Adenosine 3', 5'-cyclic monophosphate (cyclic AMP, cAMP) and guanosine 3', 5'-cyclic monophosphate (cyclic GMP, cGMP) were quantitated in the central nervous system (CNS) of the insect Manduca sexta by competitive protein binding techniques. The M. sexta CNS was found to contain a strikingly high level of cGMP, about 100-fold greater than that of mammalian brain. The level of cAMP, however, was estimated to be about one-sixth that of mammalian brain. The basal ratio of cGMP/cAMP in the insect CNS was approximately ten, whereas this ratio has been reported to be less than one in a variety of vertebrate and invertebrate tissues. Acetylcholine caused a great elevation of cGMP, but not cAMP, in the M. sexta CNS. Short-term incubation with ecdysterone (insect metamorphosis hormone) promoted
the accumulation of both cyclic nucleotides.

The existence of cyclic nucleotide-stimulable protein kinases (ATP: protein phosphotransferase, EC 2.7.1.37), a system of enzymes postulated to be instrumental in the biochemical expression of cAMP and cGMP, was demonstrated in the CNS of both larval and adult _M. sexta_. At low concentrations, cAMP was a much more effective activator of kinase activity than cGMP. Cyclic AMP lowered the $K_m$ of the CNS kinase for ATP, a phenomenon which has been reported to be unique to nervous tissue in mammals. A number of the enzymological properties of the insect kinase were similar to those reported in the literature for this enzyme in mammalian tissues. This insect CNS was also shown to possess a potent enzyme system, viz. phosphoprotein phosphatase, for the dephosphorylation of the phosphorylated products of kinase. Phosphatase activity was gauged using phosphoprotamine as substrate. Both kinase and phosphatase activities were found to be enriched in particulate fractions of the CNS.

An enzyme system for the destruction of cAMP and cGMP, cyclic nucleotide phosphodiesterase (nucleoside 3', 5'-cyclic phosphate nucleoside 5'-phosphate 31-phosphohydrolase, EC 3.1.4.c), was examined in the _M. sexta_ larval and adult CNS. Phosphodiesterase (PDE) was found in both soluble and particulate fractions of the CNS, but highest specific activity PDE was noted in a crude mitochondrial preparation, a fraction presumably containing synaptic elements.
PDE was greatly enriched in the brain relative to the other CNS ganglia, and was present at higher levels in nervous relative to non-nervous tissues. The hydrolysis of both cAMP and cGMP appeared to be the function of a single enzyme (or similar isozymes) in the larval CNS. Kinetic evidence suggested that PDE in the insect is a cooperative enzyme and is characterized by non-linear, biphasic double-reciprocal plots. PDE could be effectively inhibited by presumed physiological levels of ATP.
Cyclic Nucleotides, Cyclic Nucleotide Phosphodiesterase, Protein Kinase, and Phosphoprotein Phosphatase in the Central Nervous System of *Manduca sexta*

by

Edward Elmo Albin

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APPROVED:

Redacted for privacy

Chairman of Department of Biochemistry and Biophysics and Professor of Biochemistry in charge of major

Redacted for privacy

Dean of Graduate School

Date thesis is presented  September 10, 1973

Typed by Opal Grossnicklaus for Edward Elmo Albin
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ABBREVIATIONS USED IN THE TEXT

Ach, acetylcholine
Ap(CH$_2$)$_2$pp, $\alpha$, $\beta$-methylene-adenosine-5'-triphosphate
ATP, adenosine 5'-triphosphate
ATP$^{32}$, [$\gamma^{32}$P]ATP
BSA, bovine serum albumin
(but)$_2$ cAMP, 6-N-2'-O-dibutyryl-3', 5'-cyclic-AMP
cAMP, adenosine 3', 5'-cyclic-monophosphoric acid
cGMP, guanosine 3', 5'-cyclic-monophosphoric acid
CNS, central nervous system
DTT, dithiothreitol (Cleland's reagent)
EDTA, ethylenediaminetetraacetic acid
EGTA, ethyleneglycol-bis-(\beta-aminoethyl ether)N,N'-tetraacetic acid
GTP, guanosine 5'-triphosphate
IAA, iodoacetamide
K$_m$, Michaelis constant
LSC, liquid scintillation counting
NEM, N-ethylmaleimide
PDE, 3', 5'-cyclic nucleotide phosphodiesterase
PHMB, para-hydroxymercuribenzoic acid
PMSF, phenylmethylsulfonylfluoride
S, substrate or substrate concentration
TCA, trichloroacetic acid
Tris, tris-(hydroxymethyl)-aminomethane
V$_{max}$, maximum reaction velocity
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Purpose of the Studies Described in this Thesis

The role of cyclic nucleotides in the function of the mammalian nervous system is a subject of considerable research interest at the present time. In large measure this is because evidence has accumulated suggesting that cyclic AMP may be intimately involved in the biochemical events underlying synaptic transmission. Many studies have focused on the highly complex central nervous systems (CNS) of vertebrates, but cyclic nucleotide research on simpler nervous systems has been relatively neglected. The insect nervous system offers some distinct advantages for such studies despite the inconvenience of its small size. Insects may be readily cultured in large numbers and possess a highly developed CNS which is characterized by a short developmental time scale. The comparative lack of neurological complexity of the insect CNS commends its use in biochemical and genetic studies. Further, the nerve cord of insects is greatly restructured during pupal metamorphosis and is an attractive tissue for developmental neurochemical studies.
The purpose of the studies presented in this thesis was to examine the levels of cyclic AMP and cyclic GMP in a simple nervous system and to define in this tissue the properties of some of the enzymes involved in the metabolism and biochemical expression of these cyclic nucleotides. Specifically, some of the enzymological properties of cyclic nucleotide-dependent protein kinase, phosphoprotein phosphatase, and cyclic nucleotide phosphodiesterase were studied in the CNS of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae). With the exception of adenyl cyclase (the enzyme catalyzing the formation of cAMP from ATP), none of the enzymes related to the metabolism or function of cAMP or cGMP have yet been reported in any invertebrate nervous tissue. *M. sexta* was chosen as an experimental animal for these studies because of the relatively large size of its nervous system compared to that of other species of *Insecta* presently adapted to mass culture. Wherever possible, the characteristics of the insect CNS kinase, phosphatase, and phosphodiesterase were compared with those properties published for these enzymes in various mammalian tissues, including brain. Considerable background material will now be presented on selected aspects of the metabolism and function of cAMP and cGMP before turning to a discussion of what was learned of the *M. sexta* CNS.
Adenosine 3', 5'-cyclic monophosphate, commonly referred to as cyclic AMP (cAMP), mediates a remarkable spectrum of physiological processes, and newly-discovered actions of this cyclic nucleotide are reported almost daily. The rapidly-proliferating literature surrounding cAMP can only be described as enormous; even a casual perusal of the more general reviews on cAMP (1-9) cannot fail to nurture one's appreciation of the complex physiological manifestations and key regulatory roles of this molecule. Cyclic AMP was first discovered in rat liver by Earl Sutherland and associates as an intermediate in epinephrine-stimulated glycogenolysis and subsequently has been found in a variety of cells from higher vertebrates, bacteria, Euglena, yeast, and amoebae.

Synthesis and Degradation of cAMP

The level of cAMP in any tissue is largely controlled by the relative activities of the enzymes catalyzing its synthesis and its degradation (Figure 1). Adenyl cyclase (or adenylate cyclase), which is found in the plasma membrane of most cells, catalyzes the cyclization of ATP to form cAMP and PP$_1$ (pyrophosphate). Cyclic nucleotide phosphodiesterase, present in both particulate and soluble fractions of the cell, degrades cAMP to 5'-AMP.
Figure 1. Reactions catalyzed by adenyl cyclase and phosphodiesterase. The structural formula of cAMP is indicated.
The Second Messenger Concept

The most widely recognized role of cAMP is its fundamental involvement in Sutherland's "two messenger hypothesis of hormone action" (1, 2, 9, 10) which was proposed in 1965. This "second messenger" concept may be summarized as follows. A specific hormone or extracellular messenger (first messenger) interacts with a specific site ('receptor', or 'discriminator') on the exterior surface of the cell membrane, and this messenger-receptor interaction activates an adenyl cyclase present in the membrane. Cyclic AMP, the second messenger, is synthesized on the inner surface of the plasma membrane and then diffuses through the cell, resulting in appropriate biochemical action(s). Thus cAMP translates an extracellular message into an intracellular response. The apparent validity of this scheme has been established in a variety of biological systems (1).

The specific effect, or effects, of an increased level of cAMP will depend uniquely upon the nature of the cell in which it occurs. Many hormones (e.g., catecholamines, glucagon, ACTH, vasopressin, TSH, and others), upon interacting with their specific target cells, are known to utilize cAMP as a second messenger (1). Specificity of metabolic response in different cell types is easily rationalized, since different cells are unique in their complement of receptor sites (governing hormonal sensitivity) and in their content of
enzymes (biochemical potential). An elevated level of cAMP may, for example, promote phosphorylase activation in hepatic cells, lipolysis in adipocytes, thyroglobulin hydrolysis in the thyroid, and steroidogenesis in the adrenal cortex. It must be noted that Rasmussen et al. (4, 11) have argued that the second messenger concept in the form just presented does not adequately account for a number of observations. These authors have proposed an alternative model which includes both calcium and cAMP as mediators of cell activation by an extracellular messenger.

**Protein Kinases and Phosphoprotein Phosphatases**

**Reactions Catalyzed by Kinases and Phosphatases**

Protein kinases (nucleoside triphosphate:protein phosphotransferase) catalyze the transfer of the γ-phosphate group of nucleoside triphosphates (NTP's) to appropriate protein acceptors (Eq. 1).

\[
\text{protein} + \text{NTP} \xrightarrow{\text{kinase}} \text{protein} - \text{P} + \text{NDP}
\]

Thus, protein kinases have two substrates, a phosphate donor and a phosphate acceptor. Serine and threonine are the only protein amino acid residues which have been identified as phosphate acceptors. Most kinases appear to be relatively specific in their requirement for ATP as a phosphate donor, but a "GTP-protein kinase" has been reported
in murine adipose tissue (12). Protein kinases, particularly those which are activated by cAMP and which utilize ATP as a substrate, are ubiquitous and are capable of phosphorylating a wide variety of particulate and soluble proteins (13, 14, 15).

Another class of enzymes known as phosphoroprotein phosphatases (or simply, protein phosphatases) catalyze the release of orthophosphate from phosphoserine and phosphothreonine residues in various protein substrates (Eq. 2).

\[ \text{protein - P + H}_2\text{O} \xrightarrow{\text{phosphatase}} \text{protein + P}_i \]

The net effect of Eq. 1 and Eq. 2 is that of an NTPase system comprised of three different proteins. Protein phosphatases have been studied in several tissues (16-22), but compared to protein kinases, relatively little is known of their enzymological and regulatory properties.

**Turnover of Phosphoprotein Phosphate**

The phosphorus of cellular phosphoproteins is present primarily as an O-serine ester, and an early estimate places the tissue concentration of phosphoprotein phosphate at 10 to 20 percent of that of inorganic phosphate (23). A noteworthy feature of phosphoproteins in general is an extremely rapid turnover of their phosphate (20, 21, 24). Upon incubation with $^{32}$P-labeled ATP, many proteins from all
locations of the cell are rapidly phosphorylated; when ATP\textsuperscript{32} is depleted or removed from the incubation medium, a very active period of dephosphorylation is observed with loss of \textsuperscript{32}P from the protein. This turnover of phosphate presumably results from the combined action of protein kinases and phosphatases on specific protein substrates. Such a system of enzymes permits a dynamic variation in the state of phosphorylation of cellular proteins which is independent of their biosynthesis and degradation.

**Cyclic Nucleotide-Dependent Protein Kinases**

In 1968 Walsh, Perkins, and Krebs (25) described a protein kinase from skeletal muscle which was completely dependent upon cAMP for activity. This kinase ('phosphorylase b kinase kinase') catalyzed the phosphorylation of phosphorylase b kinase (which subsequently has been shown to be its \textit{in vivo} substrate) as well as the phosphorylation of proteins not found in the tissue from which the enzyme was derived (e.g., casein and protamine). Because this kinase did not appear to be specific for phosphorylase b kinase, it was termed a "cyclic AMP-dependent protein kinase." This terminology has been carried forward into the current literature. In the last few years protein kinases which undergo pronounced activation in the presence of low levels of cAMP have been identified in a great number of tissues and organisms, including mammals,
invertebrates, bacteria, and viruses (see 14, 26, 27, and references therein).

The term "cAMP-dependent" is generally used to describe those kinases activated by cAMP and to distinguish them from "cAMP-independent" enzymes. In some cases the description "cAMP-stimulable" may be preferable because some kinases activated by cAMP exhibit appreciable activity with no cAMP present. Protein kinases specifically activated by low concentrations of cGMP have also been discovered (14, 26, 28, 29), and these have been termed "cGMP-dependent" protein kinases.

A Fundamental Hypothesis

Based upon (1) the ubiquitous occurrence in nature of cAMP-dependent protein kinases, and (2) the apparent validity of Sutherland's second messenger concept, Greengard and colleagues have advanced a general hypothesis suggesting how cAMP may function as a universal regulatory agent (14, 30). According to this hypothesis, all the diverse effects of cAMP in various tissues and species are mediated through the regulation by cAMP of the activity of protein kinases. A great deal of evidence in support of this postulate has accumulated. It is believed that the distinctive effects of cAMP in a given tissue would be governed by the action of specific protein kinases acting upon specific substrates in that tissue. An altered state of
phosphorylation of an endogenous substrate would result in an altered functional state of this substrate, thereby resulting in a specific biochemical and physiological effect of cAMP.

The great majority of protein kinases thus far characterized in mammalian tissues are activated in the presence of cAMP. However, a number of examples have been reported (15, 31-33) in which cAMP resulted in an apparent decrease of kinase activity. Since relatively impure preparations were employed for some of these studies, the possibilities must be entertained that the net decrease in phosphorylation of the substrate protein resulted from either a cAMP-effected decrease in the activity of a protein kinase, or from a cAMP-induced increase in the activity of a protein phosphatase, or perhaps both. DeLorenzo et al. (34) have recently described one system in which cAMP apparently does directly activate a protein phosphatase, and assert that it constitutes the first example of an action of cAMP not mediated through a cAMP-dependent protein kinase. In the current study of soluble kinase and phosphatase activities from the *M. sexta* larval nerve cord, it was determined that both cAMP and cGMP were capable of stimulating protein kinase, but neither had any effect on protein phosphatase activity.

**Protein Substrate Specificity of Kinases**

It is known that many tissues, and also clonal cell lines,
contain multiple forms of cAMP-dependent protein kinase and phosphoprotein phosphatase (13, 21). The existence of several distinct kinase-phosphatase-protein substrate systems in a given cell would allow the specific phosphorylation-dephosphorylation of a variety of cellular proteins. The substrate for a particular kinase could be a nuclear protein, an enzyme, a membrane protein, or some other key protein whose properties are altered by phosphorylation. It is clear that not only the electrostatic charge, but also the molecular conformation of a substrate protein could be intimately related to its state of phosphorylation.

If elevated levels of cAMP are to bring about a tissue-specific response, this specificity must be determined by some unique properties of the protein kinase and of the endogenous substrate(s) for that protein kinase (14, 30). One would expect, then, that a given protein kinase would be extremely selective with regard to which proteins it could phosphorylate. This may well be true in vivo, but it is certainly not true in vitro. Most protein kinases from the many tissues that have been studied have very similar substrate specificities when assayed in vitro, and are able to phosphorylate a great number of exogenous protein substrates such as casein, histone, protamine, glycogen synthetase, serum albumin, and others; distinct protein kinases from a given tissue have been shown to possess quite similar exogenous substrate specificities (13). This rather broad
substrate specificity of kinases in general is, for practical reasons, fortuitous as it allows their convenient assay using readily available proteins as phosphate acceptors.

The "natural" substrates of protein kinases are for the most part unknown at the present time, but a number of "specific" protein kinases have been described (see ref. 13 for a partial review). Enzymatic phosphorylation-dephosphorylation sequences have been implicated in the regulation of glycogenolysis, lipolysis, nuclear activity, and adrenal steroid production. Also, the phosphorylation of specific proteins is believed to be involved in the regulation of pyruvic dehydrogenase activity, muscle contraction, neurosecretion, and adenohypophyseal secretory granule activity. The role of protein phosphorylation in the modulation of synaptic transmission of nerve impulses will be discussed in material that follows.

**The Mechanism of Activation of Cyclic Nucleotide-Dependent Protein Kinases**

Evidence from several laboratories (35-41) amply supports the view that cAMP-dependent protein kinases in mammals and arthropods are allosteric enzymes composed of regulatory (R) and catalytic (C) subunits and that activation of the kinase occurs after cAMP binds to the regulatory subunit (Eq. 3).

\[
(3) \quad Rc + cAMP \xrightarrow{\text{inactive}} R\cdot cAMP + C \quad \text{active kinase}
\]
The holoenzyme \((RC)\) is believed to be essentially devoid of kinase activity \((13, 42)\). Cyclic AMP promotes dissociation of the holoenzyme \((cAMP\)-dependent kinase) into a catalytic subunit \((cAMP\)-independent kinase) and a cyclic AMP-regulatory subunit complex. This situation may be viewed as one in which the active protein kinase \((C)\) is normally inhibited in the absence of cAMP by being associated with the regulatory subunit.

Miyamoto et al. \((43)\) have recently demonstrated for the first time that cGMP-dependent protein kinases may behave in a similar fashion to most cAMP-dependent protein kinases with respect to activation by cyclic nucleotide. It was observed that a purified kinase from lobster muscle could be activated by cGMP and dissociated into R and C subunits. These authors purified R and C subunits from a cAMP-dependent kinase \((\text{bovine brain})\) and also from a cGMP-dependent enzyme \((\text{lobster muscle})\). The R subunit from mammalian brain could readily be combined with the C subunit from lobster muscle to yield a cAMP-dependent "hybrid" holoenzyme. It is most interesting that an interaction between the R and C subunits occurred, since the subunits were from different tissues, different phyla, and different classes of protein kinase. This experiment serves as an excellent example of biochemical similarity despite evolutionary disparity. In this vein, evidence will be presented later in this thesis demonstrating that protein kinase, phosphoprotein phosphatase, and cyclic nucleotide
phosphodiesterase in the *M. sexta* larval nerve cord are similar to these enzymes in mammals with respect to a number of enzymological properties.

**Protein Kinase Modulator**

Several tissues are known to contain a heat-stable, trypsin-labile inhibitor of cAMP-dependent protein kinase (42, 44-47). The highest levels of inhibitor have been found in brain tissue (45, 47). As will be demonstrated, such an inhibitor was also found in the CNS of *M. sexta*. This inhibitory factor was first reported in muscle and was shown to antagonize the cAMP-promoted phosphorylation of phosphorylase kinase and glycogen synthetase, as well as inhibiting cAMP-dependent protein kinases from a variety of biological systems (13, 47). The inhibitor has been highly purified from a number of tissues (including bovine heart and brain, rabbit skeletal muscle, and lobster tail muscle) and has been characterized as a small, highly-charged acidic protein (45, 48). The inhibitor (I) has been demonstrated (45, 47) to interact reversibly with the catalytic subunit (C) of cAMP-dependent protein kinases according to Eq. 4:

\[ C + I \rightleftharpoons CI \]

The protein inhibitor and the R subunit of cAMP-dependent protein kinases exhibit similar abilities to interact with the C subunit, but it
is well-documented that these proteins are distinct entities.

Recent research (48, 49) has established that the "inhibitor" should in fact be regarded as a "modulator" of protein kinase activity in that its effect under appropriate experimental conditions may be either stimulatory or inhibitory depending upon the nature of the protein kinase, the kind and concentration of cyclic nucleotid e, the source and concentration of the modulator, the type of substrate protein, and other factors. Although the modulator inhibits enzyme activity in the presence of some substrates, it stimulates activity in the presence of others (48). These dual properties have been observed with kinase modulators obtained from a variety of tissues, and the biochemical mechanism of action of the modulator appears quite complex. For example, Donnelly et al. (49) have reported that when protamine was used as a substrate, a highly-purified modulator from lobster muscle stimulated the activity of both cAMP-dependent and cGMP-dependent protein kinases. When arginine-rich histone served as substrate, the modulator inhibited the activity of cAMP-dependent protein kinase but stimulated the activity of cGMP-dependent protein kinase. When either mixed histones or lysine-rich histones were used as substrate, both classes of enzyme were inhibited by the modulator. It was suggested that the modulator functions in vivo by binding to the catalytic subunit of, and thereby regulating the activity of, cAMP-dependent and cGMP-dependent protein kinases through
modification of their substrate specificities.

The Role of Cyclic AMP and Protein Phosphorylation in the Synaptic Transmission of Nerve Impulses

There has accumulated recently a large body of evidence which implicates cAMP in the events of synaptic transmission (29, 50-53). This cyclic nucleotide may perform both a pre- and a post-synaptic role in the transmission of nerve impulses in the central and peripheral nervous systems. The formation and degradation of cAMP appears to be regulated by the same factors that govern impulse transmission by neurons. Alterations in the excitability of the postsynaptic membrane caused by cAMP are very likely mediated through the stimulation of protein kinases.

A number of experimental observations are consistent with the idea that cAMP may be associated with the biochemical events underlying neuron-neuron and neuron-muscle communication. For example, it is known (54, 55) that the formation of cAMP in incubated mammalian brain slices can be greatly stimulated by various putative transmitters of the central nervous system such as norepinephrine, epinephrine, histamine, and serotonin. It is believed that the neurohormones bind to specific receptors on the cell surface and stimulate the activity of adenylate cyclase. Membrane depolarizing agents such as ouabain, batrachotoxin, veratradine, and high concentrations of
potassium ions can also elevate cAMP levels (56, 57), as can electrical stimulation (58).

Daly et al. (59) have examined the relationship between the level of cAMP in brain slices and the electrical state of neuronal membranes and suggest that this nucleotide plays a role in the regulation of membrane potential and electrical activity in the brain. It has been demonstrated that exogenous cAMP mimics the postsynaptic inhibitory effect (hyperpolarization) of norepinephrine on cerebellar Purkinje neurons (60). A similar hyperpolarization of the postsynaptic neurons of the superior cervical ganglion has been observed, and this effect modifies the response of these cells to subsequent activity in the preganglionic fibers (31). McAfee et al. (61, 62) have shown that brief periods of synaptic activity in autonomic ganglia resulted in an accumulation of cAMP primarily in the postsynaptic neurons of the ganglia. By way of contrast, there occurred no increase in cAMP as a result of activity in nervous tissue devoid of synapses. Breckenridge et al. (63) and Singer and Goldberg (64) observed that exogenous cAMP can modify transmission at the neuromuscular junction by enhancing the release of acetylcholine from prejunctional nerve endings.

The enzymes catalyzing the synthesis and degradation of cAMP have been found to be unusually abundant in brain and have been implicated in the process of neurotransmitter release (54, 58, 63, 64).
Higher adenyl cyclase activities in brain than in any other mammalian tissue have been reported (65), and it has been suggested that this activity is located primarily at postjunctional sites (66). Cyclic nucleotide phosphodiesterase also is present at much greater levels in nervous tissues than elsewhere (67, 68). The level of both enzymes is higher in grey than in white matter (69, 70). Assuming that the distribution of these two enzymes in brain might suggest the loci at which cAMP acts, a number of subcellular fractionation and histological studies of brain material have been conducted (66, 69, 70, 71).

DeRobertis et al. (69) and Cheung and Salganicoff (72) have shown that the specific activities of both phosphodiesterase and adenyl cyclase were very high in those subcellular fractions enriched in synaptic elements. A cytochemical study by Florendo et al. (71) of the rat cerebral cortex indicated that phosphodiesterase was particularly concentrated in postsynaptic (dendritic) nerve endings, most of it immediately adjacent to the synaptic membrane. These authors cited evidence that the bulk of phosphodiesterase was not concentrated in prejunctional nerve fibers, yet a later report by Johnson et al. (73) demonstrated a high level of phosphodiesterase in synaptic vesicle fractions.

In exocrine and endocrine cells, as well as in nerve endings, the secretory product is stored in small vesicles which upon stimulation are believed to fuse with the surface membrane and release the
product into the extracellular space. It is believed that neurotubules (microtubules) are involved in the mechanism whereby the migration of these vesicles is controlled in some nerve cells. Goodman et al. (74) have suggested a presynaptic role for cAMP in this process on the basis of the finding that neurotubular subunit protein from the bovine cerebral cortex serves as an excellent substrate for a cAMP-dependent protein kinase closely associated with the subunit protein.

The distribution of cAMP among various fractions of nervous tissue has been investigated, and it has been reported that a large amount of cAMP could be recovered in particulate fractions containing microsomes and nerve endings (75, 76). A recent report by Johnson et al. (73) demonstrated that synaptic vesicles from mouse brain have the ability to store cAMP (5,000-7,500 molecules per vesicle). These vesicles are morphologically the basic subcellular unit of presynaptic nerve terminals and are known to contain quanta of neurotransmitter which are released following nerve stimulation. Johnson et al. also detected high levels of phosphodiesterase in the vesicle fractions and suggested this enzyme was located on the exterior surface of the vesicle. The finding that cAMP is concentrated within these vesicles, which are present only in presynaptic nerve terminals, constitutes the best evidence yet obtained that supports a presynaptic role for cAMP.

Evidence will now be presented which strongly implicates
cAMP in a postsynaptic role in nervous transmission. Kuo et al. (32) examined the properties of cAMP-dependent protein kinases in various bovine tissues, including brain. The brain enzyme appeared unique in that cAMP had a much greater effect on the $K_m$ for ATP than it did for the kinases from other tissues. Most of the protein kinase of brain tissue was shown to be associated with particulate fractions (77), whereas kinase activity of all non-neural tissues appeared to be localized largely in the cytosol (31). Fractions from rat cerebrum with highest specific exogenous kinase activities were those enriched with synaptic plasma membranes, synaptic vesicles, and microsomes. In those fractions in which there was a high concentration of protein kinase, there was a high concentration of endogenous substrate for the kinase as well (31). Distribution of substrates paralleled the distribution of protein kinase activity, as well as those of adenyl cyclase and phosphodiesterase. Johnson et al. (24) examined the intrinsic kinase activity (phosphorylation of endogenous protein by endogenous kinase) in various membranous subcellular fractions from rat cerebrum and found that only those fractions containing synaptic membranes showed significant stimulation by cAMP of the phosphorylation of a protein constituent of the membrane. The selective stimulation of a single minor protein component of the synaptic membrane fraction was observed. The intrinsic phosphorylation of this, but no other membrane protein, implies that it is located in the immediate
vicinity of a kinase within the synaptic membrane and that it may be an important natural substrate.

Since the dephosphorylation of the phosphoprotein products of protein kinase would presumably be important in regulating the effects of cAMP in the nervous system, the subcellular distribution of phosphoprotein phosphatase was studied by Maeno and Greengard (21). The highest specific activity of this enzyme was noted in synaptic membrane fractions, and the subcellular distribution of the phosphatase paralleled that of cAMP-phosphodiesterase reported by DeRobertis et al. (69). Endogenous synaptic membrane phosphoprotein was found to be a much better substrate for the membrane-associated phosphatase than any of the exogenous substrates examined. A particularly high rate of phosphate turnover in synaptic membranes was observed in vitro, due to the concerted effects of endogenous kinase and phosphatase. Weller and Rodnight (20) have also noted a rapid turnover of phosphoserine phosphate in ox brain cerebral cortex membrane fractions.

All of these studies indicate that the synaptic plasma membrane contains high concentrations of adenylate cyclase, cyclic nucleotide phosphodiesterase, cAMP-dependent protein kinase, phosphoprotein phosphatase, and substrate for the kinase and phosphatase. Thus, the synaptic membrane contains the entire enzymatic apparatus for the formation, destruction, and biochemical action of cAMP, and
this would appear to be a highly compartmentalized system.

The following mechanism has been proposed (24, 29, 31, 61, 71, 77, 78) for the postsynaptic modulation by cAMP of some types of neural transmission. Activity in presynaptic fibers leads to passage of neurotransmitter through the synaptic cleft, resulting in depolarization (excitation) of the postsynaptic membrane. Cyclic AMP, which is generated in postsynaptic neurons in response to the neurotransmitter, activates a protein kinase which catalyzes the phosphorylation of a key protein substituent of the postsynaptic plasma membrane. The phosphorylation of this protein leads to a change in the ionic permeability of the membrane, a change in membrane potential, and a modification of the excitability of the postsynaptic membrane. Subsequent synaptic transmission could either be inhibited or facilitated, depending upon whether the membrane is hyper- or hypopolarized following phosphorylation. Upon removal of phosphate from the acceptor protein by a protein phosphatase present in the membrane, the synaptic membrane potential could return to its initial value. An altered membrane polarization state could be maintained for a prolonged period of time, or exist only transiently, depending on the balance of kinase and phosphatase activities.

Cyclic GMP

The ever-expanding body of evidence that cAMP mediates the
actions of many hormones and neurotransmitters has stimulated interest in other 3', 5'-cyclic nucleotides to elucidate whether they also are instrumental in hormonal or metabolic control mechanisms. At the present time only one other cyclic nucleotide, cGMP, is known to occur naturally (1) and is believed to be of biological importance. The potential physiological relevance of cyclic nucleotides other than cAMP or cGMP has been argued by Blecher et al. (79), but there is presently no evidence to substantiate this contention.

Enzymes which form cGMP from GTP have been investigated (80, 81, 82) and on the basis of substrate specificity, metal requirements, and subcellular distribution, guanyl cyclase and adenyl cyclase are generally believed to be distinct enzyme systems. However, there exists evidence suggesting that in some instances a single enzyme system may have the capability of synthesizing both cyclic nucleotides (3). The possibility that adenyl cyclase has the capability of synthesizing cGMP cannot be dismissed (1, 81). Adenyl cyclase activity has recently been reported in insect nervous tissue (83, 84) but detailed properties are not known. In particular, the hormonal sensitivity, subcellular distribution, and substrate preference of this enzyme system remain to be explored. With regard to the degradation of cGMP, in many cells the same phosphodiesterase seems able to hydrolyze both cAMP and cGMP,
and in some instances other cyclic nucleotides as well (144). However, Russel et al. have reported a cGMP-specific phosphodiesterase (182). As will be shown, substrate-specific phosphodiesterases were not evident in the current study of the *M. sexta* CNS. Evidence that cGMP may influence cAMP levels through modulation of phosphodiesterase will be treated later in this thesis.

Cyclic GMP has been demonstrated in many mammalian tissues and fluids, as well as in the lower phyla (1, 80, 81, 85). With few exceptions, it is present in most mammalian tissues in concentrations at least 10- to 100-fold lower than cAMP (80, 86, 88) and levels of cGMP in tissues of the rat are approximately $10^{-11}$ to $10^{-10}$ moles per gram wet weight (1). Until recently, the lack of sufficiently sensitive assays for this nucleotide has precluded careful studies of its properties. Cyclic GMP has been found to be particularly enriched in rat lung and cerebellum, and in the latter tissue is present at almost 70% of the level of cAMP (88). Steiner et al. (86, 87) have reported that cGMP levels in mouse cerebellum were almost ten-fold higher than in other areas of mouse brain. The possibility of an important role for cGMP in insect tissues has been especially dramatized by the finding of Ishikawa et al. (80) that, of all tissues examined in several animal phyla, the cGMP content of whole crickets was exceptionally high (about 50-fold more concentrated than in rat brain) and was two to three times greater than cAMP.
Mention has been made previously of the thesis of Kuo and Greengard that the wide spectrum of effects of cAMP are mediated via regulation of cAMP-sensitive protein kinases. The discovery of a class of kinases in various tissues and species more sensitive to activation by cGMP than by cAMP has led to a similar hypothesis for the biological function of cGMP (14, 26, 28, 88). This class of protein kinase is principally found in arthropods, although small quantities of cGMP-sensitive protein kinase have been found in several mammalian tissues (29). These enzymes have been partially separated from cAMP-dependent kinases present in the same tissue (26, 28, 29) and have $K_a$ values for cGMP in the range of $2 \times 10^{-8}$ to $3 \times 10^{-7}$ M. Cyclic GMP is capable of activating cAMP-dependent protein kinases, but generally only at a much higher concentration than is required for cAMP (89, 90). Hofmann and Sold (91) have concluded that there exists in rat cerebellum a protein kinase specifically regulated by cGMP, but after a careful scrutiny of the data presented by these authors, one must judge this conclusion to be without substantial justification. Although the biological significance of cGMP is not presently known, the very fact that there exist both cAMP-stimulable and cGMP-stimulable protein kinases in the same tissue raises a number of questions about the biochemical relationship of these two classes of enzyme. It is interesting that a protein modulator (discussed previously) has been found in many tissues which is
capable of inhibiting cAMP-dependent protein kinase activity and also of stimulating cGMP-dependent activity.

Evidence is gradually accumulating that implicates cGMP in a second messenger system distinct from that involving cAMP (85, 88, 92-94). Moreover, there are indications that cGMP is involved in the mediation of events antagonistic to those controlled by cAMP (94, 95). In a very recent and interesting paper by Hadden et al. (93), it was proposed that cGMP, in addition to eliciting other effects, serves as a membrane to nucleus messenger and that this nucleotide serves as an intracellular "mitogenic trigger" in promoting histone acetylation, phosphorylation of nuclear proteins, and increased nuclear RNA synthesis. Cyclic GMP, in addition to cAMP, has been implicated as an intracellular mediator of growth hormone release (96).

Substantial evidence is now available which indicates that levels of cGMP and cAMP are under separate regulatory control (88, 94, 97). The independent variation of these levels strongly suggests the two nucleotides do not share the same functional role in a given tissue (95). Hormones which elevate tissue cAMP levels generally have little effect on cGMP, but changes in extracellular fluid levels of cGMP associated with the action of cAMP-stimulatory agents have been observed (1).

In comparison to cAMP, the neurochemical role of cGMP is
much less well defined, but there is some evidence that the latter nucleotide is involved in nervous tissue metabolism. Ferrendelli et al. (98) have reported that drugs which influence mammalian CNS monoaminergic neurotransmitter systems (e.g., d-amphetamine, reserpine, and chlorpromazine) alter levels of cGMP in mouse cerebellum but do not influence cAMP levels. Recent studies suggest that cGMP may be intimately involved in cholinergic neurotransmission in heart and brain. Stimulation of cGMP levels in mammalian cerebral cortex, heart ventricle, and ileum by various cholinomimetic agents has been reported (94). Perfusion of the heart with acetylcholine (ACh) markedly stimulates cGMP accumulation while having little effect on cAMP; increased cGMP levels are paralleled by the negative inotropic but not the negative chronotropic effects of ACh (95). Similar stimulatory effects of ACh on cGMP, but not cAMP, have been observed in liver and thyroid (97).

Kuo et al. (88) demonstrated that ACh antagonized the increase of cAMP in rat ventricular slices elicited by isoproterenol or glucagon. Conversely, these agents lowered the increase in myocardial cGMP caused by ACh. These observations, coupled with similar findings in rabbit brain tissues, support the concept that cAMP and cGMP are under separate, and sometimes reciprocal, regulatory control (88, 94). ACh increased cGMP and decreased cAMP levels in rabbit cerebellum, but stimulated cGMP without affecting cAMP
in the cerebral cortex. Norepinephrine, however, lowered cGMP levels while greatly elevating cAMP concentrations in the cerebellum. The tentative hypothesis was proposed (88) that the (sometimes antagonistic) physiological actions of cholinergic and adrenergic agents are mediated through the regulation of the relative concentrations of cAMP and cGMP. Predominantly adrenergic effects would be accompanied by high cAMP/cGMP ratios, whereas cholinergic effects would be mediated by high cGMP/cAMP ratios.
GENERAL EXPERIMENTAL MATERIALS AND METHODS

Materials

The sources of a number of chemicals used routinely in these studies will be listed here. Additional reagents employed in specific assays will be noted in material that follows. Highly purified reagents for polyacrylamide gel electrophoresis were purchased as a kit from Bio-Rad Laboratories. This included acrylamide monomer, N, N'-methylenebisacrylamide monomer (BIS), tetramethylenediamine (TEMED), and ammonium persulfate.

Grace's insect tissue culture medium (without insect plasma) was purchased from the Grand Island Biological Company. Cellulose ester filters (HAWP 02500, 0.45µ) were from the Millipore Corp. Ethylene glycol monomethyl ether (methyl cellosolve) was obtained from J. T. Baker. These materials were from Mann Research Laboratories: Amido Schwartz (Naphthol Blue Black), Bromphenol Blue, Coomassie Brilliant Blue R-250, and sucrose (special density gradient grade).

[^3H(G)] adenosine-3', 5'-cyclic phosphate,[^3H(G)] guanosine-3', 5'-cyclic phosphate, carrier-free ^32P-phosphoric acid, and aquasol (a xylene-based scintillation solution) were acquired from New England Nuclear. The tritiated cyclic nucleotides were either used directly or purified as described in Appendix I.
The following materials were purchased from the Sigma Chemical Company: polyvinylpyrrolidone (PVP-360), β-glycerophosphate, dithiothreitol, ethyleneglycol-bis(β-aminoethyl ether)N,N'-tetra acetic acid, phenylmethylsulfonyl fluoride, p-hydroxy mercuribenzoate, iodoacetamide, N-ethyl maleimide, bovine serum albumin, trypsin (Type I), trypsin inhibitor (Type I-S), theophylline, alloxan, and 6-N-2'-O-dibutyryl-3',5'- (cyclic)-AMP. Additionally, adenosine 3', 5' cyclic monophosphoric acid (cAMP), guanosine 3', 5'-cyclic monophosphoric acid (cGMP), and all other nucleosides and nucleotides used in these studies were obtained from Sigma and used without further purification.

Spectrophotometrically standardized solutions of every nucleoside and nucleotide investigated (ATP, ADP, GTP, etc.) were prepared by measuring the absorbance at the known wavelength of maximum absorbance in neutral solution. Concentrations were determined using published values of extinction coefficients (213) or those supplied by the manufacturer.

Insect Cultures

M. sexta eggs were graciously shipped to this laboratory on a weekly basis by Mr. A. H. Baumhover, U. S. D. A., Oxford Research Station, Oxford, North Carolina. After hatching, larvae were reared at 28°C at approximately 70% relative humidity under a 16 hour
photoperiod on a synthetic diet according to the procedures described by Yamamoto (99). Approximately 16-18 days after hatching, fifth-instar larvae were collected, and the healthiest and largest animals were selected without regard to sex for dissection. Some insects were allowed to enter the pre-pupal stage (as evidenced by development of a brown cuticle coloration) and were then placed in wooden pupal cells (described by Hoffman et al., ref. 100) in total darkness. The time required for development from pre-pupa to adult was approximately three weeks. Between 12 and 36 hours after emergence from the pupal case, adult moths were taken for dissection.

A few experiments were performed with larval and adult central nervous tissues of the greater wax moth, *Galleria mellonella*. All developmental stages of this insect were maintained in the laboratory and were cultured essentially as described by Pipa (101).

**Dissection of Insect Nervous Tissues**

The morphological characteristics of the *M. sexta* larval and adult central nervous systems are similar, though not identical, to those of the wax moth (*Galleria mellonella*) which have been described by Ashhurst and Richards (102). The *M. sexta* larval central nervous system consists of a two-lobed brain (B), a suboesophageal (SO) ganglion, three thoracic (T1, T2, T3), and eight abdominal (A1, A2, ..., A6, A7, 8) ganglia, with the last two abdominal ganglia being
fused. These ganglia are joined in series by connective tissue.

During larval-to-adult neurometamorphosis (which is completed in a matter of days) there occurs a pronounced shortening of the interganglionic connectives and a fusion of some of the ganglia. The adult central nervous system possesses only seven ganglionic masses (B, SO, T, A1, ..., A4).

Larval and adult insects were chilled before dissection by placing them on ice. The wings and legs of adults were removed, and these animals were brushed under running water to remove their scales. Insects were pinned ventral surface down in a Petri dish containing charcoal-blackened Paraffin wax. During the subsequent operation under a stereo dissecting microscope, all tissues were immersed in an ice-cold saline of the composition described by Pipa (103). A longitudinal dorsal incision was made from posterior to anterior, and special care was exercised to avoid damaging the brain when the head capsule was opened. Fat bodies were washed from the body cavity with a forceful stream of saline from a wash bottle, and the gut was removed. All lateral connectives of the ventral nerve cord were transected with iridectomy scissors. The nerve cord was always manipulated by grasping with watchmaker's forceps at the central section of an interganglionic connective.

The excised nerve cord was transferred to another Petri dish containing fresh saline, and all adhering non-neural tissues were
removed. It was then thoroughly rinsed with saline followed by glass-distilled water. In most experiments, each nerve cord was immediately frozen after dissection by placing it upon a microscope slide situated on a block of dry ice. The number of nerve cords taken for each experiment ordinarily ranged from 10 to 60, and where convenient, the pooled tissues were homogenized immediately after collection. Otherwise, they were stored at -80°C until use. For experiments requiring fresh, unfrozen nerve cords, these tissues were placed in Grace's (104) insect tissue culture medium after dissection.

The adult CNS was considerably more difficult to dissect than the larval CNS, principally because the thoracic ganglia were enmeshed in flight muscle. Therefore the larval nerve cord was employed for the majority of the studies reported in this thesis. Pupal nerve cords were virtually impossible to dissect because of their very soft nature, and these tissues were, regrettably, not examined. A number of experiments were performed on larval and adult brains to afford a comparison of enzymatic activities in the brain with those of the bulk CNS. Especially careful microscopic dissection and handling techniques were essential to avoid crushing these tissues during removal from the insect. The larval brain, in particular, is very fragile. After pooling a number of brains on a microscope slide held on dry ice, quantitative transfer to a
homogenizing vessel was most easily accomplished by touching a liquid nitrogen-cooled glass rod or homogenizer pestle to the tissue mass, followed by insertion of the rod into a tissue grinder.

**Protein Determination**

The protein content of soluble and particulate protein samples was routinely estimated by the method of Lowry et al. (105), using bovine serum albumin as standard. In some experiments, a modification of this technique published by Schachterle and Pollack (106) was employed.

**Polyacrylamide Gel Electrophoresis**

Analytical disc electrophoresis in polyacrylamide gels for the separation of proteins was performed by a modification of the method of Ornstein and Davis (107, 108). The following stock solutions were prepared in glass-distilled H₂O from Bio-Rad ultra-pure reagents;

A: 3.0 M Tris·HCl, 0.23% TEMED, pH 8.9; A': same as A, except the pH = 8.0; B: 0.49 M Tris·HCl, 0.46% TEMED, pH 6.7;

C: 28% acrylamide, 0.735% BIS; D: 10% acrylamide, 2.5% BIS;

E: 4 mg% riboflavin; F: 40% sucrose; and G: 0.14% ammonium persulfate. Reagents F and G were prepared immediately before

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1Abbreviations: Tris, tris(hydroxymethyl)aminomethane; TEMED, tetramethylenediamine; BIS, methylenebisacrylamide.
use, while the remaining stock solutions were passed through a Millipore filter, deaerated, and stored in darkness at 4°C. Filtration removed considerable amounts of particulate material from the solutions and resulted in gels of great optical clarity. The separating (small pore) gel consisted of A (or A'):C:H₂O:G (1:2:1:4 by volume) and was polymerized at room temperature for 45 min. The stacking (large pore) gel was composed as B:D:E:F (1:3:1:3 by volume), and was photopolymerized for 45 min. One volume of stacking gel was used with four volumes of separating gel. In some instances riboflavin (0.0005%) was used instead of persulfate to catalyze the polymerization of the separating gel, as it was feared that the highly reactive persulfate ion might result in loss of enzyme activities (109). When the substantial resolving power of the disc technique was not required, the stacking gel was omitted entirely. If necessary, protein samples were concentrated before electrophoresis by dialysis against powdered sucrose or polyvinylpyrrolidone.

The stock running buffer solution (10X Tris-gly) contained 0.05 M Tris and 0.384 M glycine, pH 8.3. For electrophoretic separations of short duration, either 1X or 2X buffer was used in the cathodal chamber, and 2X was employed in the anodal compartment. For prolonged runs (> 2 hours) much of the buffering capacity is lost, and for this reason 10X buffer was placed in the lower (anodal) chamber, with 2X being used in the upper compartment. Buffers of
higher ionic strength (and buffering capacity) were never employed in the cathodal chamber, as they interfere with the migration of proteins and greatly prolong separation times. Bromphenol blue (.01% in 32% sucrose, filtered before use) was routinely used as a tracking dye, and 2 µl of this solution mixed with 100 µl of protein sample was sufficient to visually define the front.

A high-resolution, temperature-regulated slab-type electrophoresis apparatus, \(^2\) similar in some respects to that described by Raymond (110), was fabricated from Plexiglas by the OSU Physics shop. This vertical gel unit features a well (area 7 cm × 19 cm, thickness 3.5 mm) in which the gel is cast between two parallel, epoxy-faced plates around which coolant may be circulated. Ice-cold water was pumped through the cooling channels, and inconvenient operation of the unit in a cold room was unnecessary. The ample cross-sectional area of the upper surface of the slot (660 mm\(^2\)) could accommodate large protein loads for small-scale preparative electrophoresis; alternatively, many different samples could be simultaneously electrophoresed in separate slots (1 cm × 1.5 mm) pre-formed in the gel slab with a Plexiglas "comb" (Transidyne General Corp. slot formers were machined to fit this apparatus). The volume of each buffer chamber was 750 ml. This slab-gel unit was used for

\(^2\)I am grateful to Dr. A. W. Anderson of the OSU Dept. of Microbiology for providing detailed construction plans of a similar unit he designed.
most of the experiments to be described, as the cooling option permitted operation at high current densities with adequate dissipation of ohmic heat.

In some experiments, complete physical separation of samples from one another was desirable, and electrophoresis was conducted in 5 mm × 12.6 cm glass tubes in a Hoefer model DE 102 gel unit. Since this apparatus is not equipped with a cooling jacket surrounding the gel tubes, electrophoresis was always performed in a cold room at substantially lower current densities than those used for the slab apparatus. Additionally, a number of preparative-scale cylindrical gel columns (nominally 1 cm diam. × 20 cm long) were constructed and used in the course of this study; those fabricated from polystyrene proved much more satisfactory than columns made from glass. In general, protein bands obtained with these units were not nearly as sharp as those found with the slab gel apparatus.

All electrophoretic separations were performed at constant current using a Hewlett-Packard 6448B current-regulated power supply. After polymerization and before sample loading, the slab gel was pre-electrophoresced for 5 min at 100 ma to move the persulfate ion away from any possible contact with the sample. Before the protein sample was loaded via capillary pipette or Hamilton syringe, it was mixed with a small volume of tracking dye and a few crystals of solid sucrose to confer density and
prevent convective mixing with the electrode buffer. Whenever possible, the ionic strength of the buffer in which the protein was applied was kept as small as possible in order to facilitate zone sharpening (111). If the sample has a lower conductivity than the surrounding buffer, there occurs a sharpening of protein bands because (1) migration velocities are higher in media of low $\Gamma/2$, and (2) high electrophoretic mobilities result from the large potential differences seen across a low-conductivity zone.

After loading the sample (<10 $\mu$g/mm$^2$ of slot area), stacking was effected at 50 ma until the tracking dye entered the small-pore gel, at which time the current was increased and maintained at 100 ma for the duration of the run. When the glass-tube (Hoefer) unit was used, stacking and running currents were 2 ma and 5 ma/tube, respectively.

Immediately after electrophoresis, the gels were fixed for 30 min-1 hour in ice cold 12.5% (w/v) trichloroacetic acid (TCA). A variety of staining methods were evaluated for *M. sexta* proteins, and it was found that Amido Schwartz is not nearly as sensitive as Coomassie Brilliant Blue R250 (112). The most successful technique involved staining for 1-24 hours at room temperature in freshly-prepared 0.05% (w/v) aqueous Coomassie, followed by diffusion destaining in distilled H$_2$O. Proteins stained by this technique and stored in the cold for as long as two years in dilute TCA did not fade
appreciably. Other commonly employed staining procedures, such as Coomassie in methanol-acetic acid, or Coomassie in TCA, did not yield as intensely colored bands as the method described. The longer the staining time, the greater was the sensitivity toward detecting minor protein bands—with, however, increased probability of blurring the heavier bands and a requirement of longer destaining times. After staining, maximal band intensity/background ratios were achieved after 2-3 days of storage in the cold.

Stained gels were scanned for absorbance at 550 nm using a Gilford model 2410-S linear transport assembly mated to a Beckman DU spectrophotometer. Optimal band resolution was achieved with a 0.05 mm slit. Direct scanning for protein at 280 nm using either unfixed, or stained and extensively washed gels met with failure due to the high content of non-protein 280 nm-absorbing materials in the gel (which could not be diffused out even after weeks of storage in buffer). After scanning, the relative electrophoretic mobilities (Rf's) of the bands were calculated from the strip-chart record as the ratio of the distance travelled by the band to that migrated by the tracking dye.

When the distribution of enzyme activities over the length of the gel was desired, unstained strips were manually sectioned on a pre-cooled glass plate or block of paraffin with a scalpel or microtome blade. When very fine slices were required, it was most convenient
to section the gel with a 60-razor blade (adjustable slice thickness) cutting device constructed for this purpose. Before cutting, the gel strip was rendered relatively nondeformable by rapidly freezing with an aerosol can of liquid freon ("Freeze UP," Mann Research Laboratories). Individual gel slices were homogenized in buffer and, where appropriate, an aliquot of the slurry was assayed directly for activity; otherwise, the slurry was centrifuged, and the supernatant was assayed.
Intracellular levels of cAMP frequently achieve their maximal elevation within seconds after hormonal stimulation and may decline rapidly thereafter. Removal of a tissue from an animal may itself produce an increase or decrease in cAMP level due to a change in environment, interruption of blood supply, alteration of neuronal stimulation, or other factors. These points have been discussed by Goldberg et al. (113). Most investigators choose quick-freeze procedures followed by extraction with trichloroacetic acid (TCA) to minimize perturbations of tissue cyclic nucleotide levels after removal of the organ from the animal. It is particularly important to arrest the activity of phosphodiesterase by rapid freezing and then denaturation in TCA.

Advances in cyclic nucleotide research have been seriously hindered by the absence of rapid, simple, and reliable assays for these compounds. Cellular levels of cAMP and cGMP are often in the $10^{-6}$ M to $10^{-9}$ M range or lower, and tremendous amounts of tissue are required to purify them for measurement by physical methods. Additionally, the most sensitive biochemical methods of assay often require prior purification of cyclic nucleotides from tissue extracts, employ complicated multienzyme or immunoassay systems, and may be susceptible to interference by structurally
related nucleosides and nucleotides, or other materials (1, 113, 114). Utilization of techniques requiring many complicated analytical steps or demanding purification and separation of cyclic nucleotides before assay was not feasible for the present research in view of the limited amounts of insect nervous tissue available. A compromise between specificity and convenience of assay was struck, and the so-called "competitive binding" assays for cAMP and cGMP were selected as most appropriate to the task.

Materials and Methods

Materials

Cyclic AMP-binding protein purified from bovine muscle by the method of Gilman (115) was purchased from Diagnostic Products Corporation. This preparation contained 1.8 µg binding protein and 36 µg of bovine protein kinase inhibitor per 10 µl of solution. Upon receipt, this material was divided into 10 µl aliquots and stored at -80°C. This avoids repetitive thawing and freezing of a stock solution, which results in loss of binding activity. A cGMP-binding protein (20 µg protein per 10 µl) derived from lobster tail muscle by the procedure of Kuo and Greengard (88) was also acquired from the same supplier, divided into 10 µl aliquots, and stored at -80°C. [3H] cAMP (24.1 C/mM) and [3H] cGMP (4.47 C/mM) were purchased from New England Nuclear and used without further purification.
Tissue Incubation and Cyclic Nucleotide Extraction Procedure

Insects were dissected as described in General Methods, and the nerve cords were placed on dry ice. In some experiments the nerve cords were incubated at 30°C in Grace's insect medium containing various test substances. After incubation, they were rinsed briefly two times in fresh medium and once in distilled water before immediately freezing on dry ice. In view of the small size of the nerve cords, it is likely that the entire tissue mass was frozen almost instantaneously and that the hazard of cyclic nucleotide destruction through phosphodiesterase activity was minimal.

Dependent upon the amount of tissue available, homogenization was conducted in 0.2 to 1.0 ml of ice-cold 5% (w/v) TCA. After the addition of 0.10 ml of 1N HCl per ml of TCA, the material was again briefly homogenized. After this step, $[^{3}\text{H}]$ cAMP was added to some samples to estimate recoveries through the extraction procedure. The homogenate was centrifuged at 20,000 $\times$ g for 30 min, resulting in a very compact pellet. The supernatant was removed and extracted five times with two to three volumes of water-saturated diethyl ether to remove lipids and TCA. After lyophilization, the residue was dissolved in 50 mM Na acetate buffer, pH 4.0. Insoluble material was removed by centrifugation. Whenever possible, this extract was employed immediately in the binding assays.
Otherwise, it was stored at -20°C until use.

**Cyclic AMP Assay**

The principle of the "competitive protein binding" assay for cAMP, first described by Gilman (115), involves reacting a known amount of \(^{3}\text{H}\) cAMP and an unknown quantity of unlabeled cAMP with a cAMP-dependent protein kinase. Labeled and unlabeled cAMP compete for binding sites on the protein, and quantitation of the radioactivity in the equilibrium cAMP-protein complex determines the amount of unlabeled cAMP added. A gain in sensitivity, and also increased stability of the cAMP bound vs. time equilibrium plateau, may be realized in this method by inclusion in the reaction mixture of an inhibitor of protein kinase, which increases the affinity for cAMP (115). The commercially-available protein kinase used in this work was derived from bovine muscle and had a very high binding affinity for cAMP. As employed, the assay procedure was sensitive to at least 0.2 pmol of cAMP.

The binding assay was conducted in a 10 × 75 mm polystyrene culture tube at pH 4.0 in a total volume of 50 µl. Glass vessels proved entirely unsatisfactory as the various reagent additions would adhere to the inner surface without mixing; in contrast, the hydrophobic nature of the plastic tubes promoted coalescence of reagents into a single drop. For each incubation, 20 µl of \(^{3}\text{H}\) cAMP (0.5 pmol,
approx. $2.7 \times 10^4$ dpm) in 50 mM Na acetate (pH 4.0) was pre-mixed with 20 μl of tissue extract or standard cAMP in the same buffer. This mixture was then transferred via capillary pipette to a culture tube held on ice which contained 10 μl of a mixture of freshly-thawed binding protein (1.8 μg) and kinase inhibitor (36 μg). Unlabeled cAMP standards (0.125 to 32 pmol per 20 μl) were prepared by serial dilution in acetate buffer of a spectrophotometrically standardized solution.

The reaction mixture was incubated on ice with continuous shaking for 60-65 minutes. At the end of the incubation period, each sample was diluted to 1.5 ml with cold 20 mM potassium phosphate buffer (pH 6.0). Separation of free from protein-bound cAMP was accomplished by rapidly passing the diluted reaction mixture through a cellulose ester filter (Millipore Corp., 0.45 μ, 24 mm). The filter and filtration apparatus had been rinsed and pre-cooled with cold phosphate buffer. Each filter was quickly washed with 10 ml of cold buffer, dried, then heated at 50°C in a scintillation vial with 0.5 ml of methyl cellosolve. After dissolution of the filter was complete, 10 mls of Aquasol were added and agitated vigorously with a Vortex mixer. At least 4 hours were allowed for equilibration before liquid scintillation counting. A tritium counting efficiency of this system in a Packard 2425 LSC spectrometer ($^3$H-preset) was 45.6%; in a Beckman LS-230 counter it was 40.4%. For each experiment a
standard curve was constructed from which the apparent cAMP content of unknown samples could be determined.

In the absence of binding protein the blank was 120 cpm. $[^3\text{H}]$ cAMP associated with the binding protein and trapped by the filter amounted to $1.2 \times 10^4$ dpm in the absence of competition by unlabeled nucleotide. This accounted for almost half of the radioactivity added to the reaction mixture.

**Cyclic GMP Assay**

Cyclic GMP was measured by a modification of the competitive protein binding assay of Murad et al. (116). The principle of this method and the experimental protocol are similar to those described for the cAMP assay. Samples and cGMP standards were prepared in 50 mM Na acetate buffer, pH 4.0. Each reaction mixture contained, in a total volume of 50 µl, 10 µl (20 µg) of lobster tail muscle cGMP-binding protein, 20 µl of $[^3\text{H}]$ cGMP (3 pmol, $3.0 \times 10^4$ dpm), and tissue extract or cGMP standards (0, 3 pmol to 30 pmol). After incubation of the reaction mixture on ice for 75-80 min, they were diluted with phosphate buffer, filtered, and washed as described previously. The assay was sensitive to approximately 1 pmol of cGMP.

Observed blanks in the absence of binding protein were reproducibly 65 ± 10 dpm, whereas the activity of $[^3\text{H}]$ cGMP-binding
protein trapped by the filter was approximately 540 dpm with no unlabeled cGMP present. Only a small percentage (2%) of the added $[^{3}H]$ cGMP was bound by the protein, reflecting a relatively small affinity constant. As discussed by Murad et al. (116), the magnitude of the binding constant of the lobster muscle protein, but not the specific activity of the radioactive cGMP, limits the sensitivity of this assay.

**Results and Discussion**

Typical standard curves constructed for the cAMP and cGMP assays are shown in Figures 2 and 3. The curve for cAMP binding departed from linearity, but was quite reproducible from assay to assay. Theoretically, a perfectly straight line is expected (115), but such was not observed with the sample of binding protein used. The standard curve for cGMP binding was linear. Recovery of $[^{3}H]$ cAMP carried through the tissue homogenization and extraction procedure was at least 96%. The recovery of cGMP was not determined. A single Millipore filter was sufficient to quantitatively trap the nucleotide-protein complex since, (1) additional radioactivity (above background) was not detected on a second filter placed in tandem with the

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3 Dr. Robert Ban (Diagnostic Products Corp.), supplier of the binding protein, obtained similar results with this preparation.
Figure 2. Standard curve for the assay of cAMP. Experimental conditions were as described in the text.
Figure 3. Standard curve for the assay of cGMP. Experimental conditions were as described in the text.
first, and (2) re-filtration of the initial filtrate did not deposit additional radioactivity. Bound cyclic nucleotide was invariant between 60-120 min (cAMP) and 75-100 min (cGMP) of incubation. Replicate samples agreed very well and varied less than 10%.

The cGMP-binding protein was relatively specific for this nucleotide, since 8 pmol of cAMP produced an assay response equivalent to approximately 1 pmol of cGMP. Thus, levels of cAMP present in the insect CNS do not interfere with cGMP analyses. The cAMP-binding protein was even more specific in that 5 pmol of cGMP incorporated into the reaction mixture did not alter cAMP binding at all; 40 pmol of cGMP, however, masqueraded as 1 pmol of cAMP in this assay system. The effects of such potentially interfering substances as ATP, ADP, AMP, GTP, GDP, and GMP were not assessed in either binding assay.

When very concentrated tissue extracts were assayed for cyclic nucleotide, there resulted with increasing amounts of unknown sample a pronounced non-linearity in assay response. Non-linearity could largely be circumvented by diluting the tissue extract to contain approximately 0.5 to 2 pmol of cAMP per 10 µl, and assaying 2 to 10 µl of this solution. At least a portion of the analytical difficulties experienced in the use of concentrated tissue extracts presumably is due to endogenous nucleotides present in the extract at perhaps a thousand times greater concentration than that of the cyclic nucleotide
itself. Additionally, high concentrations of salts resulting from lyophilization may cause interference (116, 117).

If sufficient quantities of tissue were available, these difficulties could be obviated by separation of the cyclic nucleotides from other components of the extract by thin-layer or column chromatography (113). Experiments designed to separate cAMP and cGMP from interfering nucleotides and salts in M. sexta extracts by the Dowex-formate chromatographic method of Murad et al. (116) were unsuccessful.

Preceding the establishment of M. sexta cultures in this laboratory, a number of cAMP analyses were performed on CNS tissues from the greater wax moth, Galleria mellonella. Table 1 demonstrates a greater cAMP content in the adult CNS compared to the larval CNS and that incubation of the adult CNS with ecdysterone for 30 min in Grace's insect medium results in nearly a four-fold increase in cAMP. Short-term incubation of the adult CNS with $10^{-4}$ M norepinephrine (NE) was without effect. In contrast, a similar experiment with G. mellonella fat body indicated that NE elevated cAMP in this tissue by approximately 22%. A single analysis for cGMP in G. mellonella extracts yielded these values: 0.64 pmol cGMP per larval CNS, and approximately 1 pmol cGMP per adult CNS. Therefore, in both developmental stages cGMP is present in excess over cAMP.
Table 1. Cyclic AMP content of *G. mellonella* nervous tissues.

<table>
<thead>
<tr>
<th><em>G. mellonella</em> tissue</th>
<th>Incubation conditions</th>
<th>Number of CNS extracted</th>
<th>cAMP pmol/CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larval CNS</td>
<td>Control</td>
<td>39</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1)</td>
</tr>
<tr>
<td>Adult CNS</td>
<td>Control</td>
<td>28</td>
<td>0.36±0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Adult CNS</td>
<td>Control</td>
<td>36</td>
<td>0.30±0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td>Adult CNS</td>
<td>Ecdysterone, 50 µg/ml</td>
<td>18</td>
<td>1.39±1.11</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Adult CNS</td>
<td>L-Norepinephrine, 10^{-4} M</td>
<td>16</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td></td>
<td>(1)</td>
</tr>
</tbody>
</table>

Incubations and cyclic nucleotide analyses were performed as described in the text. Values shown are average contents per nerve cord ± the average deviation. The number of analyses is indicated in parentheses.
Experimental data on cAMP and cGMP contents of the larval nerve cord of M. sexta after various treatments are collected in Table 2. Theophylline elevated cAMP by about two-fold but decreased cGMP levels slightly. Theophylline is one of many methylxanthines capable of inhibiting mammalian phosphodiesterase, and as will be shown, effectively inhibits the hydrolysis of both cAMP and cGMP by the M. sexta enzyme in vitro. It is not clear why theophylline produced opposite effects upon cAMP and cGMP in the insect. This compound is reported to stimulate cGMP accumulation by several fold in rat intestine (80). Additionally, in rat hear perfused with theophylline, slightly augmented cGMP levels were accompanied by a fall in cAMP content (95).

Acetylcholine (ACh) resulted in a pronounced elevation of M. sexta CNS cGMP levels with a concomitant depression of cAMP. As discussed in the introduction, similar findings have been reported in some mammalian tissues. Whether stimulation of cGMP by ACh is an essential feature of neurotransmission in the insect CNS remains to be established. The fact that cGMP was selectively elevated in response to ACh implies that the levels of cGMP and cAMP in the insect CNS may be independently controlled. A priori, this regulation could occur at the level of either cyclase or phosphodiesterase. Since preliminary evidence reported later in this thesis suggests both cAMP and cGMP may share a common degradative pathway, it is
Table 2. *M. sexta* larval CNS cAMP and cGMP contents after various treatments.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Number of CNS extracted</th>
<th>cAMP pmol/CNS</th>
<th>cGMP pmol/CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>1.16±.20</td>
<td>11.1±.70</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>1.15±.05</td>
<td>---</td>
</tr>
<tr>
<td>Theophylline, 25 mM, 30 min</td>
<td>8</td>
<td>2.32±.37</td>
<td>8.0±.9</td>
</tr>
<tr>
<td>L-Norepinephrine, 10^-4 M, 10 min</td>
<td>8</td>
<td>1.16±.28</td>
<td>12.4±.7</td>
</tr>
<tr>
<td>Ecdysterone, 50 µg/ml, 20 min</td>
<td>8</td>
<td>2.40±.20</td>
<td>22</td>
</tr>
<tr>
<td>Ecdysterone, 50 µg/ml, 60 min</td>
<td>8</td>
<td>0.93±.30</td>
<td>9.5±2.3</td>
</tr>
<tr>
<td>Acetylcholine, 5 x 10^-2 M, 10 min</td>
<td>9</td>
<td>0.60</td>
<td>26 ±2</td>
</tr>
</tbody>
</table>

Incubations and cyclic nucleotide analyses were performed as described in the text. Values shown are average contents per nerve cord ± the average deviation. The number of analyses is indicated in parentheses.
inferred that the observed selectivity operates through stimulation of a cGMP-specific cyclase. Such an enzyme has not been reported in any insect tissue. The mechanism whereby ACh is coupled to cyclase response is not known (must ACh enter the cell to exert its effects?).

The apparent lack of effect of NE upon G. mellonella adult CNS or M. sexta larval CNS (see Tables 1, 2) was surprising, since insect nervous tissue (G. portentosa brain) has been reported to possess adenyl cyclase stimulable by this compound (83). NE is known to occur in insect nervous tissue, and at a concentration of 30 μM has been shown by Nathanson and Greengard (84) to half-maximally activate cyclase activity in thoracic ganglia homogenates of P. americana. Yet, these authors could find no evidence for an NE-specific receptor in the cockroach nerve cord and concluded the stimulatory effect of NE on adenyl cyclase was due to a partial activation of receptor sites specific for octopamine, dopamine, and serotonin. The absence of stimulation of M. sexta cyclic nucleotide levels by NE suggests that in the Insecta there may be species variation with regard to types of CNS cyclase receptors. It is important to emphasize that the experiments on G. portentosa and P. americana nervous tissues were performed with homogenates and that possible effects on phosphodiesterase in intact cell preparations, as employed in the present work, were not examined. Data will be presented later demonstrating
an apparent induction by NE of phosphodiesterase in the larval CNS of *M. sexta*.

A potentially physiologically relevant phenomenon observed in the present study of the insect CNS was the stimulation of both cAMP and cGMP by ecdysterone, the most potent insect metamorphosis hormone known. Twenty minutes of incubation with ecdysterone promoted the accumulation of both cyclic nucleotides in *M. sexta* CNS, yet after 60 minutes in the presence of this hormone both cAMP and cGMP had returned to approximately basal levels. A literature search of the influence of this hormone upon cyclic nucleotides revealed that this stimulatory effect has been reported previously in non-neural insect tissues.

The cAMP-elevating effect of molting hormone was first alluded to in an obscure report by Gilbert et al. (118) in 1971. It was stated that, upon injection of ecdysterone into diapausing pupae, cAMP was stimulated seven-fold and that juvenile hormone appeared to antagonize this effect. A subsequent report by Applebaum and Gilbert (119) showed that ecdysterone, in addition to producing effects apparently independent of the cAMP system, stimulated the formation of both cAMP and cGMP in *Hyalophora gloveri* pupae and in the pupal wing epidermis. Cyclic nucleotide stimulation, observed both in vivo and in vitro, preceded increased rates in synthesis of RNA and protein and was claimed to be the earliest discernible effect of the
molting hormone. Applebaum and Gilbert concluded, without justification, that ecdysone directly stimulated adenyl cyclase and guanyl cyclase. In a similar study of adenyl cyclase in G. portentosa brain homogenates, Rojakovick and March (83) concluded that ecdysterone weakly inhibited adenyl cyclase. The resolution of these conflicting reports awaits further research.

Ecdysterone is a steroid, and cyclic AMP has been implicated in the effects of various mammalian steroid hormones (ref. 1, p. 392 ff.). Although the precise mechanisms are poorly understood, it has generally been concluded that steroid hormones (in contrast to polypeptide hormones and neurotransmitters) do not directly stimulate cyclase (1). However, reports to the contrary have been cited by Gilbert (119). Leenders et al. (120) have shown that ecdysterone, in conjunction with exogenous cAMP, greatly enhances chromosomal puff formation in vitro in Drosophila hydei salivary gland nuclei. Cyclic AMP by itself did not promote puff formation, and ecdysterone by itself was not nearly as effective as ecdysterone plus cAMP. No direct effect of ecdysterone upon adenyl cyclase was demonstrated, although the data presented could be interpreted as supporting such an interaction. Interestingly, acetylcholine counteracted the steroid effect.

From the data in Table 2 one may calculate a basal cGMP/cAMP ratio of approximately 9.5 in the M. sexta larval nerve cord. For
comparison, this ratio in other tissues has been reported to be: rat heart, 0.12 (88); whole rat brain, 0.02 (80, 88); rat cerebellum, 0.69 (88); lobster muscle, 0.14 (88); earthworm, 0.14 (80); silkmoth fat body, 0.42 (88); and whole cricket, 2.5 (80). The latter two citations are presently the only reported values for insect tissues. It is readily seen that the insect CNS appears unique in having a cGMP/cAMP so much greater than unity. The mean wet weight per M. sexta larval nerve cord was gravimetrically determined to be approximately 2 mg. Assuming that the water content of this tissue is 80% and that cAMP and cGMP are uniformly distributed throughout this allowed volume, basal intracellular levels of the cyclic nucleotides may be estimated from the data of Table 2; cAMP, 0.7 µM; and cGMP, 7 µM. Comparing published cyclic nucleotide levels in rat brain (80) with those observed in the insect CNS on a pmol/mg wet weight basis, cAMP levels in the insect are about one-sixth of those of rat brain whereas cGMP concentrations are approximately 100-fold greater.

It would be worthwhile determining if the strikingly high level of cGMP in the insect CNS is in part due to a ready availability of substrate for its formation, GTP. The absolute levels of ATP and GTP, in conjunction with the Michaelis constants for their formation, may be important determinants of the intracellular ratio of these two nucleotides (assuming, of course, comparable rates of degradation). Additionally, systems requiring or utilizing GTP, for example
ribosomal protein synthetic systems or GTP-phosphotransferase reactions, should be scrutinized in great detail.

It cannot be over-emphasized that the apparent cyclic nucleotide levels in the insect CNS measured by the protein binding assay must be viewed with caution pending substantiation by a more accurate and highly specific technique. When these studies were initiated the binding assays of Gilman (115) and Murad et al. (116) were the only practical approaches to quantitation of minute amounts of cyclic nucleotide in crude extracts using readily available reagents. More specific radioimmunoassays for cyclic nucleotides have subsequently been described (121, 122), but not until very recently have the requisite antibodies become commercially available. With these presumably superior techniques, small, physiologically-relevant changes in cyclic nucleotide levels promoted by putative neurotransmitters and other agents can perhaps be more reliably determined. Further studies in this direction of the insect CNS may contribute to our understanding of hormonal evolution and comparative neurochemistry.
Kuo, Wyatt, and Greengard (26) have reported that a number of arthropod tissues (e.g., cecropia silkmoth larval body wall and pupal fat body, M. sexta and cockroach fat bodies, whole Drosophila, and various lobster tissues) are rich sources of cGMP-dependent protein kinases. In contrast, only small amounts of this type of kinase has been found in mammalian tissues (29). Kuo et al. were able to effect a partial resolution of cAMP- from cGMP-dependent activities by chromatographic techniques and found that the various arthropod tissues differed widely in their relative contents of these two activities. For example, in some lobster and crab tissues, as well as in those of cockroach and D. melanogaster, cAMP-dependent kinases were relatively more abundant. The lobster gill was apparently unique in that it contained exclusively cAMP-dependent activity. In most tissues of Lepidoptera cGMP-dependent kinases predominated, and in H. cecropia and M. sexta fat bodies only cGMP-stimulable kinases were detectable. The idea was advanced that in certain insect tissues cGMP may perform the role played by cAMP in other (i.e., mammalian) tissues, but there is presently available very little evidence to support this concept. In fact, it will be shown that kinase activities in soluble and particulate fractions of the M. sexta larval CNS are activated by low concentrations of cAMP, but not by
cGMP. This contrasts with the behavior of other Lepidopteran kinases reported by Kuo et al. (26).

Before proceeding, an important clarification of terms is necessary to specify just what is meant by "protein kinase activity." The ability of any given kinase to phosphorylate a specific protein of endogenous origin may be termed the intrinsic or the endogenous activity of that kinase. A priori, one might speculate that a given kinase would have but one, or perhaps only a few, preferred endogenous substrates, but in the absence of detailed knowledge of this natural substrate one is forced to gauge kinase activity utilizing exogenous protein substrates. A particular kinase may be capable of phosphorylating any of a variety of extraneous substrates presented to it in vitro, and this activity should be clearly understood as its extrinsic or exogenous activity under the conditions employed. For example, if protamine (or casein) is phosphorylated by a given enzyme preparation) this is termed its "protamine (or casein) kinase" activity.

When a kinase preparation is assayed in vitro in the presence of some chosen extraneous substrate, what is experimentally measured is the sum total of its endogenous activity (phosphorylation of proteins derived from the enzyme preparation) and its exogenous activity. To estimate the activity of a given protein kinase with respect to exogenous substrates, most workers subtract endogenous activities (those determined with no protein other than enzyme protein present) from
those obtained in the presence of the exogenous substrate. Upon reflection, this would not seem to be a reasonable correction to make in some cases, since "endogenous" activities will probably be diminished to an unknown extent by the presence of the exogenous substrate.

Materials and Methods

Materials

\[^\gamma-^{32}P\] ATP was synthesized from \(^{32}P\)-orthophosphate and unlabeled ATP as described in Appendix II. The following protein substrates for the kinase assay were purchased as lyophilized powders from Sigma: histone Type II-A (histone mixture); histone Type III (lysine rich); histone Type IV (arginine rich); \(\alpha\)-casein; bovine serum albumin (BSA), fraction V (approx. 98% albumin); and protamine sulfate, Grade I from salmon. All histones were derived from calf thymus. A cAMP-dependent protein kinase inhibitor purified from bovine heart was also acquired from Sigma.

Enzyme Source for Kinase Assays

Nerve cords were dissected and collected on dry ice as described in General Methods. Homogenization of tissues was performed in all-glass tissue grinders in 50 mM Tris·HCl (pH 7.0)-1 mM \(\text{MgSO}_4\)-1 mM DTT (approx. 25-40 \(\mu\)l homogenizing buffer per nerve
cord), unless noted otherwise. A high speed supernatant (105,000 X g for two hr) was employed for most of the studies reported herein and was prepared by centrifuging the homogenate in a screw-capped tube filled with N₂ to minimize oxidation (insect kinase activities were sulfhydryl-sensitive).

Assay of Protein Kinase

Protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) activity was assayed by measuring the enzyme catalyzed transfer of ³²P from [γ-³²P]ATP to phosphate acceptor protein. Activity determined in the presence of enzyme protein but with no exogenous phosphate acceptor present will be termed "endogenous" activity. Histone Type II-A (mixed histones) was employed as the exogenous protein substrate for most of the experiments described. The standard reaction was conducted for five min at 35°C and pH 7.0 in a total volume of 0.20 ml containing 50 mM Tris·HCl, 5 mM MgSO₄, 5 mM dithiothreitol (DTT), 250 µg of histone, ordinarily less than 40 µg of enzyme protein, and 125 µM ATP³² (approx. 1-4 x 10⁸ cpm/µmole) in the presence or absence of 10⁻⁵ M cAMP or other test substances. Background radioactivities for exogenous kinase experiments were determined for every set of assays by omitting enzyme from the reaction mixture or by using a boiled enzyme sample. Endogenous kinase backgrounds were determined by adding enzyme to a reaction
mixture containing TCA.

Depending upon the type of experiment to be performed, two different methods were used to initiate the kinase reaction. When pre-incubation of the enzyme preparation with test substances was not required, the assay was begun by addition of enzyme protein to the remaining reaction mixture components at 4°C. This technique was employed in the majority of the assays to minimize degradation of cAMP and ATP by phosphodiesterase and ATPase present in the extract. When prior incubation of the enzyme preparation with miscellaneous test substances was necessary, all components (buffer, histone, enzyme, test substances) were first mixed, pre-incubated, and then the kinase reaction was initiated by the addition of cAMP and ATP\(^{32}\) in rapid succession. The complete reaction mixture was then transferred to a shaking water bath maintained at 35°C and incubated for exactly five min.

After incubation, the reaction vessels were plunged into an ice bath, and to each tube was added 2.0 ml of ice-cold 20% (w/v) TCA-1 M H\(_3\)PO\(_4\), followed by the addition of 0.05 ml of 0.1 M ATP (unlabeled). The mixture was allowed to stand at 0-4°C for at least ten, but not more than 60 minutes, before rapidly passing it through a Millipore cellulose ester filter (0.45 μ). The filter was then washed with 20 ml of ice-cold 10% TCA, dried, and heated in a scintillation vial with 1.0 ml of methyl cellosolve. After the filter had completely
dissolved, 10 ml of Aquasol were added, and the solution was mechanically agitated for two hours before liquid scintillation counting. One unit of kinase activity is defined as that amount which transfers one picomole of phosphate from $^{32}$P-labeled ATP to protein acceptor in five min at 35°C under the assay conditions described. This conforms to units of activity employed by other workers.

**Choice of Protein Substrate**

Calf thymus histone (mixed fractions) was employed as the exogenous phosphoryl acceptor for routine assays. This choice stemmed in large measure from the favorable solubility properties of histones as compared to those of protamines, although the latter class of proteins served as superior substrates for the insect protein kinase. Many published assays for protein kinase utilize protamines as substrate, presumably because of their ready availability and inexpense. At the beginning of the present study protamine was also used as substrate, but a number of spurious observations (nonlinear assay response with increasing substrate, lack of precision in replicate experiments, and other disconcerting effects) suggested that phosphorylated protamine was not being quantitatively precipitated by TCA. Therefore, a detailed comparative study was undertaken of the precipitability of protamines, and also of histones, under
different conditions (variations in concentration of TCA, temperature, salt concentration). In capsule, protamines were found to be incompletely precipitated (and redissolved after precipitation) under a variety of conditions and could not be quantitatively trapped with a Millipore filter. Histones, on the other hand, could be completely precipitated with TCA and retained by the membrane filter.

Comments on Experimental Technique

Only a small fraction (<0.5%) of the total radioactivity present in each reaction mixture was incorporated into the histone substrate, and considerable efforts were expended to develop a technique for effectively and conveniently separating $^{32}$P-histone from the large excess of ATP$^{32}$ and $^{32}$P. The assay method of Kuo and Greengard (15), viz. centrifugation of the precipitated $^{32}$P-protein followed by multiple resuspension and washing of the pellet (and, finally, dissolution in NaOH before liquid scintillation counting), was uncommonly tedious and led to a recovery of activity only 60% of that obtained by the Millipore filtration method. Passing the precipitated reaction mixture through glass fiber filters (Whatman GF/C) according to the procedure of Erlichman et al. (41) led to incomplete recovery of $^{32}$P-protein and also to high radioactive backgrounds. Provided careful experimental techniques were exercised, the Millipore filtration method developed for the present study proved far superior to
assay methods reported in the literature.

To establish the optimum ratio of sample response to background radioactivity, a number of variations in protein precipitation and filtration techniques were investigated. Precipitation of histone with 20% TCA containing 1 M $\text{H}_3\text{PO}_4$ was found to result in a three- to four-fold reduction in background compared to precipitation with 20% TCA alone. The inclusion of unlabeled ATP carrier (5 μmole) further yielded a slight reduction in background radioactivity. It was of crucial importance to filter the precipitated protein just as rapidly as possible and to never allow the filter to become dry during the initial stages of washing. A delay of only a few seconds after pouring the reaction mixture into the filter assembly invariably led to greatly (ten- to 100-fold) increased background radioactivities. Co-precipitation or post-precipitation of additional protein carrier (250 μg of serum albumin or histone) with the substrate protein was unnecessary and only served to reduce filtration rates, leading to elevated backgrounds.

Results and Discussion

Miscellaneous Observations

Both endogenous and exogenous kinase activities were completely destroyed by heating for five min at 100°C. The $^{32}\text{P}$-incorporated
into histone by enzymatic phosphorylation was stable to boiling in neutral buffer, but was completely released from the protein by heating for ten min at 100°C in 1 N NaOH. It is known (38, 123) that phosphate esterified to protein serine and threonine residues is released as inorganic phosphate by this alkaline treatment.

The inclusion of 8-16% (w/v) sucrose or 2.5 mg/ml serum albumin into the assay mixture had a slight protective effect upon kinase activity. Repetitively freezing and thawing a freshly-prepared high speed supernatant had no effect upon the magnitude of histone kinase activity, its Mg$^{2+}$ requirement, cAMP stimulability, or behavior under high ionic strength conditions. Triton-X-100, when present in the reaction mixture at concentrations up to 5% (v/v), did not affect the activity or cyclic nucleotide stimulability of kinase in high speed supernatants. However, this detergent was capable of unmasking considerable additional kinase activity from particulate material present in low speed (10,000 x g for ten min) supernatants. This particulate material presumably consists of small synaptosomes and fragments of the plasma membrane and endoplasmic reticulum.

**Endogenous Phosphorylation**

In different analyses of larval nerve cord high speed supernatants, endogenous kinase activity varied from 5-30% of the total activity observed with 250 µg of histone in the presence of cAMP.
Since endogenous protein was present under standard assay conditions in much smaller quantity than the exogenous substrate, the fact that endogenous phosphorylation frequently accounted for such a large fraction of the total (endogenous plus exogenous) activity indicates that some protein components of the *M. sexta* CNS supernatant are much better substrates for protein kinase than is histone. The identity of these "natural" substrates is not presently known, but one may speculate that they include, among other proteins, enzymes such as glycogen synthetase and phosphorylase kinase.

The extent of phosphorylation of endogenous substrates in 11 different experiments on high speed supernatants was highly variable and ranged from 350 to 950 units per mg of endogenous protein. Likewise, the effect of cAMP upon the phosphorylation of supernatant proteins was very inconsistent from preparation to preparation. In the majority of freshly-prepared insect CNS 105,000 X g supernatants, endogenous activities determined in the presence and absence of $10^{-5}$ M cAMP agreed within 3% or better. This indicates that phosphorylation of endogenous proteins, in contrast to histone kinase activity, was not stimulable by cAMP. However, in two experiments $10^{-5}$ M cAMP or cGMP reproducibly decreased endogenous phosphorylation by about 20%; in still another experiment, either cyclic nucleotide stimulated activity by about 25%. Other workers have noted that reproducibility of the stimulatory effect of cAMP upon endogenous,
but not exogenous, phosphorylation is difficult to achieve (77, 124).

Although endogenous activities varied widely among the different preparations, exogenous activities were fairly uniform. It seems very likely that the variability in magnitude of endogenous phosphorylation is related to the instability of endogenous substrate proteins. In this regard, it should be noted that there resulted an approximate 40-50% loss in endogenous activity after rapidly freezing and thawing a freshly-prepared supernatant once. Since histone kinase activity was unaffected by this process, it is concluded that the freeze-thaw cycle in some way decreased the ability of endogenous proteins to be phosphorylated but did not impair the activity of protein kinase.

Sulfhydryl Sensitivity

Both endogenous phosphorylation of \textit{M. sexta} CNS proteins and exogenous histone kinase activity were greatly enhanced by including DTT in the homogenizing and assay buffers. Cysteine was not nearly as effective. Omission of DTT in the preparation and assay of one 105,000 \(\times\) g supernatant resulted in specific histone kinase activity that was four-fold lower than that observed when 1 mM DTT was present in both buffers. For high speed supernatants prepared in the presence of DTT, the inclusion of 1 mM DTT in the assay buffer effected a greater stimulation of cAMP-independent histone kinase activity (\(\times\) 1.80) compared to its stimulation of activity in the presence
of cAMP (× 1.18). In supernatants prepared in 0.1 mM DTT-containing buffer, further restoration of cAMP-dependent activity was possible by including the following concentrations of DTT in the assay mixture: control (0.01 mM DTT contributed by the sample), 100%; 1 mM, 148%; 10 mM, 257%; and 50 mM, 110%. Mercaptoethanol at 1-10 mM had comparatively little effect on histone kinase activity.

Para-hydroxymercuribenzoic acid (PHMB), which is capable of forming mercaptides with reactive protein -SH groups, inhibited cAMP-dependent histone kinase activity in 105,000 × g supernatants; at 10⁻⁵ M PHMB, 10% inhibition; 10⁻⁴ M, 78%; and 1 mM, 96%. At a given concentration of PHMB, cAMP-independent activity was more inhibited than cAMP-dependent activity. It is thus evident that the soluble kinase(s) of the insect nerve cord require a reduced sulfhydryl group for maximum activity. This property has not been reported as a salient characteristic of most cAMP-dependent protein kinases (13), but Jergil et al. (125) noted that a protamine kinase from trout testis was strongly stimulated by thiols. Also, Donnelly et al. (48) reported that a cGMP-dependent protein kinase derived from lobster muscle and a cAMP-dependent protein kinase from bovine heart were inactivated by PHMB.
Comparison of the Effectiveness of cAMP and cGMP to Activate Soluble Protein Kinase

The effects of various concentrations of cAMP and cGMP on the phosphorylation of histone by enzyme activity present in a larval CNS 105,000 x g supernatant are shown in Figures 4 and 5. It is readily seen that the insect kinase system could be activated by relatively low concentrations of cAMP. At a sufficiently high level of cAMP (ca. 1 μM), the rate of phosphorylation could be increased by about four-fold in the presence of 125 μM ATP, and by about six-fold in the presence of 12.5 μM ATP (smaller apparent degrees of stimulation would have been calculated if the data presented had not been corrected for endogenous phosphorylation). Figure 4 (ATP = 125 μM) indicates that slight stimulation of kinase activity by both cAMP and cGMP was first evident at a concentration of 10^{-8} M. At 10^{-8} to 10^{-6} M levels of cyclic nucleotide, cAMP was more effective than cGMP in promoting kinase activity. At a level of 10^{-7} M, the magnitude of stimulation by cAMP was approximately 80% of the maximum value attainable at higher concentrations of this compound. When the concentration of cAMP was greater than 10^{-5} M, the degree of stimulation rapidly declined, possibly due to competition by cAMP with ATP for catalytic sites of the kinase(s). At concentrations higher than 10^{-4} M, cGMP became more effective than cAMP in activating soluble kinase in the presence of 125 μM ATP. Although not enough data
Figure 4. Cyclic nucleotide dependence of soluble histone kinase activity at ATP = 125 μM. Each reaction mixture contained 19.6 μg of a 105,000 x g larval CNS supernatant, 250 μg of histone, and the specified concentration of cAMP or cGMP. Values have been corrected for endogenous phosphorylation in the absence of histone substrate (14.5 units). Incubation conditions were as described in the text, and the amount of cAMP (A) and cGMP (G) varied from 10^{-9} to 10^{-2} M.
Figure 5. Cyclic nucleotide dependence of histone kinase activity at ATP = 12.5 µM. Experimental conditions were the same as described in the legend to Fig. 4, and the correction for endogenous phosphorylation was 5.1 units.
were gathered in the $10^{-8}$ to $10^{-5}$ M concentration range to permit accurate kinetic analyses, it is clear that the $K_m$ (that concentration of cyclic nucleotide required to give one-half maximal stimulation of activity) of cAMP was between ten- and a hundred-fold lower than that of cGMP.

At 12.5 μM ATP (a concentration much less than presumed physiological levels), the activation profiles shown by cAMP and cGMP (Figure 5) were qualitatively similar to those at 125 μM ATP, but at concentrations of cyclic nucleotide greater than $10^{-5}$ M, cGMP was no longer appreciably more effective than cAMP in stimulating kinase activity. At very low concentrations, cAMP was still more effective than cGMP.

It should be remarked that, in three similar experiments using different enzyme preparations, the activation curves (at an ATP level of 125 μM) obtained with cAMP and cGMP in the $10^{-9}$-$10^{-6}$ M range were essentially identical to those depicted in Figure 4. However, the relative efficacy of kinase stimulation by cAMP and cGMP at concentrations greater than $10^{-6}$ M varied somewhat from preparation

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4 In many mammalian cells, ATP levels are believed to be in the millimolar range. The intracellular concentration of ATP in the insect CNS is, however, not known. It is conceivable that local concentrations of ATP could be as low as 10 μM, in which case the activation curves shown in Figure 5 would be more reflective of the potential for kinase activation than those curves shown in Figure 4.
to preparation. In one of these experiments, cAMP and cGMP at 10^-5 M were equipotent, but in two other experiments cGMP was marginally more effective (5-15%) at this concentration.

Activation profiles with similar features to those shown in Figures 4 and 5 have been reported for a variety of partially purified cAMP- and cGMP-dependent protein kinases (15, 26, 28, 32, 123, 126, 127). In particular, Figure 5 closely resembles activation curves of purified cAMP-dependent kinases derived from a number of bovine tissues (15, 32) in which cAMP was about 50 to 200 times more effective than cGMP in stimulating activity. It has frequently been observed that maximal activation of a given type of kinase (for example, cAMP-dependent) can be effected by a sufficiently high concentration of the "heterologous" cyclic nucleotide (cGMP in the case of a cAMP-dependent enzyme). The data of Figure 5 (12.5 µM ATP) could be interpreted as indicating the existence in the larval nerve cord of kinase specifically activated by low concentrations of cAMP, but cGMP at higher concentrations would be capable of the same degree of stimulation. However, the activation profiles of Figure 4 (125 µM ATP) are more suggestive of mixture of two enzymes, each specific for one of the two cyclic nucleotides.

The existence in the insect CNS of cAMP-specific, cGMP-specific, and possibly cyclic nucleotide-independent protein kinases remains to be established. Since considerable kinase activity was
measured in vitro in the absence of added cyclic nucleotide, one might infer the presence of kinases not regulated by cAMP or cGMP; yet these activities could also be attributed to catalytic kinase subunits dissociated from the holoenzyme either in vivo or during the process of preparation. It may be recalled that the basal intracellular levels of cAMP and cGMP in the larval nerve cord were estimated to be 0.7 μM and 7 μM, respectively. Local concentrations of these cyclic nucleotides may, however, be much higher or lower than these values. In any case, levels of cAMP and cGMP of this order of magnitude would be more than sufficient to appreciably activate soluble cyclic nucleotide-stimulable kinase in the CNS of M. sexta.

Having established the activation profiles of soluble kinase activity in the presence of various concentrations of the two cyclic nucleotides, a concentration of $10^{-5}$ M cAMP was chosen for all further routine assays. This level of cAMP is approximately a hundred-fold greater than that ($10^{-7}$ M) required to achieve appreciable kinase activation under the incubation conditions adopted, but is not so high as to inhibit enzyme activity. It was desirable to include as high a concentration of cAMP in the reaction mixture as possible when assaying crude preparations in order to minimize the effect of cAMP degradation by phosphodiesterase during the incubation period. Subsequent studies of the enzymatic properties of M. sexta CNS cyclic nucleotide phosphodiesterase revealed that 125 μM
ATP would inhibit cAMP hydrolysis by about 50% in the standard kinase reaction mixture.

**Time Course of Phosphorylation**

The time course of phosphorylation at various ATP concentrations is shown in Figure 6. At 12.5 µM and 125 µM ATP$^{32}$, enzyme activity (histone substrate) in a high speed supernatant was proportional to time up to 6-10 min of incubation. On the other hand, endogenous activity did not follow a linear time course. In the presence of 500 µM ATP$^{32}$, histone kinase activity was decidedly nonlinear with reaction time; this observation will be discussed later when kinase activity is shown as a function of ATP concentration. If DTT were omitted from the homogenization and reaction mixture buffers, histone kinase activity was proportional to time only up to about three minutes of incubation. The time course of histone phosphorylation in the presence of 125 µM ATP$^{32}$ by a low speed (10,000 x g) supernatant was linear for only a few minutes before reaching an apparent plateau level by 20 minutes of incubation (data not shown).

**Effect of Varying Enzyme Concentration**

The data presented in Figure 7 indicate that the phosphorylation of histone (± cAMP) was proportional to the amount of supernatant protein added up to a concentration of about 20 µg per reaction mixture.
Figure 6. Protein kinase activity as a function of incubation time at various ATP concentrations. The presence (+) or absence (-) of $10^{-5}$ M cAMP is indicated. Incubation conditions were as described in the text. Each mixture contained 20.6 µg protein from a 105,000 x g supernatant which had been stored for two days before assay. Activities were not corrected for endogenous (End) phosphorylation.
Figure 7. Dependence of exogenous and endogenous phosphorylation upon the amount of enzyme. Each reaction mixture contained the specified quantity of enzyme (105,000 x g supernatant, stored frozen before assay) in the presence or absence of 10^{-5} M cAMP.
(0.1 mg/ml) and slowly departed from linearity at higher concentrations. Identical endogenous activities were observed in the presence and absence of $10^{-5}$ M cAMP, and linearity with increasing amounts of added supernatant was noted only up to 20 µg of protein.

**Effect of Varying the Amount of Histone**

Kinase activity as a function of the amount of histone substrate is shown in Figure 8. A double-reciprocal plot of the data indicated an approximate $K_m$ for histone of 80 µg per reaction mixture (0.4 mg/ml) both in the presence and absence of cAMP. That the stimulation of activity by cAMP does not affect the $K_m$ for histone has also been reported in some mammalian kinase preparations (15, 27). In the presence of cAMP, the $V_{max}$ of the insect enzyme was increased by a factor of 2.6.

**Kinase Activity as a Function of ATP Concentration**

Data are presented in Figure 9 illustrating the dependence of reaction velocity upon the concentration of ATP when Mg$^{2+}$ (5 mM) and the second substrate for the kinase (histone) were held constant. In the presence of $10^{-5}$ M cAMP and a high concentration of ATP (500 µM) an apparent stimulation of kinase activity occurred; this was especially apparent from a Lineweaver-Burk plot of the data. It is instructive to recall the time course of phosphorylation (Figure 6).
Figure 8. Dependence of histone kinase activity upon amount of substrate. Each reaction mixture contained 18.2 µg of protein from a freshly-prepared 105,000 × g supernatant and the indicated amount of histone in the presence (○) or absence (□) of 10⁻⁵ M cAMP. Activities observed in the presence of histone (endogenous plus exogenous) were corrected for endogenous activities determined in a parallel experiment.
Figure 9. Kinase activity as a function of ATP concentration. Assay conditions were as described in Methods, except for the variation of ATP. Each reaction mixture contained 20.6 μg of protein from a high speed supernatant which had been stored three days at -20°C before assay. Exogenous plus endogenous activities are shown in the presence (Θ) and absence (ο) of 10^{-5} M cAMP. Endogenous phosphorylation (Δ) is also shown as a function of ATP concentration.
observed under these conditions (+cAMP, 500 μM ATP) in which a marked deviation from linearity became evident after four minutes of incubation. It is possible that these effects at high ATP concentrations are due to a high \( K_m \) protein kinase which is not kinetically observed at the low ATP levels (125 μM) used for routine assays. Alternatively, a low \( K_m \) kinase in the presence of cAMP and high levels of ATP may undergo a time-dependent activation.

After correction for endogenous phosphorylation, double reciprocal plots of the data of Figure 9 and also of data from similar experiments were multiphasic (progressive downward curvature at high ATP concentrations). One possible explanation for this behavior is that there are present at least two enzymes with disparate Michaelis constants for ATP. From the activities observed at ATP concentrations in the range 12.5-250 μM, \( K_m \) values in the presence and absence of 10\(^{-5}\) M cAMP were approximately 10 μM and 50 μM, respectively. Cyclic AMP, in addition to lowering the apparent \( K_m \) by a factor of five, resulted in an approximate doubling of the \( V_{max} \).

The same qualitative effect has been observed with a purified kinase from bovine brain (15), but cAMP has been reported to have little effect on the \( K_m \) for ATP in kinase preparations from bovine epididymal spermatozoa (128) and bovine tracheal muscle (27). Kuo et al. (32) reported that cAMP had a far greater effect on the \( K_m \) for ATP of a bovine brain kinase than it did upon kinases purified from a
number of other bovine tissues. This effect appears, then, to be a nervous tissue-specific property in mammals, and it is noteworthy that the same phenomenon was observed in the present study of the M. sexta central nervous system.

**Kinase Activities in the Larval and Adult CNS**

Attempts to measure CNS homogenate kinase activities using the assay described (125 μM ATP, 10^-5 M cAMP, 5 min incubation) yielded unsatisfactory and non-reproducible results. It appears that the use of very short incubation periods, high substrate levels, and relatively small amounts of enzyme will be required to develop a satisfactory assay (specifically, one linear with time and amount of enzyme) for particulate samples. It was found in preliminary experiments that increasing the amount of homogenate assayed actually decreased the incorporation of ³²P into histone, and that greater apparent activities were obtained after 2 min of incubation than after 5 min. These findings were not surprising, because it is known that membranous preparations contain high levels of ATPase, cyclic nucleotide phosphodiesterase, and phosphoprotein phosphatase, all of which materially interfere with the kinase assay. Because of these difficulties, only soluble kinase activity (i.e., that in supernatants) was investigated in the present study.

In Table 3 are collected representative data from different
Table 3. Protein kinase activities in larval and adult CNS supernatants.

<table>
<thead>
<tr>
<th>Exp't.</th>
<th>No. of CNS</th>
<th>Enzyme source</th>
<th>10-5 M cAMP</th>
<th>Specific activity units/mg protein</th>
<th>Activity per nerve cord units/CNS</th>
<th>Ratio +/-</th>
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<td></td>
<td></td>
<td>Larval CNS</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>la</td>
<td>30</td>
<td>10,000 X g supernatant</td>
<td>+</td>
<td>4411</td>
<td>601</td>
<td>-</td>
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<td>lb</td>
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<td>+</td>
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<td>313</td>
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</tr>
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<td>240</td>
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<td>3</td>
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<td>5495</td>
<td>559</td>
<td></td>
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<td>259</td>
<td>2.05</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>105,000 X g supernatant</td>
<td>-</td>
<td>4072</td>
<td>305</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult CNS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>105,000 X g supernatant</td>
<td>+</td>
<td>3556</td>
<td>257</td>
<td>2.07</td>
</tr>
</tbody>
</table>

Assays were performed as described in Methods, and each reaction was initiated by the addition of enzyme protein. Activities were not corrected for endogenous phosphorylation.
experiments on soluble kinase activities (in 10,000 × g 10 min and 105,000 × g 2 hr supernatants) in the *M. sexta* larval CNS. A single experiment performed on the adult CNS is also shown. Larval CNS low speed (10,000 × g) supernatants, which contain appreciable amounts of particulate material sedimentable at higher centrifugal forces, exhibited somewhat greater specific activities than high speed (105,000 × g) supernatants. If a freshly-prepared low speed supernatant (in hypotonic buffer) were allowed to "age" at 25°C for various periods of time before assay, histone kinase activity increased almost linearly with time until 60 min had elapsed, at which time cAMP-dependent activity had almost doubled relative to that measured immediately after preparation of the supernatant. Increases occurred in both cAMP-dependent and cAMP-independent activities, but the magnitude of stimulation by cAMP at zero time (× 2.56) was much greater than that after 60 min of aging (× 1.50). This aging phenomenon did not occur in high speed supernatants. One explanation for these observations is that kinase activity is either activated in, or released by, particulate material present in the 10,000 × g supernatant in a time-dependent fashion.

Among those experiments compiled in Table 3, the mean specific kinase activity of larval CNS high speed supernatants was 3574 and 1575 units/mg protein, respectively, in the presence and absence of cAMP. The ratio of activities (+ cAMP/-cAMP) ranged from 2.05
to 2.59. In comparison, the adult CNS supernatant specific kinase activity with cAMP present was considerably higher (8625 units/mg), but cAMP-independent activity in this tissue (1587 units/mg) was nearly the same as that of the larval CNS. The ratio of activities in the adult CNS supernatant was 5.43, more than twice that of the larval nerve cord.

An experiment was performed to determine if additional soluble activity could be released from particulate material by detergent treatment. The 105,000 × g pellet from 30 larval nerve cords (the supernatant from this pellet contained 313 units/CNS of cAMP-dependent activity) was homogenized for one minute at 4 °C in buffer containing 1% (w/v) Triton X-100 and centrifuged at 105,000 × g for 2 hr. This supernatant contained additional cAMP-dependent protein kinase activity (218 units/CNS), 70% of that found in the original supernatant before Triton-extraction of the pellet. The membrane-associated but detergent extractable kinase exhibited a cyclic nucleotide activation profile (Figure 10) quite distinct from that described previously for soluble kinase (see Figure 4). The ascending portion of the cAMP activation curve (in the range 10⁻⁸ - 10⁻⁶ M) for the solubilized particulate preparation was similar to that noted for the soluble kinase activity, as was the concentration of cAMP (ca. 10⁻⁶ M) which promoted greatest kinase activity in both samples, but in the case of the kinase extracted from the membranes, (a) activation began at the very
Figure 10. Cyclic nucleotide dependence of kinase activity extracted from *M. sexta* larval CNS membranes by Triton X-100. Incubation conditions were as described in the text, and the amount of cAMP (A) and cGMP (G) varied from $10^{-9}$ to $10^{-5}$ M.
low cAMP concentration of $10^{-9}$ M, and (b) concentrations of cAMP greater than $10^{-6}$ M were inhibitory to enzyme activity. These features distinguish the membrane-associated kinase from the soluble kinase. An activation profile almost identical to that depicted in Figure 10 has been reported for a partially purified "cAMP-specific" protein kinase from lobster gill (26).

It is evident that a substantial amount of histone kinase activity is present in particulate fractions of the insect CNS. Of emphasis is the fact that in the present studies most tissues were stored frozen and homogenized in hypotonic buffer, and it is probable that vesicular structures were lysed. Assay of membranous fractions prepared in this fashion would result in a conservative estimate of that activity associated with particulate material in vivo. Even so, latent activity could still be unmasked in these particulate fractions by incubation with Triton X-100, which presumably lyses organelles and/or releases kinase embedded in membrane matrices. Further studies of the insect protein kinase should be conducted on soluble and particulate fractions prepared in iso- or hypertonic media. It is known (77) that most of the protein kinase activity of rat brain is associated with particulate fractions, and much of this was found in synaptosomes, synaptic membrane fragments, and synaptic vesicles.
Effects of Bivalent Cations and EDTA

The effects of various bivalent cations and EDTA upon the histone kinase activity of a high speed supernatant are presented in Table 4. These assays were conducted in the presence of $10^{-5}$ M cAMP. The effect of Mg$^{2+}$, both in the presence and absence of cAMP, will be further described in material that follows. The strong inhibition of activity by EDTA, and its stimulation by Mg$^{2+}$, demonstrate that the insect protein kinase is Mg$^{2+}$-dependent. Many other protein kinases reported in the literature exhibit a similar absolute metal requirement (e.g., 15, 27, 28, 32, 39, 91, 124, 129, 130, 131). None of the metals examined were as effective as Mg$^{2+}$ at a 5 mM level although Co$^{2+}$ seemed to partially substitute for Mg$^{2+}$. Ca$^{2+}$ and Zn$^{2+}$ inhibited the insect kinase. This effect has also been described in other kinase preparations (27, 32, 130).

In Figure 11 is shown the relationship between protein kinase activity ($\pm$ cAMP) and Mg$^{2+}$ concentration at an initial ATP$^{32}$ level of 125 $\mu$M. The enzyme source for this experiment was the same 105,000 $\times$ g supernatant as used in the above experiment, except that it had been stored frozen overnight before assay. Both endogenous and exogenous activities were activated by Mg$^{2+}$. Optimal histone kinase activity in the presence or absence of cAMP occurred at approximately 5 mM MgSO$_4$ and declined at higher concentrations.
Table 4. Effects of bivalent cations and EDTA on protein kinase activity.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration mM</th>
<th>Histone kinase activity (+10^{-5} M cAMP) units</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
<td>7.3</td>
</tr>
<tr>
<td>MgSO_4</td>
<td>0.5</td>
<td>37.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>46.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>22.2</td>
</tr>
<tr>
<td>CaCl_2</td>
<td>5</td>
<td>1.7</td>
</tr>
<tr>
<td>MnCl_2</td>
<td>5</td>
<td>7.3</td>
</tr>
<tr>
<td>CoCl_2</td>
<td>5</td>
<td>22.1</td>
</tr>
<tr>
<td>ZnSO_4</td>
<td>5</td>
<td>3.0</td>
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<td>CuCl_2</td>
<td>5</td>
<td>7.8</td>
</tr>
<tr>
<td>FeCl_2</td>
<td>5</td>
<td>11.6</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Assay conditions were as described in Methods except for varying the type and amount of cation. Each reaction was initiated by the addition of 18 μg of protein from a freshly-prepared larval CNS 105,000 × g supernatant (50 mM Tris, 1 mM DTT). Activities have not been corrected for endogenous phosphorylation (4.7 units in the absence of Mg^{2+}). The initial ATP concentration was 125 μM.
Figure 11. Kinase activity as a function of Mg$^{2+}$ concentration. Reaction conditions were as described in the legend to Table 4, except that the supernatant (18 ug protein per assay) had been stored overnight before assay. Ex = exogenous, End = endogenous kinase activity.
This decline may be due to inhibitor of kinase activity under high ionic strength conditions (for comparison, see the relationship between activity and univalent salt concentration in Figure 14). With no added \( \text{Mg}^{2+} \), rates of histone phosphorylation with cAMP present or absent were identical (i.e., \( \text{Mg}^{2+} \) is obligatory for cyclic nucleotide stimulability). \( \text{Mg}^{2+} \) concentration vs. kinase activity profiles qualitatively similar to that of Figure 11 have been demonstrated in a variety of enzyme preparations, including those from calf thymus nuclei (129), bovine muscle (27), and bovine brain (15).

All protein kinases thus far characterized require \( \text{Mg}^{2+} \) as a bivalent cation in order to form the true molecular substrate, a \( \text{Mg}^{2+} \)-ATP complex (13). The stability constant of this complex is very large at neutral pH, and one might expect optimum kinase activity when the \( \text{Mg}^{2+} \) concentration becomes approximately equal to that of ATP. This was not observed in the present study (ATP = 125 \( \mu \text{M} \)), nor has it been observed in other investigations in which a molar ratio of \( \text{Mg}^{2+} \) to ATP much greater than one was employed (15, 27, 39, 129). In part, this may be due to a reduction in the availability of \( \text{Mg}^{2+} \) for ATP by buffer components and metal-binding proteins of the enzyme sample. More likely, however, is that the enzyme itself may possess \( \text{Mg}^{2+} \)-binding sites whose occupancy is essential for catalytic function. Thus, \( \text{Mg}^{2+} \) may serve two roles in promoting kinase activity.
**Temperature and pH Dependence**

Figure 12 indicates the dependence upon pH of kinase activity in the presence of cAMP. Activities as a function of pH with no cAMP present were not determined. The nerve cords for this experiment were homogenized in a solution of low buffering capacity (10 mM Tris·HCl, pH 7.0-0.1 mM DTT) and diluted 1:10 into the reaction mixture to avoid disturbing the pH of the test buffer. Activity was observed over a wide range of pH values and in Tris·HCl was relatively invariant from pH 7 to 10. Appreciable activity remained even at pH 11 (data not shown). Activity in 63 mM sodium glycyl-glycine buffer at pH 7.5 was 31% greater than that in Tris·HCl of the same molarity at the same pH. A number of published assays for protein kinase employ glycerophosphate buffer, but the activity of the insect kinase in this buffer was considerably less than in Tris·HCl. This is possibly due to the complexing of Mg^{2+} by glycerophosphate, thereby reducing the availability of Mg^{2+} for ATP. At pH 7.0, activity in 63 mM Tris·HCl was about 17% lower than in 31 mM Tris·HCl. This dependence of activity upon ionic strength will be treated in more detail later.

The effect of temperature on kinase activity with or without 10^{-5} M cAMP present is shown in Figure 13. The reaction mixtures for these assays were composed in thin-walled culture tubes (to promote
Figure 12. pH dependence of kinase activity in the presence of cAMP. Each reaction mixture contained 4.50 μg of a 105,000 × g supernatant and 10^{-5} M cAMP. Endogenous activities were not determined. Buffers (each 63 mM): O, Tris·HCl; □, Tris·maleate; △, sodium acetate; ▲, sodium glycerophosphate. Also ●, 31 mM Tris·HCl.
Figure 13. Effect of temperature on kinase activity. Assays were conducted as described in Methods, except that the ATP concentration was 100 μM and DTT was absent. Each reaction mixture contained 34.2 μg of protein from a 105,000 × g supernatant which had been stored frozen for two days prior to assay. Corrections were not determined for endogenous activity.
rapid thermal equilibration) and held on ice until initiation of the reaction by the addition of enzyme, followed by incubation for five min in a shaking water bath at the specified temperature. The slope of activity (+cAMP) vs. T was relatively constant in the range 10°-50°C and was somewhat greater than the mean slope of activity (-cAMP) vs. T. Enzymatic activity continued to increase up to 50°C but rapidly fell past that temperature. The ratio of activities in the presence and absence of cAMP was computed at each temperature (R(+/-) = 1.64, 2.78, 2.07, 2.44, 1.78, 1.46 from 10° to 60°C in 10 degree increments) and followed no discernible pattern. If one hypothesizes that higher temperatures should promote the dissociation of regulatory and catalytic kinase subunits (with loss of cAMP-stimulability), it would be expected that the ratio (+/-) of activities should decrease with increasing temperature. The present data do not support this hypothesis.

Effects of Salts

Weller and Rodnight (20) observed that intrinsic protein kinase activity of ox brain synaptosomal membrane preparations was, with no cAMP present, inhibited by 20-30% in the presence of 10-100 mM NaCl or KCl, provided that incubations were conducted for 1-10 minutes; little inhibition was caused by these salts for incubation periods less than one minute. However, Ahmed and Judah (132) showed that
Na$^+$ stimulated kinase activity during short-term (5s) incubations, but Weller and Rodnight were unable to corroborate this report. Reimann et al. (39) found that casein phosphorylation in the presence of cAMP by a soluble protein kinase purified from rabbit skeletal muscle was severely inhibited by 0.25 M salt, and Corbin et al. (133) have shown that protein kinase from the same source which is bound to histone-Sepharose 4B affinity columns can be eluted by the addition of salt. It is clear that in these preparations salt decreased the affinity of the enzyme for the protein substrate. These inhibitory effects were also noted for soluble protein kinase from the insect nerve cord (Figure 14).

The enzyme source for the insect kinase experiment was a 105,000 x g supernatant from 40 larval nerve cords prepared in 50 mM Tris·HCl-1 mM MgSO$_4$-1 mM DTT, and assays (five min incubation) were conducted as described in Methods. It is readily seen (Figure 14) that low concentrations of salt were much more deleterious to cAMP-dependent activity than to cAMP-independent activity. With 10$^{-5}$ M cAMP present, KCl was more inhibitory than NaCl at any given concentration, but these two salts were indistinguishable in their influence upon cAMP-independent protein kinase activity. LiCl was even more potent than KCl in antagonizing cAMP-dependent activity (data not shown). As the salt concentration was increased, the ratio of activity (+cAMP)/activity (-cAMP) steadily declined,
Figure 14. Inhibition by NaCl and KCl of kinase activity. Each reaction mixture contained 19.6 μg of protein from a 105,000 × g supernatant which had been stored frozen before assay. The sum of endogenous plus exogenous (histone) kinase activities is indicated. Endogenous activity in the absence of salt was 15.2 units in the presence of cAMP.
which may indicate that high ionic strength conditions inhibit cAMP binding to a regulatory subunit of protein kinase.

Abilities of Various Proteins to Serve as Kinase Substrates

A number of different proteins were examined for their abilities to serve as phosphate acceptors in the protein kinase reaction, both in the presence and absence of cAMP. One reason for undertaking this study was to enable a comparison of the properties of the insect kinase(s) with those of other kinases reported in the literature. Also, the information gained from such experiments may at some later date prove useful for distinguishing various insect kinases on the basis of their substrate specificities.

All of the exogenous proteins examined were capable of being phosphorylated (Table 5). On a weight basis, protamine served by far as the best substrate for kinase activity in the high speed supernatant, both in the presence and absence of cAMP. With 250 μg of substrate protein present in the standard assay, the following order of ability to accept a phosphoryl group from ATP was observed: protamine ≫ histone II > histone IV > histone III > casein > serum albumin. This trend obtained in the presence or absence of cAMP. At a lower substrate level (50 μg, ± cAMP), protamine still served as the best substrate and albumin as the worst, but the relative
<table>
<thead>
<tr>
<th>Exogenous substrate</th>
<th>Amount (ug)</th>
<th>Kinase activity (units)</th>
<th>Ratio +/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−cAMP</td>
<td>+10⁻⁵ cAMP</td>
</tr>
<tr>
<td>None</td>
<td>14.5</td>
<td>15.3</td>
<td>1.05</td>
</tr>
<tr>
<td>Histone II-A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>250</td>
<td>54.0</td>
<td>124.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>30.1</td>
<td>65.5</td>
</tr>
<tr>
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<td>250</td>
<td>45.4</td>
<td>88.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>39.7</td>
<td>47.7</td>
</tr>
<tr>
<td>Histone IV&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
<tr>
<td></td>
<td>50</td>
<td>30.1</td>
<td>53.2</td>
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<tr>
<td>Protamine</td>
<td>250</td>
<td>300.4</td>
<td>477.3</td>
</tr>
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<td></td>
<td>50</td>
<td>101.5</td>
<td>184.1</td>
</tr>
<tr>
<td>Bovine serum albumin (fraction V&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>250</td>
<td>31.5</td>
<td>32.4</td>
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<tr>
<td></td>
<td>50</td>
<td>16.9</td>
<td>19.8</td>
</tr>
<tr>
<td>α-casein</td>
<td>250</td>
<td>44.0</td>
<td>44.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>32.1</td>
<td>26.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>For brevity of description, Sigma "Type" designations are indicated with Roman numerals. II = histone mixture, III = lysine-rich, IV = arginine-rich histone.

<sup>b</sup>98% albumin

Incubations were conducted as described in the text, except for the variation of kind and amount of substrate. Each reaction mixture contained 36.4 ug of a 105,000 x g supernatant. Activities in the presence of exogenous substrate have not been corrected for endogenous phosphorylation.
efficacy of phosphorylation of the remaining proteins was altered.
The various types of histone were roughly comparable as substrates for kinase in M. sexta high speed supernatants.

A great variation in the effect of cAMP on the phosphorylation of the different proteins was observed. Stimulation of kinase activity by $10^{-5}$ M cAMP was greatest for histone II (mixed histones), and decreased in the order histone II > histone IV > histone III > protamine > serum albumin > casein. If endogenous kinase activity were subtracted from the total (endogenous + exogenous) activity in the presence of added substrate, even greater values than those reported in Table 5 for the degree of stimulation by cAMP would be calculated. Cyclic AMP had little effect on the phosphorylation of albumin and casein; in fact, cAMP appeared to slightly inhibit the phosphorylation of casein. This latter effect has been reported for protein kinases from rabbit reticulocytes (38) and ox brain (134), but cAMP was without noticeable influence on casein phosphorylation by enzymes from human erythrocytes (124) and rat brain (31). In still other kinase preparations (14, 25, 27, 135) stimulation of casein phosphorylation by cAMP to varying degrees has been reported. It is not clear why cAMP promotes the phosphorylation of some substrates but not others. However, the ability of cAMP to either stimulate or inhibit kinase activity, depending on the nature of the substrate, is very likely of physiological importance.

Protamine is a poorer substrate than histone for kinases
derived from a number of mammalian sources including bovine tracheal muscle (27), rat liver (123), rat brain (31), and rabbit liver and muscle (136). This is also true for leukaemia virus and stomatitis virus kinase activities (137). A highly purified cAMP-dependent protein kinase from rat brain cytosol showed the following substrate preferences (15): histone > protamine > casein > serum albumin. The insect enzyme differs from this scheme only in that protamine was more readily phosphorylated than histone. Although it initially appeared that the M. sexta kinase was distinguished from mammalian enzymes by virtue of its preference for protamine, very recent reports (124, 138, 139) indicate that some mammalian kinases also phosphorylate protamine more readily than histones.

It has generally been observed in a variety of mammalian kinase preparations that casein and bovine serum albumin are phosphorylated to a much lesser extent than histone (15, 27, 31, 43, 130, 135). In a limited survey of some nonmammalian kinase preparations (viz. lobster muscle, roundworm, and fish), Kuo and Greengard (14) observed that casein apparently served as a more effective substrate than histone. These authors suggested that this differential ability of casein and histone to serve as substrates might reflect a basic difference between the protein kinase of mammals and that of lower species. The present investigation demonstrates that casein is a less effective substrate than histone for the insect kinase preparation. Thus, in at
least one nonmammalian tissue, the *M. sexta* larval CNS, the hypothesis advanced by Kuo and Greengard is not valid.

**Effects of Various Compounds on Kinase Activity**

A number of compounds were tested for their effect upon cAMP-stimulated protein kinase activity in high speed supernatants (Table 6). Adenine and adenosine had little effect; at a level of 1 mM, these compounds were present at eight times the initial concentration of ATP in the reaction mixture. It is interesting that 5'-AMP, but not 3'-AMP, interfered with cAMP-dependent kinase activity. At both a millimolar and sub-millimolar concentration, ADP served as a relatively potent inhibitor. The inhibition by 5'-AMP and ADP may be due to their ability to compete with cAMP at a regulatory site, or with ATP at a catalytic site of the kinase(s).

In studies of partially purified cAMP-dependent protein kinases from 15 bovine tissues, Kuo et al. (32) reported that adenine, adenosine, and 5'-AMP were very weak inhibitors in most cases, but that ADP significantly inhibited enzyme activity. Miyamoto et al. (43) observed strong inhibition by ADP of the catalytic subunits of lobster muscle cAMP-dependent and cGMP-dependent protein kinases. These authors also found that a number of highly purified kinases (both holoenzymes and catalytic subunits) from bovine brain were inhibited 50-60% by 50 µM ADP (ATP = cAMP = 5 µM in their assay system).
Table 6. Effects of various compounds on kinase activity.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Kinase activity (±10^{-5} M cAMP) percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>none (control)</td>
<td></td>
<td>100%</td>
</tr>
<tr>
<td>adenine</td>
<td>1 mM</td>
<td>86</td>
</tr>
<tr>
<td>adenosine</td>
<td>1 mM</td>
<td>90</td>
</tr>
<tr>
<td>3′-AMP</td>
<td>1 mM</td>
<td>102</td>
</tr>
<tr>
<td>5′-AMP</td>
<td>1 mM</td>
<td>53</td>
</tr>
<tr>
<td>ADP</td>
<td>0.01 mM</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>0.10 mM</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>26</td>
</tr>
<tr>
<td>phosphoserine</td>
<td>1 mM</td>
<td>91</td>
</tr>
<tr>
<td>phosphothreonine</td>
<td>1 mM</td>
<td>92</td>
</tr>
<tr>
<td>p-nitrophenyl-P</td>
<td>1 mM</td>
<td>85</td>
</tr>
<tr>
<td>glucose-1-P</td>
<td>1 mM</td>
<td>86</td>
</tr>
<tr>
<td>glucose-6-P</td>
<td>1 mM</td>
<td>79</td>
</tr>
<tr>
<td>potassium phosphate</td>
<td>1 mM</td>
<td>92</td>
</tr>
<tr>
<td>theophylline</td>
<td>10 mM</td>
<td>90</td>
</tr>
<tr>
<td>GTP</td>
<td>25 µM</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>125 µM</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>250 µM</td>
<td>45</td>
</tr>
</tbody>
</table>

Assays were conducted as described in Methods (ATP = 125 µM, cAMP = 10 µM), and each reaction was initiated by the addition of 14.7 µg of enzyme protein (105,000 x g supernatant). The control activity was units/mg enzyme protein. Endogeneous activity was not determined in this experiment.
In another study (15), Miyamoto et al. reported that 5' -AMP was a very weak inhibitor of purified bovine brain kinase. The insect enzyme system appears similar to that of bovine brain with respect to inhibition by ADP, but is dissimilar as regards inhibition by 5' -AMP.

Under standard assay conditions, 10 mM theophylline inhibited kinase activity in the presence of cAMP only to a small extent (10%). This compound was somewhat more inhibitory (23%) to kinase activity measured with no cAMP present. In future studies it may be desirable to include theophylline in the reaction mixture when assaying crude enzyme fractions in order to inhibit cyclic nucleotide phosphodiesterase activity (see Table 19 for inhibition of M. sexta diesterase by this compound). This was not done in the experiments described in this thesis because preliminary assays (in glycerophosphate buffer using a protamine substrate) indicated greater than 40% inhibition of cAMP-dependent protein kinase activity in the presence of 8 mM theophylline. It was felt that this magnitude of kinase inhibition was too great a price to pay in return for inhibiting cyclic nucleotide hydrolysis.

Phosphoserine and phosphothreonine at 1 mM had very little effect on kinase activity. Experiments with these substances were conducted with the aim of inhibiting phosphoprotein phosphatase activity present in the extract, and thereby (possibly) enhancing
the level of $^{32}$P esterified to histone. Subsequent studies of *M. sexta* soluble phosphatase activity, however, established that these compounds had no influence upon phosphatase activity (see Table 11). Para-nitrophenylphosphate, glucose-1-phosphate, and glucose-6-phosphate were only slightly more inhibitory to the kinase than potassium phosphate.

The phosphorylation of histone by ATP$^{32}$ was greatly reduced by including unlabeled GTP in the reaction mixture (Table 6). When GTP was present in equal amounts to ATP$^{32}$ (each 125 μM), the incorporation of $^{32}$P into histone was reduced almost exactly by a factor of two. GTP would seem to be a very effective competitor with ATP for kinase catalytic sites, but it remains to be determined whether or not GTP serves as a phosphate donor. In this regard, it is worth mentioning what has been learned in other studies when ATP was replaced by GTP. Kuo and Greengard (28) have reported that [γ-$^{32}$P] GTP had little or no ability to serve as a phosphate donor for histone phosphorylation by purified cAMP-dependent protein kinases from rat adipose cells, bovine brain, bovine heart, and bovine skeletal muscle, or by cAMP-dependent or cGMP-dependent protein kinases from lobster muscle. The same ATP-specificity has also been reported for a kinase from bovine tracheal muscle (27). In these experiments GTP did not affect ATP:protein phosphoryltransferase reactions. However, Kleinsmith and Allfrey (129)
γ-\textsuperscript{32}P labeled ATP, GTP, ITP, CTP, and UTP were all capable, to varying degrees, of serving as substrates for a kinase from rat liver nuclei. Furthermore, nucleoside triphosphates other than ATP have been shown to compete with ATP for kinases in human erythrocyte ghosts (126). And finally, a protein kinase specifically requiring GTP for its substrate has recently been described in adipose tissues of the rat (12). The finding in the present study of the insect CNS that GTP can compete quite effectively with ATP strongly indicates that further studies should be undertaken to establish the properties in this tissue of GTP-specific kinases, ATP-specific kinases, and/or ATP, GTP-nonspecific kinases.

**Protein Kinase Inhibitor**

As discussed in the general introduction, a number of tissues have been shown to contain a protein inhibitor of cAMP-dependent protein kinase. It was desirable to establish if such a factor were present in the insect CNS. A prominent characteristic of kinase inhibitors thus far characterized, and one which has proven quite useful during their purification, is stability at elevated temperatures. For example, the protein kinase inhibitor from lobster muscle is extraordinarily stable to heat, resisting destruction by boiling at 100°C for five hours (48). A simple procedure, essentially that employed by Appleman et al. (44) and Gilman (115), was used to
prepare a crude inhibitor fraction from *M. sexta* larval nerve cords. In brief, 52 larval nerve cords were homogenized in 2.0 ml of 10 mM Tris·HCl (pH 7.0)-2 mM EDTA, boiled for 10 min, and centrifuged. The precipitate that formed after the addition of TCA (final concentration, 5%) to the supernatant was collected by centrifugation, suspended in buffer, and dialyzed for 21 hours against distilled water. The pH of the dialyzed solution was adjusted to 7.5 with 1 N NaOH, and this solution was lyophilized. The powder was carefully weighed on a microgram balance, and 0.30 ml of buffer was added to yield a solution containing 7.3 mg of the crude inhibitor fraction per ml.

To investigate the effect of mammalian protein kinase inhibitor upon the insect system, a crude inhibitor fraction was prepared from 940 g of rabbit skeletal muscle through the dialysis step of the procedure described by Walsh et al. (47). This procedure is similar to that described above (boiling, TCA precipitation), with only minor modification. Additionally, a protein kinase inhibitor from beef heart prepared according to Gilman (115) was purchased from Sigma. The insect nerve cord inhibitor fraction, as well as the mammalian preparations, were devoid of protein kinase and phosphoprotein phosphatase activities.

Figure 15 indicates that some component of the crude insect inhibitor fraction seriously interfered with soluble cAMP-dependent protein kinase activity of the larval CNS. This particular experiment
Figure 15. Effect of various amounts of the insect crude inhibitor fraction upon soluble cAMP-dependent kinase activity. Each reaction mixture contained 46 μg of enzyme protein (105,000 × g supernatant), 250 μg of histone substrate, and 27 μM ATP$^{32}$. Activities are expressed as percentages of that observed with no inhibitor present.
was performed before optimum assay conditions were established for the insect protein kinase, and the composition of the reaction mixture (10 mM Mg$^{2+}$, 50 mM glycerophosphate buffer (pH 7.0), 27 μM ATP$^{32}$) differed from that employed for the bulk of these studies. Additionally, it should be noted that the protein content of the inhibitor fraction was not determined, and that the data are presented in terms of the weight of this fraction included in the reaction mixture; only a fraction of this material represents protein. The degree of inhibition of the insect kinase activity rose sharply with increasing amounts of inhibitor and then apparently reached a plateau level (about 10% of the activity measured with no inhibitor present) at higher inhibitor concentrations. Almost identical inhibition curves have been reported for a number of mammalian cAMP-dependent protein kinases (42, 47). When present at 250 μg per ml in the reaction mixture, the mammalian kinase inhibitor fractions derived from bovine heart and rabbit muscle were totally without effect upon the insect kinase cAMP-dependent activity.

Assuming a 100% recovery of inhibitor activity in the lyophilized inhibitor fraction (i.e., all of the activity present in the nerve cord starting material), it was calculated that the amount of this fraction required to produce 50% inhibition of high speed supernatant cAMP-dependent protein kinase activity was only about twice that amount already contributed to the reaction mixture by the quantity of
supernatant assayed. It is thus apparent that kinase activity in the crude soluble fractions used for the present studies of the *M. sexta* CNS was highly suppressed by endogenous inhibitor. Other investigators (15, 32, 46) have experienced difficulties in assaying the protein kinase activity of crude extracts due to the presence of inhibitor.

As an alternative to the rather drastic procedures (boiling, precipitation with TCA) that were used to obtain an inhibitor fraction from the insect CNS, it would be desirable to employ less stringent and more conventional protein purification procedures. The method used was, however, ideally suited for the small amount of tissue available, but conceivably could have resulted in appreciable loss of inhibitor activity. It has been reported that inhibitors from rabbit muscle (45) and lobster muscle (48) are almost quantitatively recovered after prolonged boiling and TCA precipitation, but that an inhibitor from human polymorphonuclear leucocytes is destroyed after heating at 80°C for 3 min (46).

The biochemical and functional significance of the protein kinase inhibitor is largely unknown. It is apparent, however, that selective interactions of such inhibitors with specific protein kinases could effectively regulate the activity of these kinases and that variations in either the level of kinase or of the kinase inhibitor could markedly influence the metabolism of the cell. The available evidence (42) suggests that the inhibitor interacts with the active species of
cAMP-dependent protein kinase (C subunit) but does not significantly influence the activation process itself (RC → R + C). Therefore it is possible that one function of the inhibitor could be the termination of cAMP-mediated events. Due to time limitations, the properties of the insect kinase inhibitor were not examined in detail. Further research will be required to establish if this inhibitor can function as a "modulator" of kinase activity (see introduction).

Polyacrylamide Disc Gel Electrophoresis

A sample of soluble kinase from the larval CNS was prepared for polyacrylamide gel electrophoresis by homogenizing 14 nerve cords in 0.20 ml of 50 mM Tris·HCl (pH 7.0)-1 mM DTT-1 mM MgSO₄ and centrifuging at 35,000 × g for 30 min. The supernatant was then mixed with powdered sucrose and a small quantity of bromphenol blue tracking dye. Electrophoresis was conducted in an 8 mm × 25 cm glass tube which contained 8 ml of separating gel and 1 ml of stacking gel. The compositions of the gels (using reagent A' in the small pore gel) and electrophoresis buffer were described previously (general experimental methods). The separating gel was polymerized with persulfate, and the entire gel column was pre-electrophoresed at 500 volts (current = 2 ma) for 10 min before changing anodal and cathodal buffers and applying the protein sample. The loaded column was then electrophoresed for 19 hr at 500 volts.
in a cold room. After carefully removing the polyacrylamide gel
cylinder from the glass tube, the 15 cm separating gel was frozen
with liquid freon and cut into 30 equal 0.5 cm slices. Each slice was
homogenized in 0.5 ml of 100 mM Tris·HCl (pH 7.0)-1 mM DTT-2
mM MgSO₄, centrifuged, and a 50 µl aliquot of each supernatant was
assayed for kinase activity.

Histone kinase assays were conducted in the presence of 10⁻⁵ M
cAMP as described previously, except that the incubation time was
increased to 30 min. The distribution of kinase activity across the
separating gel is depicted in Figure 16. Activity was evident all
the way from the separating gel-stacking gel interface up to slice
no. 17, but peak activities were found in slices 10 and 11 (R_f = 0.33-
0.37) and in slices 15-16 (R_f = 0.50-0.53). It is not possible to
estimate the number of discrete soluble protein kinases from the
activity pattern observed, but in view of the extreme resolving power
of the disc electrophoretic technique and the great spread of activities
over the gel, it seems likely there are more than three. Each slice
may, in fact, contain a discrete protein kinase.

Having established the loci of maximum activities in the gel,
slices 10, 11, 15, and 16 were selected for assay in the presence
and absence of 10⁻⁵ M cAMP to determine which, if any, of the kinase
activities were stimulable by cAMP. The following data were
obtained:
Figure 16. Distribution of soluble kinase activity across a polyacrylamide gel. Electrophoresis and kinase assays (+10^{-5} M cAMP) were conducted as described in the text. Arrows indicate those gel slices which were later analyzed both in the presence and absence of cAMP.
<table>
<thead>
<tr>
<th>Gel slice no.</th>
<th>Ratio of activities (+cAMP/-cAMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.35</td>
</tr>
<tr>
<td>11</td>
<td>0.93</td>
</tr>
<tr>
<td>15</td>
<td>1.13</td>
</tr>
<tr>
<td>16</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Allowing (at most) a possible experimental uncertainty of 0.10 in the calculated activity ratios, only the activity in slice no. 10 was clearly stimulable by cAMP. Activity in slice no. 15 was weakly stimulated by cAMP, but that in the remaining slices was not. These observations are compatible with the hypothesis that those slices possessing cAMP stimulability represent kinase holoenzymes (catalytic plus regulatory subunits), whereas those not affected by cAMP represent either catalytic subunits of cAMP-dependent enzymes or non-cyclic nucleotide-sensitive protein kinases. The polyacrylamide gel disc electrophoretic technique is especially suited to separation of very small amounts of proteins, and it would be highly worthwhile pursuing these gel experiments on a larger scale. In particular, acrylamide gel-separated kinase activities should be examined for both cAMP- and cGMP-stimulability, and also for substrate specificity (ATP or GTP), in the hope of conclusively establishing if there exist in the insect CNS ATP-specific and/or GTP-specific kinases regulated by one or both of these cyclic nucleotides.
Summary

Evidence has been presented establishing the existence of cyclic nucleotide-stimulable protein kinase in the central nervous systems of both larval and adult *M. sexta*. The specific activity of cAMP-dependent protein kinase, and the magnitude of activation by cAMP, were much greater in the adult than in the larval CNS. It was shown that an effective inhibitor of cAMP-dependent kinase could be prepared from the larval CNS, and that some physical properties of this factor (heat stability, acid precipitability) are similar to those described for inhibitors present in other tissues. Polyacrylamide gel electrophoresis of soluble CNS proteins indicated the presence of multiple kinase activities in the larval CNS, and the distribution of activity over the gel could be interpreted as indicating the presence of separated kinase holoenzymes and catalytic subunits. A considerable amount of protein kinase activity was associated with particulate material and could be unmasked by treatment with Triton X-100.

In general, the enzymological properties of the larval CNS kinase were similar to those reported for other (mammalian and non-mammalian) kinases with respect to cyclic nucleotide stimulability, metal requirements, inhibition by ADP, $K_m$ for exogenous protein substrate, influence of univalent salts, inhibition by a heat-stable factor (protein kinase inhibitor), and lack of stimulation by cAMP of
casein phosphorylation. The insect enzyme system appeared to
der differ from mammalian kinases with the finding that GTP was a very
effective inhibitor of ATP: protein phosphotransferase activity. The
exogenous protein substrate specificity of the insect CNS kinase was
similar to that of rat brain with the sole exception that protamine was
a preferred phosphate acceptor for the insect kinase. A particularly
interesting observation was that cAMP lowered the $K_m$ of the larval
nerve cord kinase for ATP, and this phenomenon is known to be a
nervous tissue-specific property in mammals.

At low concentrations, cAMP was a much more potent activator
of soluble and particulate kinase activities than was cGMP. The
cyclic nucleotide activation profile determined for the Triton-ex-
tracted particulate kinase activity suggested that this activity was
cAMP-specific. However, the activation profiles observed for
soluble protein kinase could be interpreted as indicating either (1) a
cAMP-dependent enzyme which could be stimulated by higher concen-
trations of cGMP, or (2) a mixture of cAMP-dependent and cGMP-
dependent enzymes. Separation and purification of the insect CNS
activities will be required to resolve this uncertainty.
Phosphoprotein phosphatase, which catalyzes the hydrolysis of orthophosphate esterified to serine and threonine residues of various proteins, has been studied in several tissues (16-22). Of these studies, the most comprehensive is that of Maeno and Greengard (21) defining the subcellular distribution and other properties of phosphatase in rat cerebral cortex. Extrinsic phosphatase activity was much greater, and more concentrated in particulate fractions, in brain than in any non-nervous tissue. At least four distinct protein phosphatases were present in the cerebral cortex, and the particulate enzyme was distinguished from the cytosolic phosphatases by its kinetic properties and its activation by Mn$^{2+}$. Multiple forms of phosphatase were also found in cultured neuroblastoma and neuroglial cells. Greater than 50% of the total activity of rat brain cortex was particulate, and of the particulate preparations examined, the highest specific activity was found in fractions containing synaptic membranes. Among several phosphorylated proteins examined, endogenous membrane protein served by far as the best substrate for membrane-bound cerebral protein phosphatase.
Materials and Methods

Materials

The phosphoprotein substrate for the phosphatase assay, $^{32}$P-labeled protamine, was prepared as described in Appendix III. All the experiments described herein were performed within two $^{32}$P half-lives (28 days) after preparation of the substrate. O-phospho-l-serine and dl-0-phosphothreonine were products of Sigma. All materials used in the phosphomolybdate extraction procedure (ammonium molybdate, benzene, isobutyl alcohol) were of analytical reagent quality.

Enzyme Preparation

Larval nerve cords from *M. sexta* were used exclusively for this study. After dissection, all tissues were rapidly frozen on dry ice until 20-30 nerve cords had been collected. Immediately after thawing, the nerve cords were homogenized in 50 mM Tris·HCl (pH 7.0) buffer containing, where specified, 1 mM EDTA and/or 1 mM DTT (dithiothreitol).

Assay for Phosphoprotein Phosphatase

Phosphatase activity (extrinsic activity) was assayed by measuring the enzyme-catalyzed release of radioactive orthophosphate
from $^{32}$P-labeled protamine. The reaction mixture was composed of 50 mM Tris·HCl (pH 7.0), 10-100 μg $^5$ of $^{32}$P-protamine, 1 mM DTT, and ordinarily less than 25 μg of enzyme protein in a cumulative volume of 0.10 ml. All reagent additions were made with a Hamilton syringe, and various test substances were incorporated into the assay mixture as indicated. Since preliminary experiments indicated that the majority of protamine phosphatase activity was soluble after homogenization in hypotonic Tris buffer, either 34,000 X g or 105,000 X g supernatants were employed for most of these studies. However, subsequent experiments to be described established that a considerable amount of additional "latent" activity could be unmasked through incubation of particulate samples with the non-ionic detergent Triton X-100.

The reaction was normally initiated by the addition of enzyme, incubated in a shaking water bath at 30°C for 10 min, and terminated with 1.0 ml of 20% TCA. Following the addition of 0.10 ml of 0.5% serum albumin (protein carrier), the suspension was thoroughly mixed and allowed to stand at room temperature for 5 to 10 min. Precipitated protein was removed by centrifugation at 10,000 X g for 10 min. The deproteinized supernatant was transferred to a

$^5$All data presented here were obtained using the same preparation of $^{32}$P-protamine, and at the beginning of these studies standard reaction mixtures contained 100 μg of substrate. As the stock of protamine was depleted, it became necessary to employ less material in each assay.
$13 \times 100$ mm culture tube and mixed with 0.05 ml of $10^{-2} \text{ M } \text{KH}_2\text{PO}_4$ (carrier phosphorus), then with 1.0 ml of 5\% ammonium molybdate in 3N $\text{H}_2\text{SO}_4$. This solution was periodically agitated over a 10 min period, and then the $^{32}\text{P}$-containing phosphomolybdate complex was extracted once with 2.0 ml of water-saturated isobutyl alcohol-benzene (1:1, v/v). A 1.0 ml aliquot of the organic phase was delivered into a scintillation vial, well-mixed with 10 ml of Aquasol, and counted after three hours in a liquid scintillation spectrometer.

In parallel with almost every experiment, a sample of the $^{32}\text{P}$-substrate was counted to verify the predicted radioactivity calculated from the known activity at the time of preparation. Phosphomolybdate-extractable background radioactivities (in the absence of enzyme) were approximately 25-50 cpm, whereas enzymatically hydrolyzed $^{32}\text{P}$ amounted to 5,000-30,000 cpm. Replicate samples agreed within 3\%. As is indicated in Appendix III, 16 nanomoles of phosphate were incorporated into each mg of protamine by enzymatic phosphorylation to synthesize the phosphoprotein substrate. Thus, with 100 $\mu$g of phosphoprotamine present in the standard assay, the initial concentration of hydrolyzable phosphate was 16 $\mu$M. Neglecting the phosphate already present in the protamine prior to its phosphorylation allows a definition of one unit of protamine phosphatase activity as that amount which hydrolyzes one picomole of phosphate from phosphoprotamine in 10 minutes under the assay conditions employed.
In experiments designed to evaluate the efficacy of the iso-
butanol-benzene extraction procedure, it was established that 99.0-
99.4% of the total $^{32}$P orthophosphate extractable in three successive
treatments was recovered in the first extract. Subsequent extractions,
or further addition of ammonium molybdate and phosphate carrier
followed by re-extraction, resulted in negligible supplementary ac-
tivity. A small and non-extractable amount of radioactivity remained
in the aqueous phase, this presumably being due to unprecipitated
$^{32}$P-protamine or $^{32}$P-peptides formed by proteolytic enzymes
present in the M. sexta sample.

**Choice of Substrate**

Protamine was chosen as a substrate for the present research
for two principal reasons. First, it is known to be readily phos-
phorylated and dephosphorylated in vivo and in vitro (19, 140). And
second, it is a relatively inexpensive protein commercially-available
in great quantities. Proteins other than protamines which have re-
ceived attention as phosphatase substrates include casein (19, 21),
phosvitin (19), and various histone subfractions (19, 21). Of these,
phosphorylated histones and protamines (both very basic proteins)
appear to be the best substrates for assessing extrinsic mammalian
phosphatase activity. Because of the relatively great expense of
histones, they were not investigated in the present study.
Protamines are exceptionally basic, low molecular weight proteins having high isoelectric pH values (pI  12; ref. 141, p. 54). They typically consist of approximately 70% arginine and therefore, for values of pH < pI, they are highly positively charged. Because of this great charge at physiological pH values necessary for assaying phosphatase, it was expected that enzyme activity would be strongly dependent upon the ionic strength of the assay medium. "Ionic effects" were, in fact, observed in the present study. Further research (for example, with less highly charged substrates such as histone) will be necessary to ascertain if observed variations of enzyme activity with ionic strength are properties of the phosphatase per se, or if they reflect primarily the highly-charged nature of the substrate.

Results and Discussion

Miscellaneous Observations

With sufficiently long incubation periods, the radioactive P i esterified to protamine by enzymatic phosphorylation could be quantitatively hydrolyzed by the M. sexta enzyme. Phosphatase activity in a 105,000 x g supernatant was unstable to freezing and thawing, and whenever possible, samples were assayed immediately after homogenization and centrifugation. Overnight storage of supernatants
at -20°C after rapidly freezing them in liquid nitrogen resulted in a 10-15% loss of activity. With a freshly prepared supernatant there occurred nearly a 40% reduction in activity after three rapid freeze-thaw cycles. For a high speed supernatant prepared in the absence of DTT, the inclusion of bovine serum albumin (BSA, 100 µg per 100 µl) into the reaction mixture resulted in an apparent increase in activity of 39%. BSA may have protected the enzyme(s) from denaturation or from degradation by proteases present in the extract. Glycerol at 10% (v/v) caused an 11% decrease in activity, but sucrose at 10% (w/v) was without effect. Triton X-100 at 0.01% did not affect soluble phosphatase activity, but if present at 0.1% in the reaction mixture, this detergent inhibited activity by about 15%. Heating at 100°C for 5 min completely destroyed enzymatic activity.

**Time, Enzyme, and Substrate Dependences of Reaction Velocity**

With 100 µg of $^{32}$P-protamine substrate and 19.1 µg of 105,000 × g supernatant protein present in the reaction mixture, the rate of dephosphorylation was linear with time up to ten minutes of incubation (Figure 17). The downward curvature observed past ten minutes may reflect substrate depletion, \(^6\) or it may be due to loss of enzyme

\(^6\)Also, it seems possible that some of the protamine phosphoserine residues would be more accessible to enzyme than others, and that these would preferentially be dephosphorylated early in the incubation period.
Figure 17. Time course of phosphoprotamine dephosphorylation. Enzyme source was a 105,000 X g 2 hr supernatant prepared from 20 M. sexta larval nerve cords. The tissue was homogenized in buffer containing no DTT, and the specific activity of this preparation was relatively low. 19.1 μg protein was present in each reaction mixture.
activity under the conditions employed (no DTT present). A separate experiment established that $^{32}$P-release was proportional to the amount of supernatant protein at least through 20 µg. The dependence of reaction velocity upon substrate concentration is presented in Figure 18. The enzyme source for this experiment was a 34,000 X g supernatant, and aliquots were assayed in the presence of 10-160 µg phosphoprotamine. As is indicated in the inset to Figure 18, a double-reciprocal plot of the data showed non-ideal Michaelis-Menten behavior. Two $K_m$ values (20 µg and 40 µg of protamine per 100 µl) were obtained by extrapolation of the two linear segments.

Sulfhydryl Sensitivity

Preliminary experiments indicated a progressive loss in protamine phosphatase activity during prolonged centrifugation of supernatants prepared in Tris buffer containing no protective reagents for sulfhydryl groups. An increase of almost 40% in activity could be effected in such supernatants by including 1 mM DTT in the reaction mixture. However, 10 mM DTT was less effective than 1 mM DTT in restoring activity. Cysteine was also capable of stimulating activity (18% at 1 mM). Para-hydroxymercuribenzoic acid (PHMB) resulted in 94% inhibition of the insect enzyme when present at $10^{-4}$ M. After the sulfhydryl sensitivity of the phosphatase was discovered, in all subsequent experiments 1 mM DTT was included
Figure 18. Phosphatase activity as a function of substrate concentration. Enzyme source was a 34,000 X g supernatant containing 1 mM EDTA and 1 mM DTT. Each reaction mixture included 30 μg of enzyme protein and 10-160 μg of 32P-protamine. Inset: double reciprocal plot of the data.
in the homogenization buffer and in the assay mixture.

**Effects of Salts and Ionic Strength**

A relatively large activation of soluble protamine phosphatase activity occurred in the presence of NaCl or KCl. In view of the well-known flux and active transport of these ions accompanying neuronal transmission, the possibility became apparent that fluctuations in intracellular levels of these ions might regulate phosphatase activity in vivo. Visions of specificity were dispelled, however, with the findings that a variety of other univalent salts were also capable of stimulating phosphatase activity (Table 7). At 100 mM concentrations, all salts tested were approximately equipotent. The stimulatory effect appeared to be independent of the anion, since acetate and chloride salts of sodium and potassium behaved similarly. Tetramethylammonium chloride (TMAC), although slightly less effective than NaCl or KCl, was also capable of enhancing protamine phosphatase activity. Phosphatase activity was also dependent upon the concentration, and hence, the ionic strength of the buffer employed. The relative activities of identical amounts of enzyme protein assayed in 50 mM, 80 mM, 135 mM, and 430 mM Tris·HCl buffers (pH 7.0) were 1:1.35:1.68:1.89.

Phosphatase activity in a 34,000 × g supernatant was further examined as a function of NaCl and KCl concentrations (Figure 19).
Table 7. Effect of nonvalent cations on phosphatase activity in a 105,000 x g M. sexta larval CNS supernatant.

<table>
<thead>
<tr>
<th>Salt (100 mM)</th>
<th>Protamine phosphatase activity (percent of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100%</td>
</tr>
<tr>
<td>NaCl</td>
<td>132</td>
</tr>
<tr>
<td>KCl</td>
<td>129</td>
</tr>
<tr>
<td>NaCl + KCl (each (100 mM)</td>
<td>159</td>
</tr>
<tr>
<td>NaC$_2$H$_3$O$_2$</td>
<td>135</td>
</tr>
<tr>
<td>KC$_2$H$_3$O$_2$</td>
<td>141</td>
</tr>
<tr>
<td>LiCl</td>
<td>137</td>
</tr>
<tr>
<td>TMAC$^1$</td>
<td>121</td>
</tr>
</tbody>
</table>

$^1$Tetramethylammonium chloride

Twenty larval nerve cords were homogenized in 50 mM Tris-HCl (pH 7.0) - 1 mM EDTA and centrifuged at 105,000 x g for two hr before assay of the supernatant. The enzyme sample contributed 0.1 mM EDTA to the reaction mixture.
Figure 19. Effect of NaCl, KCl, and NaF on phosphatase activity in a 34,000 × g supernatant.
The behavior of these two salts was qualitatively very similar, with KCl being marginally more stimulatory at concentrations greater than 75 mM. Activity was optimal in the presence of 0.2-0.4 M salt and declined at higher concentrations. When phosphatase was assayed in the presence of various concentrations of both NaCl and KCl (each present at 50-200 mM), the effect was one of additivity, and no Na\(^+\) + K\(^+\) synergism was observed. Detailed studies of the influence of salts upon any phosphatase preparation have not been reported in the literature, but Meisler and Langan (19) have noted that histone and protamine phosphatase activities derived from rat liver were activated by 0.1 M-0.2 M NaCl. However, Weller and Rodnight (20) observed a relatively strong inhibition of intrinsic phosphatase activity in ox brain synaptosomal and microsomal preparations by levels of NaCl and KCl as low as 10 mM.

The simplest explanation of the observed salt effects in the insect preparation is that high ionic strength allows a more favorable interaction between the phosphatase and its positively charged substrate. This might be due to a reduction of charge repulsion effects between enzyme and substrate. Still another possibility is that high salt conditions induce a conformational change in the protamine such that certain phosphoserine residues are more readily dephosphorylated. On the other hand, activation by salts may in part be a property of the enzyme itself, but further research with less highly charged substrates
will be required to examine this possibility.

In Figure 19 is also shown the strongly inhibitory influence of low concentrations of NaF, an effect which has been reported in rat brain cerebral cortex (21) and liver (19) preparations. Protamine phosphatase activity in _M. sexta_ CNS supernatants was severely inhibited in the presence of 0.1 M NaF and could not be restored by the addition of NaCl.

**Effects of Bivalent Metals and EDTA**

As shown in Table 8, the chelating agent ethylenediaminetetraacetic acid (EDTA) promoted an impressive activation of phosphatase activity in high-speed supernatants. The higher the concentration of EDTA employed, the greater was the magnitude of its effect. Concentrations greater than 25 mM could not be tested using the phosphatase assay described since they interfered with phosphomolybdate complex formation. In supernatants prepared from _M. sexta_ nerve cords homogenized in the absence of EDTA, incorporation of 25 mM EDTA into the assay mixture produced greater than a four-fold increase in activity. In samples homogenized in the presence of EDTA, further activation was still possible through inclusion of this compound into the reaction mixture. In Table 9 further information is presented on phosphatase activities found in larval CNS homogenates and supernatants after homogenization in the presence or absence
Table 8. Effect of EDTA\(^1\) on protamine phosphatase activity.

<table>
<thead>
<tr>
<th>EDTA added to assay mixture</th>
<th>Relative phosphatase activity in 105,000 x g supernatant prepared in Buffer A(^2)</th>
<th>Buffer B(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>1 mM</td>
<td>167%</td>
<td>133%</td>
</tr>
<tr>
<td>5 mM</td>
<td>248%</td>
<td>--</td>
</tr>
<tr>
<td>10 mM</td>
<td>362%</td>
<td>199%</td>
</tr>
<tr>
<td>25 mM</td>
<td>455%</td>
<td>206%</td>
</tr>
</tbody>
</table>

Assays were conducted as described in the text, using 100 µg of phosphoprotamine substrate.

\(^1\)EDTA, ethylenediaminetetraacetic acid. The stock solution of this compound was prepared by neutralizing the fully acidic form with Tris. Since phosphatase activity was dependent upon univalent cations, an additional variable would be introduced by employing disodium EDTA.

\(^2\)Thirty *M. sexta* larval nerve cords were homogenized in 50 mM Tris·HCl (pH 7.0). Activity of the supernatant in the absence of added EDTA was 2.1 × 10\(^3\) units/mg protein.

\(^3\)Thirty nerve cords were homogenized in 50 mM Tris·HCl (pH 7.0) -1 mM DTT-1 mM EDTA, and the enzyme sample contributed 0.2 mM each of EDTA and DTT to the reaction mixture. Activity in the absence of added EDTA was 7.3 × 10\(^3\) units/mg protein.
Table 9. Phosphatase activities of larval CNS homogenates and supernatants as a function of homogenizing buffer composition.

<table>
<thead>
<tr>
<th>Exp't</th>
<th>No. of CNS</th>
<th>Enzyme source</th>
<th>Homogenizing buffer</th>
<th>Specific activity units/mg protein</th>
<th>Activity per nerve cord units/ CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>la</td>
<td>20</td>
<td>Homogenate</td>
<td>Tris</td>
<td>--</td>
<td>389</td>
</tr>
<tr>
<td>lb</td>
<td>20</td>
<td>34,000 x g supernatant</td>
<td>Tris</td>
<td>$2.6 \times 10^3$</td>
<td>342</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>34,000 x g supernatant</td>
<td>Tris-DTT</td>
<td>$4.9 \times 10^3$</td>
<td>621</td>
</tr>
<tr>
<td>3a</td>
<td>30</td>
<td>Homogenate</td>
<td>Tris-DTT-EDTA</td>
<td>$5.0 \times 10^3$</td>
<td>1544</td>
</tr>
<tr>
<td>3b</td>
<td>30</td>
<td>34,000 x g supernatant</td>
<td>Tris-DTT-EDTA</td>
<td>$1.1 \times 10^4$</td>
<td>1403</td>
</tr>
</tbody>
</table>

The basic buffer was 50 mM Tris·HCl (pH 7.0), which contained (where indicated) 1 mM EDTA and 1 mM DTT, or both. All assays were conducted in the absence of DTT using one-tenth volume (10 μl) of enzyme protein and 100 μg of $^{32}$P-protamine. One unit of enzyme activity is that amount which hydrolyzes 1 pmol of phosphate from phosphoprotamine in 10 min.
of DTT and EDTA. Highest activities were attained with both of these compounds present. The insect CNS homogenate specific activity recorded in Table 9 is slightly higher than that reported by Maeno and Greengard (21) for a rat cerebral cortex homogenate assayed under similar conditions to those reported here.

EDTA is known to form very stable 1:1 complexes with almost all bi- and trivalent metal ions, and chelates univalent ions as well (142). The activation of phosphatase by EDTA is presumably due to removal of inhibitory metals from the enzyme, allowing a full expression of activity. In support of this contention, it was also noted in the present study that a 20% activation of phosphatase occurred in the presence of 1 mM 1,10-phenanthroline. This compound is known to effectively complex Fe$^{2+}$, Fe$^{3+}$, Zn$^{2+}$, Cu$^{2+}$, and other polyvalent metal ions (142). Further investigation of phosphatase in the presence of more specific and potent metal chelating agents is warranted in hopes of determining which endogenous metal(s) might exert deleterious effects upon its activity.

The influence of various bivalent metal ions upon phosphatase activity was examined (Table 10). The tissue for this experiment had been homogenized in buffer containing 1 mM EDTA to complex potentially inhibitory metals of endogenous origin, and the sample contributed 0.1 mM EDTA to the reaction mixture. Thus, a small fraction of the added metal ions was complexed by this chelating
Table 10. Effect of bivalent metals on phosphatase activity.

<table>
<thead>
<tr>
<th>Addition (mM)</th>
<th>Relative phosphatase activity of $34,000 \times g$ supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100%</td>
</tr>
<tr>
<td>MgCl$_2$, 1</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>10.18</td>
</tr>
<tr>
<td></td>
<td>1.63</td>
</tr>
<tr>
<td>CaCl$_2$, 1</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>1.18</td>
</tr>
<tr>
<td>MnCl$_2$, 1</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>1.30</td>
</tr>
<tr>
<td>CuCl$_2$, 1</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>ZnSO$_4$, 1</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>FeCl$_3$, 1</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Thirty larval nerve cords were homogenized in 50 mM Tris-HCl-1 mM DTT-1 mM EDTA and centrifuged at 34,000 $\times g$ for 30 min before assay of the supernatant. The sample contributed 0.1 mM EDTA to the assay mixture. The reaction was initiated by the addition of 100 $\mu$g of $^{32}$P-protamine. Activity is expressed relative to that observed in the absence of added metal ions (1.1 x $10^4$ units/mg protein).
agent. At a concentration of 1 mM, neither Mg\(^{2+}\), Ca\(^{2+}\), nor Mn\(^{2+}\) exerted any significant effect, and at 10 mM, they were mildly stimulatory. Cu\(^{2+}\), Zn\(^{2+}\), and Fe\(^{3+}\) strongly inhibited enzyme activity, possibly through reaction with sensitive-SH groups. The metal ion dependences of the insect preparation shown in Table 10 are qualitatively similar to those reported for rat cerebral cortex protamine phosphatase (21), with one exception: soluble phosphatases of rat brain were activated approximately 100% by 2.5 mM MnCl\(_2\). It seems possible that the small elevation of _M. sexta_ CNS phosphatase activity caused by bivalent metal ions may simply be due to ionic effects discussed previously. However, the situation appears more complex in that preliminary experiments on the insect enzyme in the presence of both salt and Mg\(^{2+}\) (or Mn\(^{2+}\)) indicate that bivalent metals may antagonize the stimulatory effect of salts. In fact, in one experiment millimolar concentrations of Mn\(^{2+}\) in the presence of optimally stimulatory concentrations of NaCl greatly inhibited phosphatase activity.

The stimulatory effects of EDTA and 1,10-phenanthroline coupled with the relative ineffectiveness of added bivalent metals strongly indicate that the _M. sexta_ CNS phosphoprotamine phosphatase is not dependent upon bivalent metals for activity. A similar circumstance prevails in the case of rat liver histone and protamine phosphatase, whose activities are unaffected by 2 mM EDTA (19).
However, Paigen and Griffiths (16) reported that a crude preparation from mouse liver was metal dependent and that low concentrations of 1,10-phenanthroline were strongly inhibitory. Also, Rose and Heald (17) have reported an inhibitory effect of EDTA upon ox brain phosphatase.

Temperature and pH Dependence

Figure 20 indicates the profile of protamine phosphatase activity in 80 mM sodium acetate, Tris·HCl, and Tris·maleate buffers as a function of pH. Only a small volume (10 μl, 20 μg) of phosphoprotamine substrate was used in these assays to minimize perturbing the pH of the test buffer. Optimal activities were observed in Tris buffers at pH values near neutrality. Similar pH profiles were noted in Tris·HCl and acetate buffers prepared at constant ionic strength (data not shown). At pH 7.0, activities in potassium phosphate and β-glycerophosphate buffers were 41% and 46%, respectively, of that observed in Tris·HCl.

The effect of temperature on soluble phosphatase activity in the presence or absence of salt is shown in Figure 21. With no NaCl present, optimal activity was observed at the relatively low temperature of 30°C. In the presence of 200 mM NaCl, activity steadily increased until approximately 40°C, beyond which temperature activity rapidly declined.
Figure 20. pH dependence of protamine phosphatase activity. Assays were conducted as described in Methods, except each reaction was initiated by the addition of 20 μg of protamine. Each assay contained 33.1 μg of a 34,000 × g supernatant, and activities are expressed relative to that noted for Tris·HCl, pH 7.0 (3.5 × 10³ units/mg protein). Buffers (each 80 mM); ○, Tris·HCl; □, Tris·maleate; Δ, sodium acetate.
Figure 21. Protamine phosphatase activity (± NaCl) as a function of temperature. Each reaction mixture contained 20 µg of protamine and 33.1 µg of a 34,000 X g larval CNS supernatant. Activities are expressed relative to that measured at 30°C in the absence of salt.
Effects of Various Compounds on Phosphatase Activity

The sulfhydryl sensitivity of the phosphatase and its inactivation by PHMB, and also inhibition by NaF have been noted previously. A variety of phosphate-containing compounds were also tested for their influence upon activity (Table 11). Orthophosphate and β-glycerophosphate were capable of appreciable inhibition at 5 mM levels, but gave only small interference at 1 mM. Glucose-1-phosphate and glucose-6-phosphate were without considerable effect at the 1 mM level. Cyclic AMP, cGMP, and adenosine exerted no great influence under the conditions employed. The very minor activation of phosphatase by 1 mM phosphoserine and phosphothreonine is very likely due to ionic effects mentioned earlier. Both ATP and GTP at millimolar levels were capable of considerably reducing phosphatase activity, but at $10^{-4}$ M were only slightly inhibitory. γ-aminobutyric acid and acetylcholine at 5 mM, and ecdysterone (0.1 mg/ml) were without effect (data not shown). It is exceedingly unlikely, therefore, that the insect protamine phosphatase activity is identical with "non-specific" phosphatase, glucose-1 or 6-phosphatase, ATPase, or cyclic nucleotide phosphodiesterase activities.

Distribution of Phosphatase Among Soluble and Particulate Fractions

Early experiments with M. sexta larval nerve cords showed that
Table 11. Effects of various compounds on protamine phosphatase.

<table>
<thead>
<tr>
<th>Addition (mM)</th>
<th>Protamine phosphatase activity (percent of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>100%</td>
</tr>
<tr>
<td>adenosine, 1</td>
<td>99</td>
</tr>
<tr>
<td>ATP, 0.1</td>
<td>95</td>
</tr>
<tr>
<td>1.0</td>
<td>41</td>
</tr>
<tr>
<td>GTP, 0.1</td>
<td>89</td>
</tr>
<tr>
<td>0.86</td>
<td>20</td>
</tr>
<tr>
<td>cAMP, 0.1</td>
<td>96</td>
</tr>
<tr>
<td>cGMP, 0.1</td>
<td>95</td>
</tr>
<tr>
<td>phosphoserine, 0.1</td>
<td>102</td>
</tr>
<tr>
<td>1.0</td>
<td>105</td>
</tr>
<tr>
<td>phosphothreonine, 0.1</td>
<td>96</td>
</tr>
<tr>
<td>1.0</td>
<td>107</td>
</tr>
<tr>
<td>glucose-1-phosphate, 1</td>
<td>93</td>
</tr>
<tr>
<td>glucose-6-phosphate, 1</td>
<td>94</td>
</tr>
<tr>
<td>β-glycerophosphate, 1</td>
<td>87</td>
</tr>
<tr>
<td>5</td>
<td>47</td>
</tr>
<tr>
<td>potassium phosphate, 1</td>
<td>91</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
</tr>
</tbody>
</table>
equal volumes of an homogenate prepared in Tris buffer and of the supernatant derived therefrom contained approximately the same amount of phosphatase activity. The provisional conclusion was drawn, therefore, that under the conditions employed the majority of activity was soluble. The following description pertains to an experiment undertaken to evaluate in further detail the distribution of protamine phosphatase activity among soluble and particulate fractions. In particular, it was of interest to determine if additional activity could be unmasked by detergent treatment of particulate material.

Thirty larval nerve cords were thoroughly homogenized in 1.0 ml of 50 mM Tris·HCl (pH 7.0)-1 mM DTT-1 mM EDTA in an all-glass tissue grinder, and an aliquot of the homogenate was stored under $N_2$ on ice. The remaining homogenate was transferred to a polycarbonate centrifuge tube, blanketed with $N_2$, and sedimented at 34,000 $\times$ g for 30 min. One portion of the stored homogenate was incubated on ice for 20 min with 0.1% (v/v) Triton X-100, and another with 0.1% (w/v) sodium deoxycholate. Still another was frozen and thawed three times. After centrifugation of the bulk homogenate was complete, the pellet was stored at -20°C, and the supernatant and homogenate samples were assayed for phosphatase activity (results presented in Table 12).

The enzyme activity in the 34,000 $\times$ g pellet was further
Table 12. Phosphatase activities in a larval CNS homogenate and supernatant, and the effect of Triton X-100.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Protamine phosphatase activity units / CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>780</td>
</tr>
<tr>
<td>Homogenate + 0.1% Triton&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1046</td>
</tr>
<tr>
<td>Homogenate + 0.1% deoxycholate&lt;sup&gt;1&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>Homogenate, freeze-thaw 3x</td>
<td>642</td>
</tr>
<tr>
<td>34,000 × g supernatant</td>
<td>806</td>
</tr>
<tr>
<td>Triton extract of washed 34,000 × g pellet&lt;sup&gt;2&lt;/sup&gt;</td>
<td>70</td>
</tr>
<tr>
<td>Washed, Triton-extracted 34,000 × g pellet</td>
<td>130</td>
</tr>
</tbody>
</table>

<sup>1</sup> Final concentration of the detergents in the assay mixture was 0.01%.

<sup>2</sup> The 34,000 × g pellet had been stored at -20°C for six days prior to washing and extraction.

Thirty <i>M. sexta</i> larval nerve cords were treated as described in the text. Each fraction was assayed in the presence of 40 µg of 32P-protamine. One unit of phosphatase activity equals one picomole of phosphate hydrolyzed from phosphoprotamine in 10 min.
examined at a later date. Some loss of activity may have occurred during storage. After 6 days of storage at -20°C, the pellet was resuspended in 2.0 ml of cold 50 mM Tris-1 mM DTT and magnetically stirred on ice for 30 min. After sedimentation at 34,000 × g for 30 minutes, the supernatant was discarded and the washed pellet was incubated at 0-4°C with 0.5 ml of 50 mM Tris- HCl (pH 7.5)-1 mM DTT-0.1% Triton for one hour. This material was again centrifuged at 34,000 × g, and the supernatant was assayed for phosphatase. The pellet was suspended in Tris containing DTT and the suspension (washed, Triton-extracted membranes) was also assayed (Table 12).

The phosphatase activity of the untreated homogenate and its 34,000 × g supernatant were, within possible experimental error, approximately equal. Incubation of the homogenate with Triton resulted in a 34% increase in activity relative to the untreated sample, but deoxycholate (an anionic detergent) destroyed enzyme activity. Freezing and thawing three times caused an 18% reduction in homogenate phosphatase activity; a separate experiment indicated that such treatment also resulted in considerable loss of activity in a 105,000 × g supernatant. Brief sonication of an homogenate sample caused, approximately a 35% loss of activity. As is indicated in Table 12, after Triton extraction of the washed 34,000 × g pellet, additional phosphatase activity was found in the soluble fraction. Further,
there remained even more activity in the membranes which could not be extracted with Triton.

To briefly examine if any differences existed between the soluble and particulate enzymes with regard to metal requirement and stimulability by salt, the phosphatase extracted from the washed 34,000 × g pellet with Triton was assayed in the presence of NaCl, EDTA, and Mn$^{2+}$. The magnitudes of activation by 200 mM NaCl and 10 mM EDTA were similar to those reported previously for the soluble enzyme, but Mn$^{2+}$ effected a much greater stimulation of the Triton-extracted activity (220% at 10 mM).

In 1961 Rose and Heald (17) suggested that phosphoprotein phosphatase might require a stabilizing material of a protein or lipoprotein nature, but no further information in this regard has appeared.

To investigate the possible influence of endogenous lipids upon soluble phosphatase from *M. sexta* CNS, a chloroform-methanol extract of the 105,000 × g pellet from 20 larval nerve cords was prepared, lyophilized, and suspended in 0.10 ml of 0.1% Triton X-100. This material was itself devoid of phosphatase activity, but 20 μl of the extract included in the standard assay mixture stimulated the activity of a 34,000 × g supernatant by about 10%. Further research will be required to establish if, in fact, some lipid component of membranous fractions is required for optimal phosphatase activity.

On the basis of the limited data available, it would not be
possible to estimate the particulate/soluble distribution of protamine phosphatase in the M. sexta larval CNS. The present studies do indicate, however, that a considerable quantity of phosphatase is associated with particulate fractions and may be unmasked in the presence of the detergent Triton X-100. It should be clear that the apparent subcellular distribution of phosphatase may be strongly dependent upon a variety of experimental variables such as tonicity of the homogenizing medium, stability of the various fractions during their preparation, and the presence or absence of detergent. As has been discussed by Maeno and Greengard (21), it may well depend on the nature of the phosphoprotein substrate. The tissues for the present study were stored frozen before homogenization in hypotonic media, and it is probable that mechanical and osmotic rupture of labile structures has occurred.

Of particular interest in future studies would be an examination of intrinsic phosphatase activity, both in soluble and particulate fractions of the insect CNS. This will require prior phosphorylation of endogenous proteins with ATP$^{32}$ by endogenous protein kinase in the presence of cAMP or cGMP. It seems likely, as suggested by the experiments of Maeno and Greengard (21), that endogenous proteins would be dephosphorylated much more readily than exogenous substrates.
CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN THE CENTRAL NERVOUS SYSTEM OF M. SEXTA

Introduction

In most tissues the intracellular concentration of cAMP is determined by the relative activities of hormone-sensitive adenyl cyclase, which catalyzes cAMP formation from ATP, and of one or more nucleoside 3', 5'-monophosphate phosphodiesterases which degrade it to 5'-AMP. Although several distinct phosphodiesterases are present in many biological systems, they are loosely referred to as phosphodiesterase (PDE), irrespective of their individual properties and substrate preferences. With the exception of one preliminary report (192), there is presently no firm evidence which suggests that PDE is under direct hormonal control. Since PDE is the only known enzyme capable of destroying cAMP, it emerges as a key regulatory enzyme responsible in part for the magnitude and duration of hormone action. Similar considerations apply to cGMP, whose intracellular levels are determined by the relative activities of phosphodiesterase and the soluble (cytosolic) enzyme guanyl cyclase. A study of the factors influencing phosphodiesterase activity is therefore important in understanding the regulation of all cyclic nucleotide-dependent events in the cell. Mechanisms which have been proposed for the regulation of PDE activity include inhibition by nucleoside
triphosphates and pyrophosphate (153), allosteric modulation (145, 183), protein-protein interactions (155, 189), and protein-lipid interactions (149).

In general, prolonged elevation of intracellular cAMP levels would be deleterious (or lethal) to cell function, and it is to be expected that there exists a potent enzyme system to rapidly reduce cAMP concentrations to basal levels. A continued accumulation of cAMP after its influence had been exerted would be disadvantageous and energetically wasteful. In brain tissue phosphodiesterase is apparently present in great excess over adenyl cyclase (65, 67). Weiss and Costa (198) have found that in most areas of rat brain, PDE activity exceeds that of adenyl cyclase by almost a 100-fold. Viewed simply, this excess of PDE could protect the cell against sustained high levels of cAMP.

It is presently believed that cAMP is generated primarily in particulate locations of the cell (by membrane-associated adenyl cyclase) but that at least a portion of the biological expression of cAMP occurs in the cytosol. PDE is present in both particulate and soluble fractions of the cell and thus is capable of terminating the action of cAMP in both of these cellular locations. In most tissues approximately 50% of the PDE activity is firmly associated with particulate fractions. The particulate and soluble activities have been reported to be either kinetically similar or dissimilar, depending
upon the tissue and the method of preparation (1, 144, 190, 191).

Cyclic AMP phosphodiesterase was first discovered in dog liver by Sutherland and Rall (214) and later was partially purified from bovine heart muscle by Butcher and Sutherland (67). Subsequent studies of PDE in several sources have suggested multiple enzymes for the hydrolysis of cyclic nucleotides. In many tissues it appears there exist at least two, and probably more, forms of PDE (144, 145, 148, 167, 169, 172, 191). Pending a physical separation and characterization of the activities, it cannot be conclusively decided whether these are entirely distinct enzymes, isoenzymes, or perhaps different conformational states or degrees of aggregation of the same enzyme. Superficially, it would seem relatively straightforward to experimentally determine the number of phosphodiesterase activities present within a given tissue, and for each of the activities to ascertain its substrate specificity. With the exception of a series of studies by Appleman et al. (182, 183, 190, 191), this has not been accomplished because of formidable difficulties encountered during separation and purification of the enzymes. These include large losses of enzyme and activity during purification and instability of PDE in dilute solutions (144, 153, 190, 194).

On the basis of kinetic evidence, Beavo, Hardman and Sutherland (144) suggested the presence of two cAMP-PDE activities (K_m ≈ 0.8, 25 µM) and one cGMP-PDE activity (K_m ≈ 20 µM) in a washed
1000 × g pellet from bovine heart. Kinetic analyses of a partially purified cytosolic preparation from the same tissue revealed PDE activity having a $K_m$ of about 20 µM for cAMP and 2 µM for cGMP. In this soluble preparation, each cyclic nucleotide exerted an apparently competitive inhibitory effect upon the hydrolysis of the other, and it seemed both were capable of being hydrolyzed by the same enzyme. Kinetic data obtained by Beavo et al. on the particulate and supernatant fractions from both murine and bovine heart were most easily rationalized by considering that there were at least two PDE's present in homogenates and that a low $K_m$ cAMP-PDE was associated with the particles. That there are apparently great differences in PDE activities among mammalian tissues was indicated by the findings of Beavo et al. that: (a) the ratio of cAMP to cGMP hydrolysis varied several fold among particulate and soluble fractions from bovine and rat heart when assayed at a 1 µM and a 1 mM substrate level, but (b) in rat liver, brain, or skeletal muscle the ratio was nearly the same at either substrate level. Of interest was the observation that in many fractions from these mammalian tissues, cGMP was hydrolyzed much more rapidly than cAMP. It is known that cAMP is present in great excess over cGMP in these tissues.

Cyclic nucleotide phosphodiesterase activities from various tissues of the rat (liver, skeletal muscle, heart muscle, kidney, epididymal fat pad, and brain cortex) have been partially resolved
without substantial purification by agarose gel chromatography
(Thompson and Appleman, ref. 191). Each of these tissues con-
tained a high molecular weight phosphodiesterase of apparent cyto-
plasmic origin which was capable of hydrolyzing both cAMP and cGMP,
with greater affinity for the latter nucleotide. The kinetics of cGMP
hydrolysis by soluble activities were "ideal" in the Michaelis-Menten
sense, whereas a double-reciprocal representation of cAMP hydroly-
sis was, for every tissue except liver, markedly nonlinear. Under
certain conditions, the hydrolysis of each substrate by soluble activity
from the brain cortex appeared to be competitively inhibited by the
other, suggesting the presence of a single enzyme capable of degrad-
ing both cyclic nucleotides. However, the biphasic kinetic plot of
cAMP hydrolysis could also be interpreted as indicating multiple
diesterase activities in this fraction. In addition to the soluble high
molecular weight enzyme, certain tissues (brain, muscle, kidney,
and adipose) contained a smaller molecular weight enzyme with a
low $K_m$ (Michaelis constant) that apparently is associated with the
plasma membrane in vivo. This latter activity appeared to be spe-
cific for cAMP hydrolysis, was noncompetitively inhibited by cGMP,
and kinetic studies suggested both regulatory and interacting substrate
sites on the molecule. No cGMP-specific diesterase was evident in
any of those tissues examined, and rat liver appeared to be unique
since it was the only organ devoid of a low $K_m$ enzyme for cAMP
It will prove instructive to illustrate the difficulties one may encounter in attempting to characterize multiple phosphodiesterase activities by briefly reviewing what has been learned in very recent studies of rat liver (Russel et al., 1973; ref. 182). This information will provide a background for interpretation and comparison of results obtained in the present study of the insect nervous system. On the basis of experiments described above (191), rat liver appeared less complicated than other tissues with respect to its PDE activities; agarose gel chromatography of rat liver supernatants performed immediately after preparation of the extract indicated only a single high M. W. enzyme capable of hydrolyzing both cyclic nucleotides ($K_m$ (cGMP) = 7 µM; $K_m$ (cAMP) = 92 µM; linear double-reciprocal plot for both enzymes). That the actual situation is in fact considerably more complex will now be indicated.

If liver supernatant were allowed to "age" at 4°C for 24 hrs before assay, an additional chromatographically-separable activity (a low $K_m$ cAMP-specific PDE) became kinetically evident from a biphasic double-reciprocal plot (182). This low $K_m$ (∼10 µM) activity observed in "aged" preparations was also found after mild trypsin treatment of a newly-prepared extract. Gel filtration data indicated the two activities (low and high $K_m$ cAMP-PDE's) were of approximately the same molecular size, and the possibility was raised that
there occurred a transition of higher \( K_m \) forms to those of lower \( K_m \).

Other workers have noted a transformation from low \( K_m \) to high \( K_m \) phosphodiesterase in the slime mold *Dictyostelium discoideum*, a process which appears to involve sulfhydryl modification (151, 177).

DEAE-cellulose chromatographic data obtained by Russel et al. (182) with sonicated soluble and particulate rat liver fractions indicate that there are at least three separable phosphodiesterase activities (I, II, III) of comparable molecular weight, and these differ greatly in their intracellular location, substrate affinity, and substrate specificity:

I. This enzyme is soluble, specific for cGMP (\( K_m = 6 \mu M \)), exhibits linear kinetics, and accounts for about 6% of the cGMP-PDE activity of the homogenates.

II. This fraction is soluble, degrades both nucleotides, represents about 50% of the homogenate activity of both, has moderate affinities for them (\( K_m [\text{cAMP}] = 40 \mu M \), \( K_m [\text{cGMP}] = 20 \mu M \)), and shows nonlinear kinetics for both cAMP and cGMP.

III. This activity is entirely particulate, specific for cAMP (\( K_m = 6 \mu M \)), and exhibits very anomalous kinetic behavior. It accounts for approximately 36% of the activity of the homogenates.

Fraction II is apparently the major activity kinetically observed in sonicated homogenates and supernatants, and has a higher affinity for cGMP than for cAMP. This activity is kinetically very complicated in that distinctly nonlinear Michaelis-Menten plots are observed, and each nucleotide acts as a noncompetitive inhibitor of the hydrolysis
of the other. Interestingly, cGMP at low concentrations serves as a potent activator of cAMP hydrolysis, but at higher concentrations inhibits cAMP-PDE activity. In contrast, cAMP at any concentration inhibits cGMP hydrolysis. The activation of cAMP-PDE by low, but not high, concentrations of cGMP has been noted by others in rat liver, brain, kidney, heart, and thymus (145), and in rat thymic lymphocytes (162). This phenomenon could not be demonstrated in a variety of preparations from the *M. sexta* CNS.

Elucidation of the apparently complex regulatory properties of phosphodiesterase(s) will ultimately depend upon separation and characterization of the various activities, but this class of enzymes has thus far proven exceptionally difficult to purify. Many workers have discovered progressive losses in enzyme activity with increasing purification of cAMP-PDE, and common preparative techniques (pH and \([\text{NH}_4\text{SO}_4]\) fractionation, ion exchange chromatography, etc.) result in very low yields (68, 153, 155, 164). Cheung (154, 155) and Teo et al. (189) established that this inactivation during purification was in part due to the loss of a specific protein activator (A) from the enzyme unit; this heat-stable glycoprotein (M. W. \(\approx 20,000\)) has been purified from bovine heart muscle to apparent homogeneity. Recombination of partially purified phosphodiesterase with the activator effectively reconstitutes activity according to Eq. 5.

\[
(5) \quad E + A \quad \underset{\text{EA}}{\longrightarrow}
\]
The reversible interaction of free activator (A) with free enzyme (E) of low activity is accompanied by an enhancement of \( V_{\text{max}} \) and a decrease of \( K_m \) for cAMP. Teo et al. (189) suggested that E and A are entirely separate proteins rather than subunits of an active enzyme, and that the equilibrium situation may be strongly dependent upon the intracellular environment. Wang, Teo, and Wang (189, 196) have shown that cAMP itself may slowly enhance the interaction between E and A, and therefore PDE may be termed a hysteretic enzyme.

The fact that cAMP can apparently modify the activity of cAMP-PDE by continuously regulating the amount of active complex (EA) formed may be physiologically significant in that elevated levels of cAMP could serve as a signal for the destruction of this nucleotide. Since the substrate-activation of PDE appears to be a slow process relative to the rate of enzyme catalysis, such hysteretic regulation would allow cAMP to perform its biochemical functions before being degraded.

In mammals, cyclic nucleotide phosphodiesterase has been found to be much more abundant in brain than in any other tissue.

Frieden ("Kinetic aspects of regulation of metabolic processes," J. Biol. Chem. 245, 5788 (1970)) has defined "hysteretic enzymes" as those which respond slowly to a change in ligand, either substrate or modifier, concentration.
For example, the specific activity of cAMP-PDE in rat brain cortex is at least ten times that of liver (67). The considerable enrichment of this enzyme in nerve ending particles (69, 153), coupled with the previously-cited observations of neurotransmitter-stimulated elevation of cAMP levels, has motivated numerous investigations of mammalian brain PDE (148, 153, 154, 169, 171, 173, 184, 190, 194, 197). With the exception of a unique electrophoretic pattern (173), no evidence is presently available which suggests any special properties of brain PDE compared to that in other tissues.

As is the case with any other tissue, there is no agreement about the number of phosphodiesterase activities in mammalian brain. Kinetic analyses have suggested two forms of the enzyme in rat brain (144, 148, 190) having $K_m$'s of approximately 5 µM and 100 µM, while agarose gel and Sephadex chromatography have disclosed either two or three forms (169, 190). Four bands of phosphodiesterase activity were detected by starch-gel electrophoresis of a soluble supernatant fraction from whole rat brain (173), but these were not isolated or characterized. Recently, fully six distinct forms of cAMP-PDE from a supernatant fraction of rat cerebellum have been separated by preparative polyacrylamide disc gel electrophoresis (194). These six forms of PDE had markedly different stabilities. No kinetic constants for the separated activities were reported, although it was
stated that no low \( K_m \) (high affinity) form was detectable in any
fraction. One may speculate that the electrophoretic process has
somehow modified phosphodiesterase activity, since a low \( K_m \) cAMP-
PDE is kinetically evident in crude preparations before separation.
Uzunov et al. (194) have speculated that the high affinity form of the
enzyme may become unstable during purification and separation from
its activator.

Cyclic nucleotide phosphodiesterase appears to be an inducible
enzyme. This was first established by D'Armiento et al. (159) in
cultured mouse fibroblasts. In normal or SV40-transformed cells,
cAMP-PDE activity could be stimulated many-fold over a 48 hour
period by treatment of the cultures with \((\text{but})_2\) cAMP and theophylline.
The experiments on PDE induction in 3T3 fibroblasts indicated that
the accumulation of enzyme began quickly after stimulation of cAMP
levels, and that both new RNA and protein synthesis were required.
Since \((\text{but})_2\) cAMP and theophylline markedly elevate cAMP levels
through inhibition of PDE, it was suggested that cAMP itself functions
as an inducer of the enzyme. The findings of D'Armiento et al. are
firmly supported by the more comprehensive experiments of Uzunov,
Shein, and Weiss (193) on cloned rat C-2A astrocytoma cells. These
workers demonstrated that a six hour incubation of these cells with
norepinephrine, which is known to elevate the intracellular level of
cAMP, resulted in a three-fold increase in one of the two
electrophoretically separable forms of cAMP-PDE present in the C-2A glial cell line. The over-all increase in PDE activity was relatively small, and unfortunately no kinetic parameters or substrate specificities of the stimulated PDE were reported.

There has appeared one other report in the recent literature by Schwartz et al. (186) relating to PDE induction, namely that stimulated in the C-6 rat glial tumor cell line by 5-bromodeoxyuridine or (but)$_2$cAMP. After two days of culture in the presence of (but)$_2$cAMP, there resulted in these cells an approximate four-fold increase in cAMP-PDE. Apparently, both PDE activities present in this cell line were induced by the elevated cAMP levels. The possibility of early induction of one form, followed by transformation to another, was not examined. Evidence will be presented demonstrating that PDE in the M. sexta CNS also appears to be an inducible enzyme. In contrast to the mammalian systems described above, this process in the insect nerve cord seemingly does not require protein synthesis and may be due to an activation of pre-existing enzyme.

Materials and Methods

Materials

Tritium-labeled cAMP and cGMP were purchased from New England Nuclear and purified by thin-layer chromatography on
cellulose as described in Appendix I. Spectrophotometrically standardized solutions of unlabeled cAMP and cGMP in neutral 10 mM Tris·HCl buffer were mixed with the appropriate amount of lyophilized, tritiated nucleotide to yield \(5.0 \times 10^{-4}\) M stock solutions of the following specific activities: cAMP, 400 to 600 dpm/pmol; cGMP, 100 to 200 dpm/pmol. The specific activities were determined both by liquid scintillation counting (LSC) of a measured volume of the stock solution and also by LSC of the nucleoside, 5'-nucleotide, and cyclic nucleotide spots obtained after paper chromatographic separation of the phosphodiesterase assay mixture (see "Assay II"); the specific activities thus determined always agreed within 2%.

Anion exchange resin (Bio-Rad Laboratories AG1-X2, 200 to 400 mesh) was washed twice in 0.5 N NaOH, acetone, and 0.5 N HCl, and finally rinsed extensively with glass-distilled water to pH 6. The resin (essentially free of fines) was stored as a 1:2 slurry in glass distilled water. Snake venoms (Ophiophagus hannah, king cobra; Crotalus atrox, western diamondback rattlesnake) were obtained from Sigma and prepared as 1 mg/ml solutions in water.

4-(3, 5-dimethoxybenzyl)-2-imidazolidinone (Ro20-2926), a synthetic phosphodiesterase inhibitor, was kindly provided by Dr. Herbert Sheppard of Hoffman-La Roche, Department of Pharmacology. 1-ethyl-4-(isopropylidenehydrazino)-1H-pyrazolo-(3, 4-b)-pyridine-5-carboxylic acid, ethyl ester (SQ20009), also a phosphodiesterase
antagonist, was supplied by Dr. Sidney Hess of the Squibb Institute for Medical Research.

**Cyclic AMP Phosphodiesterase Assays**

Two standard assay systems were routinely employed to estimate cAMP phosphodiesterase activity. The first of these, "Assay I," was used in situations not requiring extreme accuracy but only relatively rapid, semi-quantitative estimations, whereas the second more laborious procedure, "Assay II," was utilized to obtain an accurate measure of diesterase activity. Total incubation volumes ordinarily comprised 0.1 ml, but occasionally it was necessary to halve this volume in order to conserve the limited amount of enzyme protein available.

**Assay I**

This assay consists of a modification of the two-step methods of Butcher and Sutherland (67) and Thompson and Appleman (190) and is summarized in Table 13. In the first stage of the reaction $[^3H]$ cAMP is hydrolyzed to $[^3H]$ 5'-AMP by tissue phosphodiesterase; a portion of the 5'-AMP so formed may be further degraded to adenosine and phosphate by endogenous 5'-nucleotidase activity present in the tissue extract. In the second stage of the reaction, 5'-AMP is further converted to adenosine and phosphate by a potent 5'-nucleotidase
Table 13. Protocol for phosphodiesterase assay ("Assay I").

**STEP 1**

- Tris·HCl, 50 mM, pH 7.5
- MgSO₄, 5 mM
- DTT, 1 mM
- [³H] cAMP (ordinarily 50 µM for standard assays)
- ± test substances
- Enzyme (pre-incubated in absence of cAMP)
  - 0.10 ml reaction volume
  - Incubate 10 min at 30°C
  - Boil 3 min, cool

**STEP 2**

- + 0.1 ml of 1 mg/ml snake venom (containing 5′-nucleotidase)
- Incubate 30 min at 30°C
- + 1.0 ml of 1:2 slurry of AG1-X2 anion exchange resin
- Centrifuge 1,000 x g for 5 min
- Withdraw 0.10 ml aliquot of supernatant for scintillation counting
(5'-ribonucleotide phosphohydrolase; E. C. 3.1.3.5) present in the added snake venom. The stage 1 reaction mixture consisted of 50 mM Tris·HCl (pH 7.5), 5 mM MgSO₄, 1 mM DTT, various concentrations of [³H]cAMP as specified in the figure legends, and an appropriate quantity of enzyme in a total volume of 0.1 or 0.2 ml. The volume of enzyme protein was at most one-tenth to one-fifth of the total incubation volume, thus minimizing the perturbation of the system's buffering capacity. Miscellaneous test substances were incorporated in the reaction mixture wherever specified. Relative levels of enzyme and substrate were chosen to produce less than 30% conversion of cAMP to 5'-AMP; in many cases less than 15% of the substrate was consumed at the end of the incubation period.

After equilibration of the enzyme for 10 min at 30°C in the reaction mixture (minus substrate), the reaction was initiated by the addition of [³H]cAMP and further incubated in a shaking water bath. After exactly 10 min at 30°C the reaction was terminated by boiling the incubation tubes for 3 min. An aluminum block containing wells (Scientific Products Temp-Blok) maintained at 100°C proved invaluable for this purpose and was much more convenient than a boiling water bath, primarily because of the greatly reduced hazard of spattering water into the tubes and creating an uncertainty in volume.

8A substrate concentration of 50 µM was frequently employed for routine assays.
The condensate which formed on the walls of the reaction tubes as a result of boiling was coaxed down into the vessel with a gentle stream of nitrogen gas, insuring a constant volume for each reaction mixture.

In the second stage of the assay procedure, 100 μg of snake venom (Ophiophagus hannah) in 0.1 ml of H₂O was added to the cooled stage reaction vessels, well mixed, and incubated for another 30 min at 30 °C to effect quantitative conversion of 5'-AMP to adenosine. The 5'-nucleotidase activity of the venom was at least 100 μmoles/mg/hr under the conditions employed. This latter reaction was terminated by the addition of 1.0 ml of 1:2 slurry in H₂O of AG1-X2 anion exchange resin, which preferentially binds all charged nucleotides, thus largely removing the unreacted [³H] cAMP substrate and leaving [³H] adenosine in the aqueous phase. The slurry was agitated at room temperature for a minimum of 10 min, and after centrifugation at 1000 × g for 5 min, an aliquot (0.1 ml) of the supernatant was taken for liquid scintillation counting (LSC) in 10 ml of Aquasol.

Whenever sufficient enzyme was available, the assays were performed at least in duplicate, under conditions in which the reaction rate was linear with respect to both time of incubation and concentration of protein. Blanks for each experiment (ordinarily performed in triplicate) were of the following nature: (i) omission of enzyme, (ii) heat-inactivated enzyme, or (iii) addition of [³H] cAMP
substrate after the stage I incubation. All data were corrected for the averaged blank value, and a knowledge of (1) the ratio of final effective aqueous assay volume/volume of sample measured by LSC, (2) the specific activity of substrate (dpm/pmol), (3) the counting efficiency of the LSC system (determined by internal standardization with $[^3H]$ toluene), and (4) the protein content of the sample,$^9$ allowed a calculation of apparent specific enzyme activities. The fact that 1.0 ml of resin slurry contains 0.68 ml (experimentally determined) of $H_2O$ is integral to this calculation. As will be discussed later, Assay I provides minimal (too low) estimates of diesterase activity; therefore specific enzyme activities obtained by this method should be regarded as apparent picomoles of cAMP hydrolyzed per mg of protein per minute under standard assay conditions. 

Assay II

The composition of the reaction mixture for this chromatographic assay procedure is identical to that described for step 1 in "Assay I" (Table 13). After 10 min of incubation at 30° C, the 100 μl assay solution was boiled for 3 min, cooled, and 20 μl of a carrier solution containing 0.01 to 0.05 μmole each of cAMP, $5'-AMP$, and adenosine was added. A suitable aliquot (ordinarily 25 μl) of the resultant mixture was spotted on Whatman No. 1 (chromatography grade) paper and evaporated almost to dryness with a draft of warm air. For the sake of...
of convenience, boiled reaction mixtures were sometimes stored as long as two weeks at -20°C before spotting on paper; in some experiments it was advantageous to terminate the diesterase reaction by rapidly freezing the mixtures in liquid nitrogen, followed by the addition of carrier before the boiling step. Results obtained by these various procedures were entirely commensurate.

Chromatograms were developed in a closed equilibrated chamber for 16-24 hours in an ascending fashion at room temperature using 1.0 M ammonium acetate-95% ethanol (3:7, v/v). Observed $R_f$ values were 0.19 for 5' -AMP, 0.48 for cAMP, and 0.65 for adenosine. The spots were visualized under UV light, cut into many small pieces, and transferred into culture tubes containing 3.0 mls of 0.01 N HCl. After incubation with frequent agitation for 15 min at approximately 80°C, an aliquot of the 0.01 N HCl extract was withdrawn for LSC in 15 mls of Aquasol. Better than 99% of the radioactivity in the spots could be extracted by this simple one-step procedure.

In addition to 5' -AMP, a non-negligible amount of $[^3H]$ adenosine was formed when crude phosphodiesterase preparations were assayed, and it was necessary to measure the radioactivity in both products to accurately gauge diesterase activity. It will be shown that the 5'-nucleotidase activity is primarily particulate in the Manduca CNS and that an appreciable fraction (in some cases, as
great as 50%) of the 5'-AMP diesterase product may be hydrolyzed
to adenosine in the course of the standard assay. At a substrate
level of 50 μM, it was determined that conversion of label into
adenosine could be totally suppressed by inclusion of 0.1 mM 5'-AMP
in the incubation medium. This was not routinely done in the present
research, as an inhibition of both cAMP-PDE and cGMP-PDE activi-
ties by 5'-AMP could be demonstrated.

Control tubes (without enzyme) were incubated with each set of
experimental tubes, thereby establishing a blank correction for both
the 5'-AMP and adenosine spots. For all samples assayed, the sum
of the radioactivities observed in the 5'-AMP, adenosine, and (unre-
acted) cAMP sections of the chromatogram were equal to the radio-
activity of the [3H]cAMP initially added; thus a stoichiometric con-
version of cAMP to 5'-AMP and adenosine is indicated, and very little
conversion to other radioactive products occurs under the conditions
employed.

Units of diesterase activity are defined as the sum of the pico-
moles of 5'-AMP plus picomoles of adenosine formed in 1 minute at
30°C, and specific enzyme activities are reported as units per mg
of protein.

Cyclic GMP Phosphodiesterase Assays

Cyclic GMP phosphodiesterase activity was quantitated via the
two assay systems described for cAMP-PDE, utilizing identical
incubation conditions. For paper chromatographic separations, .05
µmole each of cGMP, 5'-GMP, and guanosine were added to the incu-
bation mixture before application to Whatman No. 1 paper. A variety
of solvent systems were examined for optimal resolution of the three
compounds, but none proved as satisfactory as the ethanol-ammonium
acetate mixture described for cAMP-PDE. The observed \( R_f \) values
in this system were 0.12 for 5'-GMP, 0.40 for cGMP, and 0.58 for
guanosine; a very slight tailing of the guanosine spot occasionally
occurred but proved of little consequence. As was the case for
5'-AMP-phosphohydrolase activity, 5'-GMP-phosphohydrolase activ-
ity was non-negligible and found primarily in particulate fractions,
thus necessitating quantitation of both \([^3\text{H}]5'\text{-GMP}\) and \([^3\text{H}]\) guanosine
to accurately measure cGMP phosphodiesterase activity.

In all cases the observed total radioactivities in the 5'-GMP,
cGMP, and guanosine areas of the chromatogram were equivalent to
the activity of the \([^3\text{H}]\) cGMP added; therefore no detectable conver-
sion to other metabolites was indicated. Units and specific activities
for cGMP-PDE are those defined for cAMP-PDE, always taking the
sum of 5'-GMP and guanosine as the correct measure of cGMP-PDE.
It is unfortunate that a simultaneous determination of both cAMP- and
cGMP-diesterase activities was not possible using the paper chro-
matographic method (Assay II) due to the close proximity and over-
lapping of spots.
General Comments on Phosphodiesterase Assays

Boiling for three minutes sufficed to completely destroy both cAMP-and cGMP-phosphodiesterase activities, and in no instance did a heat-killed blank differ significantly from one obtained in the absence of enzyme. For unexplained reasons, chromatographic blank values were somewhat lower when carrier was added to the reaction before the boiling step, rather than adding carrier after the boiling step. It was desirable to re-purify the \[^{3}\text{H}]\text{cAMP}\) and \[^{3}\text{H}]\text{cGMP}\) stock solutions (see Appendix I) monthly or whenever the total nucleoside + 5'-nucleoside phosphate content approached 1% of the apparent cyclic nucleotide titre. After much of this research was completed, a somewhat faster and superior chromatographic technique for separation of cGMP, 5'-GMP, and guanosine were found (200), namely descending development with 1.0 M ammonium acetate (pH 7.5)-95% ethyl alcohol (2:5, v/v). This method was used only once, and its main advantages are reduction of development time and guanosine-tailing; in other respects it is equivalent to the entirely satisfactory development technique already described.

As noted previously, the more time-consuming chromatographic Assay II was used wherever practicable in preference to the resin method, Assay I. This was principally because of the greatly increased accuracy, sensitivity, and reproducibility of the former
method, and also because more information could be obtained (estimates of both diesterase and nucleotidase activities). Replicate samples assayed by the paper chromatographic method frequently agreed within 0.5% or less, whereas those measured by Assay I often varied 5 to 10%. Although substantial information on mammalian diesterase utilizing the resin method has been reported in the literature (182, 183, 190, 191), considerable caution should be exercised in comparing results obtained by this technique with those acquired by more accurate methods. When newly thin-layer chromatographed cyclic nucleotide (99.9% pure) was used at a 50 μM substrate level, the minimal observed backgrounds (expressed as a percent of the total substrate present) were 4.4% for cAMP and 19.2% (!) for cGMP. When one considers that only 10-30% of the substrate is consumed during a standard assay, the very serious limitations of Assay I become apparent. For comparison, maximal 5' -nucleotide backgrounds observed using Assay II were 0.36% and 0.40% for cAMP and cGMP, respectively. The time- and labor-saving features of Assay I are most attractive, and for this reason considerable effort was expended attempting to perfect the method, with disappointing results. No reduction in background could be effected with any of the following variants: substitution of miscellaneous other anion exchange resins for AG1-X2, increasing the amount of resin three-fold, longer incubation time with resin, lower
and higher incubation temperatures, or slowly passing the reaction mixture through a column of resin followed by elution with water.

Purified 5'-nucleotidase was substituted for *Ophiophagus hannah* venom in some experiments, but no increase in sensitivity or reduction in background resulted from this modification. In summary, estimates of diesterase activity obtained with the resin method ranged for three- to seven-fold too low.

**Collection of Non-neural Tissues**

For comparative purposes, phosphodiesterase activities were examined in a variety of *Manduca* tissues, viz. larval fat body, haemolymph, muscle, gut, and CNS; additionally, adult flight muscle and adult CNS were assayed for both cAMP-PDE and cGMP-PDE. Newly emerged adults and fifth-instar larvae were selected without regard to sex and chill-immobilized before dissection.

Intact larval fat bodies were collected by vacuum aspiration into 0.32 M sucrose buffered with 50 mM Tris·HCl (pH 7.5), and after centrifugation at 10,000 × g for 10 min, the floating layer was collected for homogenization. The central portion of the larval gut was dissected and transferred to a petri dish, cut longitudinally and rinsed exhaustively with cold saline. Every effort was made to free the mid-gut from extraneous tissues, but it was impossible to remove the finely-enmeshed network of nerve fibers. Larval muscle
was obtained by scraping the extensively-washed body wall with a fine scalpel; these fibers were rinsed once before homogenization. Larval haemolymph was procured from a small dorsal puncture, care being exercised to avoid contamination from fat bodies; the haemolymph was allowed to drip into a small container on ice and was quickly diluted with two volumes of ice-cold buffer. Small bundles of adult dorsal flight muscle fibers were excised from the thorax and rinsed extensively with cold saline. Additionally, both larval and adult CNS tissues were dissected in cold saline, rinsed briefly in distilled water, and temporarily stored on a microscope slide placed on dry ice.

Each tissue was homogenized thoroughly in 50 mM Tris–HCl (pH 7.5)-1 mM MgSO₄ with an all glass tissue grinder (motor-driven pestle), followed by centrifugation of a portion of the homogenate at 34,000 × g for 30 min. Both the homogenates and supernatant fluids were examined for cAMP-PDE and cGMP-PDE at a 50 μM substrate level according to Assay II. The results of this experiment are presented in Table 14.

CNS Preparations

The complete central nervous systems of larval and adult animals were dissected as described in General Methods. In view of the very high diesterase activity in *Manduca* CNS, pilot experiments
could be performed using only 1-3 nerve cords; in fact, in one experiment a single larval brain was assayed. However, considerably larger amounts of tissue were required for most experiments in which sufficient protein was needed for 10-20 diesterase determinations in addition to a Lowry protein estimation (for calculation of specific enzyme activities). A relatively long time period was required for collection of 20-60 nerve cords, and to guard against deterioration of the tissue upon removal from the animal, it was deemed important to freeze each nerve cord on dry ice immediately after dissection. This freezing step before homogenization had no apparent deleterious effect on gross diesterase activities, but it is possible that the subcellular distribution of diesterase was influenced by this freeze-thaw cycle.

Homogenization was generally performed in all-glass micro tissue grinders in either (a) 0.32 M sucrose-10 mM Tris·HCl (pH 7.5)-1 mM MgSO₄ (hypertonic buffer) or (b) 10 mM Tris·HCl (pH 7.5)-1 mM MgSO₄ (hypotonic buffer). Depending upon the experiment, 1 mM DTT was sometimes included in either buffer. It was found that measurable diesterase activity, particularly cAMP-PDE, was a function of the osmolarity of the homogenizing buffer. All operations (dissection, homogenization, centrifugation, and assay) were performed as rapidly as possible. Enzyme material not needed for immediate assay was divided in small, pre-measured aliquots.
into a number of culture tubes, rapidly frozen in liquid nitrogen, and stored at -20°C.

In view of reports (67, 68, 198) that cyclic nucleotide phosphodiesterase is particularly abundant in mammalian brain (relative to non-neural and peripheral nervous tissues), it was of interest to compare the gross diesterase activity of larval brain with the activities of the remaining ganglia.

The entire CNS from each of 20 larvae was removed from the animal, freed of extraneous tissue, and placed in ice-cold 0.32 M sucrose buffered with neutral 10 mM Tris. Exercising extreme care to avoid crushing the ganglia, all lateral fibers and interganglionic connectives were trimmed away as close to the neural mass as possible. Corresponding ganglia were pooled in small vessels placed on dry ice, and after all nerve cords had been partitioned (approx. 4 hrs), each composite sample was transferred without thawing to an all-glass micro tissue grinder. Homogenization of each sample was conducted in exactly 200 µl of a hypotonic buffer (10 mM Tris, pH 7.5-1 mM MgSO₄) for one minute, followed by slow magnetic stirring on ice for five minutes. The total homogenate volume was accurately measured with a calibrated capillary pipette, and two 10 µl aliquots from each sample were saved for protein determination. The distribution of cAMP-PDE and cGMP-PDE among larval CNS ganglia is presented in Table 23 and Figure 30.
To investigate the particulate-soluble nature of PDE activities in larval brain, an homogenate was prepared in hypertonic sucrose solution, followed by sedimentation and examination of the pellet and supernatant for PDE activity. Unfortunately, there was available insufficient material to readily conduct a comprehensive subcellular fractionation, as has been done using the entire larval CNS as starting material. Larval nerve cords were dissected in 10 mM Tris·HCl (pH 7.5)-.32 M sucrose-1 mM DTT (Buffer A), followed by removal of the brains and temporary storage on dry ice. After 18 brains had been collected, they were vigorously homogenized in an all-glass micro tissue grinder in 150 µl of Buffer A. The volume of the homogenate was measured, and samples were withdrawn for protein and PDE activity determinations. One aliquot of the homogenate was incubated at 0°C with 0.5% Triton X-100 for 10 min before assay to investigate possible unmasking or solubilization of particulate enzyme activities. The remaining homogenate was centrifuged in the cold at 34,000 x g for 30 minutes, and the supernatant was removed with an Hamilton syringe. The pellet was gently rinsed with Buffer A and the wash discarded, followed by resuspension of the pellet in 0.5 ml of the same buffer. After another centrifugation at 34,000 x g for 30 minutes, the supernatant was discarded and the once-washed pellet suspended in 100 µl of Buffer A. All fractions were assayed immediately upon collection according to Assay II. Experimental data are
shown in Table 24 and Figure 31.

**Preparation of Subcellular Fractions from the Larval CNS**

Fifty-five larval nerve cords were dissected over a three-day period, pooled, and stored at -80°C. They were dispersed in a pre-cooled Teflon-glass tissue homogenizer (Kontes Duall 20) in 1.2 mls of ice-cold 10 mM Tris·HCl (pH 7.5) - .32 M sucrose - 1 mM MgSO₄ - 1 mM DTT (Buffer A). After vigorous homogenization by motor-driven pestle for 30 seconds, the homogenate was centrifuged at 700 X g for 10 minutes in the tissue grinder and the supernatant was carefully pipetted away. The pellet was resuspended by homogenization in one volume (equal to that originally added) of Buffer A and centrifuged again under the same conditions. The following particulate fractions were collected after centrifugation in Buffer A at the specified forces and times: nuclear fraction, 700 X g for 10 min (.7N10); mitochondrial fraction, 10,000 X g for 20 min (10 M 20); microsomal fraction, 105,000 X g for 60 min (105 µ 60). All g-values are referenced to the bottom of the tube, and all centrifugations (except the initial sedimentation) were performed at 0-4°C in a polycarbonate screw-capped centrifuge tube blanketed with nitrogen gas to retard possible oxidation. The volume of each pooled supernatant was accurately measured with a 1 in 1/100 glass pipette and a Hamilton syringe.
Every particulate fraction, with the exception of microsomal pellet, was washed once by thorough suspension (small magnetic stirring bar) in 0.5 to 1.0 volumes of Buffer A, then centrifuged again at the same force and for the same time, the wash supernatant being pooled with that initially collected. The crystal clear post-microsomal supernatant was termed the soluble fraction, S. After gently rinsing the walls of the vessel with Buffer A and discarding the wash, each particulate fraction was suspended in 1 volume of 10 mM Tris·HCl (pH 7.5) - 1 mM MgSO$_4$ - 1 mM DTT by vigorous magnetic stirring for 5 minutes on ice. Immediately after collection, each sample was assayed for cAMP-PDE and cGMP-PDE (Assay II 50 μM substrate) without further delay. An aliquot of each sample was analyzed in duplicate for protein content, permitting a calculation of specific enzyme activities for the soluble and particulate fractions. Data are presented in Table 26 and Figure 32.

Ammonium Sulfate Fractionation

A 10,000 X g supernatant fraction served as the starting material for a simple one-step ammonium sulfate precipitation. Directions are provided for a 60 nerve cord preparation, although in one instance the procedure was carried out with only 10 nerve cords, with a proportional diminution of the volumes specified. Sixty larval nerve cords were homogenized vigorously for 5 min with intermittent cooling.
in 3.0 mls of 50 mM Tris·HCl (pH 7.5) - 10% (w/v) sucrose - 1 mM MgSO$_4$ - 1 mM DTT in an all-glass tissue grinder. The homogenate was stirred on ice for 5 min and centrifuged at 10,000 × g for 10 min. The supernatant was transferred to another vessel on ice, and an equal volume of 100% saturated (at room temperature) of ammonium sulfate was added dropwise over a 3 min period. The mixture was gently stirred on ice for 30 min then allowed to stand an additional 15 min in the cold. The resulting precipitate was collected by centrifugation at 20,000 × g for 20 min, whereupon it was suspended in 3.0 mls of ice-cold 20 mM Tris·HCl (pH 7.5) containing 1 mM each of MgSO$_4$ and DTT. Both the supernatant and suspended pellet were dialyzed for 16 hours in the cold against 4 liters of 10 mM Tris·HCl (pH 7.5) - 1 mM MgSO$_4$ - 0.1 mM DTT. This procedure was altered slightly for experiments designed to investigate bivalent metal ion requirements of the enzyme(s), in that magnesium was omitted from all buffers.

After dialysis, the slightly turbid solution was centrifuged at 10,000 × g for 10 min, and the precipitate was discarded. The supernatant was apportioned into a number of small aliquots, frozen in liquid nitrogen, and stored at -20°C until use. This preparation will be termed the "(0-50) AS" fraction. A very light flocculence resulted upon thawing the (0-50)AS solution, but it was not removed by centrifugation before assay of this fraction.
Activity Stain for Phosphodiesterase in Polyacrylamide Gels

The development of sensitive and reliable techniques for the detection of phosphodiesterase in polyacrylamide gel matrices after electrophoresis has, with one exception (163), been relatively neglected in the literature. Such techniques would permit rapid estimations of the number and relative levels of activities in different samples and would help establish the presence of tissue-specific phosphodiesterases. The method adopted for the present work is a composite of the methods of Shanta et al. (184) for the histochemical localization of cAMP-PDE, and of Goren et al. (163) for the detection of PDE in acrylamide gels. As developed, the gel staining technique is apparently not sufficiently sensitive to permit detection of very small quantities of PDE in crude preparations, but probably would prove quite powerful for the study of purified enzyme, if such were available. Very large quantities of M. sexta protein were required to permit satisfactory visualization of diesterase activities.

If polyacrylamide gels containing PDE are incubated in the presence of cAMP and a source of 5'-nucleotidase, the 5'-AMP produced by diesterase is further converted to adenosine and phosphate. The phosphate may then react in the gel with lead acetate to produce a relatively insoluble white band of PbHPO$_4$$_2$, and this may in turn be converted to a more readily visualizable brown band of PbS by
treatment with (NH₄)₂S. Cyclic GMP-PDE may similarly be localized by incubation of the gel strip with cGMP instead of cAMP. The success of this method is dependent upon diffusion of cyclic nucleotide, nucleotidase, and Pb²⁺ into the gel matrix before appreciable diffusion (band broadening and loss of activity from the gel) of phosphodiesterase can occur.

Polyacrylamide gel electrophoresis with and without a stacking gel was performed as described in General Methods. Gel strips were never handled without using gloves, as it was found that a single fingerprint was sufficient to stain the outer surface of the acrylamide strip. Immediately after electrophoresis, gels were rinsed briefly in cold distilled water and transferred to a freshly-prepared reaction mixture containing 50 mM Tris-maleate (pH 7.5), 10 mM MgSO₄, 10 mM cyclic nucleotide, 2 mM lead acetate, and a source of nucleotidase; all reagents were prepared in glass-distilled H₂O and efforts were made to avoid any possible source of phosphate contamination, which would result in serious interference. Various sources of nucleotidase activity were tested for optimal activity staining: 0.5 mg of snake venom (Ophiophagus hannah, or Crotalus atrox), 5 units (Sigma) of alkaline phosphatase, or 5 units (Sigma) of purified 5'-nucleotidase were incubated with each gel strip in a total volume of 5 mls. Incubation times ranged from 10 min to 1 hour at 25°C. After incubation, the gel slices were stirred in 4 liters of ice-cold
glass-distilled H$_2$O for 1 hour (to diffuse unprecipitated Pb$^{2+}$ out of the gel), then placed in 5% (NH$_4$)$_2$S for varying periods of time (1-60 minutes). Excess (NH$_4$)$_2$S was removed by stirring the gel in cold H$_2$O, and then the gels were examined for activity bands on a light table and also under a microscope.

When sufficient staining was evident, the gels were scanned at 500 nm using a Gilford linear transport. Replicate gel slices were stained for protein with 0.05% Coomassie Blue, and others were cut into small sections with a scalpel, macerated with a glass rod, and incubated with [³H] cyclic nucleotide to directly measure PDE activity according to Assay I.

Results and Discussion

Tissue Distribution of Activity

Both cAMP- and cGMP-PDE activities were found in every M. sexta tissue examined (Table 14). The ratio of cGMP to cAMP activity varied greatly among the tissues, as did the apparent soluble-particulate distribution of activity. By far the greatest specific activities were found in larval and adult CNS preparations. It may be noted that both CNS samples revealed high levels of both cAMP-PDE and cGMP-PDE, whereas larval and adult muscle preparations contained predominantly only cAMP-PDE. A priori, one must
Table 14. Phosphodiesterase activities in various *M. sexta* tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity units/reaction mixture</th>
<th>Specific activity units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP-PDE</td>
<td>cGMP-PDE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cAMP-PDE</td>
</tr>
<tr>
<td>Larval CNS, a homog.</td>
<td>248.0</td>
<td>316.1</td>
</tr>
<tr>
<td>&quot; CNS, sup.</td>
<td>162.7</td>
<td>253.8</td>
</tr>
<tr>
<td>&quot; fatbody, homog.</td>
<td>73.3</td>
<td>142.3</td>
</tr>
<tr>
<td>&quot; fatbody, sup.</td>
<td>13.8</td>
<td>12.2</td>
</tr>
<tr>
<td>&quot; haemolymph, homog.</td>
<td>70.0</td>
<td>164.2</td>
</tr>
<tr>
<td>&quot; haemolymph, sup.</td>
<td>78.9</td>
<td>177.1</td>
</tr>
<tr>
<td>&quot; gut, homog.</td>
<td>78.3</td>
<td>50.2</td>
</tr>
<tr>
<td>&quot; gut, sup.</td>
<td>67.4</td>
<td>62.0</td>
</tr>
<tr>
<td>&quot; muscle, homog.</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>&quot; muscle, sup.</td>
<td>50.4</td>
<td>10.0</td>
</tr>
<tr>
<td>Adult CNS, b homog.</td>
<td>268.5</td>
<td>268.9</td>
</tr>
<tr>
<td>&quot; CNS, sup.</td>
<td>173.4</td>
<td>135.6</td>
</tr>
<tr>
<td>&quot; muscle, homog.</td>
<td>182.1</td>
<td>37.1</td>
</tr>
<tr>
<td>&quot; muscle, sup.</td>
<td>79.8</td>
<td>8.2</td>
</tr>
</tbody>
</table>

*\(^a\) seven nerve cords were homogenized

*\(^b\)five nerve cords

Tissue homogenates and 34,000 x g 30 min supernatants were prepared as described in Methods and assayed in the presence of 50 uM substrate (Assay II). Comparison of the relative activities of homogenate and supernate (equal volumes were assayed) in a given tissue is indicative of the fraction of enzyme activity that is sedimentable under the conditions employed. One unit of activity is equal to one picomole of substrate hydrolyzed per min per mg protein. Diesterase activities (units/reaction mixture) are not intercomparable among the tissues, as different amounts of protein were analyzed. Homogenate specific activities were determined only for the CNS preparations.
consider that the ratio of the two hydrolytic activities in a given tissue, and the relative levels of these activities among different tissues may strongly depend upon the assay conditions adopted (pH, ionic environment, substrate concentration, etc.); it will be seen that CNS activity in particular is relatively pH-sensitive and that the ratio of cGMP/cAMP hydrolyses is a function of substrate level. Since all tissues were assayed under conditions found especially suitable for Manduca CNS preparations, there exists the possibility that different conditions of assay would reveal altered activity patterns. For example, enzymes with Michaelis constants much greater than the substrate concentration employed (50 μM) would not be adequately saturated. With these cautions in mind, one may nonetheless draw the provisional conclusion that both the larval and adult central nervous systems appear greatly enriched in PDE activities relative to the other tissues. Whether or not these CNS activities are tissue- and function-specific remains to be examined.

Miscellaneous Properties of Larval CNS Phosphodiesterase

Larval nerve cords were dissected and frozen on dry ice, and after 15 had been collected, the tissue was thoroughly homogenized in 0.4 ml of 10 mM Tris·HCl (pH 7.5) - 1 mM MgSO₄ (hypo-tonic buffer) in an all-glass tissue grinder. Completeness of
homogenization was tested by microscopic visualization of a portion of the homogenate. The homogenate was diluted with cold buffer to a final protein concentration of 2.56 mg/ml and agitated on ice for 5 minutes with a small magnetic stirrer. A sample of this homogenate was immediately assayed (for construction of Lineweaver-Burk plot; see Figure 23 and Figure 24). While this experiment was being conducted, the remaining homogenate was stored on ice in a stoppered vessel. Approximately 40 minutes elapsed between the time of homogenization and initiation of the second set of assays. The effects of different treatments are shown in Table 15.

A significant difference in cAMP-PDE activity was noted between the sample assayed immediately after homogenization and the sample assayed approximately 40 minutes later. It appears that a fraction of the cAMP-PDE activity is unstable, or perhaps is converted to an inactive form, since there is an apparent 13% loss of activity during 40 minutes of storage on ice. In contrast to this effect, there was no appreciable difference in cGMP-PDE activity in the two samples. To examine whether the decrease in cAMP-PDE activity might be due to selective attack of proteolytic enzymes in the homogenate upon the cAMP-diesterase, a sample of homogenate (256 μg protein) was incubated at 30°C for 5 min with 2.5 μg of trypsin, followed by neutralization with 10 μg of soybean trypsin-inhibitor before assaying for both diesterase activities. As shown in Table 15, virtually no loss in
Table 15. Phosphodiesterase activities after various treatments of a larval CNS homogenate.

<table>
<thead>
<tr>
<th>Sample (15 nerve cords)</th>
<th>Protein assayed (ug)</th>
<th>p mol in 10 min per assay</th>
<th>Specific activity pmol/min/mg</th>
<th>Percent of specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5'-AMP + Adenosine</td>
<td>5'-GMP + Guanosine</td>
<td>cAMP-PDE</td>
</tr>
<tr>
<td>Homogenate+</td>
<td>51.2</td>
<td>3079</td>
<td>3166</td>
<td>6014</td>
</tr>
<tr>
<td>10,000 x g 10 min supernatant</td>
<td>47</td>
<td>1728</td>
<td>2518</td>
<td>3677</td>
</tr>
<tr>
<td>10,000 x g 10 min supernatant - Triton X-100a treated</td>
<td>42.7</td>
<td>1546</td>
<td>2261</td>
<td>3620</td>
</tr>
<tr>
<td>Homogenate* (control)</td>
<td>51.2</td>
<td>2393</td>
<td>3058</td>
<td>4674</td>
</tr>
<tr>
<td>&quot; - Triton X-100a</td>
<td>46.6</td>
<td>2807</td>
<td>3694</td>
<td>6030</td>
</tr>
<tr>
<td>&quot; - Sonicate b</td>
<td>51.2</td>
<td>2430</td>
<td>3081</td>
<td>4746</td>
</tr>
<tr>
<td>&quot; - freeze-thaw 3x c</td>
<td>51.2</td>
<td>2591</td>
<td>3361</td>
<td>5060</td>
</tr>
<tr>
<td>&quot; - heat 50°C 10 min</td>
<td>51.2</td>
<td>2291</td>
<td>2975</td>
<td>4475</td>
</tr>
<tr>
<td>&quot; - Trypsin</td>
<td>46.6</td>
<td>2096</td>
<td>2765</td>
<td>4497</td>
</tr>
</tbody>
</table>

+ Assayed immediately after homogenization (compare with *).

* This sample, and all entries below it, were assayed approximately 40 min after homogenization.

a A sample of enzyme protein was incubated on ice for 10 minutes with 0.50% (v/v) Triton X-100 before assay; the final Triton concentration in the incubation mixture was 0.50%.

b Kontes K-881440 with microprobe, setting 6; 3 periods of sonication, 10 seconds each, for every 0.10 ml of solution.

c Quick frozen in liquid nitrogen followed by slow thawing to room temperature; 3 freeze-thaw cycles.

Assays were conducted as described in the text (Assay II, 50 uM substrate, 0.2 ml reaction mixture).
either activity resulted from this treatment.

From a comparison of total apparent activities in the 10,000 × g supernatant and the homogenate, it was calculated that approximately 56% and 49% of the cAMP- and cGMP-diesterase activities, respectively, were associated with particulate fractions. That a significant portion of the sedimentable activity may be "unmasked" is shown in Table 15. Three freeze-thaw cycles, presumably by disrupting subcellular membranes, increase both activities by about 10% relative to the untreated homogenate. Brief sonication or heating at 50°C for 10 minutes are without pronounced effect. Most notably, incubation of the homogenate on ice for 10 min with the non-ionic detergent Triton X-100 (0.50%) prior to assay results in close to a 30% increase in both activities. Triton was totally without effect on soluble cAMP-PDE and and cGMP-PDE. One is led to the conclusion that Triton is acting by solubilization of membrane- or organelle-associated activities, but it does not influence the soluble activities. The particulate/soluble nature of these activities and their subcellular distribution will be discussed later in greater detail.

The ratio of cGMP to cAMP hydrolysis is greater than one for all the entries in Table 15, indicating that the former nucleotide is the best substrate when assays are conducted at the 50 μM level. Data presented later will demonstrate an inversion of this cGMP/cAMP ratio in some samples at substrate levels less than 50 μM.
Pilot experiments with larval CNS homogenates performed according to Assay 1 indicated that 50 mM salt (either NaCl or KCl), or two-fold variation in buffer concentration were without appreciable effect on cAMP- and cGMP-PDE; thus, ionic strength does not appear to be an important determinant of either activity. Inclusion of bovine serum albumin in the incubation mixture at a final concentration of 0.1 mg/ml, sucrose at 5% (w/v), or glycerol at 1% (v/v) resulted in no evident change in activity. Storage of larval CNS homogenates or 10,000 X g supernatants for as long as 3 months at -20° C led to no detectable loss of enzymatic activity. In fact, particulate samples which were stored frozen inevitably exhibited increased activities upon thawing.

The data of Table 16 demonstrate that in the crude homogenate either nucleotide may inhibit the hydrolysis of the other (compare sample 7 with 1, sample 8 with 2). When both substrates are present in equimolar amounts, cAMP hydrolysis is somewhat more inhibited by cGMP than is cGMP hydrolysis inhibited by cAMP. Although meaningful generalizations are not possible, the relative inhibitory effects of one nucleotide upon the other at the substrate concentration used are compatible with the greater rate of hydrolysis of cGMP compared to cAMP (see Figure 22). It is possible that, if lower substrate concentrations were examined, the relative inhibitory effects of the two nucleotides would be reversed. In principle,
Table 16. Inhibition by dibutryl cAMP, and influence of each cyclic nucleotide on the hydrolysis of the other.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Substrates (each 50 µM)</th>
<th>Labeled Products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm/1 ml</td>
</tr>
<tr>
<td>1</td>
<td>$^3$H-cAMP</td>
<td>15,637</td>
</tr>
<tr>
<td>2</td>
<td>$^3$H-cGMP</td>
<td>5,012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Σ 20,649</td>
</tr>
<tr>
<td>3</td>
<td>$^3$H-cAMP + cAMP</td>
<td>11,092</td>
</tr>
<tr>
<td>4</td>
<td>$^3$H-cGMP + cGMP</td>
<td>2,976</td>
</tr>
<tr>
<td>5</td>
<td>$^3$H-cAMP + (but)$_2$ cAMP</td>
<td>12,918</td>
</tr>
<tr>
<td>6</td>
<td>$^3$H-cGMP + (but)$_2$ cAMP</td>
<td>4,548</td>
</tr>
<tr>
<td>7</td>
<td>$^3$H-cAMP + cGMP</td>
<td>10,118</td>
</tr>
<tr>
<td>8</td>
<td>$^3$H-cGMP + cAMP</td>
<td>3,649</td>
</tr>
<tr>
<td>9$^a$</td>
<td>$^3$H-cAMP + $^3$H-cGMP</td>
<td>13,965</td>
</tr>
</tbody>
</table>

$^a$After development of the paper chromatogram for 26 hours, fused spots ($5'$-AMP + $5'$-GMP and adenosine + guanosine) were separated from the unreacted substrates. The sum of the radioactivities of these four products is indicated. Due to the incomplete resolution of the four spots, the simultaneous determination of both cAMP-PDE and cGMP-PDE was not possible.

51.2 µg of larval CNS homogenate protein were present in each reaction mixture (Assay II, 0.2 ml volume). The specific activities of the $[^3$H]-nucleotides were 530 dpm/pmol and 133 dpm/pmol for cAMP and cGMP, and the counting efficiency was 0.309.
detailed kinetic measurements designed to gauge the inhibition constant ($K_i$) of each nucleotide toward the other would provide information useful in deciding if there were present a single enzyme capable of utilizing both substrates. Since somewhat anomalous kinetics were observed for the hydrolysis of both cAMP and cGMP by the larval CNS homogenate (Figure 23, Figure 24), it was not possible to unambiguously estimate reliable $K_m$'s. Thus a kinetic analysis of types of inhibition (competitive or non-competitive) in this crude preparation would be difficult. It should be emphasized that even if it were possible to demonstrate an apparent competitive inhibitory effect of each cyclic nucleotide upon the other in a given preparation, this information would be necessary but not sufficient to prove both nucleotides were being hydrolyzed by the same enzyme.

Dibutryl cyclic AMP functions as a fair inhibitor of both diesterase activities in the CNS homogenate (Table 16), although it is not nearly as effective as the inhibition of one cyclic nucleotide by the other. It was hoped to selectively inhibit with (but)$_2$ cAMP one activity to a much greater degree than the other, but the experimental data demonstrate that the extents of inhibition (17% for cAMP-PDE and 10% for cGMP-PDE) are comparable in magnitude.

---

10 If both cAMP and cGMP were serving as alternate substrates for a single, "ideal" (obeying Michaelis-Menten kinetics) enzyme, then the degree of interference of one upon the other would be predictable from the relative $K_m$ values; for an enzyme with alternate substrates, the $K_i$ of one nucleotide would be the same as the $K_m$ for that nucleotide measured in the absence of the other.
The data of Table 16 conclusively rule out the possibility that the observed gross homogenate diesterase activity toward both cyclic nucleotides is due to a mixture of two enzymes, each of which has a very much greater affinity for one nucleotide than the other. If this were the case, inhibition of one activity by the other nucleotide would not be observed, and (summing samples 1 and 2) nearly 20,000 cpm of products would be expected from the two enzymes acting independently. In fact, only (approximately) 14,000 cpm are anticipated when one sums (samples 7, 8) the observed activities of each labelled nucleotide in the presence of an equimolar amount of the other (unlabelled) nucleotide. This calculated value of roughly 14,000 cpm was experimentally verified by simultaneously employing both labelled nucleotides as substrates (sample 9).

When both enzyme activities in the homogenate were assayed over a relatively wide range of substrate concentrations, a plot of velocity vs. substrate (Figure 22) appeared to crudely resemble the hyperbolic \( S \) vs. \( v \) relationship expected for an ideal Michaelis-Menten enzyme. However, when the data were inverted, the Lineeweaver-Burk double-reciprocal representations of the data (Figures 23, 24) were distinctly nonlinear. It was possible to graphically obtain two different sets of \((K_m, V_{max})\) values for each activity by extrapolation of the locally linear segments of these plots. Anomalous double-reciprocal plots obtained with a variety of cAMP-PDE
preparations have been reported by many workers (143, 150, 162, 182, 190, 191, 199); nonlinear behavior would seem the rule rather than the exception.

The kinetic analysis of the larval CNS homogenate using substrate concentrations in the range 1-500 µM indicates two $K_m$ values for cAMP-PDE (10 µM and 21 µM) and two $K_m$'s for cGMP-PDE (8.6 µM and 18.5 µM). In view of the similarity of the $K_m$'s at high and low substrate concentrations, it would be possible to argue that the hydrolysis of cAMP and cGMP are activities of a single enzyme having very nearly equal affinities for both cyclic nucleotides. Supplementary kinetic information derived from a variety of *M. sexta* preparations will be presented later.

Figure 23 and Figure 24 reveal that the $V_{max}$'s corresponding to the low $K_m$ values of cAMP and cGMP are $6.62 \times 10^3$ and $5.8 \times 10^3$, and on the basis of these values one would predict for $[S] >$ approximately 10 µM, the ratio of cGMP/cAMP hydrolysis would be less than 1. As is indicated in Figure 22, this is in fact observed up to a substrate concentration of approximately 50 µM, when the ratio becomes $>1$ for all higher substrate concentrations. This is compatible with the observation that, at high concentrations of cyclic nucleotide, $V_{max} (\text{cGMP}) > V_{max} (\text{cAMP})$. 
Figure 22. Substrate vs. reaction velocity plot of cAMP and cGMP hydrolysis. The enzyme source was a freshly-prepared larval CNS homogenate. Paper chromatographic blanks (Assay II) were run at each substrate concentration, and units of velocity are pmol substrate hydrolyzed per min per mg protein. The ratio of cGMP/cAMP hydrolysis was clearly dependent upon substrate concentration.
Figure 23. Double-reciprocal plot of the hydrolysis of cAMP by a larval CNS homogenate. Substrate concentrations range from 5 to 500 μM. Velocity is expressed as pmol cAMP hydrolyzed per min per mg protein.
Figure 24. Double-reciprocal plot of the hydrolysis of cGMP by a larval CNS homogenate. See legend to Figure 23 for additional details.

K_m1 = 8.6 μM  V_max, 1 = 5.8 \times 10^3
K_m2 = 19 μM  V_max, 2 = 8.3 \times 10^3
Phosphodiesterase Activity as a Function of Incubation Time and Concentration of Protein

The time- and protein concentration-dependences of cAMP and cGMP hydrolysis (50 µM substrate) at 30°C catalyzed by various M. sexta CNS preparations are depicted in Figure 25 A, B and Figure 26 A, B. In all of these preparations, hydrolysis of substrate appeared reasonably linear as a function of time and the stated protein concentration through at least 10 minutes of incubation, the time period utilized for all standard assays. Even when 90 µg of homogenate protein was assayed at a 50 µM substrate level (data not shown), hydrolysis was linearly time-dependent up to 10 minutes, but decreased thereafter, reflecting that an appreciable fraction of the substrate had been consumed. The longest periods of linearity were observed in those assays conducted at high substrate concentrations and containing small amounts of enzyme.

Ammonium Sulfate Precipitation

As is indicated in Table 17, ammonium sulfate precipitation of the soluble (10,000 × g supernatant) diesterase activities results in only slight increases in specific activities relative to those found in the supernatant. Although the purification factors are small, ammonium sulfate treatment serves a useful purpose in removing the enzyme activities from the original (supernatant) environment of
Figures 25 A, B. Time-dependence of cyclic nucleotide hydrolysis at 30°C.
Figures 26 A, B. Diesterase activities as a function of protein concentration.
Table 17. Ammonium sulfate precipitation of soluble larval CNS phosphodiesterase.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein mg</th>
<th>Total activity units</th>
<th>Percent of 10,000 x g supernatant activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cAMP-PDE</td>
<td>cGMP-PDE</td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>16.32</td>
<td>44,780</td>
<td>85,122</td>
<td>2,744</td>
</tr>
<tr>
<td>10,000 x g supernatant</td>
<td>8.46</td>
<td>28,890</td>
<td>42,858</td>
<td>3,415</td>
</tr>
<tr>
<td>(0-50) AS (before dialysis)</td>
<td>5.40</td>
<td>22,151</td>
<td>35,877</td>
<td>4,102</td>
</tr>
<tr>
<td>Dialyzed, cfgd. (0-50) AS</td>
<td>5.28</td>
<td>28,917</td>
<td>32,601</td>
<td>5,477</td>
</tr>
<tr>
<td>Dialyzed, 50% (NH₄)₂SO₄ supernatant</td>
<td>1.11</td>
<td>2,313</td>
<td>0</td>
<td>2,084</td>
</tr>
</tbody>
</table>

The 10,000 x g supernatant from 60 larval nerve cords was treated with (NH₄)₂SO₄ as described in Methods, and the fraction precipitating at 50% (NH₄)₂SO₄ saturation is designated (0-50) AS. All samples were measured at 50 uM initial substrate concentration according to Assay II.
cellular salts, miscellaneous nucleotides, and other potentially inhibitory substances of the cytosol. The overnight-dialyzed and centrifuged (0-50) AS fraction gave an apparent recovery of 100% for cAMP-PDE, although a small amount of activity remained in the (NH$_4$)$_2$SO$_4$ supernatant. Only 76% of the supernatant cGMP-PDE activity was recovered in the (0-50) AS fraction, but no activity was found in the (NH$_4$)$_2$SO$_4$ supernatant. Kinetic measurements of the (0-50) AS material are presented in Table 28.

**Effect of pH and Buffers**

A 10,000 × g supernatant from larval CNS was used as an enzyme source to examine the pH-dependence of the two diesterase activities (Figures 27, 28). Both activities were maximal at approximately pH 7.5 and were appreciably attenuated at higher or lower pH. In the presence of imidazole, cAMP-PDE was elevated by about 10% at pH 7.5, whereas cGMP-PDE was diminished to about the same extent. Imidazole-stimulation to varying degrees of mammalian cAMP-PDE preparations, with or without a change in pH optimum, has been reported by many workers (67, 150, 153, 175). O'Dea et al. (176) reported that imidazole, which stimulated cAMP-PDE in mammalian brain, also inhibited the hydrolysis of cGMP in the same preparation. These effects are shared by the insect enzyme. Stimulation by imidazole seems a consistently reported feature of
Figure 27. pH-dependence of cAMP phosphodiesterase activity. Samples of a 10,000 x g larval CNS supernatant were pre-incubated and assayed in the specified buffer (75 mM) at 30°C (Assay I, 50 uM substrate). Activities are expressed relative to those observed with Tris HCl, pH 7.5.
Figure 28. pH-dependence of cGMP-phosphodiesterase activity. Experimental conditions were described in the legend to Figure 27.
mammalian enzyme preparations, but the mechanism responsible for this effect has not been satisfactorily explained.

The observed pH optimum (7.5) for *M. sexta* larval CNS PDE is similar to those reported for beef heart (67), frog erythrocytes (181), bovine brain (155), rat brain (153), uterine smooth muscle (143), and human blood platelets (188). It is somewhat lower than the pH optima (8.5-9.2) reported for fish brain (201), dog heart (175), and *M. sexta* larval gut supernatants (Whitmore et al., ref. 202); it must be noted that the PDE assay utilized by Whitmore et al. is subject to some criticism. Thus the *M. sexta* CNS cAMP-PDE preparation resembles mammalian and amphibian diesterases as regards pH-optimum; furthermore, it behaves in a fashion similar to mammalian enzymes with respect to imidazole stimulation. The insect cGMP-phosphodiesterase also exhibited maximal activity near pH 7.5, and so is similar to the only reported pH-optimum for a cGMP-PDE (pH 7.5 for uterine muscle; ref. 143).

At any value of pH below 7.5, both diesterase activities were considerably lower in phosphate buffer than in either Tris·HCl or imidazole·HCl. It is evident that the intracellular pH, as well as the local concentration of inorganic phosphate, may be important modifiers of phosphodiesterase activity.
Temperature Dependence of Activity

It was of interest to determine if the rates of cAMP and cGMP hydrolysis would exhibit differential dependences upon temperature. An (NH₄)₂SO₄ fraction prepared from a 10,000 x g supernatant (see Methods) was incubated in 50 mM Tris·HCl (pH 7.5) - 5 mM MgSO₄ - 1 mM DTT at various temperatures, and PDE activity was estimated by Assay I. Reactions were conducted for only 5 minutes to minimize substrate consumption at the higher temperatures.

Figure 29 demonstrates that cGMP-PDE activity is maximal at approximately 35°C (and falls off sharply at T > 35°C), whereas optimal cAMP-PDE activity is attained at a higher temperature (50°C). Cyclic AMP-PDE activity at 30°C was 80% of that observed at 37°C. The steep slopes of activity vs. temperature in Figure 29 emphasize the necessity of careful temperature control near 30°C, the temperature chosen for all routine assays. Samples of protein pre-incubated at 50°C for 10 min and then assayed at 30°C showed no significant differences between those pre-incubated at 30°C before assay, thus ruling out the possibility that cGMP-PDE activity is irreversibly denatured at the higher incubation temperature. The considerable differences in the temperature profiles of cAMP and cGMP hydrolysis cannot be interpreted as suggesting the existence of separate enzymes for the destruction of these two substrates. It is conceivable that a
Figure 29. Temperature dependence of diesterase activities. Each reaction mixture contained 17 µg of a 16-hour dialyzed 50% (NH₄)₂SO₄ preparation. After pre-incubation of each mixture at 30°C for 10 min to equilibrate the activities, all tubes were cooled before addition of cyclic nucleotide (1000 pmol) and further incubation for 5 minutes at the specified temperature. Assay I was employed.
single enzyme having affinity for both nucleotides would, at some temperature, adopt a conformation favoring only one substrate.

**Metal Requirements**

Utilizing a dialyzed 0-40% \((NH_4)_2SO_4\) fraction from whole rabbit brain as a source of diesterase, Drummond and Perrot-Yee (68) reported that the cAMP-PDE had an absolute requirement for \(Mg^{2+}\). They found that dialyzed extracts were completely inactive in the absence of magnesium, that activity could be entirely abolished with 1 mM EDTA, and that magnesium concentrations higher than 0.8 mM were highly inhibitory. These features are not exhibited by a similarly prepared enzyme from the insect larval CNS (Table 18).

The enzyme source for the experiment summarized in Table 18 was an overnight-dialyzed 0-50% \((NH_4)_2SO_4\) fraction prepared from a larval CNS supernatant. Although appreciable cAMP-PDE activity was seen in the absence of added metal, bivalent cations appear to be required for maximal activity. PDE activity with no added \(Mg^{2+}\) was nearly two-thirds of the activity observed with 5 mM \(Mg^{2+}\). Contrary to the findings of Drummond et al. (68), Cheung (152), and Rosen (181), who examined mammalian and amphibian diesterases, the residual activity could not be abolished when the \(M.\ sexta\) preparation was assayed in the presence of 1-10 mM EDTA containing no added bivalent ions. Even when a sample of enzyme was
Table 18. Effects of bivalent cations and chelating agents on cAMP-PDE.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Millimolarity</th>
<th>Activity percent of control</th>
<th>Addition</th>
<th>Millimolarity</th>
<th>Activity percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>--</td>
<td>100%</td>
<td>Pb$^{2+}$</td>
<td>1</td>
<td>108</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0, 1</td>
<td>154</td>
<td>5</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>150</td>
<td>5</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>166</td>
<td>10</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>187</td>
<td>Cu$^{2+}$</td>
<td>1</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>254</td>
<td>Fe$^{2+}$</td>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>1</td>
<td>99</td>
<td>1, 10 phenanthroline</td>
<td>1</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>80</td>
<td>EDTA</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>Ca$^{2+}$ + Mg$^{2+}$ ea, 1 mM</td>
<td>155</td>
<td></td>
<td>2 EDTA + Mg$^{2+}$ 1 + 10</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>1</td>
<td>206</td>
<td>EGTA</td>
<td>1</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>229</td>
<td>2 EDTA + Mg$^{2+}$ 1 + 10</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>1</td>
<td>211</td>
<td>2 EDTA + Mg$^{2+}$ 1 + 10</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>200</td>
<td>EGTA + Mg$^{2+}$ 1 + 10</td>
<td>146</td>
<td></td>
</tr>
</tbody>
</table>

A dialyzed 0-50% (NH$_4$)$_2$SO$_4$ fraction of larval CNS tissue was analyzed in the presence of 50 uM [H] cAMP according to the "Assay I" procedure. DTT was omitted from the incubation mixture. Ca$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Cu$^{2+}$, and Fe$^{2+}$ were employed as their chloride salts, Zn$^{2+}$ and Mg$^{2+}$ as sulfates, and Pb$^{2+}$ in its acetate form. Activities are expressed as percentages of that found in the absence of added metal.
pre-incubated for 1 hour at 25°C in the presence of 30 mM EDTA followed by dilution and assay (final EDTA concentration was 10 mM), 51% of the control cAMP-PDE activity remained. These findings may be interpreted as indicating that either the M. sexta cAMP-PDE does not have an absolute Mg$^{2+}$ requirement, in contrast to mammalian enzymes (154), or that endogenous Mg$^{2+}$ (or other metal) ions are strongly bound and incapable of being complexed by EDTA. Experiments with the insect CNS have shown that 0-50% (NH$_4$)$_2$SO$_4$ fractions prepared in the absence of Mg$^{2+}$ are much less stable (prone to precipitation after one freeze-thaw cycle) than are similar fractions prepared in the presence of Mg$^{2+}$. Thus, although magnesium is possibly not obligatory for catalytic activity, it may be involved in stabilization of the enzyme.

In the case of the M. sexta preparation there appears to be no optimal concentration of Mg$^{2+}$, e.g. a level of magnesium which if exceeded results in highly reduced activities. Drummond et al. (68) have reported great inhibition of rabbit brain PDE beyond 0.8 mM Mg$^{2+}$, but Cheung (153) has noted only slight inhibition of a rat brain diesterase at Mg$^{2+}$ concentrations above 10 mM. Although the metal requirements of protein activator-deficient phosphodiesterase have not been reported for any mammalian tissue, it has been established that the activator (A) itself absolutely requires Mg$^{2+}$ for activity. Teo et al. (189) have shown that low levels of Mg$^{2+}$ are necessary for
activator activity and stability, that high levels are inhibitory, and
that inactivation by low concentrations of EDTA could not be reversed
by addition of Mg$^{2+}$. Therefore it is possible that the Mg$^{2+}$-require-
ment of crude mammalian PDE preparations is intrinsic to the ac-
tivator, but not to A-deficient PDE. It is shown in Table 18 that the
minor inhibition of the insect cAMP-PDE caused by EDTA could be
reversed by addition of Mg$^{2+}$. On the basis of these studies with the
M. sexta CNS using Mg$^{2+}$ and EDTA, it may be suggested there is
apparently absent in this tissue a protein activator having properties
similar to the mammalian enzyme. The seeming absence of activator
in this insect CNS will be further discussed in a later section.

Table 18 further shows that Mn$^{2+}$ and CO$^{2+}$ were even more
effective than Mg$^{2+}$ in stimulating M. sexta cAMP-PDE, that Zn$^{2+}$,
Cu$^{2+}$, or Fe$^{2+}$ were moderately inhibitory, and that calcium has little
effect at a level of 1 mM. Since Kakiuchi et al. (168, 169) have re-
ported the presence in rat brain cortex of a Ca$^{2+}$ plus Mg$^{2+}$ depend-
ent cAMP-PDE activity, these two metal ions were tested together
on the insect enzyme. No synergism was noted, and the effect of
Ca$^{2+}$ + Mg$^{2+}$ did not differ significantly from that of Mg$^{2+}$ alone. To
explore whether contaminating levels of Ca$^{2+}$ (originating from the
CNS) were influencing PDE activity, EGTA$^{11}$ was tested with or

$^{11}$EGTA, ethylene glycol-bis (beta-aminoethyl ether)-N, N'-
tetraacetic acid, is a relatively selective complexometric agent for
without Mg\(^{2+}\). By itself, EGTA was mildly inhibitory, and this inhibition could be relieved by Mg\(^{2+}\). The absence of inhibition by lead is significant because of the use of lead acetate as an integral part of the activity stain for diesterase reported later in this work. It is interesting to note in the literature that 2.4 mM Pb\(^{2+}\) resulted in 93% inhibition of mouse brain cAMP-PDE (70).

The metal requirements of the insect cGMP-PDE were not examined in detail, but it was established that the Mg\(^{2+}\)-effect upon cGMP-PDE is qualitatively similar to that upon cAMP-PDE. Cyclic GMP activity could not be abolished with EDTA.

**Inhibition Studies**

Many compounds, both physiological and non-physiological, are capable of inhibiting phosphodiesterase (150, 152, 160, 165, 187, 203). A variety of agents were tested on insect preparations in the hope of finding a potent antagonist of either (or both) cAMP and cGMP-PDE activities. Such an inhibitor would be exceedingly useful for other cyclic nucleotide-related assays in crude preparations, for example in adenyl cyclase and protein kinase assays. Unfortunately, no such agent effective at very low concentrations was found. A number of the compounds investigated were antagonistic at the millimolar level, but none specifically inhibited one activity (e.g. cAMP-PDE or cGMP-PDE) while totally sparing the other. This finding
substantiates the suspicion that cAMP and cGMP may serve as alternate substrates for a single activity in _M. sexta_ CNS.

Butcher and Sutherland (67) first demonstrated that the methylxanthines inhibit cAMP-PDE, and of these compounds the most effective was theophylline. Theophylline has been employed in many systems as a reference inhibitor, and at a 1 mM level is capable of inhibiting bovine heart cAMP-PDE 65% (2 mM substrate concentration). It is important to bear in mind that the apparent degree of inhibition is a function of the relative levels of enzyme, substrate, and inhibitor. Theophylline was fairly effective against both insect activities, and was more active than either caffeine or theobromine (Table 19). It was, however, not nearly as potent as either ATP or GTP.

A variety of synthetic inhibitors far more active against mammalian PDE than any methylxanthine have been reported in the literature (150, 158, 160, 187). Two of these experimental compounds, Ro20-2926 and SQ20009, were acquired from Hoffman-LaRoche and Squibb, respectively. Ro20-2926, which is at least 42 times more potent than theophylline against rat erythrocyte cAMP-PDE (187), was less than one-third as potent as theophylline against _M. sexta_ CNS cAMP-PDE; it was slightly more effective in inhibiting cGMP-PDE. SQ20009, which is reported to inhibit rat brain cAMP-PDE 50% at a concentration of $2 \times 10^{-6}$ M (150), was no more effective
Table 19. Inhibition of phosphodiesterase by miscellaneous compounds.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>cAMP-PDE</td>
</tr>
<tr>
<td>None (Control)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NEM</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>IAA</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>PMSF</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>theobromine</td>
<td>1</td>
<td>34\textsuperscript{R}</td>
</tr>
<tr>
<td>caffeine</td>
<td>1</td>
<td>31\textsuperscript{R}</td>
</tr>
<tr>
<td>theophylline</td>
<td>1</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>89</td>
</tr>
<tr>
<td>NaF</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>alloxan</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>54</td>
</tr>
<tr>
<td>ATP</td>
<td>0.1</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>78</td>
</tr>
<tr>
<td>GTP</td>
<td>0.1</td>
<td>50\textsuperscript{R}</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>100\textsuperscript{R}</td>
</tr>
<tr>
<td>Ap(CH\textsubscript{2})pp</td>
<td>1</td>
<td>39</td>
</tr>
<tr>
<td>ADP</td>
<td>1</td>
<td>46</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>0.1</td>
<td>30\textsuperscript{R}</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>adenosine</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>2', 3'-cAMP</td>
<td>1</td>
<td>46</td>
</tr>
<tr>
<td>3', 5'-cIMP</td>
<td>1</td>
<td>53</td>
</tr>
<tr>
<td>PP\textsubscript{1} (pyrophosphate)</td>
<td>1</td>
<td>27\textsuperscript{R}</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>53</td>
</tr>
<tr>
<td>SQ20009</td>
<td>1</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>10\textsuperscript{-2}</td>
<td>23</td>
</tr>
<tr>
<td>Ro 20-296</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>10\textsuperscript{-2}</td>
<td>0</td>
</tr>
</tbody>
</table>

Enzyme (18 μg of a 0-50% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction in 100 μl) was preincubated with the stated compound for 10 min at 30 °C before addition of substrate (50 μM). Assay II (paper chromatographis) was employed for all samples except those with superscript R, these being assayed by the resin method (Assay I). Control activities were and pmol/min/mg protein for cAMP-PDE and cGMP-PDE, respectively. The Mg\textsuperscript{2+} concentration was 1 mM. See text for discussion.
than theophylline against the insect cAMP-PDE at a concentration of 1 mM. Although the list of potent (mammalian) PDE inhibitors worthy of further exploration (see, for example, refs. 150, 187) was by no means exhausted in the present study, the relative impotency of Ro20-2926 and SQ20009 toward the insect diesterase is indicative of some fundamental difference in molecular architecture of *M. sexta* diesterase.

The sulfhydryl inhibitors N-ethylmaleimide (NEM) and iodoacetamide (IAA) were only moderately inhibitory at 1 mM concentration, and cGMP-PDE was marginally more affected than was cAMP-PDE. It is not particularly meaningful to attach significance to such -SH inhibition studies of highly impure enzyme preparations (undoubtedly many extraneous proteins are being alkylated), and one cannot conclude whether or not an -SH is essential for catalytic activity. Likewise, the observed small inhibitory effect of phenylmethylsulfonylfluoride (PMSF) does not positively indicate the involvement of a serine residue at or near the active site, but only supports the possibility. Drummond (68) has noted that crude preparations of rabbit brain cAMP-PDE are totally unaffected by long-term incubation with millimolar levels of IAA, diisopropylfluorophosphate, or cocaine.

The effects of alloxan, NaF, and α,β-methylene-adenosine-5'-triphosphate (Ap[CH₂]pp) were also tested on the insect preparation. These compounds have been reported as useful experimental modifiers
of adenyl cyclase, and it is essential to establish if they exert a simultaneous effect on PDE; the influence of alloxan or Ap(CH$_2$)$_2$pp on any phosphodiesterase preparation has not been published. The phosphonic analog of ATP, Ap(CH$_2$)$_2$pp, has been employed at millimolar levels as a competitive, hydrolysis-resistant, substrate for adenyl cyclase (204, 205). As is indicated in Table 19, it also functions as a fair inhibitor of insect phosphodiesterase. It is somewhat less antagonistic than ATP toward cAMP-PDE, but appears equipotent with ATP toward cGMP-PDE. The utilization of Ap(CH$_2$)$_2$pp as an inhibitor of adenyl cyclase therefore suffers from the standpoint of specificity; this compound apparently can also function as a phosphate donor in protein kinase reactions (206). Alloxan (2, 4, 5, 6-tetraoxypyrimidine), advertised as a selective inhibitor of cyclase which does not interfere with membrane ATPase (207), has been employed at millimolar levels in cytochemical studies (208). At 1 mM concentration, alloxan exerted a small but non-negligible effect on both cAMP-PDE and cGMP-PDE in the _M. sexta_ preparation. Fluoride ion, which is able to markedly stimulate adenyl cyclase in most multicellular organisms, is moderately inhibitory toward cAMP-PDE in _R. pipiens_ erythrocytes (181). At 1 mM, it appears to be without influence on the insect CNS activities.

Table 19 demonstrates that the hydrolysis of both cAMP and cGMP (50 $\mu$M initial substrate concentration) by the insect preparation
is quite strongly inhibited by millimolar levels of ATP and GTP. Such inhibition of cAMP-PDE by nucleoside triphosphates has been reported in other mammalian and amphibian preparations (143, 152, 153, 167). For example, Cheung (153) has noted greater than 40% inhibition of rat brain cAMP-PDE by 3 mM ATP, GTP, TTP, CTP, UTP, or ITP at a Mg\(^{2+}\) concentration of 1.8 mM. The possible physiological importance of ATP inhibition, and its dependence upon Mg\(^{2+}\) levels, will be discussed later. Both insect CNS activities were also inhibited, in decreasing order of potency, by ADP > 5'-AMP > adenosine. Goren and Rosen (164) have reported that a partially purified preparation from bovine heart was inhibited approximately 10% by 1 mM adenine, adenosine, guanine, or guanosine. Inhibition by ADP of any mammalian PDE preparation has not been reported. Rosen (181), Goren and Rosen (164), and Franks and MacManus (162) have found 5'-AMP to be totally noninhibitory toward cAMP-PDE in frog erythrocytes, bovine heart, and rat thymic lymphocytes. The insect activity may or may not be unique with respect to inhibition by ADP and AMP. The observed great inhibition of the *M. sexta* diesterase by 3', 5'-cIMP is not surprising in view of the structural similarity of this compound to both cAMP and cGMP, coupled with the fact that it was present in much larger amount (x20) than either substrate.

The inhibition of cAMP and cGMP (3', 5'-cyclic phosphates) hydrolysis in *M. sexta* by 1 mM 2',3'-cGMP was somewhat greater
than that observed with ADP, AMP, or theophylline at equivalent concentrations. Possibly, 2',3'-cGMP would exert similar or even greater effects, but this compound was not investigated. It has been reported that 2',3'-cGMP at 10^{-4} M is mildly inhibitory to rat lymphocyte cAMP-PDE (162), but Rosen (181) found that cyclic 2',3'-nucleotides were completely noninhibitory toward cAMP-PDE in *R. pipiens* erythrocytes. No information is presently available which supports the (unlikely) possibility that 2',3'-cyclic phosphates may serve as (perhaps non-hydrolyzable) substrates for the insect PDE. Yet, the observed inhibition of 3',5'-cyclic nucleotide hydrolysis suggests the insect enzyme may not be entirely specific for the 3',5'-diester linkage.

The relatively strong inhibition of rat brain phosphodiesterase by ATP and PP_i, comparable in magnitude to that observed for the insect preparation (Table 19), was first reported by Cheung (152). It has been suggested (153) that the primary effect of ATP might lie in its well-known ability to complex Mg^{2+}, thus reducing Mg^{2+} available to the enzyme. In view of the possible importance *in vivo* of PDE inhibition by ATP, the effect of this compound as a function of Mg^{2+} levels was investigated in insect CNS preparations (Tables 20, 21). Both cAMP-PDE and cGMP-PDE were progressively antagonized by ATP as the Mg^{2+} concentration was reduced. In the absence of added Mg^{2+} there resulted greater than 50% inhibition of both
diesterase activities at 1 mM ATP, a concentration which may easily be attained in vivo. Even at high Mg\(^{2+}\) levels, supra-millimolar ATP concentrations virtually abolished diesterase activity. Physiologically, an ATP/Mg\(^{2+}\) ratio greater than 1 would be expected to elevate cAMP levels through inhibition of PDE; however, an opposite effect might be an attenuation of cAMP levels via diminution of the natural substrate of adenyl cyclase, an ATP-Mg complex. The inhibitory effects of GTP as a function of Mg\(^{2+}\) concentration were also examined (data not shown), and results qualitatively similar to those found with ATP summarized in Tables 20 and 21 were observed. GTP was considerably more effective at a given concentration than ATP in inhibiting cAMP-PDE. GTP inhibition of cGMP-PDE was even greater than that of cAMP-PDE.

**Phosphodiesterase Activities in the Larval and Adult CNS**

In Table 22 are compiled data from representative experiments on phosphodiesterase activities in the entire central nervous system of both larval and adult *M. sexta*. Values obtained using fresh, unfrozen larval nerve cords (Experiment 4) compare favorably with those found using tissues frozen on dry ice before homogenization. It is clear that the measurable activities are quite sensitive to the nature of the homogenizing medium; the greater activities seen after
Table 20. Effects of Mg\(^{2+}\) and ATP on cAMP-PDE activity.

<table>
<thead>
<tr>
<th>Mg(^{2+}), mM</th>
<th>ATP, mM</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100%</td>
<td>70</td>
<td>37</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>110</td>
<td>68</td>
<td>40</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>131</td>
<td>--</td>
<td>72</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>132</td>
<td>--</td>
<td>82</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Source of enzyme was a 0-50\%(NH_4)_2SO_4 fraction from larval CNS prepared in the absence of Mg\(^{2+}\). This fraction had been stored for two months at -20° C before use. A heavy precipitate, which was not removed by centrifugation, formed upon thawing. All samples (17 µg protein) were preincubated for 10 min at 30° C with the stated concentration of Mg\(^{2+}\) and ATP before estimating cAMP-PDE activity according to Assay I, purified 5'-nucleotidase (Sigma) being substituted for snake venom. PDE activity observed in the absence of Mg\(^{2+}\) and ATP was assigned a value of 100%.

Table 21. Effects of Mg\(^{2+}\) and ATP on cGMP-PDE activity.

<table>
<thead>
<tr>
<th>Mg(^{2+}), mM</th>
<th>ATP, mM</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100%</td>
<td>76</td>
<td>48</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>114</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>125</td>
<td>--</td>
<td>56</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>125</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

Experimental conditions were as described in the legend to Table 20, except cGMP was substituted for cAMP.
<table>
<thead>
<tr>
<th>Expr't.</th>
<th>Tissue</th>
<th>No. of CNS</th>
<th>Homog. buffer</th>
<th>Enzyme source</th>
<th>Specific activity (b) units/mg protein</th>
<th>Total activity (c) units/ CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cAMP-PDE</td>
<td>cGMP-PDE</td>
</tr>
<tr>
<td>1a</td>
<td>Larval CNS</td>
<td>55</td>
<td>H+</td>
<td>Homogenate</td>
<td>2837</td>
<td>4900</td>
</tr>
<tr>
<td>2a</td>
<td>&quot;</td>
<td>60</td>
<td>H+</td>
<td>Homogenate</td>
<td>2744</td>
<td>5216</td>
</tr>
<tr>
<td>3a</td>
<td>&quot;</td>
<td>15</td>
<td>H-</td>
<td>Homogenate(c)</td>
<td>6014</td>
<td>6183</td>
</tr>
<tr>
<td>3b</td>
<td>&quot;</td>
<td>15</td>
<td>H-</td>
<td>Homogenate(d)</td>
<td>4674</td>
<td>5973</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>4</td>
<td>H-</td>
<td>Homogenate(e)</td>
<td>5589</td>
<td>5783</td>
</tr>
<tr>
<td>1b</td>
<td>&quot;</td>
<td>55</td>
<td>H+</td>
<td>700 x g supernatant</td>
<td>4227</td>
<td>5870</td>
</tr>
<tr>
<td>2b</td>
<td>&quot;</td>
<td>60</td>
<td>H+</td>
<td>10,000 x g supernatant</td>
<td>3415</td>
<td>5066</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>20</td>
<td>H-</td>
<td>34,000 x g supernatant</td>
<td>5319</td>
<td>ND</td>
</tr>
<tr>
<td>1c</td>
<td>&quot;</td>
<td>55</td>
<td>H+</td>
<td>105,000 x g supernatant</td>
<td>3517</td>
<td>6717</td>
</tr>
<tr>
<td>6a</td>
<td>Adult CNS</td>
<td>11</td>
<td>H-</td>
<td>Homogenate(f)</td>
<td>2856</td>
<td>2860</td>
</tr>
<tr>
<td>6b</td>
<td>&quot;</td>
<td>11</td>
<td>H-</td>
<td>10,000 x g supernatant</td>
<td>5192</td>
<td>4060</td>
</tr>
</tbody>
</table>

\(a\) H+, hypertonic; H-, hypotonic.

\(b\) Both substrates present initially at 50 uM

\(c\) Assayed immediately after homogenization

\(d\) Same homogenate assayed 40 min later

\(e\) Fresh, unfrozen nerve cords; protein content not determined; specific activity calculated using mean protein/CNS value found in other experiments.

\(f\) Frozen-thawed 1x

ND = not determined
preparation under hypotonic conditions are most easily rationalized
under the assumption of osmotic rupture of some subcellular organ-
elles. It is likely that combinations of hypotonicity, detergent-treat-
ment, and freeze-thaw cycles would reveal even more activity. Addi-
tionally, it would be worthwhile to investigate sonic oscillation (using
more strenuous conditions than those employed in a preliminary
experiment; see Table 15) as a means of unmasking enzyme activity.

The observed ratio of cGMP/cAMP hydrolysis (50 μM substrate)
was ≥1 for all homogenates and supernatants, with the exception of
the adult CNS 10,000 × g supernatant. In particulate samples these
differences in rates of hydrolysis of cAMP and cGMP could be ex-
plained by the exclusion of one cyclic nucleotide from some relatively
"inaccessible" phosphodiesterase activity. Yet the observations
that detergent-treatment and freeze-thawing do not markedly alter
the cGMP/cAMP ratio does not lend support for such an exclusion
phenomenon. A further possibility, one which will require extensive
purification of the diesterase(s) for conclusive proof, is that one of
the cyclic nucleotides is preferentially bound by some component of
the homogenate or supernatant--and so is inaccessible to the diester-
ase. Cellular components capable of performing this function include
membrane-associated cAMP-binding sites, regulatory subunits of
cyclic nucleotide dependent protein kinases, and perhaps other yet-
uncharacterized structures. O'Dea et al. (176) have demonstrated
that cAMP bound to the regulatory subunit of protein kinase cannot be hydrolyzed by PDE. Miyamoto et al. (15) have provided evidence for intracellular sequestration of cAMP by binding activities other than those of protein kinase. It is, however, difficult to see how an appreciable fraction of the substrate present in the standard diesterase assay could be bound by the small amounts of cellular material present.

Table 22 indicates that a considerable fraction of the homogenate PDE activity in the adult CNS is sedimentable, as is the case with larval CNS. The specific PDE activities of adult CNS homogenates are somewhat lower than those observed in similarly-prepared larval CNS samples. However, both total cAMP-PDE and cGMP-PDE (units of activity per CNS) are moderately greater in the adult form.

Distribution of Phosphodiesterase Activity Among Larval CNS Ganglia

Table 23 indicates that larval brain clearly possesses greater cAMP- and cGMP-PDE activities as compared to the remaining ganglia. Cyclic GMP-PDE activity was appreciably greater than cyclic AMP activity in all ganglia (SO to A7, 8) except in the brain, where these activities were nearly equal. Greatest cAMP activity (expressed as units of activity per ganglion) was exhibited by the brain, suboesophageal, and terminal abdominal fused ganglion,
Table 23. Distribution of phosphodiesterase activity among larval CNS ganglia.

<table>
<thead>
<tr>
<th>Ganglion</th>
<th>ug protein assayed</th>
<th>Picomoles/10 min/assay</th>
<th>Units per ganglion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenosine</td>
<td>5'-AMP</td>
<td>Guanosine</td>
</tr>
<tr>
<td>Brain</td>
<td>35.2</td>
<td>120</td>
<td>2386</td>
</tr>
<tr>
<td>SO</td>
<td>25.2</td>
<td>132</td>
<td>1436</td>
</tr>
<tr>
<td>T1</td>
<td>24.6</td>
<td>97</td>
<td>1162</td>
</tr>
<tr>
<td>T2</td>
<td>28.0</td>
<td>119</td>
<td>1279</td>
</tr>
<tr>
<td>T3</td>
<td>27.0</td>
<td>104</td>
<td>1270</td>
</tr>
<tr>
<td>A1</td>
<td>26.8</td>
<td>61</td>
<td>960</td>
</tr>
<tr>
<td>A2</td>
<td>27.0</td>
<td>96</td>
<td>1005</td>
</tr>
<tr>
<td>A3</td>
<td>25.2</td>
<td>110</td>
<td>1127</td>
</tr>
<tr>
<td>A4</td>
<td>28.4</td>
<td>115</td>
<td>1165</td>
</tr>
<tr>
<td>A5</td>
<td>24.8</td>
<td>80</td>
<td>1250</td>
</tr>
<tr>
<td>A6</td>
<td>25.7</td>
<td>93</td>
<td>1234</td>
</tr>
<tr>
<td>A7, 8</td>
<td>40.4</td>
<td>143</td>
<td>1319</td>
</tr>
</tbody>
</table>

Twenty larval ganglia of each type were homogenized in 200 ul of hypotonic buffer (see Methods), and aliquots were taken for PDE assay (50 uM substrate, Assay II) and Lowry protein determination.
whereas greatest cGMP-PDE activity was found in the brain and in A7, 8; these three ganglia (B, SO, A7, 8) are the three physically largest of the larval CNS. 5'-nucleotidase activities (see adenosine and guanosine values in Table 23) directed at either 5'-AMP or 5'-GMP appear qualitatively similar throughout the CNS.

When compared on the basis of specific diesterase activities (see Figure 30), the larval brain and suboesophageal ganglion possess greater cAMP-activity than the residual ganglia. By way of contrast, cyclic GMP phosphodiesterase activity appears to be relatively uniformly distributed through the ganglia.

**Phosphodiesterase Activities in Larval and Adult Brains**

Data obtained in a single experiment were presented in Table 23 and Figure 30 concerning total and specific PDE activities in ganglion homogenates prepared in hypotonic buffer. Supplementary information on brain PDE activities obtained in other experiments will now be presented. The material for these studies was homogenized in either hypertonic or hypotonic buffer, as specified.

The data of Tables 24 and 25 show that cGMP-PDE activity in larval brain is not nearly as suppressed by hypertonic conditions as is cAMP-PDE, and also that much additional cAMP-PDE is obtained after Triton treatment or freeze-thawing of homogenates. These treatments have comparatively little effect on larval brain homogenate
Figure 3. Specific diesterase activities of larval ganglion homogenates. Specific diesterase activities of each ganglion were calculated from data entered in Table 23. The percentage of activity exhibited by each ganglion relative to the total calculated activity (summed over all ganglia) is depicted.
Table 24. Soluble and particulate PDE activities in larval brain homogenates prepared in hypertonic buffer.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (18 brains)</th>
<th>Specific activity (S = 50 uM)</th>
<th>Ratio of specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cAMP-PDE</td>
<td>cGMP-PDE</td>
</tr>
<tr>
<td>Homogenate</td>
<td>383 ug</td>
<td>5321</td>
<td>7633</td>
</tr>
<tr>
<td>0.5% Triton + Homogenate</td>
<td>--</td>
<td>7266</td>
<td>7490</td>
</tr>
<tr>
<td>34,000 x g supernatant</td>
<td>162</td>
<td>1172</td>
<td>3168</td>
</tr>
<tr>
<td>Washed 34,000 x g particles</td>
<td>120</td>
<td>8154</td>
<td>8212</td>
</tr>
</tbody>
</table>

Experimental details were described in Methods. An aliquot of the homogenate was incubated for 10 min at 0°C with 0.5% Triton X-100. The final concentration of Triton in the reaction mixture was 0.05%. The total protein recovered in the 34,000 x g supernatant and pellet was considerably less than that noted in the homogenate. It is likely that protein (and activity) was discarded with the buffer used to wash the 34,000 x g pellet.
Figure 31. Soluble and particulate phosphodiesterase activities of larval brain determined at two different substrate concentrations. All fractions were measured at both a 1 μM and a 50 μM initial substrate concentration. As noted in the legend to Table 24, protein and activity were discarded with the buffer used to wash the pellet, thus accounting for the apparent non-conservation of homogenate activity in the supernatant and particles.
Table 25. Phosphodiesterase activities of larval and adult brain homogenates (50 uM substrate).

<table>
<thead>
<tr>
<th>Expr't</th>
<th>Tissue</th>
<th>No. of brains</th>
<th>Homog. buffer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Comment</th>
<th>Specific activity units/mg protein</th>
<th>Total activity units/brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cAMP-PDE</td>
<td>cGMP-PDE</td>
</tr>
<tr>
<td>1</td>
<td>Larval brain</td>
<td>20</td>
<td>H−</td>
<td>freeze-thaw 1x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&quot; &quot;</td>
<td>39</td>
<td>H−</td>
<td>freeze-thaw 1x&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&quot; &quot;</td>
<td>5</td>
<td>H−</td>
<td>fresh, unfrozen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>&quot; &quot;</td>
<td>18</td>
<td>H+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4b</td>
<td>&quot; &quot;</td>
<td>18</td>
<td>H+</td>
<td>freeze-thaw 3x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Adult brain</td>
<td>5</td>
<td>H−</td>
<td>freeze-thaw 1x</td>
<td>&gt;5995&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4003</td>
</tr>
</tbody>
</table>

<sup>a</sup> H+, hypertonic; H−, hypotonic.

<sup>b</sup> Tissue stored at -80°C for nine days prior to homogenization.

<sup>c</sup> Appreciable fraction of substrate consumed during assay (35%).
cGMP-PDE. The effect of Triton on the larval brain homogenate differs from its effect upon bulk CNS homogenates (see Table 15), since in the latter case detergent-treatment resulted in a substantial increase in both cAMP-PDE and cGMP-PDE activities.

Table 24 demonstrates the presence of very high specific PDE activities in the washed 34,000 × g pellet as compared to either the homogenate or supernatant. In this brain pellet cAMP- and cGMP-PDE activities were nearly equal; by way of contrast, cGMP activity was approximately twice that of cAMP-PDE in a similarly-prepared (mitochondrial) pellet from bulk CNS (see Table 26). This is interpreted as a relative enrichment of cAMP-PDE in this brain particulate fraction. The 34,000 × g particles are presumably constituted of nuclei, large cell fragments, mitochondria, some microsomal material and miscellaneous other organelles. Further fractionation and sub-fractionation of the brain material will be required to establish the origin (hence, allow a speculation about function) of this particulate activity. It was calculated that 19% and 35% of the homogenate cAMP-PDE and cGMP-PDE activity, respectively, remained in the supernatant after centrifugation. Therefore, a considerably larger fraction of brain PDE is particulate when compared to the distribution of PDE in bulk CNS. The homogenate, 34,000 × g pellet, and supernatant were each assayed at both 1 μM and 50 μM initial cyclic nucleotide substrate concentration (Figure 31).
substrate variation it was found that the ratio of cGMP/cAMP hydrolysis was \( \geq 1 \) for every fraction. By varying substrate concentrations from millimolar to micromolar, Beavo et al. (144) found that the ratio of cGMP/cAMP hydrolyses in subcellular fractions from bovine and rat heart was either greater or less than one, depending upon the substrate level. This ratio was, however, always greater than one in rat brain.

Comparison of Tables 22 and 25 indicates that about 10% of the total cAMP-PDE and cGMP-PDE of larval CNS is located in the brain, although the total protein content of the brain relative to that of the whole CNS is somewhat less (approx. 6%). In contrast, in the central nervous system of the adult a much greater proportion of the total nerve cord activity is accounted for by the brain: 43% of the cAMP-PDE, and 30% of the cGMP-PDE. Approximately 20% of the total adult CNS protein is provided by the brain. In both developmental stages, therefore, the cAMP-PDE content of the brain is nearly twice that expected assuming a uniform distribution of PDE throughout the CNS protein. The significance of this enrichment in insect brain tissue remains to be established. During larval to adult development, total cAMP-PDE activity increases from 150 units/brain to 1050 units/brain (Table 25), a seven-fold increase. A relatively smaller increase in total cGMP-PDE activity occurs, developing from approximately 160 units/brain in the larval CNS to 700
units/brain in the adult (4.4-fold increase).

It was shown that either hypotonicity, treatment with Triton X-100, or multiple freeze-thaw cycles reveal greatly increased larval brain homogenate cAMP-PDE activity, but a proportional increase cGMP-PDE was not observed. These findings suggest the existence in larval brain of a cAMP-specific phosphodiesterase that is compartmentalized or weakly membrane-bound, possibly within nerve endings or other organelles. Differential solubilization of cAMP-PDE by the mentioned treatments was not observed in bulk CNS (containing the brain), and it seems likely that any cAMP-specific activity present in small amounts would be masked by the larger amounts of cAMP- and cGMP-PDE present in the entire CNS. Kinetic evidence will be presented which does not indicate any distinguishing features of brain cAMP-PDE compared to that in the bulk CNS.

Subcellular Distribution of Phosphodiesterase in the Larval CNS

The preparation of subcellular fractions was described in Methods. As is indicated by the data collected in Table 26, considerable PDE activity is found in all fractions from the M. sexta larval CNS. In particular, the highest specific activities are found in the 10M20 (mitochondrial) and 105µ60 (microsomal) particulate preparations. This finding appears highly significant, since similarly
prepared fractions from mammalian brain are known to contain (in addition to mitochondria and microsomes) synaptic membranes, vesicles, and small nerve endings (69). Possibly the greater part of PDE activity in *M. sexta* CNS is localized in these fractions in vivo. It seems essential to bear in mind that the tissue from which these fractions were derived was frozen at -80°C before homogenization, and that possibly the freeze-thaw cycle has disrupted the particulate/soluble distribution of PDE activities. Release into the medium of particulate-associated proteins by multiple freeze-thaw processes is a well-known phenomenon. Further experimentation with unfrozen insect nervous tissue will be required to evaluate the possibility that methods employed in the present preparation have resulted in an artifactual distribution of PDE among the fractions.

Table 26 demonstrates that the extent of hydrolysis of 5'-nucleotide (produce of PDE) to nucleoside is much less in the 105,000 × g supernatant than in any of the particulate fractions. It is clear that 5'-nucleotidase activity is primarily particulate in *M. sexta* CNS and that for all fractions other than high speed supernatants, an appreciable portion of the product of diesterase will rapidly be further degraded. Account of this was taken in the present research by summing both products (5'-nucleotide and nucleoside) as a measure of PDE activity. Table 26 suggests that the microsomal fraction (105,000 × g pellet) in *M. sexta* is greatly enriched in 5'-nucleotidase
Table 26. Distribution of phosphodiesterase activities among crude subcellular fractions from larval CNS.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fraction volume (ml)</th>
<th>Protein (μg/10 μl)</th>
<th>Volume assayed (μl)</th>
<th>Picomoles in 10 min per reaction mixture</th>
<th>Specific activity units/mg protein</th>
<th>Nucleotidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adenosine</td>
<td>5'-AMP</td>
<td>Guanosine</td>
</tr>
<tr>
<td>Homogenate</td>
<td>2.4</td>
<td>70</td>
<td>10</td>
<td>416</td>
<td>1,570</td>
<td>714</td>
</tr>
<tr>
<td>Post-nuclear supernatant</td>
<td>2.38</td>
<td>44</td>
<td>10</td>
<td>267</td>
<td>1,594</td>
<td>227</td>
</tr>
<tr>
<td>Nuclear pellet</td>
<td>2.15</td>
<td>28.6</td>
<td>20</td>
<td>182</td>
<td>483</td>
<td>152</td>
</tr>
<tr>
<td>Mitochondrial pellet</td>
<td>1.0</td>
<td>12</td>
<td>20</td>
<td>474</td>
<td>1,233</td>
<td>488</td>
</tr>
<tr>
<td>Microsomal pellet</td>
<td>0.40</td>
<td>29</td>
<td>20</td>
<td>1,443</td>
<td>1,671</td>
<td>1,371</td>
</tr>
<tr>
<td>105,000 x g 60 min supernatant</td>
<td>2.88</td>
<td>20</td>
<td>20</td>
<td>75</td>
<td>1,332</td>
<td>60</td>
</tr>
</tbody>
</table>

Fifty-five larval nerve cords were fractionated as described in Methods, and diesterase assays (Assay II) were conducted in a total volume of 0.20 ml at 50 μM substrate level. Each particulate fraction, with the exception of the microsomal pellet, was washed once and re-centrifuged before suspension and assay.
activity.

At the substrate level employed (50 μM), cGMP was hydrolyzed to a greater extent than cAMP in all fractions examined. The same qualitative effect (cGMP/cAMP hydrolysis > 1) has been noted in rat brain and liver preparations at a 1 μM substrate level, but not at millimolar levels (144). Since the crude mitochondrial fraction (10 M20) possessed the greatest specific PDE activities of the fractions examined, it was further explored over a wide range of substrate concentrations (Table 27). The 10M20 pellet has been suspended in hypotonic buffer (see Methods) and was frozen and thawed once before assaying with 1 to 50 μM cAMP or cGMP. Comparison of 10M20 PDE activities at 50 μM substrate in Tables 26 and 27 shows that a single freeze-thaw cycle has resulted in increases of 55% and 26%, respectively, in cAMP-PDE and cGMP-PDE activities. This increase may be due to the rupture of synaptic elements and release into the medium of previously latent activity. It may be seen in Table 27 that the cGMP/cAMP hydrolysis ratio is always greater than one and is smallest at a 1 μM substrate level. At presumed physiological levels (ca. 1 μM) these cyclic nucleotides would be hydrolyzed at approximately the same rate.

In Figure 32 the data of Table 26 are cast in a different form: the general distribution of diesterase is presented as activity units per larval CNS found in a particular fraction. It is readily seen that
Table 27. Dependence of phosphodiesterase activity in a larval CNS mitochondrial fraction upon substrate concentration.

<table>
<thead>
<tr>
<th>Substrate concentration µM</th>
<th>Specific phosphodiesterase activity units/mg protein</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP-PDE</td>
<td>cGMP-PDE</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1,275</td>
<td>1,475</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3,425</td>
<td>5,250</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5,387</td>
<td>8,125</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>10,988</td>
<td>18,262</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>14,212</td>
<td>24,312</td>
<td></td>
</tr>
</tbody>
</table>
Figure 32. Partition of cAMP-PDE and cGMP-PDE in the *M. sexta* larval CNS. Diesterase activities are presented as units per nerve cord found in a particular fraction. The ratio of cGMP-PDE to cAMP-PDE for each fraction is indicated in parentheses.
a large part of the activity present in the homogenate remains soluble
(105,000 × g 60 min supernatant) after centrifugation. Summing
activities over the soluble and three particulate fractions, it may be
calculated that 52% of the total cAMP-PDE activity is sedimentable,
and also that 35% is found in the 10M20 and 105 µ60 pellets. Simi-
larly, 51% of the cGMP-PDE activity is particulate, and 37% is asso-
ciated with the 10 M 20 and 105 µ 60 pellets. It is exceptionally unlikely
that more than a small fraction of the observed activities in the par-
ticulate fractions could be due to contaminating supernatant fluid,
since the pellets were thoroughly washed in a large buffer volume,
sedimented, and resuspended in fresh buffer before assay.

Kinetic Studies

Preparations from the larval CNS were assayed for cAMP-PDE
and cGMP-PDE at 6 to 8 substrate concentrations in the range of 1 to
200 µM according to Assay II. Backgrounds were determined for
every sample at each substrate concentration tested, and substrate
consumption was generally less than 15%. It was previously estab-
lished that the hydrolysis of substrate was linear at least up to 10 min
of incubation, and analysis of the fixed time (10 min) assay data spe-
cified v, the initial specific reaction velocity (pmol/min/mg protein).
Lineweaver-Burk double-reciprocal plots allowed a graphical deter-
mination of $K_m'$, the Michaelis constant, and $V_{max}'$, the maximum
specific initial velocity.

Reciprocal plots for cAMP and cGMP hydrolysis were in every case nonlinear, as exemplified by the kinetic behavior of a larval CNS mitochondrial fraction illustrated in Figure 33 (see also Figures 23 and 24 for biphasic reciprocal plots shown by a CNS homogenate). A discontinuity in slope was generally observed at a substrate concentration of about 10 to 20 μM. Two apparent $K_m$ values, and two $V_{max}$'s could be derived by extrapolation of the linear portions of the Lineweaver-Burk plots. In Table 28 are compiled kinetic constants calculated for a variety of larval CNS preparations.

It clearly emerges that PDE in the Manduca sexta CNS exhibits two apparent Michaelis constants for the hydrolysis of each cyclic nucleotide, and these are relatively close in value (approximately 10 μM and 20 μM). Other workers have noted two apparent Michaelis constants in crude mammalian cAMP-PDE preparations, but these values are quite well separated: in rat brain, 4 μM and 120 μM (150); mouse retina, 0.16 and 2.82 mM (185); C-6 glial cells, 1.5 μM and 200 μM (186); rat kidney, 2.7 μM and 95 μM (167); and in human blood platelets, 70 μM and 0.7 mM (188). In these preparations it would be possible to ignore the contribution of the high $K_m$ activity by assaying at a cAMP concentration near that of the low $K_m$ component, and in effect, examine the behavior of one "kinetically separated" component. In view of the close $K_m$ values in the Manduca sexta CNS, such an
Figure 33. Lineweaver-Burk plot of cAMP and cGMP hydrolysis by a mitochondrial fraction from the larval CNS. Units of reaction velocity, \( V \), are picomoles substrate consumed/min/mg protein.
Table 28. Kinetic constants for the hydrolysis of cAMP and cGMP catalyzed by a number of larval CNS preparations.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>cAMP-PDE</th>
<th></th>
<th>cGMP-PDE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Michaelis constants</td>
<td>Maximum velocities</td>
<td>Michaelis constants</td>
<td>Maximum velocities</td>
</tr>
<tr>
<td></td>
<td>uM</td>
<td>nmol/min mg protein</td>
<td>uM</td>
<td>nmol/min mg protein</td>
</tr>
<tr>
<td>Larval CNS homogenate</td>
<td>10</td>
<td>6.6</td>
<td>8.6</td>
<td>5.8</td>
</tr>
<tr>
<td>34,000 x g 30 min supernatant</td>
<td>7.5</td>
<td>5.2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0-50% (NH₄)₂SO₄ precipitate</td>
<td>8.0</td>
<td>6.1</td>
<td>9.1</td>
<td>5.1</td>
</tr>
<tr>
<td>10,000 x g 20 min (mitochondrial) pellet</td>
<td>6.7</td>
<td>8.0</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Larval brain homogenate</td>
<td>7.7</td>
<td>7.1</td>
<td>7.4</td>
<td>6.6</td>
</tr>
</tbody>
</table>

PDE preparations were assayed in the presence of 1-200 uM substrate according to Assay II. Kinetic constants were graphically determined from slopes and intercepts of the extrapolated linear portions of each Lineweaver-Burk double-reciprocal plot.
approach would not be feasible.

In mammalian PDE preparations it has generally been observed that double reciprocal plots are distinctly non-linear for cAMP hydrolysis, but are linear for cGMP hydrolysis (150, 166, 170, 185). Two micromolar $K_m$'s for cGMP-PDE were evident in the present study of *M. sexta* CNS, and this behavior distinguishes the insect preparation from others reported. Only a single $K_m$ for hydrolysis of cGMP has been reported in these mammalian preparations: rat brain, 20 µM (150); mouse retina, .66 mM (185); and cat heart, 5.3 µM (150). Biphasic double reciprocal plots of the nature observed in the insect CNS can be interpreted as indicating two or more separate enzymes with different affinities for substrate, or alternatively, as suggesting a single enzyme with regulatory properties (there may or may not be multiple substrate sites). Until the insect CNS activities are substantially purified, judgment on these alternatives must be reserved. If there is present in the insect CNS a single enzyme (or isozymes having similar substrate affinities) the experimental data thus far obtained only allow the generalization that, kinetically, it appears susceptible to moderate substrate activation. It is possible that the low $K_m$ site is primarily responsible for maintenance of basal nucleotide levels, but upon transient elevation of these levels, the high $K_m$ site assists in attenuating cyclic nucleotide concentrations.
Double-reciprocal plots of cAMP-PDE kinetic data similar to those noted for the insect preparation have been reported for a variety of crude and partially purified mammalian enzymes. In crude enzyme preparations, this nonlinearity may be indicative of multiple enzymes with disparate affinities for cyclic nucleotide, and the downward curvature of double-reciprocal plots may simply reflect a summation of velocities. To analyze situations such as these, Thompson and Appleman (190) have synthesized a series of theoretical curves to fit experimental data by mathematically composing various mixtures of two independent enzymes of known and nonidentical $K_m$ and $V_{max}$, each of which is presumed to obey Michaelis-Menten kinetics. It was apparent that kinetic constants derived from extrapolation of the linear portions of these biphasic curves were not reliable estimators of the "true" kinetic constants of the individual components of the system. The fact that an experimentally observed broken-line reciprocal plot can be simulated by two separate enzymes acting independently only allows, but does not prove, the existence of such enzymes.

The anomalous reciprocal plots exhibited by PDE can also be interpreted as indicating a single "homotropic, cooperative regulatory enzyme," i.e. one which contains two or more binding sites for substrate, and binding of the first substrate to one of the sites affects subsequent binding to the other. Russel et al. (183) have asserted that
PDE is a "negatively cooperative" enzyme, although it appears that alternative models could equally well satisfy the experimental data. The term "negative cooperativity" has been coined by Conway and Koshland (157) to describe multiple catalytic site enzymes in which binding of the first ligand decreases the affinity for the next ligand. A biphasic Lineweaver-Burk plot would also be exhibited by a positively-cooperative enzyme, or by a single enzyme having multiple, independent substrate sites of different affinities. Further, it is possible that PDE may well be a "heterotropic regulatory enzyme," one in which activity is altered by non-substrate effectors or modulators. Possible candidates for modulators of PDE activity include ATP or other nucleoside triphosphates, pyrophosphate, "protein activators," or membrane lipids.

Comparison of Phosphodiesterase in the M. sexta With That in Various Tissues

In Table 29 are entered representative specific activities of PDE reported in a variety of tissues. Intercomparison of values published by different authors is difficult because of widely varying assay conditions (temperature, buffer, substrate concentration, etc.). The $V_{\text{max}}$ values reported for PDE in Table 29 for the insect CNS homogenate were obtained by extrapolation of kinetic data in the approximate substrate range 50-200 μM. It is conceivable that higher
Table 29. Comparison of phosphodiesterase in the *M. sexta* CNS with that in various tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzyme source</th>
<th>Assay temp. °C</th>
<th>Substrate conc.</th>
<th>Specific PDE activity nmol/min/mg protein</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. sexta</em> larval CNS</td>
<td>H</td>
<td>30</td>
<td>∞</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>Rat brain</td>
<td>H</td>
<td>not specified</td>
<td>2 mM</td>
<td>39</td>
<td>178</td>
</tr>
<tr>
<td>Rat brain</td>
<td>S</td>
<td>25</td>
<td>2 mM</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Chicken brain</td>
<td>H</td>
<td>not specified</td>
<td>2 mM</td>
<td>44</td>
<td>178</td>
</tr>
<tr>
<td>Rat brain cortex</td>
<td>H</td>
<td>37</td>
<td>4 mM</td>
<td>130</td>
<td>171</td>
</tr>
<tr>
<td>Rat brain cortex</td>
<td>S</td>
<td>25</td>
<td>2 mM</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Mouse brain cortex</td>
<td>H</td>
<td>37</td>
<td>2 mM</td>
<td>64</td>
<td>185</td>
</tr>
<tr>
<td>Rat cerebrum</td>
<td>H</td>
<td>30</td>
<td>1 mM</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Bovine cerebrum</td>
<td>H</td>
<td>30</td>
<td>2 mM</td>
<td>54</td>
<td>155</td>
</tr>
<tr>
<td>Mouse brain synaptic vesicles</td>
<td>--</td>
<td>30</td>
<td>6 x 10^-9 M</td>
<td>0.5</td>
<td>73</td>
</tr>
<tr>
<td>Mouse C-6 glioma</td>
<td>H</td>
<td>30</td>
<td>2.3 mM</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Mouse retina</td>
<td>H</td>
<td>37</td>
<td>4 mM</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Rat liver</td>
<td>H</td>
<td>30</td>
<td>10 uM</td>
<td>---</td>
<td>191</td>
</tr>
<tr>
<td>Mouse adipocytes</td>
<td>S</td>
<td>37</td>
<td>∞</td>
<td>6.5</td>
<td>170</td>
</tr>
<tr>
<td>Rat pancreatic islet</td>
<td>H</td>
<td>37</td>
<td>18 uM</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

aH = homogenate; S = supernatant.
b∞ indicates calculated or reported maximum specific velocity, $V_{max}$. 
substrate concentrations would have resulted in even greater downward curvature of the double reciprocal plots, in which case larger maximum velocities would have been estimated. Specific PDE activities of *M. sexta* CNS homogenates appear to be about one-fifth those of mammalian neural preparations.

**Phosphodiesterase Distribution in Polyacrylamide Gels**

In view of the small amounts of insect CNS tissue available for conventional methods of purification ([NH$_4$]$_2$SO$_4$ fractionation, column chromatography, etc.), it was optimistically expected that disc electrophoresis might provide a simple one-step, many-fold purification of PDE using relatively little starting material. Although these studies have not yet been carried to completion, data presented here indicate that electrophoretic separation of diesterase activities in *M. sexta* would be feasible. The polyacrylamide gel staining technique developed for these studies suffers from a poor limit of detectability and also, for unexplained reasons, from nonreproducibility. Pending refinement of the technique, it appears that more laborious detection methods (e.g., direct assay of fractions) will be required for these small amounts of tissue.

Preliminary experiments indicated no visual PDE bands when *M. sexta* CNS supernatants were applied to standard Ornstein-Davis disc gels; the pH of the separating gel in this system is 8.9,
and of the stacking gel, 6.7. Attempts to reveal phosphodiesterase bands by varying the pH of the stacking gel (pH 7.2) and of the separating gel (pH 8.0, 7.0) were not successful. Even though the gels were pre-electrophoresed before application of the sample, it was feared that the persulfate employed to polymerize the separating gel might interfere with PDE activity. For this reason, separating gels were photopolymerized in glass tubes with riboflavin as an alternative to chemical polymerization with persulfate; still no positive PDE-staining bands were revealed.

Successful staining patterns were obtained in two experiments in which the stacking gel was totally omitted; the pH of the separating gel in both experiments was 8.0. One of the separating gels was polymerized with persulfate and run in the slab gel apparatus described in General Methods. The other was photopolymerized with riboflavin and electrophoresed in 0.5 × 13 cm cylindrical glass column. Both gels were extensively pre-run before application of the sample, and a much superior staining pattern was obtained with the slab-gel apparatus. Alkaline phosphatase yielded better patterns than either 5'-nucleotidase or snake venom (see Methods for conditions). In Figure 34 are shown the protein pattern and diesterase stain obtained with a 34,000 × g larval CNS supernatant. One gel strip was stained for cAMP-PDE by incubation at room temperature for 45 minutes in 10 mM cAMP, 2 mM lead acetate, and 10 units of alkaline
Figure 34. Comparison of duplicate gel strips stained for protein with Coomassie Blue (A) and for cAMP-PDE activity (B). A 34,000 X g supernatant (prepared in hypertonic buffer) from larval CNS was applied to the gel (17 µg protein/mm²) and electrophoresed for three hours. Vertical lines in A indicate principal peak positions calculated from a densitometer scan at 550 nm. C represents a densitometer scan at 500 nm of activity-stained gel B; peak positions correspond to Rf's of 0.21, 0.24. Vertical lines in C indicate the approximate positions where a replicate gel strip was sliced and further assayed for diesterase activity (Figure 35).
phosphatase. A replicate strip was not examined for cGMP-PDE staining activity since large quantities of cGMP (relatively expensive) would be required.

Two exceptionally sharp bands of cAMP-PDE activity were observed, corresponding to $R_f$'s of 0.21 and 0.24; in addition, a very diffuse background stain was observed further in the gel ($R_f$ approximately 0.50 to 0.65). A very faint stain was seen on the beginning surface of the gel, possibly indicating that some large molecular weight material was unable to penetrate the polyacrylamide matrix. Immediately after electrophoresis, an unstained 6.5 cm gel strip was cut into 13 equal slices. After maceration of each piece in 10 volumes of buffer, followed by centrifugation, an aliquot of the supernatant was assayed for both cAMP-PDE and cGMP-PDE activity according to the resin method (Figure 35). Greatest cAMP-PDE activity was found in those slices (Nos. 3, 4) corresponding to the two discrete bands observed in the stained gel strip; no activity was detected in those slices corresponding to the apparently non-specific stain seen further in the gel. When examined for cGMP-PDE activity by the admittedly insensitive resin assay, the sectioned gel demonstrated appreciable activity in slices 2 and 3, none in 4, and somewhat lower activities (background?) further along in the gel. The phosphodiesterase in slices 3 and 4 was further examined by the more sensitive chromatographic technique (Assay II, 50 μM substrate), and calculated
Figure 35. Apparent distribution of PDE over a polyacrylamide gel as estimated by Assay I. Sections 3 and 4 correspond to visually discernible activity bands staining positively with cAMP. Although not revealed by the resin method, slice 4 in fact contained cGMP activity.
activities were as follows:

<table>
<thead>
<tr>
<th>Gel section</th>
<th>Activity Units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP-PDE</td>
</tr>
<tr>
<td>3</td>
<td>282</td>
</tr>
<tr>
<td>4</td>
<td>125</td>
</tr>
<tr>
<td>Σ</td>
<td>407</td>
</tr>
</tbody>
</table>

Knowledge of the total activity applied to the gel (approximately 1800 units of cAMP-PDE and 2400 units of cGMP-PDE) allowed an estimation of recoveries. Nearly 23% of the cAMP-PDE and 38% of the cGMP-PDE activity applied to the gel was recovered in slices 3 and 4. Both substrates could be hydrolyzed by the activity in each slice (recall that the resin method indicated no cGMP-PDE activity in slice 4) and the ratio of cGMP/cAMP activities in both was > 1.

These preliminary results imply there may be only two discrete phosphodiesterase activities, similar in electrophoretic mobility, which are present in the soluble fraction of M. sexta CNS. In view of the insensitivity of the staining technique employed, the possibility that there are more than two bands cannot be discounted. The close adjacency on the gel of the two distinct bands seen after staining ($\Delta R_f = 0.03$) indicates these proteins differ only slightly in charge and/or size. Proteins of identical shape and molecular weight but distinguished by only one or two charges would be expected to behave
similarly.

**Phosphodiesterase Activators?**

The existence of a protein activator of cAMP-PDE has been established in rat, bovine, and human brain (155, 168, 194), in bovine heart (164, 189), and human blood (188). This activator apparently lacks both tissue and mammalian species specificity (155). The presence of such an activator has not yet been implicated in any non-mammalian tissue. A distinguishing feature of the protein activator is its exceptional stability to boiling. To investigate whether the insect CNS possessed an activator having similar physical properties (i.e., stability at 100°C), a crude "activator fraction" was prepared from 55 larval nerve cords according to the procedure of Cheung (155). After homogenization, boiling for 5 min, centrifugation, and boiling for 5 min more, the supernatant was dialyzed overnight. The dialysate was then concentrated against powdered sucrose. Control experiments showed this preparation was totally devoid of PDE activity. This "activator fraction" neither stimulated or diminished cAMP-PDE or cGMP-PDE activity in a 0-50% (NH₄)₂SO₄ CNS preparation or a 105,000 x g supernatant. It likewise had no effect upon polyacrylamide-gel separated activities. It may be noted that Uzunov and Weiss (194) observed, following electrophoretic separation of rat cerebellar PDE, that there resulted a resolution of activity
into six distinct PDE-containing bands and one activator band; cAMP-PDE in two of the six bands was effectively stimulated after admixture with the activator fraction.

On the basis of these experiments with the *M. sexta* CNS, one may conclude:

(1) there is not present in the insect CNS a protein activator that is required for activity, or

(2) an activator, if extant, was destroyed during boiling and thus is dissimilar to the mammalian protein as regards heat stability. Or,

(3) there was already present excess or tightly-bound activator in all of the preparations examined.

The presence of an activator in the insect system has not been discounted, but further research will be required to prove its existence. It would be interesting to examine what effect, if any, would be exerted upon *M. sexta* PDE by purified mammalian activator (189).

The stimulation of cAMP hydrolysis in rat liver extracts by submicromolar and micromolar concentrations of cGMP was first reported by Beavo et al. (144). This interesting phenomenon was later observed in crude preparations of other rat tissues, including brain (145). The stimulatory effect was reversed at higher concentrations of cGMP, where this nucleotide began to inhibit cAMP
hydrolysis. At any concentration of cAMP, however, cGMP hydrolysis was only decreased. Whether or not the modulation of cAMP-PDE by cGMP is physiologically operative has not been determined. As has been discussed by Beavo, Hardman, and Sutherland (145), the concentrations of cGMP required to maximally stimulate mammalian cAMP-PDE in vitro are approximately those found in many tissues. The concentration-dependent influence of cGMP would partially explain the opposite effects of low and high concentrations of exogenous cGMP on cell proliferation (162). A number of experiments were designed to investigate the effect of cGMP upon cAMP hydrolysis, and vice versa, in the insect nervous tissue.

Cyclic AMP hydrolysis (50 μM substrate) by various larval CNS preparations was measured in the presence and absence of 1 to 10 μM cGMP. Conversely, cGMP hydrolysis was gauged in the presence or absence of low cAMP concentrations. In homogenates, (NH₄)₂SO₄ precipitates, or polyacrylamide gel-separated fractions the influence of one nucleotide upon the hydrolysis of the other was purely inhibitory. It appears therefore, that the previously discussed stimulability of mammalian PDE is not shared by the insect enzyme system.

Further experimentation with partially purified PDE and a broader range of substrates is in order. It is important to recall that in the insect, cGMP is present in great excess over cAMP. Thus, the hydrolysis of cAMP in vivo would in all likelihood be greatly inhibited,
and the observed lack of stimulation of cAMP-PDE by cGMP is not too surprising.

**Phosphodiesterase Induction**

As discussed in the introduction, elevated levels of cAMP appear to promote the induction of cAMP-PDE in a number of mammalian cells adapted to tissue culture (159, 186, 193). Experiments were designed to establish if elevated intracellular cyclic nucleotide levels could induce the accumulation of either cAMP-PDE or cGMP-PDE, or both, in the *M. sexta* larval nerve cord. Since long-term culture conditions have not been established for this nervous tissue, incubation periods with various test substances were necessarily limited to only a few hours' duration. Longer incubation times invariably led to extensive lysis of the CNS. It must be emphasized that in the induction experiments to be described, parallel determinations of cAMP and cGMP were not made. The data presented must be regarded as preliminary findings, and further research will be required to quantitatively correlate the observed effects of the various compounds tested with changes in intracellular cyclic nucleotide concentrations.

The selection of appropriate agents to stimulate cAMP levels in the insect CNS was influenced by a recent report of Nathanson and Greengard (84). These authors established the presence of
discrete adenylate cyclases in the thoracic ganglia of Periplaneta americana (cockroach) which could be selectively and additively activated by low levels of octopamine, dopamine, and serotonin (5-hydroxytryptamine); at high concentrations, norepinephrine was also capable of stimulating adenyl cyclase in this tissue. Dopamine, serotonin, and norepinephrine are known to occur in insect nervous tissue (209), but it has not been established if they function as neurotransmitters. Octopamine has been found in both vertebrate and invertebrate nervous systems, and evidence has been provided suggesting that this compound may function as a transmitter (210). All of the above compounds were capable of appreciably elevating cAMP in the P. americana CNS, but octopamine was by far the most effective (84). The effects of any of these substances on cGMP levels were not determined.

M. sexta larval nerve cords were rapidly dissected and stored until use in either (a) the saline-bicarbonate-glucose Ringer solution described by Nathanson et al. (84), or (b) Grace's insect medium. Each incubation was initiated by transferring the pooled nerve cords (5-10 per experiment) into 5.0 ml of fresh, oxygen-saturated medium containing the test substance under examination. Serotonin, norepinephrine, octopamine, (but)₂ cAMP, theophylline, and the protein synthesis inhibitors cycloheximide and actinomycin D were tested singly or in various combinations, as specified. Incubations were
conducted for six hours at 37°C with periodic agitation in an atmosphere of 100% O₂. Following incubation, the tissues were thoroughly washed three times with ice-cold 100 mM Tris·HCl (pH 7.5) - 0.32 M sucrose and once briefly with cold distilled water before homogenization in 10 mM Tris·HCl (pH 7.5) - 1 mM MgSO₄ (hypotonic buffer). After quickly freezing and thawing three times, the homogenate was assayed for cAMP-PDE and/or cGMP-PDE. Experimental results are presented in Table 30.

Dibutryl cAMP, which previously was shown to inhibit cAMP-PDE and cGMP-PDE in the M. sexta CNS (and presumably elevates the levels of both cyclic nucleotides), resulted in about a 20% increase in both PDE activities during the six hour incubation period. Serotonin and octopamine were somewhat less effective, but the influence of these compounds was clearly distinguishable above control PDE activities. Norepinephrine (1 mM) promoted an appreciable increase in cAMP-PDE activity (33%) but caused an even greater elevation of cGMP-PDE activity (61%). It may be recalled (Table 2) that 10⁻⁴ M norepinephrine apparently did not affect cAMP or cGMP levels during a 10 min incubation of the M. sexta larval CNS, but further experiments will be necessary to establish if this is true or not. Norepinephrine is known to greatly stimulate cAMP levels in other insect nervous tissues (83, 84). As is indicated in Table 30, octopamine and dibutryl cAMP resulted in increased PDE activity
Table 30. Induction of phosphodiesterase in the larval M. sexta nerve cord.

<table>
<thead>
<tr>
<th>Experiment (Substrate concentration)</th>
<th>Incubation medium</th>
<th>Addition b</th>
<th>PDE activity units/CNS</th>
<th>Increase over control units/CNS</th>
<th>Percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>cAMP-PDE</td>
<td>cGMP-PDE</td>
<td>cAMP-PDE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 R (50 uM)</td>
<td>none (control)</td>
<td>423</td>
<td>---</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>(but)_2cAMP</td>
<td>503</td>
<td>---</td>
<td>80</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>octopamine</td>
<td>516</td>
<td>---</td>
<td>93</td>
<td>---</td>
</tr>
<tr>
<td>2 G (100 uM)</td>
<td>none (control)</td>
<td>769</td>
<td>1051</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(but)_2cAMP</td>
<td>938</td>
<td>1249</td>
<td>169</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>octopamine</td>
<td>873</td>
<td>1187</td>
<td>104</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>(but)_2cAMP + cycloheximide</td>
<td>1087</td>
<td>1520</td>
<td>318</td>
<td>469</td>
</tr>
<tr>
<td>3 G (100 uM)</td>
<td>none (control)</td>
<td>738</td>
<td>1084</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>serotonin</td>
<td>829</td>
<td>1131</td>
<td>91</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>norepinephrine</td>
<td>983</td>
<td>1746</td>
<td>245</td>
<td>662</td>
</tr>
<tr>
<td></td>
<td>cycloheximide</td>
<td>928</td>
<td>1493</td>
<td>190</td>
<td>409</td>
</tr>
<tr>
<td></td>
<td>actinomycin D</td>
<td>1013</td>
<td>1840</td>
<td>275</td>
<td>756</td>
</tr>
<tr>
<td></td>
<td>(but)_2cAMP + cycloheximide</td>
<td>1136</td>
<td>1851</td>
<td>398</td>
<td>767</td>
</tr>
<tr>
<td></td>
<td>(but)_2cAMP + actinomycin D</td>
<td>908</td>
<td>1301</td>
<td>170</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>(but)_2cAMP + theophylline</td>
<td>993</td>
<td>1523</td>
<td>255</td>
<td>439</td>
</tr>
</tbody>
</table>

a R = saline-bicarbonate-glucose Ringer solution; G = Grace's insect medium (contains salts, amino acids, sugars, and vitamins).

b Final concentrations in the incubation medium were: 1mM for (but)_2cAMP, octopamine, theophylline, serotonin, and norepinephrine; 0.5 mM for cycloheximide; and 1 ug/ml for actinomycin D.

Larval nerve cords (5-10 per experiment) were incubated in the stated medium with the specified test substance for six hours at 37°C in a 100% O_2 atmosphere. PDE assays were as described in the text (Assay II) using a thrice frozen-and-thawed homogenate as source of enzyme.
when the nerve cords were incubated in either (a) Ringer's solution, which contained no amino acids, or (b) Grace's tissue culture medium (containing amino acids). This demonstrated that amino acids were not required for the observed increase in PDE levels to occur, and suggested that either the amino acid pool size of the nerve cords was quite large or that protein synthesis was not required for induction (see below).

Cycloheximide or actinomycin D could not prevent the apparent induction of PDE by dibutryl cAMP. In fact, these protein synthesis inhibitors themselves promoted great increases in PDE activity. These observations are in marked contrast to those of D'Armiento et al. (159), who noted that induction of PDE in 3T3 fibroblasts could be totally blocked by cycloheximide or actinomycin D. Assuming these inhibitors are capable of blocking protein synthesis in the \textit{M. sexta} CNS (this has not been demonstrated), as they are known to do in a variety of biological systems, it can only be concluded that the "induction" (this term is used loosely) of PDE in the insect does not depend upon protein or RNA synthesis. This would be possible, for example, if there occurred a protein synthesis-independent conversion from a precursor or inactive form of PDE to an active form. It could also result from a diminution in the rate of degradation of active enzyme during the incubation period, or from the inhibition of synthesis of some rapidly turning-over component (an inhibitor
or some other protein) which normally interferes with PDE activity. It is conceivable that cycloheximide and actinomycin D themselves promote an elevation of intracellular cyclic nucleotide levels, and this in turn effects a protein synthesis-independent activation of PDE.

Further research must be conducted with the insect CNS to determine (1) if the activation process requires an intact cell system, (2) the time course of PDE activation, (3) the extent of inhibition of \textit{M. sexta} protein synthesis effected by various transcriptional and translational level protein synthesis inhibitors, (4) the relationship between elevated cAMP (cGMP) levels and induction, (5) the subcellular distribution of activated PDE (is soluble or particulate enzyme activity more susceptible to activation?), (6) the kinetic properties of the induced activity, and (7) whether or not elevated PDE activity returns to basal levels after removal of the stimulus. If the induction (or activation) of PDE proves to be correlated with elevated cyclic nucleotide levels (either cAMP or cGMP, or both), this could be interpreted as a physiologically important control mechanism which would allow the cell to protect itself against prolonged elevated levels of cAMP or cGMP.

\textbf{General Discussion}

Investigation of PDE in the CNS of \textit{M. sexta} has led to the following conclusions:
(1) As is the case with mammals, PDE in *M. sexta* is present at highest levels in nervous tissue.

(2) The total and specific PDE activities of larval and adult brains are greater than those of the remaining ganglia.

(3) Specific PDE activities of the adult CNS are somewhat lower than those of the larval CNS, although both protein content and total PDE activity are considerably greater in the adult CNS.

(4) Measurable enzymatic activities are very dependent upon the method of tissue preparation (buffer tonicity, presence or absence of detergent, freezing and thawing).

(5) The metal requirement of the insect PDE differs from that of other reported bivalent metal ion-requiring PDE preparations. Mg$^{2+}$ does not appear to be absolutely required, and EDTA cannot abolish enzymatic activity.

(6) The *M. sexta* CNS PDE is vulnerable to inhibition by a variety of physiological and non-physiological compounds, nucleoside triphosphates being the most potent. Some agents capable of acute antagonism of mammalian PDE's are comparatively ineffective toward the insect enzyme system.

(7) PDE was found in both soluble and particulate fractions of the larval CNS. Subcellular distribution studies indicated very high specific activity PDE in a crude mitochondrial preparation, a fraction presumably enriched in synaptic elements.
(8) Kinetic studies of larval CNS preparations revealed non-linear double-reciprocal plots for the hydrolysis of both cAMP and cGMP, and two Michaelis constants (approximately 10 μM and 20 μM) could be derived from the data. Similar kinetic behavior of all particulate and soluble fractions was observed.

(9) The larval brain appeared to contain cAMP-specific PDE activity, but this could not be confirmed by kinetic analyses.

(10) The hydrolysis of both cAMP and cGMP appears to be the function of a single enzyme in the larval CNS. The intracellular level of one cyclic nucleotide may in part determine the concentration of the other by inhibiting its degradation. No stimulatory effect of cGMP upon the hydrolysis of cAMP, or vice versa, was observed.

(11) The presence of a protein activator of cAMP-PDE, known to occur in mammalian systems, could not be established in the insect CNS.

The greater specific PDE activity of the insect brain relative to that of the other ganglia may reflect enhanced levels in the brain of PDE enzymes relative to other proteins or an enrichment in the brain of particular cell types containing high levels of these enzymes. It remains to be determined if the high PDE levels in brain are paralleled by greater cyclic nucleotide levels in this ganglion compared to the remainder of the CNS. Although it has not been done in the present
study, it would be highly desirable to re-evaluate the data in terms of enzyme activity per unit of DNA. Expression of specific activities in terms of units per unit of protein limits one to an evaluation of changes in enzyme activities relative to those of other proteins. A knowledge of enzyme activity per unit of DNA, hence per cell, would convey more information.

The functional significance of the increase in PDE activity during larval to adult development and of the greater activity in brain relative to the other ganglia is not known. It is possible that PDE activity is correlated with the absolute number of synapses or with the magnitude of synaptic activity. Electron microscopy might prove useful in examining the former possibility; the latter possibility could well be tested by assaying PDE in two groups of animals, one maintained in a low-stimulus environment, and the other provided with high levels of neural input (light, shock, agitation, etc.). Relatively little information has been gained in the present study by examining PDE in only the initial (larval) and final (adult) states of neural transformation. An appraisal of the functional significance of PDE might be aided by an examination of the ontogenetic development of this enzyme system during pupal neurometamorphosis. It may be expected that PDE activity will be modified at certain critical periods of neural development, and these alterations may be correlated with morphological or neurochemical changes, perhaps with the establishment of new synaptic structures. It would be particularly
desirable to examine the temporal development of cyclic nucleotide levels in the insect CNS during pupation. It is conceivable that elevations in cAMP or cGMP levels promoted by endogenous hormones may themselves be instrumental in regulating the development of PDE.

Only two electrophoretically-separable forms of PDE were detected in the *M. sexta* larval CNS, and this may reflect the relatively small number of cell types present in this tissue. In comparison, mammalian brain (or even localized regions thereof) is constituted of an inordinately complex association of morphologically and functionally dissimilar cell types and contains multiple forms of PDE. In view of its comparative simplicity, the insect CNS would seem an attractive tissue for further neurological research, particularly in the area of developmental neurobiochemistry.

With regard to the desirability of a relatively homogeneous population of neural cells for investigating multiple molecular forms of PDE, the very recent experiments of Uzunov et al. (193-195) serve as an excellent example. Six electrophoretically separable forms of cAMP-PDE could be detected in one area of the rat brain, viz. the cerebellum. In another area of this brain, the cerebrum, only four separable activities (designated I to IV) were distinguished (193). On the suspicion that these different molecular forms might be localized in specific cell types, cAMP-PDE was examined in cloned neuroblastoma cell lines (N1E and N18) and in a C-2A astrocytoma cell
culture. In both lines of neuroblastoma only a single activity, corresponding to peak III from the cerebrum, was found. On the other hand, the C-2A astrocytes revealed two activities with electrophoretic mobilities similar to peaks I and IV of the whole cerebrum. Although clonal lines of insect neural cells have not been established, it may be possible to specify the cellular origin(s) of PDE by an examination of neuronal- and glial-enriched fractions.

Kinetic analyses of various fractions from the larval *M. sexta* nerve cord disclosed two Michaelis constants for the hydrolysis of cAMP, and two for cGMP. These constants did not differ appreciably in soluble and particulate preparations, ammonium sulfate and subcellular fractions, and brain homogenates. Cyclic AMP or cGMP hydrolytic activities could not be distinguished by their metal requirements, pH optima, or subcellular distribution; nor was there any pronounced differential susceptibility of the two activities to a variety of inhibitors. It seems likely that the degradation of both cAMP and cGMP in the insect nerve cord may be a function of a single enzyme (or kinetically similar isozymes). This conclusion is advanced with caution, however, as it is entirely possible that some forms of PDE present in small quantities were kinetically masked. The existence in the insect CNS of cAMP-specific or cGMP-specific PDE's, which have been reported in mammalian systems, cannot be disallowed. Conclusive proof must await large-scale separation and purification
of the various activities, followed by a detailed kinetic and enzymological study of their properties.

It was shown that millimolar levels of ATP and PP\textsubscript{1} effectively inhibit the insect PDE. This modulation of PDE activity may be physiologically relevant since ATP and PP\textsubscript{1} are the substrate and product, respectively, of adenyl cyclase. Although the intracellular concentrations of ATP and PP\textsubscript{1} in the \textit{M. sexta} CNS are not known, Cheung (152) has estimated that levels of ATP in rat brain may reach 4 mM; this concentration of ATP, particularly in conjunction with low intracellular levels of Mg\textsuperscript{2+}, would severely inhibit both cAMP-PDE and cGMP-PDE in the nerve cord. It seems likely that circumstances favorable to the formation of cAMP, i.e., activated adenyl cyclase in the presence of moderate levels of ATP, would also lead to suppression of phosphodiesterase (through inhibition by ATP, PP\textsubscript{1}, and also 5\textsuperscript{'-}AMP). Thus it appears that a transient elevation of cAMP concentrations in the presence of a great excess of phosphodiesterase would be rendered possible, at least in part, by the susceptibility of PDE to inhibition. The present research on \textit{M. sexta} CNS phosphodiesterase has indicated that it is capable of being regulated by endogenous substances. Of these, nucleoside triphosphates would appear to be the most potent, although numerous other compounds (PP\textsubscript{1}, ADP, AMP, adenosine, etc.) may participate in the general framework of control mechanisms.
In mammalian tissues, phosphodiesterase activity (as gauged \textit{in vivo}) has been reported to be present in great excess over adenyl cyclase (65, 198). It will be necessary to accurately quantitate adenyl cyclase activity in \textit{M. sexta} to determine whether this apparent disparity obtains in the insect. However, under the assumption that cyclase activity in the \textit{M. sexta} CNS is comparable in magnitude to that reported in other insect tissues (83, 84, 119), the provisional conclusion may be drawn that diesterase levels greatly exceed cyclase levels in the insect central nervous system. How, then, may cAMP be expected to accumulate under such apparently catabolic circumstances? It appears likely, as suggested by Cheung (152, 153), that phosphodiesterase may exist \textit{in vivo} in a greatly inhibited state.

After the present research was completed, there appeared in the literature a report of PDE activity in another insect tissue, \textit{Hyalophora cecropia} (silkmoth) larval fat body (161). Detailed properties of this enzyme were not established, but two $K_m$ values for cAMP hydrolysis (3.5 $\mu$M, 190 $\mu$M) and two for cGMP (2.7 $\mu$M, 180 $\mu$M) were reported. The similarity of high and low $K_m$ values for both cyclic nucleotides in the silkmoth preparation suggests that, analogous to the case of the \textit{M. sexta} CNS, there may be present a single enzyme capable of utilizing both substrates.
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APPENDIX I

Purification of \(^{3}\)H cAMP and \(^{3}\)H cGMP

In some instances it was necessary to purify the commercially obtained tritiated cyclic nucleotides before use in radiometric assays, as they frequently contained radioactive impurities in the range of 5 to 10\%. For example, in the case of paper chromatographic assays for phosphodiesterase, it was particularly important that the substrates be free from contaminating levels of \(^{3}\)H nucleoside and \(^{3}\)H 5'-nucleotide. These substances were formed after long-term storage of the \(^{3}\)H cyclic nucleotides, and required chromatographic removal.

Materials

\(^{3}\)H cyclic adenosine 3', 5'-monophosphate (specific activity 24 Curies/mMole) and \(^{3}\)H cyclic guanosine 3', 5'-monophosphate (specific activity 4 Curies/mMole) were obtained as ammonium salts in 50\% ethanol from New England Nuclear. Precoated thin layer microcrystalline cellulose chromatographic plates, 5 cm X 20 cm X 250 \(\mu\), were purchased from Applied Science Laboratories, Inc. Before use, these plates were pre-washed two times by development with distilled water, air dried, activated at 90\°C for 30 minutes, and stored until use in a desiccated chamber. 2-propanol
(spectroquality) and ethanol (100%) were from Matheson, Coleman, and Bell, and from Commercial Solvents Corporation, respectively.

**Purification Procedure**

The $[^{3}H]$ cyclic nucleotide solution was spotted in a wide horizontal band approximately 3-4 cm from the bottom of the plate and evaporated with warm air from a hair dryer; up to 2.0 ml of the solution ($<10^{-7}$ moles of nucleotide) were applied to a single plate. Unlabeled cAMP or cGMP (ca. 0.02 μmole) was chromatographed on the same plate, well-separated from the labeled nucleotide by a vertical channel inscribed in the adsorbent. The plates were developed (ascending) at room temperature with 2-propanol-$\text{NH}_{4}\text{OH-H}_{2}\text{O}$ (7:1.5:1.5, v/v/v). After approximately six hours, during which the solvent front migrated within 2-3 cm from the top of the plate, the chromatograms were removed from the tank and air dried.

**Recovery of Cyclic Nucleotides**

The area of the chromatogram containing the marker nucleotide (unlabeled) was determined under UV light, and a broad segment (approximately 3 cm in width) of the parallel $[^{3}H]$ nucleotide-containing channel was scraped with a spatula and the adsorbent brushed onto glassine paper. After transfer to a 13 × 100 cm centrifuge tube, the adsorbent was covered with one volume (equal to that applied to the
plate) of 50% ethanol, and the slurry was warmed briefly to 50°C. Separation of the eluted nucleotide from the adsorbent slurry was simply effected by low speed centrifugation through an Amicon CF50A ultrafilter fitted in a Centriflo conical support. The clear solution thereby obtained was divided into a number of tubes, rapidly frozen in liquid nitrogen, and stored at -80°C until use. When further concentration of the nucleotide solution was required, it was accomplished by lyophilization in a 5 ml conical centrifuge tube. An appropriate volume of the desired solvent or buffer was then added to the residue, and the walls of the tube were gently rinsed by swirling in a Vortex mixer.
APPENDIX II

Preparation of $\gamma^{32}$P ATP

There are available in the literature a number of methods for the synthesis and purification of ATP labeled with $^{32}$P in the terminal phosphate position (131, 211, 212). These methods capitalize upon the exchange reaction between $^{32}$P-orthophosphate and the $\gamma$-phosphate group of ATP which occurs in the presence of phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglyceric acid (212). The published methods are relatively noncommital as regards time of incubation, reaction pH, temperature, and presence of cofactors (such as mercaptoethanol, NAD, and cysteine). The preparative procedure finally adopted for the present work may be considered a composite of the methods of Glynn and Chappell (212) and of Post and Sen (211), with modification. The reaction volume was kept as small as possible to enhance the rate of terminal phosphate exchange.

Materials

Carrier-free $^{32}$P-phosphoric acid in 0.02 N HCl was purchased from New England Nuclear. ATP, ADP, 3-phosphoglycerate, and NAD were all obtained from Sigma. Yeast 3-phosphoglycerate kinase (2 mg/ml) was purchased from Calbiochem as a suspension in 2.7 M
(NH₄)₂SO₄/0.04 M Na₄P₂O₇. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (10 mg/ml) was obtained from Sigma as a suspension in 2.6 M (NH₄)₂SO₄ containing 0.001 M EDTA.

Preparative Procedure

The following two solutions were prepared immediately before use. Solution I (total volume 1.30 ml) was composed of 50 µmoles ATP, 0.5 µmole ADP, 10 µmoles 3-phosphoglyceric acid, 0.1 µmole β-diphosphopyridine nucleotide (NAD), 5 µmoles cysteine (free base), 20 µl of 10% (v/v) 2-mercaptoethanol in H₂O, 100 µg (50 µl) of 3-phosphoglycerate kinase suspension, 500 µg (50 µl) of glyceraldehyde-3-phosphate dehydrogenase suspension, and 50 µl of 0.10 M MgCl₂. Solution II was constituted of 0.50 ml of 0.5 M Tris, pH 8.0, 1.0 ml (10 mC) H₃P³²O₄ in 0.02 N HCl, and 30 µl of 1.0 M Tris (to neutralize the HCl in the H₃P³²O₄).

Solution I was carefully mixed with solution II in the screw-capped vial in which the H₃P³²O₄ was shipped. A tiny drop of the resultant solution (total volume = 2.8 ml) was added to narrow-range pH test paper to check that the final pH was in the range of 7 to 8. The reaction mixture was incubated at 30°C for 1 hour in a shaking water bath.
Separation of the Labeled ATP

A 1 × 4 cm Dowex column (200-400 mesh, chloride form) was prepared in a Kontes 2A glass funnel; the resin had been extensively pre-treated as described by Post and Sen (211). The entire reaction mixture was transferred without deproteinization to the column, and then the incubation vial was rinsed with 2.0 ml of water, this washing also being added to the column. Column elution followed closely the schedule published by Glynn and Chappell (212). The column was first eluted with 40 ml of 20 mM NH₄Cl-0.02 N HCl which served to remove traces of AMP, ADP, and orthophosphate. Elution was then effected with 40 ml of glass-distilled water, followed by 15-18 ml of 0.25 N HCl, the HCl fraction (containing ATP³²) being collected in a small graduated cylinder immersed in ice. The ATP³² fraction in 0.25 N HCl was then neutralized with 2 M Tris, divided into small aliquots, and stored in -80°C until use.

Purity of the Product

A 1 to 200 dilution of the ATP³² fraction was made in 50 mM Tris·HCl (pH 7.0) and the UV absorbance was measured at 250, 259, 260, and 280 nanometers. Spectral ratios (A₂₅₀/A₂₆₀ and A₂₈₀/A₂₆₀) were calculated and compared with published literature values (213); agreement was excellent. The absorbance at 259 nm allowed a
determination of the concentration of ATP, assuming a molar extinction coefficient of $15.4 \times 10^3$ at pH 7.0.

**Recovery and Specific Activity**

In three separate preparations the recovery of ATP ranged from 75.0 to 88.5%. The incorporation of $^{32}$P into recoverable ATP varied from 80.0 to 90.2%, and the final concentration of the ATP$^{32}$ Dowex column fraction fell in the range $2.10$ to $2.50 \times 10^{-3}$ M. Specific activities varied from $3.1$ to $4.8 \times 10^{11}$ cpm per mmole.
APPENDIX III

Preparation of $^{32}$P-labeled protamine

The $^{32}$P-labeled protamine substrate for the protein phosphate assay was prepared by enzymatic phosphorylation of protamine. $[^\gamma-^{32}\text{P}]$ ATP was prepared as described (Appendix II). Protamine sulfate from salmon (Grade I, histone-free) and cAMP-dependent protein kinase from beef heart were obtained from Sigma. The latter material was in fact an impure fraction containing $<0.01$ $\mu$M units/mg ATPase and approximately 0.5 $\mu$M units/mg phosphodiesterase, but proved entirely satisfactory for the phosphorylation reaction. The preparative reaction employed is similar in some aspects to those described by Meisler and Langan (19) and Maeno and Greengard (21), but it is sufficiently different to warrant complete description here.

The reaction was most conveniently carried out in a heavy-walled 15 ml Corex centrifuge tube and was composed as follows:

1. 46 ml of 50 mM Tris·HCl (pH 7.0)
2. 0 ml of 10 mg/ml protamine sulfate in 50 mM Tris·HCl (pH 7.0)
1.0 mg of cAMP-dependent protein kinase
200 $\mu$l of 40 mM theophylline (phosphodiesterase inhibitor)
20 $\mu$l of 2.0 M MgSO$_4$
80 $\mu$l of 50 mM DTT
40 $\mu$l of 1 mM cAMP
200 $\mu$l of 2.1 mM $[^\gamma-^{32}\text{P}]$ ATP (approximately 10$^8$ cpm)
4.0 ml total volume

Reagents were added in the order noted, and this incubation mixture contained 20 mg of protamine sulfate, 1.0 mg of kinase,
2 mM theophylline, 10 mM MgSO$_4$, 1 mM DTT, 10 μM cAMP, and 105 μM ATP$^{32}$. The crude protein preparation employed (Sigma) lent considerable turbidity to the solution, but this proved of little consequence. The reaction vessel was flushed with nitrogen gas, sealed, and incubated at 35-37°C for 2 hours. At this time, 40 μl of 1 mM cAMP and 200 μl of 2.1 mM ATP$^{32}$ were added to replenish these compounds in the reaction mixture (the kinase preparation was contaminated with phosphodiesterase and ATPase). After four hours of further incubation, 1.06 ml of 10% TCA was added dropwise with continuous mixing so that the final TCA concentration was 2%.

Meisler and Langan (19) have reported this step precipitates calf liver protein kinase but not protamine. Separate experiments performed in this laboratory confirmed this observation for the bovine heart enzyme. After centrifugation at 10,000 X g for 10 min and transfer of the supernatant to another vessel, phosphorylated protamine was precipitated by the addition of 5.0 ml of 40% TCA (final concentration, 20%). The mixture was placed on ice for 5 min and then collected by centrifugation in the cold at 10,000 X g for 20 min. The supernatant was decanted, and the precipitate was twice washed by suspension in 10 ml of cold 10% TCA followed by centrifugation. The washed pellet was dissolved in 4 ml of 10 mM Tris·HCl (pH 7.0) by stirring for 60 min in a 40°C water bath, followed by precipitation with an equal volume of 40% TCA; this process was repeated once.
After dissolution of the pellet in 5 ml of warm mM Tris·HCl (pH 7.0), the solution was dialyzed in the cold for 15 hours against eight liters of water, followed by lyophilization.

After lyophilization, the residue was stirred for one hour at 40 °C in 40 ml of 50 mM Tris·HCl (pH 7.0) to form a clear solution. Two aliquots (20 µl, 40 µl) were assayed for protein content by a micro-modification of the method of Lowry et al. (105) using unlabeled protamine as a standard. Recovery of protamine carried through the phosphorylation reaction was 100%, and the final solution contained 2 mg protein per ml. Incorporation of $^{32}$P from ATP$^{32}$ into protamine was 38% and amounted to 16 nanomoles of phosphate per mg of protamine. Immediately after preparation, the specific activity was $2.9 \times 10^6$ cpm per mg of protamine. It has been demonstrated that serine is the only amino acid residue of protamine which is phosphorylated by rat liver and bovine brain kinases (19, 21). Presumably this is true also for the bovine heart kinase used in the present preparation.