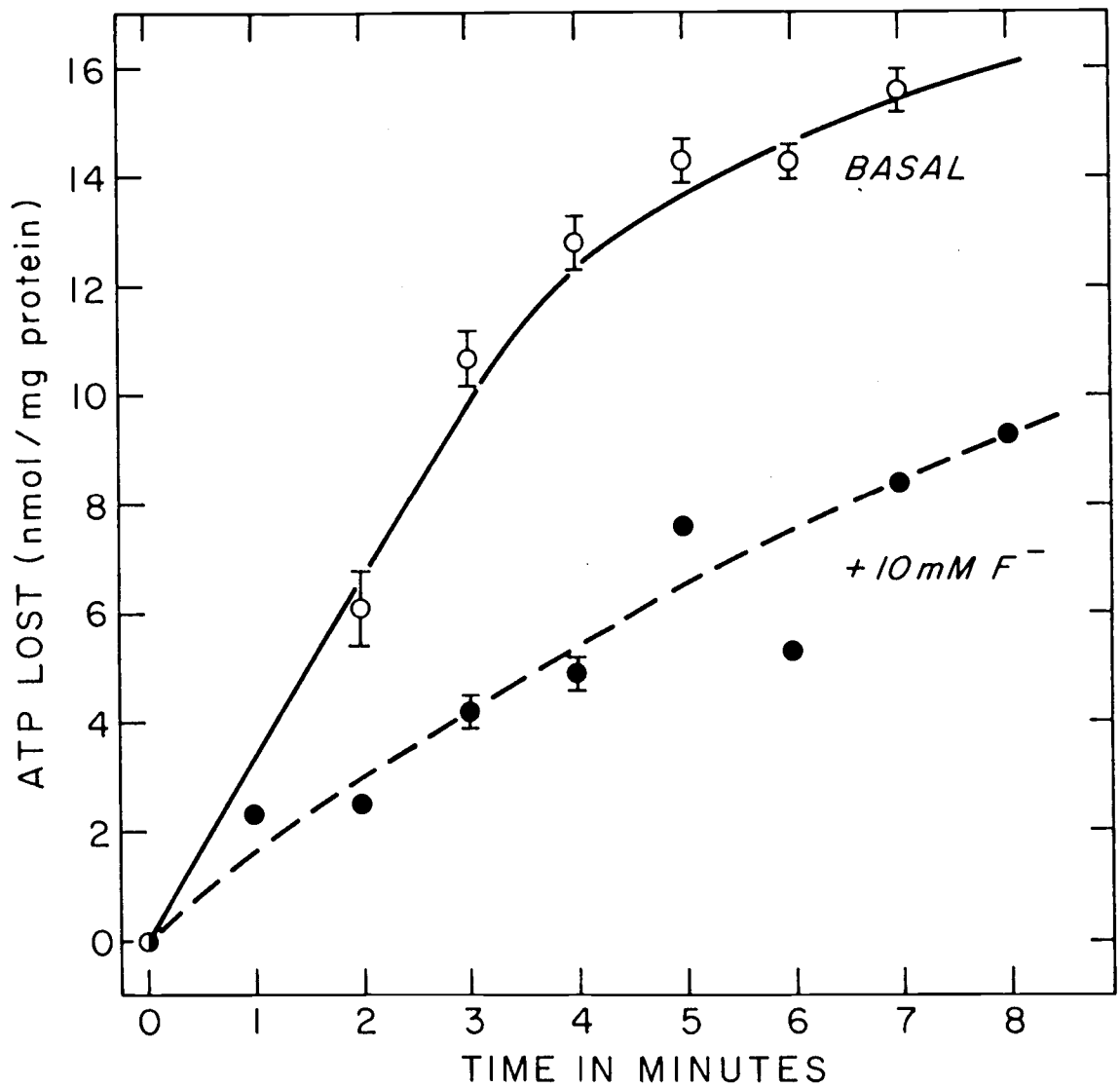

ATP LOST (nmol / mg protein)
TIME IN MINUTES
BASAL
+ 10mM F⁻
0
2
4
6
8
10
12
14
16
0
1
2
3
4
5
6
7
8

Figure 7. Formation of cyclic AMP with time.

The synthesis of cyclic AMP is shown in the presence (filled circles, broken line) or absence (open circles, solid line) of 10 mM sodium fluoride. Each point represents the mean of four replicate experiments, and bars indicate SEM. Other conditions are as detailed in the legend to Figure 1.

Figure 7.

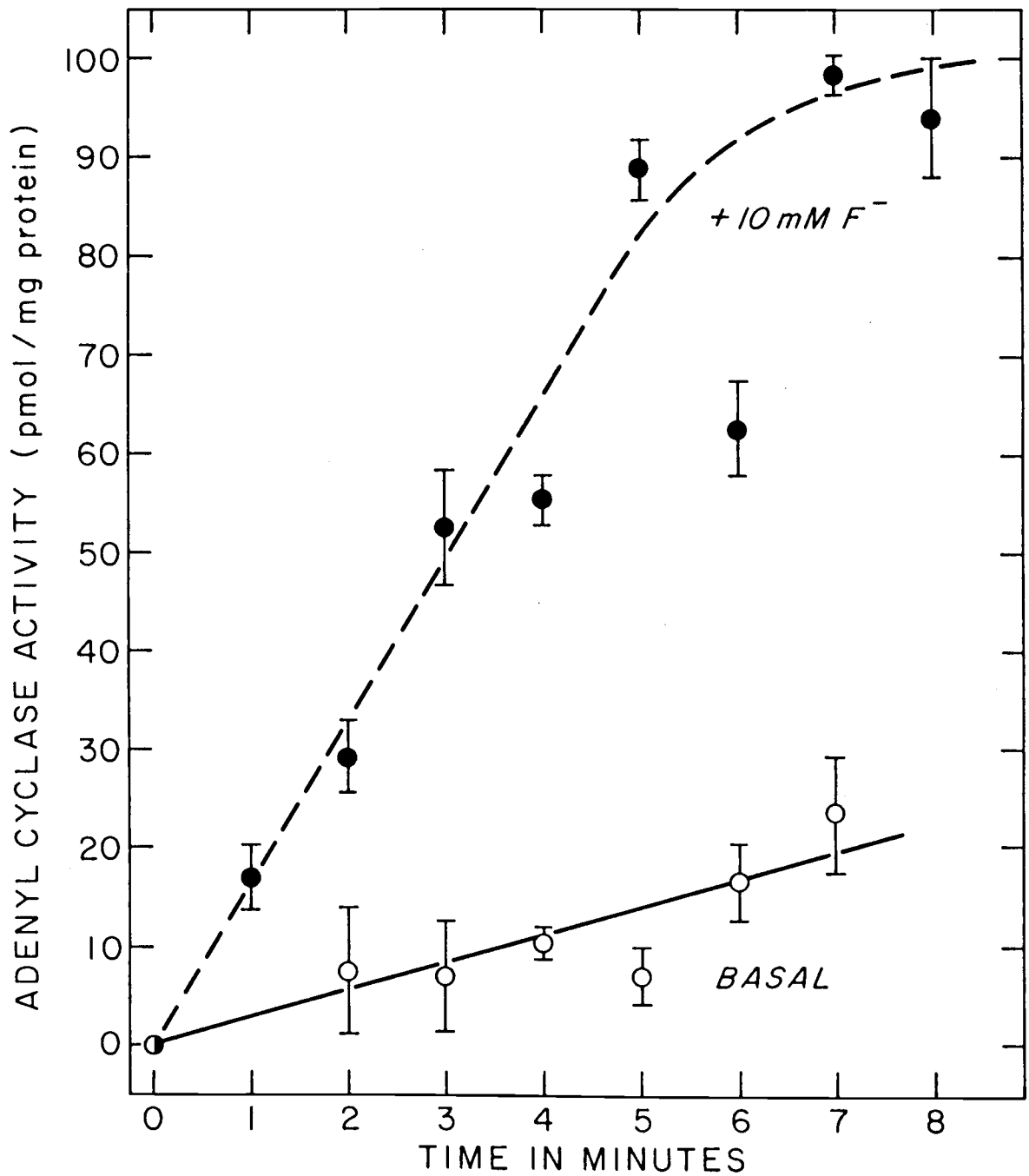
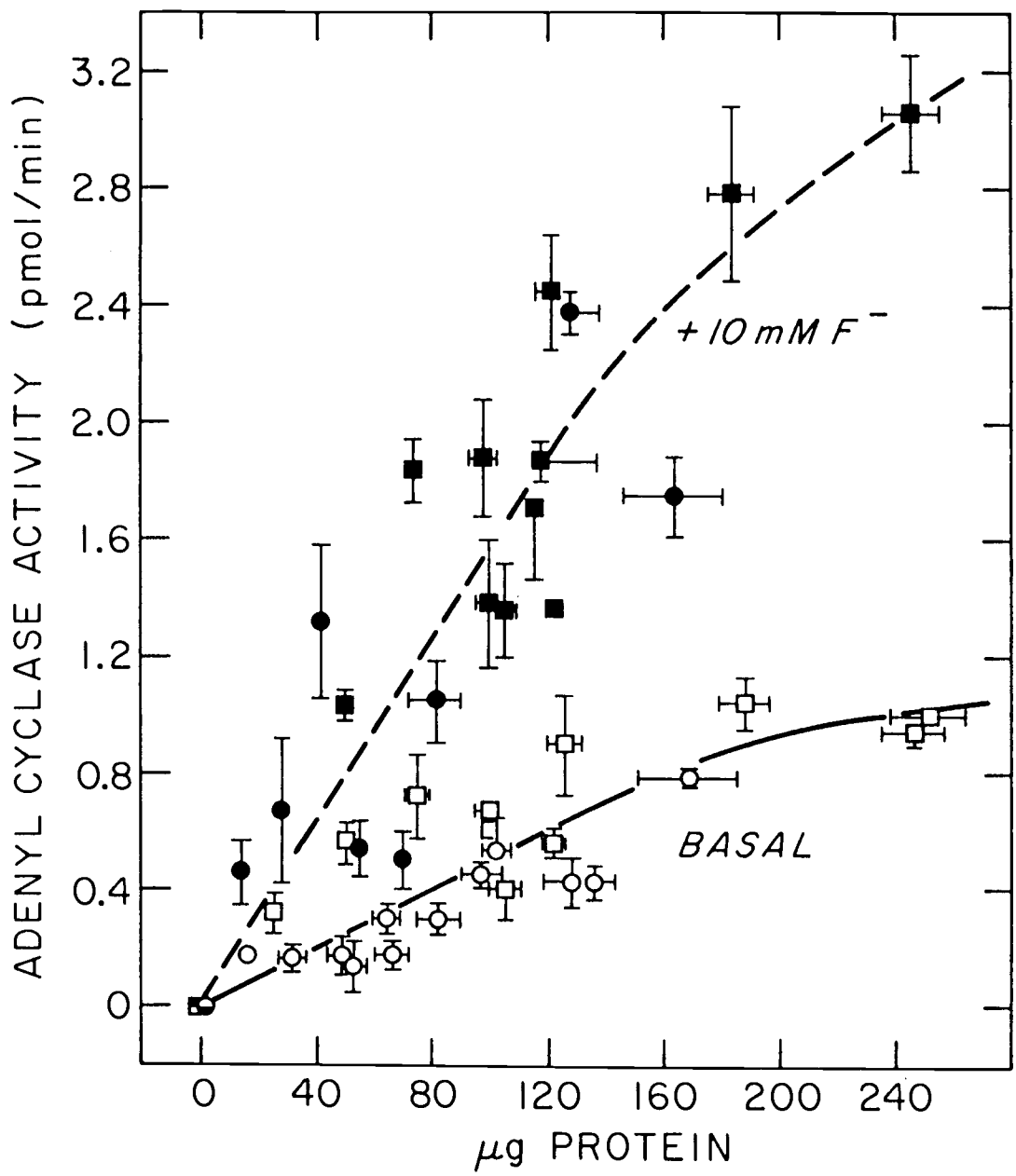

ADENYL CYCLASE ACTIVITY (pmol / mg protein)
100
90
80
70
60
50
40
30
20
10
0
+ 10 mM F⁻
BASAL
0
1
2
3
4
5
6
7
8
TIME IN MINUTES

Figure 8. Variation of cyclic AMP production with enzyme protein.

Open circles and squares represent basal activity of fresh and frozen preparations, respectively, and filled circles and squares represent 10 mM fluoride-stimulated activity of fresh and frozen preparations, respectively. Each point is the mean of four replicate experiments ± SEM. Assays were incubated five minutes with an initial substrate concentration of 100 µM. The lines were plotted using least-squares fitting. For the fluoride-stimulated enzyme, the average values obtained using fresh nerve cords have a coefficient of determination of 0.817, and average values from frozen enzyme preparations have a coefficient of determination of 0.899. The combined data, up to 130 µg protein, has a coefficient of determination of 0.878. The coefficients of determination for the basal curve are 1.156, 0.781, and 0.864 for fresh preparations, frozen preparations, and all preparations respectively up to 170 µg protein.

Figure 8.


ADENYL CYCLASE ACTIVITY (pmol/min)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
+10 mM F⁻
BASAL
0
40
80
120
160
200
240
μg PROTEIN

cyclic AMP in the presence and absence of ten mM fluoride. The slopes of the least-squares-fitted curves for basal and fluoride-stimulated enzymes correspond to maximal activities of 5.0 and 15.6 pmoles cyclic AMP formed per minute per mg protein. Analysis using Student's t-test revealed that these slopes differ with a $P<0.001$. Because linearity was lost with more than 130 µg protein present, all subsequent assays were conducted with less than this amount of enzyme present. No statistical difference was observed in using either fresh, unfrozen or frozen tissue for basal or fluoride-stimulated enzyme preparations.

Stimulation by Fluoride

Fluoride ion had no effect on adenyl cyclase activity, 5.8 pmoles per minute per mg protein basally, until a concentration of 8 mM was attained (see Figure 9). At that point, activity rapidly accelerated to a plateau value of about 19 pmoles cyclic AMP formed per minute per mg protein. This plateau begins at ten mM fluoride and extends at least to 25 mM fluoride. At 50 mM fluoride, no stimulation of adenyl cyclase activity is seen.

Effect of Substrate Concentration

As pointed out above, ATP is degraded rapidly by the crude homogenate. Hence, it was not possible to obtain linear production of cyclic AMP at low concentrations of substrate. This difficulty is depicted in Figure 10. The curves have been drawn shifted to the left toward the lower substrate concentrations which prevail in the face of the great ATPase activity present in the crude homogenates. Only the higher values on this curve could be used for double reciprocal plots. Such a Lineweaver-Burk analysis is depicted in the inset in Figure 10. For the basal enzyme the apparent K_M is 97.2 μM, and the apparent V_{MAX} is 12.8 pmoles cyclic AMP formed per minute per mg protein. The apparent K_M is 90.4 μM, and the apparent V_{MAX} is 22.3 pmoles per minute per mg for the fluoride-stimulated enzyme preparation.

Stimulation by 5-Hydroxytryptamine

Figure 11 presents the effect of varying 5-HT concentration on adenyl cyclase activity. Activity increases with 5-HT until a maximum of two-fold stimulation is reached at 2 μM 5-HT. Activity remains at a plateau, then returns to an activity similar to basal at 4 mM 5-HT. The EC_{50} for 5-HT is 0.5 μM.

Figure 9. Effect of fluoride ion concentration on formation of cyclic AMP.

Each point represents the mean of at least four replicate experiments, and error bars indicate S.E.M. Assays were incubated five minutes with an initial substrate concentration of 100 μM.

Figure 9.

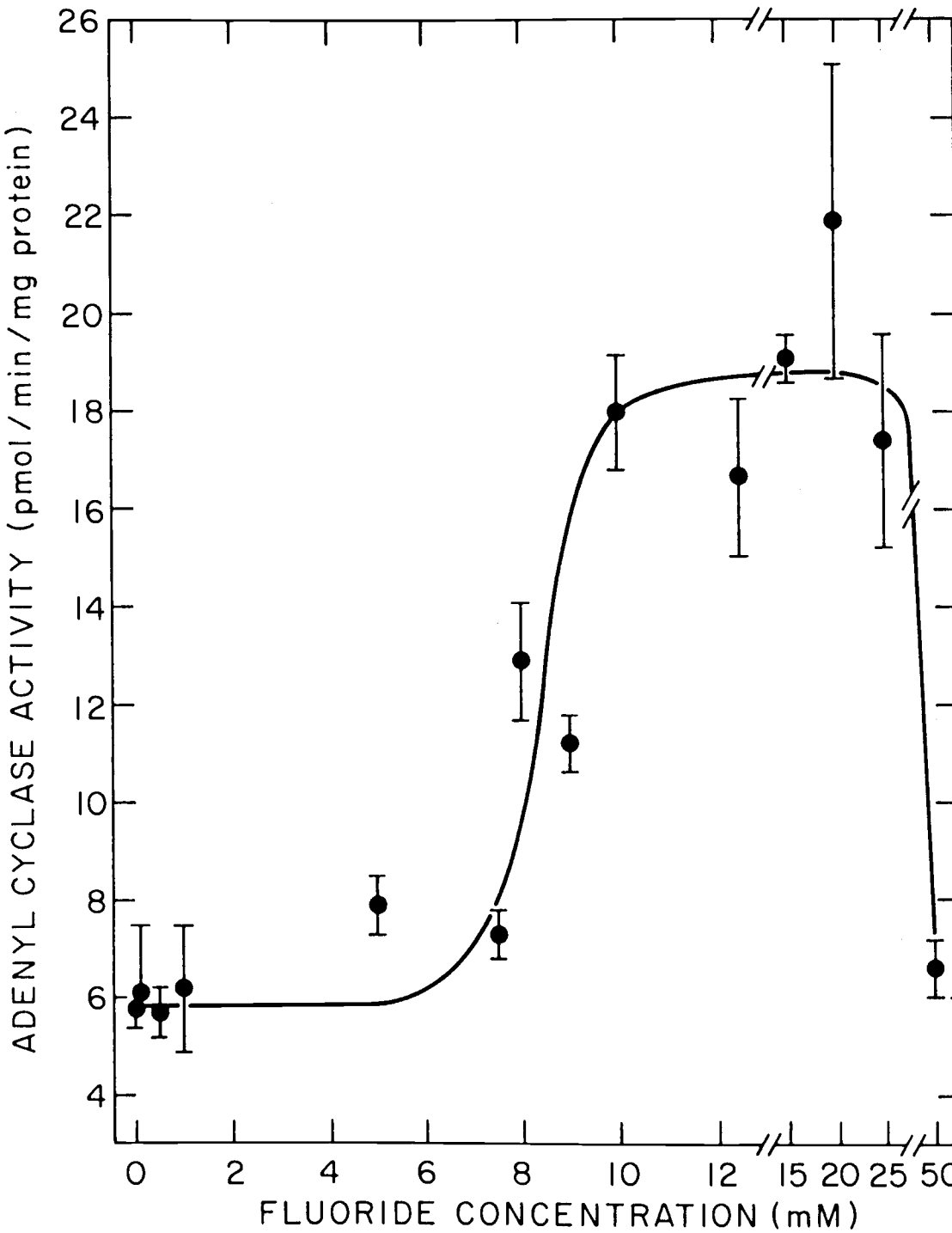

ADENYL CYCLASE ACTIVITY (pmol / min / mg protein)
26
24
22
20
18
16
14
12
10
8
6
4
0
2
4
6
8
10
12
15
20
25
50
FLUORIDE CONCENTRATION (mM)

Figure 10. Effect of substrate concentration on cyclic AMP production.

Open circles and the solid line represent basal activity, and filled circles and broken line represent activity in the presence of 10 mM fluoride. Each point is the mean of four replicate experiments ± SEM. Assays were incubated for five minutes with 122.4 ± 3.0 (SEM) µg protein. Curves were drawn to simulate activity if substrate degradation were considered (see text). The inset shows Lineweaver-Burk plots of some of these data. Again, open circles depict basal activity and filled circles represent fluoride-stimulated activity. Lines were drawn using least-squares linear regression of the means: Coefficients of determination were 0.969 and 0.832 for basal and fluoride-stimulated enzymes respectively. The x and y intercepts are -0.0103 and 0.078 for the basal enzyme and -0.0111 and 0.045 for the fluoride-stimulated enzyme.

Figure 10.

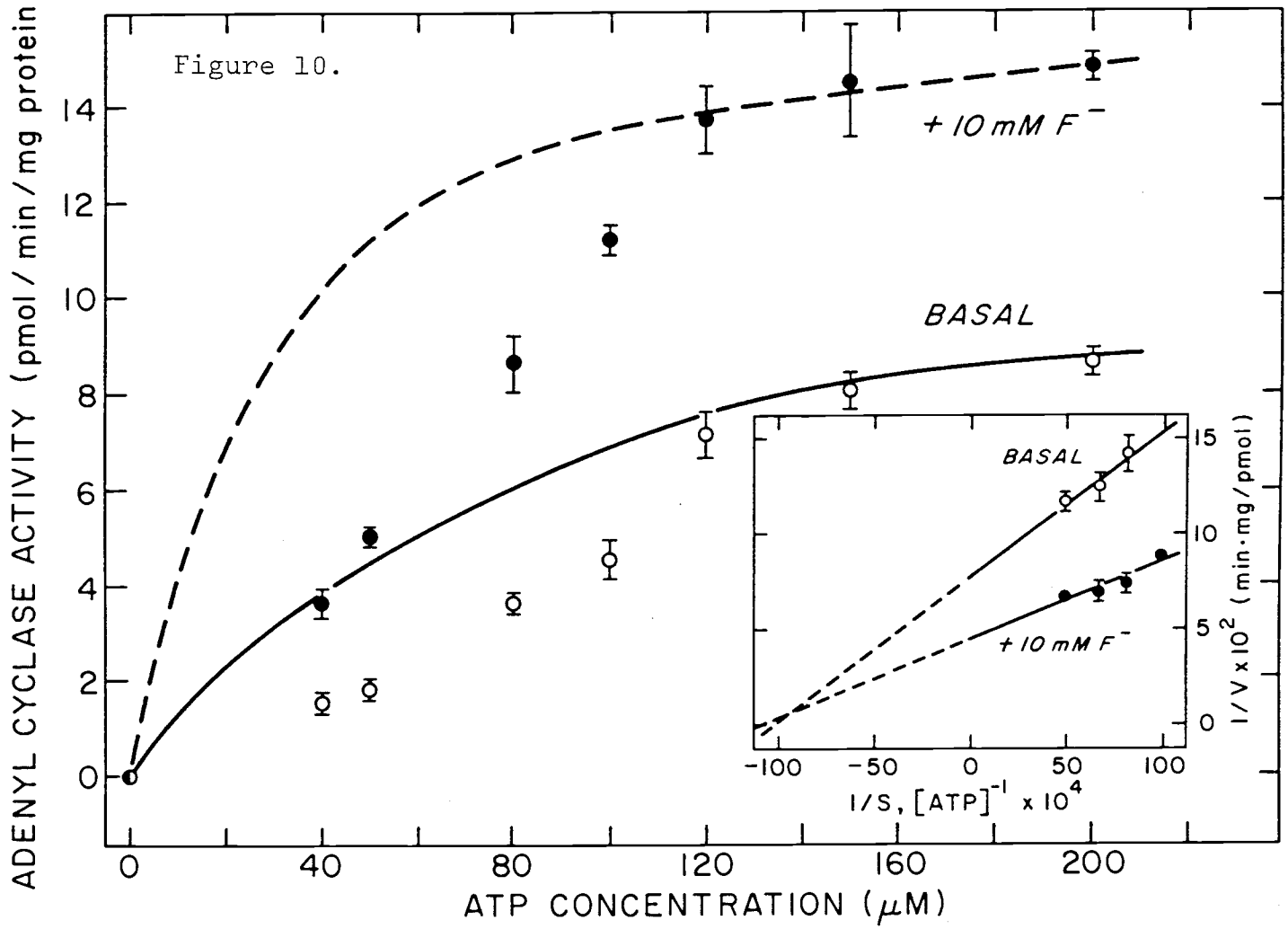

ADENYL CYCLASE ACTIVITY (pmol / min / mg protein)
ATP CONCENTRATION (μM)
+ 10 mM F⁻
BASAL
BASAL
+ 10 mM F⁻
1/V x 10² (min·mg/pmol)
1/S, [ATP]⁻¹ x 10⁴

Figure 11. Effect of 5-hydroxytryptamine on adenyl cyclase activity.

Each point represents the mean of three replicate experiments and bars indicate the SEM. Assays were incubated for five minutes using 100 μM ATP initially and 70 to 110 μg protein per assay obtained from fresh, unfrozen nerve cords.

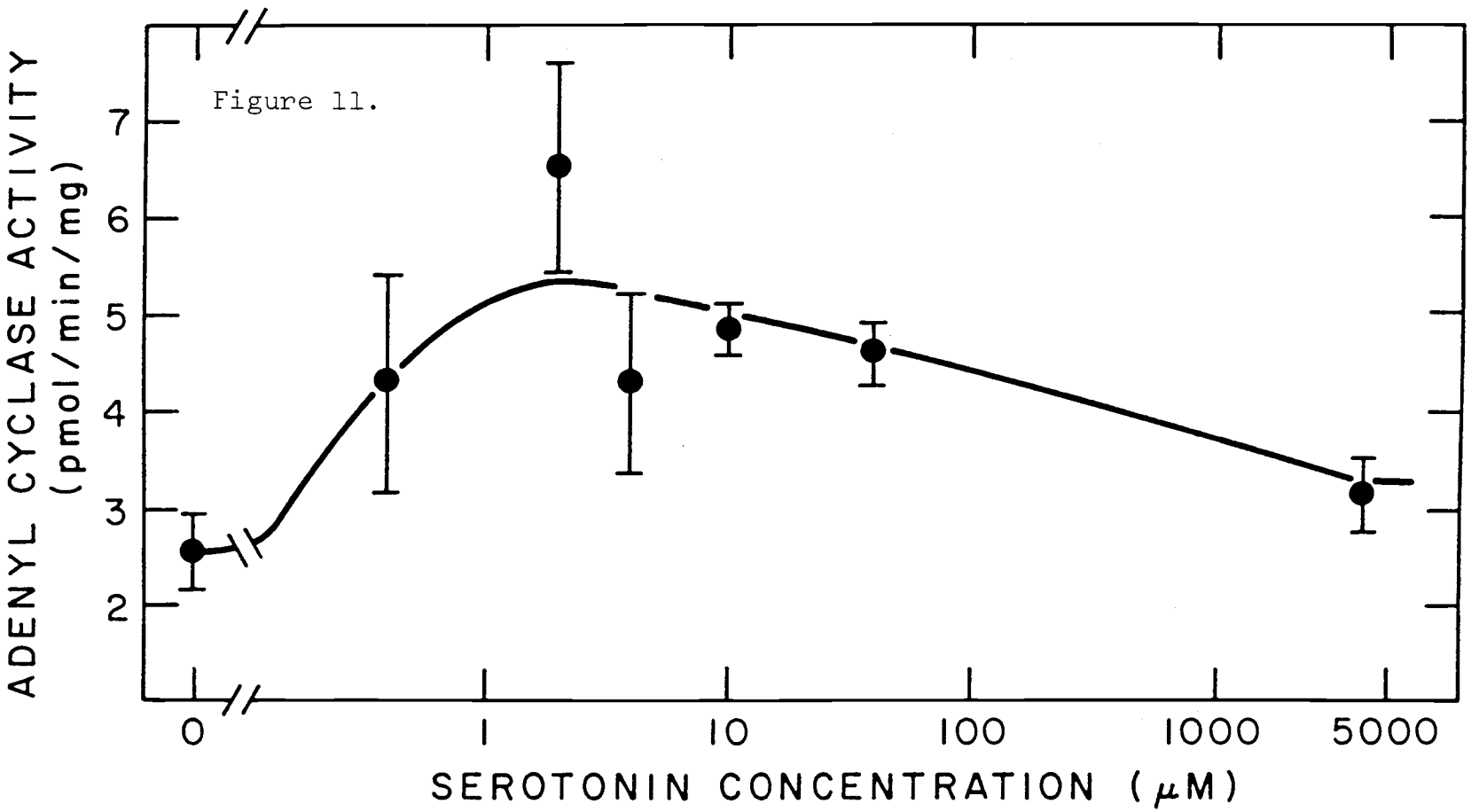

ADENYL CYCLASE ACTIVITY
(pmol/min/mg)
2
3
4
5
6
7
0
1
10
100
1000
5000
SEROTONIN CONCENTRATION (μM)

Figure 11.

Table 12. Effect of Putative Neurotransmitters on Adenyl Cyclase Activity.

Results are given as the mean pmoles of cyclic AMP formed per minute per mg protein ± SEM, and the number of replicate experiments is given in parentheses. Assays involved incubation for five minutes with 100μM ATP initially and included 102 - 135μg enzyme protein from fresh, unfrozen nerve cords. Drugs, when added, were present at 100μM.

Addition	Specific Activity
None	3.48 ± 0.63 (11)
Dopamine	4.22 ± 0.95 (4)
Octopamine	3.97 ± 0.53 (4)
Norepinephrine	1.70 ± 0.31 (8)
Histamine	2.77 ± 0.31 (4)
Taurine	2.33 ± 0.37 (4)
β-Alanine	2.80 ± 0.31 (4)
Adenosine	2.26 ± 0.46 (8)
β-Ecdysone	1.79 ± 0.37 (8)
γ-Aminobutyric acid	3.58 ± 0.16 (4)
Glutamic acid	1.90 ± 0.73 (4)
Glycine	1.04 ± 0.23 (3)
Aspartic acid	2.64 ± 0.34 (4)
Acetylcholine	2.03 ± 0.31 (4)

Effects of Other Putative Neurotransmitters

As shown in Table 12, no other tested putative neurotransmitter aside from 5-HT (above) increased adenyl cyclase activity in crude homogenates significantly. The insect molting hormone, β-ecdysone, did not increase adenyl cyclase activity.

Adenyl Cyclase in Neuronal- and Glial-Enriched Fractions

The results of a preparation of neuronal- and glial-enriched fractions are listed in Table 13. No significant difference in basal or fluoride-stimulated adenyl cyclase activity was seen between fractions, but CNPase, AChE, and PDE all varied significantly with the greater activity present in the glial-enriched fraction.

Discussion

The lack of sufficient removal of labeled ATP by various minicolumn chromatography systems was only slightly less disheartening than the variability observed in alumina results obtained with materials from different sources. In an isolated instance such figures might be considered due to some form of operator error, but in general it appears that there was some deeper flaw. Albin (1973) has reported a similar lack of success. The high degree of impurity found in a stock solution of ^{3}H-ATP (50% occurred as adenosine on

Table 13. Various Parameters of Neuronal- and Glial-Enriched Fractions Obtained from Nerve Cords.

Results are given as mean ± SEM in the units indicated in parentheses with the enzyme name. The number of assays is given in parentheses with the values of the enzyme activities.

Enzyme Parameter	Neuronal Fraction	Glial Fraction
CNPase (μmol/min/mg)	0.243 ± 0.006 (3)	0.541 ± 0.087 (3)
Adenyl cyclase: (pmol/min/mg)		
Basal	5.07 ± 0.81 (4)	4.88 ± 0.76 (4)
10mM fluoride-stimulated	16.55 ± 2.71 (4)	18.39 ± 1.88 (4)
Cyclic AMP Phosphodiesterase (nmol/min/mg)	0.422 ± 0.121 (3)	0.619 ± 0.093 (3)
Acetylcholinesterase (nmol/min/mg)	19.1 ± 0.4 (3)	29.6 ± 0.5 (3)

chromatography), the failure to coprecipitate all ATP with inorganic salts, and the success of paper chromatographic techniques provided the impetus to pursue further investigations of the adenyl cyclase using the latter system. Thus, assay systems which were excluded were the classic techniques of Krishna et al. (1968) and Ramachandran (1971) and the more recent modifications of Nakai and Brooker (1975).

The R_f values obtained in the paper chromatography system were not significantly different from those reported by Tao (1974). No procedure used in the assay had any ill effect on the accuracy, authenticity, or reproducibility of the separations.

The time dependence of the assay is similar to the work of Harmar and Horn (1977) which explored cockroach brain adenyl cyclase. Crude homogenates in their hands produced linear formation of cyclic AMP for up to five minutes.

The maximal activity of the basal enzyme is slightly lower than that observed in other insects and tissues. Sutherland et al. (1962) first noted that whole "fly larvae" contained an activity of less than 50 pmoles cyclic AMP formed per minute per mg protein. Nathanson (1973) and Harmar and Horn (1977) have reported values for enzymes from cockroach

thoracic ganglia and brain as 10-11 pmoles per minute per mg and 18 pmoles per minute per mg respectively. Filburn and Wyatt (1976) found an activity of about 17 pmoles per minute per mg in Cecropia fat body. The work of Arch and Newsholme (1976) gives activities two orders of magnitude in excess of these for the adenyl cyclase from Schistocerca cerebral ganglion, but values for various insect flight muscles are much lower. Basal activity is much higher in the brain of Gromphadorhina (Rojakovich and March, 1972). Adenyl cyclase from larval and day one adult wing pads and wings from Locusta have a lower activity than observed in M. sexta nerve cords (Achazi et al., 1977).

Fluoride stimulation gives an enzyme preparation which is three-fold more active than the basal enzyme at 100 μM ATP. This activation does not occur in intact nerve cords (see previous work) nor in broken-cell preparations at low concentrations of fluoride- but the effect rapidly rises to a plateau which occurs from 10 to 25 mM fluoride ion, and gradually returns to basal levels at 50 mM fluoride. Analysis by substrate concentration variation suggests that the maximal velocity of the fluoride-stimulated enzyme is only about twice that of the basal enzyme at saturating substrate concentrations and that stimulation is

non-competitive as the affinity for substrate is unaltered while V_{MAX} is increased by fluoride. Two- to three-fold increases in adenyl cyclase activity due to fluoride stimulation are common for insect cyclases (Arch and Newsholme, 1976; Achazi et al., 1977) and some mammalian ones as well (Perkins and Moore, 1971), although the increases can be greater (Rojakovick and March, 1972; Nathanson, 1973). The appearance of an activity plateau for intermediate fluoride concentrations is known for mammalian enzymes where a decrease at high levels is also seen (Birnbaumer et al., 1969; Perkins and Moore, 1971; Menon et al., 1973; Kalish et al., 1974; Johnson et al., 1975). The latter phenomenon has been reported in an insect preparation as well (Rojakovick and March, 1972). Drummond et al. (1971) also found that fluoride non-competitively stimulates the enzyme, increasing V_{MAX} without affecting the K_M for the substrate (see also Robison et al., 1971).

In view of other work (see previous chapters) which has suggested that 5-HT may act to elevate cyclic AMP levels in the M. sexta CNS, with a high probability that this rise is mediated via adenyl cyclase, a dose-response curve of enzyme activity was obtained. This revealed that maximal stimulation, about two times

basal activity of the cyclase, occurred at 2 μM 5-HT, and it would appear that half-maximal stimulation occurred at 0.5 μM. This latter figure is in agreement with the "K_a" cited by Nathanson (1973) for the adenyl cyclase in cockroach thoracic ganglia. Several recent articles have disclosed work on serotonin-sensitive adenyl cyclases in mammalian brain tissues (Kocur et al., 1975; Pagel et al., 1976; Fillion et al., 1977).

No other putative transmitter stimulated adenyl cyclase activity in the hornworm nerve cord. It has been suggested (Albin, 1973) that adenyl cyclase receptors may vary with insect species. Most interestingly, β-ecdysone does not stimulate the adenyl cyclase in agreement with results with the cockroach brain (Rojakovick and March, 1972), silk moth fat body (Filburn and Wyatt, 1976), and locust wing pad preparations (Achazi et al., 1977). Previous reports of positive effects should therefore be noted with care for tissues in which effects occur (Applebaum and Gilbert, 1972; Castillón et al., 1973; Vedeckis and Gilbert, 1973)

Palmer (1973) found that neuronal- and glial-enriched fractions from rat and rabbit brain both contained adenyl cyclase activity which was fluoride-stimulable and that there was no difference in these

activities between fractions. Slight activation was seen by Palmer with NE, DA, or histamine in the neuronal fraction and not in the glial fraction. The preliminary experiment reported here extends Palmer's first finding to the M. sexta nerve cord. It would be preferable to investigate cyclase activation by putative neurotransmitters using a preparation made from unfrozen nerve cords. In view of the small yields of the cell-enrichment process, such an investigation awaits the establishment of the methodology for femtomole-sensitive cyclic AMP assays in this laboratory (Cailla et al., 1973).

SUMMARY

The role of adenosine 3',5'-cyclic monophosphate (cyclic AMP) in neurotransmission in the ventral nerve cord of Manduca sexta has been investigated chemically. The findings of these studies are as follows: (1) Serotonin dramatically elevated cyclic AMP levels over controls in incubated cell suspensions of neurons bulk-isolated from intact nerve cords. (2) Cyclic AMP and cyclic GMP were present in intact nerve cords at levels comparable to those found in other insect nervous tissues. (3) In contrast to mammals and carnivorous insects, high potassium in the incubation medium decreased basal, serotonin (5-HT), and theophylline-stimulated accumulations of cyclic AMP in intact nerve cords. (4) In general, one mM ascorbate impaired cyclic AMP accumulation in basal, 5-HT- or theophylline-stimulated intact nerve cords, while a 100 μM concentration had no effect on basal accumulation at pH 5.3. (5) 5-HT stimulated cyclic AMP accumulation at pH 3.5 and 5.3; at pH 6.5 theophylline-stimulated accumulation is potentiated by 5-HT which had no effect alone. (6) Aspartic acid, gamma-aminobutyric acid (GABA), norepinephrine (NE), and isoproterenol were effective in elevating cyclic AMP levels at only one of the tested pH's. (7) β-Ecdysone had no effect on cyclic AMP levels

in intact nerve cords, but markedly increased cyclic GMP levels after ten and thirty minutes incubation. (8) Acetylcholine (ACh), aspartic and glutamic acids, GABA, glycine and theophylline stimulated cyclic GMP accumulation in intact nerve cords. (9) Synthesis of serotonin from radiolabeled tryptophan and ACh from labeled choline by intact isolated nerve cords in vitro was demonstrated. (10) Brain contained twice the level of free amino acids as did the nerve cord and was particularly enriched in glutamate. (11) The brain contained five times as much NE and 5-hydroxytryptophan as did the nerve cord, which contained an equivalent amount of 5-HT and was three-fold enriched in dopamine. (12) The adenosine triphosphate level in the nerve cord was determined and corresponds to an intracellular concentration of one mM. (13) A fluoride-stimulable adenyl cyclase was demonstrated in the nerve cord which was stimulated at μmolar concentrations by 5-HT but not by any other putative neurotransmitters tested.

CONCLUSION

The results reported in this thesis contribute further evidence to support the suggestion that cyclic AMP has a functional role in the insect nervous system. Furthermore, these results are consistent with the hypothesis that 5-HT may be a neurotransmitter in the M. sexta CNS which exerts its effects via cyclic AMP and that ACh and amino acids act via cyclic GMP. In view of the work by Daley and Vande Berg (1976), it is tempting to speculate that a push-pull mechanism is at work in this system as well. It may well be, though, that different neurons or cell types respond to different transmitters or neurohormones with different cyclic nucleotides or other second messengers.

It must be pointed out, as a final note, that experimental work utilizing electrophysiological observation is clearly required to substantiate a role for cyclic nucleotides in neurotransmission. Do cyclic nucleotide levels rise in response to presynaptic electrical stimulation? Does this stimulation result in the release of a neurotransmitter at the synaptic cleft? Does this neurotransmitter elevate cyclic nucleotide levels and can its effect on cyclic nucleotide elevation and the postsynaptic potential

be blocked or reversed by classical pharmacological neural blocking agents? Many of the criteria proposed by Beam and Greengard (1974) await fulfillment.

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