MONOAMINE-SENSITIVE ADENYLATE CYCLASE IN THE NERVOUS SYSTEM OF THE LOCUST SCHISTOCERCA GREGARIA

by

ANDREW THOMAS KILPATRICK

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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ABBREVIATIONS

App(NH)p	Adenylyl-imidodiphosphate
ATP	Adenosine-5'-triphosphate
Cyclic AMP (cAMP)	Adenosine-3':5'-cyclic phosphate
EDTA	Ethylenediaminetetra acetic acid
EGTA	1,2-Di(2-aminoethoxy)-ethane-N N N'N'-tetra acetic acid
GDP	Guanosine-5'-diphosphate
Gpp(NH)p	Guanylyl-imidodiphosphate
Gpp(CH_2)p	Guanylyl-[$\beta\gamma$-methylene]-diphosphate
GTP(γ)S	Guanosine-5'-O-(3-thiotriphosphate)
GTP-ase	Guanosine triphosphatase
NADH	Reduced nicotinamide-adenine dinucleotide
TRIS	2-Amino-2-hydroxymethylpropane-1,3-diol

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SUMMARY

The observation that dopamine, octopamine and serotonin represent the major monoamines present in insect nervous tissue has led to speculation about their role. For the monoamines to act as neurotransmitters, receptors should exist. Moreover, if chemical neurotransmission involves cyclic AMP as a "second messenger", then monoamine receptors linked to adenylate cyclase should also be present. The aim of this work was to determine whether locust cerebral ganglia possessed an adenylate cyclase that was affected by these monoamines present in Schistocerca gregaria nervous tissue and, if so, to see if the response was mediated by separate receptors. In addition, a comparison of the properties of the locust adenylate cyclase with those from other organisms was carried out.

Cerebral ganglia of S. gregaria contain an adenylate cyclase stimulated maximally, in the presence of 10^{-4}M GTP, by octopamine (10^{-4}M), dopamine (10^{-4}M) and serotonin (10^{-5}M). The preferential inhibition of the octopamine-, dopamine- and serotonin-stimulated adenylate cyclase by phentolamine (8 μM), chlorpromazine (10 μM) and cyproheptadine (10 μM) respectively, together with the ability of any combination of saturating amounts of the monoamines to stimulate in an additive manner suggests that activation occurs via separate receptors.

The replacement of GTP (10^{-4}M) by Gpp(NH)p (10^{-4}M) leads to a greater efficiency of activation of locust adenylate cyclase in a pellet fraction. Furthermore, this activation appeared to be independent of octopamine, dopamine or serotonin. However,

additional washing of the membrane preparation revealed an octopamine-sensitive component which could be inhibited by phentolamine (80 μM). The failure of phentolamine to reduce the Gpp(NH)p activation suggests that endogenous octopamine is not primarily responsible for the activity.

Gpp(NH)p (10^{-4}M) forms an active stable enzyme complex with S. gregaria adenylate cyclase similar to that observed with cyclases from other organisms. Complete reversal of this locust enzyme complex required the simultaneous addition of octopamine (10^{-4}M) and GTP (10^{-4}M).

Time course experiments showed that a lag occurred in the activation of rat liver adenylate cyclase in the presence of Gpp(NH)p. Furthermore, this lag was abolished by glucagon. Thus, it was suggested that a slow isomerisation of adenylate cyclase from an inactive to active form of the enzyme was responsible for the lag. The failure of the locust enzyme in time course experiments to display a lag in the presence of Gpp(NH)p (10^{-4}M) suggests that if a transition state exists, its interconversion must be rapid.

Pre-incubation of locust adenylate cyclase with GTP (10^{-4}M) followed by the addition of Gpp(NH)p (10^{-4}M) led to a lag in activation in time course experiments. This lag was abolished by octopamine. However, although the evidence is not conclusive this lag most likely represents the time necessary for Gpp(NH)p to remove the tightly bound nucleotide from its binding site. Furthermore, octopamine would appear to facilitiate its exchange.

The inhibition of Gpp(NH)p activation by GDP (10^{-4}M and 10^{-3}M) together with the reduced stimulation observed with octopamine (10^{-4}M) and GDP (10^{-4}M) in the presence of App(NH)p suggests that GDP may produce an inactive form of the enzyme.

INTRODUCTION

Many of the biochemical and physiological responses of target cells to circulating hormones depend on the action of adenosine-3':5'-cyclic phosphate (cyclic AMP). For example, cyclic AMP, regarded as a "second messenger" which translates the extra-cellular message of the hormone into an intra-cellular response, is involved in glycogenolysis, lipolysis and the release of thyroid stimulating hormone (Robison et al, 1971).

The observation that the CNS has the highest activity of adenylate cyclase, the enzyme which catalyses the conversion of ATP to cyclic AMP of any mammalian tissue studied (Sutherland et al, 1962) and that the activity varied considerably in different brain regions (Weiss and Costa, 1968) prompted research into its involvement in brain function (Greengard et al, 1972). In non-neural tissue, it was suggested that cyclic AMP-mediated responses occurred through binding of the hormone to specific receptors on the target cell surface (Robison et al, 1967). If cyclic AMP acts as a "second messenger" in the nervous system, then a "primary messenger" (neurotransmitter) and receptor should exist. Evidence has accumulated that some putative neurotransmitters (noradrenaline, dopamine, serotonin, histamine, glutamate, asparate, substance P) can alter cyclic AMP levels, both in intact cells and cell-free systems (Daly, 1976).

Before describing the evidence for a possible role of cyclic AMP in brain function, the distribution of the monoamines, noradrenaline, dopamine, serotonin and octopamine in vertebrate and invertebrate nervous systems will be discussed.

Distribution of Noradrenaline, Dopamine, Octopamine and Serotonin

1. Noradrenaline

Noradrenaline, first identified as the neurotransmitter substance of adrenergic nerves in the peripheral nervous system (Von Euler, 1946), was later identified as a normal constituent of mammalian brain (Holtz, 1950) and a specialised function for noradrenaline was suggested by its non-uniform distribution (Vogt, 1954). Thus, the highest concentration of noradrenaline was found in the hypothalamus (Table 1). In rat brain, noradrenaline-containing neurons originate from cell bodies situated in the pons and medulla oblongata. These cell bodies give rise to the ascending pathways to the cortical area, limbic system and hypothalamus and the descending pathways to the spinal cord (Cooper et al, 1978).

In many invertebrate nervous tissues, noradrenaline, although present, exists at lower concentrations than that observed for dopamine and octopamine (Table 2). The absence of evidence for the presence of specific noradrenergic neurons in insects has suggested that the noradrenaline detected in insect nervous tissue is either due to the presence of a small class of as yet unidentified noradrenergic neurons or is the result of a metabolic artefact (Evans, 1980). Such an artefact could arise if released dopamine was taken up by octopaminergic neurons and converted to noradrenaline by tyramine-β-hydroxylase.

TABLE 1

Monoamine Distribution in Vertebrate Central Nervous System

Species	Brain Region	NA tissue (µg/g)	DA tissue (µg/g)	OCT tissue (ng/g)	SER tissue (µg/g)	OCT/ NA Ratio
Dog	Whole Brain	0.16	0.19	-	-	-
	Medulla	0.37	0.13	-	0.55	-
	Cerebellum	-	-	-	0.07	-
	Hypothalamus	1.00	-	-	1.75	-
	Cerebral Cortex	0.05	-	-	0.17	-
	Caudate Nucleus	0.10	5.90	-	-	-
Cat	Whole Brain	0.22	0.28	-	-	-
	Medulla	-	-	-	0.55	-
	Cerebellum	-	-	-	0.27	-
	Hypothalamus	1.40	-	-	1.78	-
	Cerebral Cortex	-	-	-	0.24	-
	Caudate Nucleus	0.10	9.90	-	-	-
Rat	Whole Brain	0.49	0.60	-	-	-
	Medulla	0.72	-	5.38	-	0.007
	Cerebellum	0.17	-	3.38	-	0.02
	Hypothalamus	1.29	0.14	13.52	-	0.01
	Cerebral Cortex	0.28	0.01	4.04	-	0.023
	Caudate Nucleus	0.27	6.39	-	-	-

Data for noradrenaline (NA) and dopamine (DA) — Iversen and Iversen, 1967

Data for octopamine (OCT) — Buck *et al*, 1977

Data for serotonin (SER) — Udenfriend *et al*, 1957

TABLE 2

Monoamine Distribution in Invertebrate Brain Tissue

Species	NA tissue (μg/g)	DA tissue (μg/g)	OCT tissue (μg/g)	SER tissue (μg/g)	OCT/NA Ratio
Periplaneta americana	0.37[1]	2.35[1]	4.52[2]	-	11.5
Schistocerca gregaria	0.11[3]	0.87[3]	2.43[3]	1.40[4]	22.0
Locusta migratoria	0.24[5]	1.31[5]	-	2.34[5]	-
Helix aspersa	0.07[6]	5.16[6]	0.13[7]	5.10[6]	1.8
Octopus vulgaris	4.40[8]	11.90[8]	1.20[8]	3.70[8]	0.3

Superscript numbers indicate references.

1. Frontali and Haggendal, 1969
2. Robertson and Steele, 1973
3. Robertson, 1976
4. Klemm and Axelsson, 1973
5. Hiripi and S-Rozsa, 1973
6. Juorio and Killick, 1972
7. Robertson and Juorio, 1976
8. Juorio and Molinoff, 1974

2. Dopamine

Dopamine, identified in sympathetic nerves and ganglia (Schuman, 1956), was also demonstrated to be present in mammalian brain (Table 1). Dopamine distribution differed markedly from that of noradrenaline. Unlike noradrenaline, the highest level of dopamine was not in the hypothalamus, but in the caudate nucleus suggesting that dopamine existed independently of noradrenaline and that the dopamine present was not merely as a precursor for noradrenaline. Two dopaminergic pathways in mammalian brain have been identified using fluorescence spectroscopy.

a) The nigro-striatal DA fibres which originate in the substantia nigra and ascend through the lateral and mid hypothalamus, fanning out to innervate the neostriatum.

b) The mesolimbic DA fibres which originate principally from DA cell bodies surrounding the interpenduncular nucleus and ascend to innervate the limbic forebrain (Livett, 1973).

In invertebrates, dopamine is present in specific neurons of the molluscs Helix aspersa (Sedden et al, 1968), Planorbis corneus (Marsden and Kerkut, 1970) and Lymnaea stagnalis (Cottrel et al, 1979) and has been identified in a wide variety of invertebrate species (Table 2). In insects, dopamine is a major phenylethylamine when compared with noradrenaline.

3. Octopamine

Octopamine, identified in sympathetic nerves of rats (Molinoff and Axelrod, 1969, 1972), has been shown to have a non-uniform distribution in rat brain (Buck et al, 1977). Octopamine has only been found in areas of the brain that contain noradrenaline. This, together with the failure to detect specific octopaminergic cells, has suggested that the small amounts of octopamine found in mammalian brain are a result of a metabolic mistake, since the enzymes, aromatic amino acid decarboxylase and β-hydroxylase, which are thought to be involved in the synthesis of octopamine, are present in noradrenergic neurons. The highest level of octopamine (13.5 ng/g tissue) present in the hypothalamus is only 1% of the level of noradrenaline (Table 1).

Octopamine is widely distributed in many invertebrate species (Table 2) and it is suggested that the medial group of dorsal unpaired cells of the locust (Evans and O'Shea, 1977, 1978) and the cockroach (Dymond and Evans, 1979) are octopaminergic. A phylogenetic relationship between the ratio of the levels of octopamine and noradrenaline in nervous tissue has been suggested (Robertson and Juorio, 1976). In protostomes (insects, crustaceans, annelids and gastropods), octopamine predominates over noradrenaline whereas in deuterostomes (echinoderms and mammals), the situation is reversed (Tables 1 and 2).

4. Serotonin

Serotonin, first identified as a naturally occurring vasodilator in serum (Rapport, 1949), was later identified in the nervous system of the dog (Amin et al, 1954). In mammalian brain, serotonin was not randomly distributed but localised in definite areas (Udenfriend et al, 1957). Thus, the hypothalamus contained the highest level of serotonin (Table 1). Fluorescence microscopy has identified that serotonin-containing neurons are localised in the raphe nuclei which are a series of nuclei in the lower midbrain and upper pons (Aghajanian, 1972).

Serotonin is present in a wide variety of invertebrates (Table 2) and serotonin-containing neurons have been identified in the nervous system of the annelid Lumbricus terrestris (Rude, 1966; Myhrberg, 1967). Although present in many insect species, it has not been detected in the caddis fly (Bjorklund et al, 1970; Klemm and Bjorklund, 1971).

Role of Monoamines in Nervous System

The evidence presented suggests that dopamine, octopamine and serotonin represent the major monoamines present in insect nervous tissue. This has led to speculation about their role. For the monoamines to act as neurotransmitters, receptors should exist. Moreover, if neurotransmission involves cyclic AMP as a "second messenger", then monoamine receptors linked to adenylate

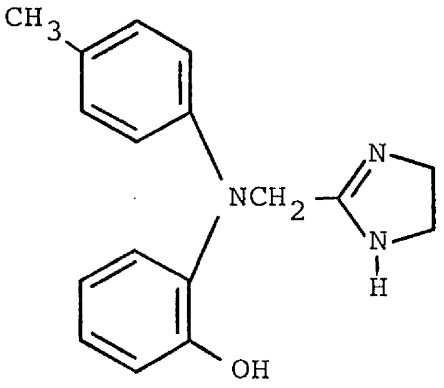
cyclase should also be present. Before describing the possible involvement of cyclic AMP in mediating neurotransmission and the evidence for monoamine-sensitive adenylate cyclases, the classification and identification of monoamine receptors in vertebrate and invertebrate nervous tissues will be discussed.

1. Receptor Classification

The characterisation and classification of receptors have depended historically upon the relative effects of various compounds on biological activity e.g. noradrenaline in the sympathetic nervous system stimulated the contraction of vascular smooth muscle and relaxation of smooth muscle in the intestine. Both types of receptor respond to the catecholamines noradrenaline, adrenaline and isoproterenol, but the effectiveness of these agonists on the two receptors is quite different. For example, with α-adrenergic receptors, adrenaline > noradrenaline >isoproterenol and for β-receptors, isoproterenol > adrenaline > noradrenaline (Alquist, 1948). Antagonists which are relatively selective for either α- or β-receptors are well known. Phentolamine, whose structure is given in Figure 1, is an α-antagonist whereas propranolol is a β-antagonist (Table 3). Classical antagonists of α- or β-adrenergic receptors are ineffective as antagonists of dopamine receptors (Kebabian et al, 1972) but anti-psychotic drugs are potent antagonists (Clement-Cormier et al, 1972; Karobath and Leich, 1974). Cyproheptadine, lysergic acid, diethylamine are suggested serotonin antagonists (Nathanson and Greengard, 1974).

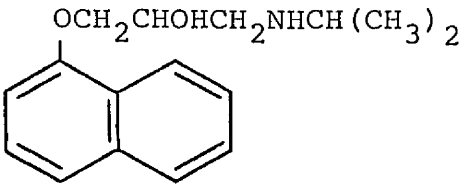
FIGURE 1

Structures of the Antagonists:
Phentolamine, Propranolol, Cyproheptadine and Chlorpromazine



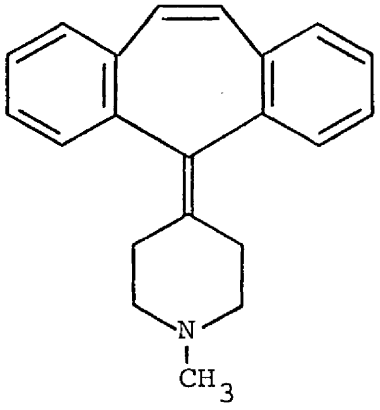
Phentolamine

(α-antagonist)



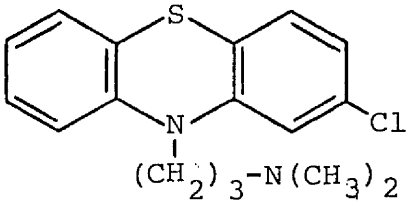
Propranolol

(β-antagonist)



Cyproheptadine

(serotoninergic antagonist)



Chlorpromazine

(dopaminergic antagonist)

TABLE 3

Monoamine Antagonists

Monoamine Antagonists	
Receptor Type	Antagonist
α-Receptors	Phentolamine, tolazoline, phenoxybenzone
β-Receptors	Propranolol, pronethalol
Dopamine Receptors	Phenothiazines (e.g. fluphenazine, chlorpromazine)
	Thioxanthenes (e.g. clothiapine, clozapine)
	Butyrophenones (e.g. haloperidol, spiroperidol)
Serotonin Receptors	Cyproheptadine, lysergic acid diethylamide

2. Identification of Monoamine Receptors

2.1 Dopamine

Pharmacological evidence indicates the existence of different classes of dopamine receptors, for example, the neuroleptic drug haloperidol competes with dopamine for dopamine binding sites (Seeman et al, 1975; Creese et al, 1975) but is relatively weak as an inhibitor of dopamine-sensitive adenylate cyclase. In rat striatum, the neurotoxin kainic acid, almost totally depleted dopamine-sensitive adenylate cyclase activity but only elicited a 40-50% decline in [^{3}H]-haloperidol binding (Schwartz et al, 1978). Based on studies such as these, Kebabian and Calne, 1979 classified the dopamine receptors as D-1 and D-2. D-1 receptors have effective agonists in the μmolar range, are linked to adenylate cyclase and are not affected by the anti-psychotic drug sulpiride. D-2 receptors, however, are stimulated by agonists in the nmolar range, are not linked to adenylate cyclase and are antagonised by sulpiride.

In invertebrate nervous tissue, studies on the mollusc Helix aspera have identified neurons that are inhibited (Woodruff and Walker, 1969; Woodruff, 1971) and excited (Stryker-Boudier et al, 1974) by dopamine.

2.2 Octopamine

Iontophoretic application of octopamine to unidentified neurons of Aplysia californica resulted in a slow hyperpolarisation suggesting the existence of octopamine receptors. Dopamine had no effect while noradrenaline gave a small response (Carpenter and Gaubatz, 1974). However, the response to noradrenaline was considered negligible as A. californica nervous tissue does not contain noradrenaline (Saavadra et al, 1974). From studies on cell E26 in the sub-oesophageal ganglia of H. aspersa, octopamine was found to be excitatory and dopamine and noradrenaline inhibitory (Batta et al, 1979). Phentolamine (2×10^{-7}M) blocked the excitatory action of octopamine without affecting the dopamine and noradrenaline response.

In the locust, the extensor-tibiae muscle of the hind leg is a large and powerful muscle used in walking, kicking and jumping. It is innervated by the fast and slow extensor motoneurons and an inhibitory motoneuron (Hoyle and Burrows, 1973). The muscle also receives a fourth axon from the dorsal unpaired median extensor tibiae (DUMETi) neuron (Hoyle et al, 1974) which originates from the region of the DUM cells in the meta thoracic ganglia. Cells from the DUM region, when incubated with labelled tyrosine, synthesised tyramine and octopamine but there was no evidence for the synthesis of either noradrenaline or

dopamine (Hoyle and Barker, 1975). The identification of octopamine (0.1 pmole/cell soma) in DUMETi cells (Evans and O'Shea, 1977) and the observation that stimulating the DUMETi neuron potentiated the extensor muscle tension generated by the slow extensor motoneuron (Evans and O'Shea, 1977) and that this potentiation was mimicked by exogenously applied octopamine (10^{-6}M) and blocked by phentolamine, suggested that the DUMETi neuron was octopaminergic (Evans, 1980).

2.3 Serotonin

In the rat, serotonin appears to exert an inhibiting effect both on serotonin-containing neurons of the raphe nuclei as well as neurons receiving serotonergic connections from the raphe nuclei (Haigler and Aghajanian, 1974).

In invertebrates, certain gastropod neurons may be depolarised and excited by serotonin (Gershenfeld and Stefani, 1966). In H. aspersa, serotonin has two inhibitory actions (Gershenfeld, 1971) as it stimulates either an influx of chloride ions or an efflux of potassium.

Before cyclic AMP can be considered as a "second messenger", a biochemical and physiological response to cyclic AMP must be demonstrated. Much of our

understanding of the possible role of cyclic AMP in brain function is derived from studies on the mammalian superior cervical ganglion.

Cyclic AMP - Role in Synaptic Transmission

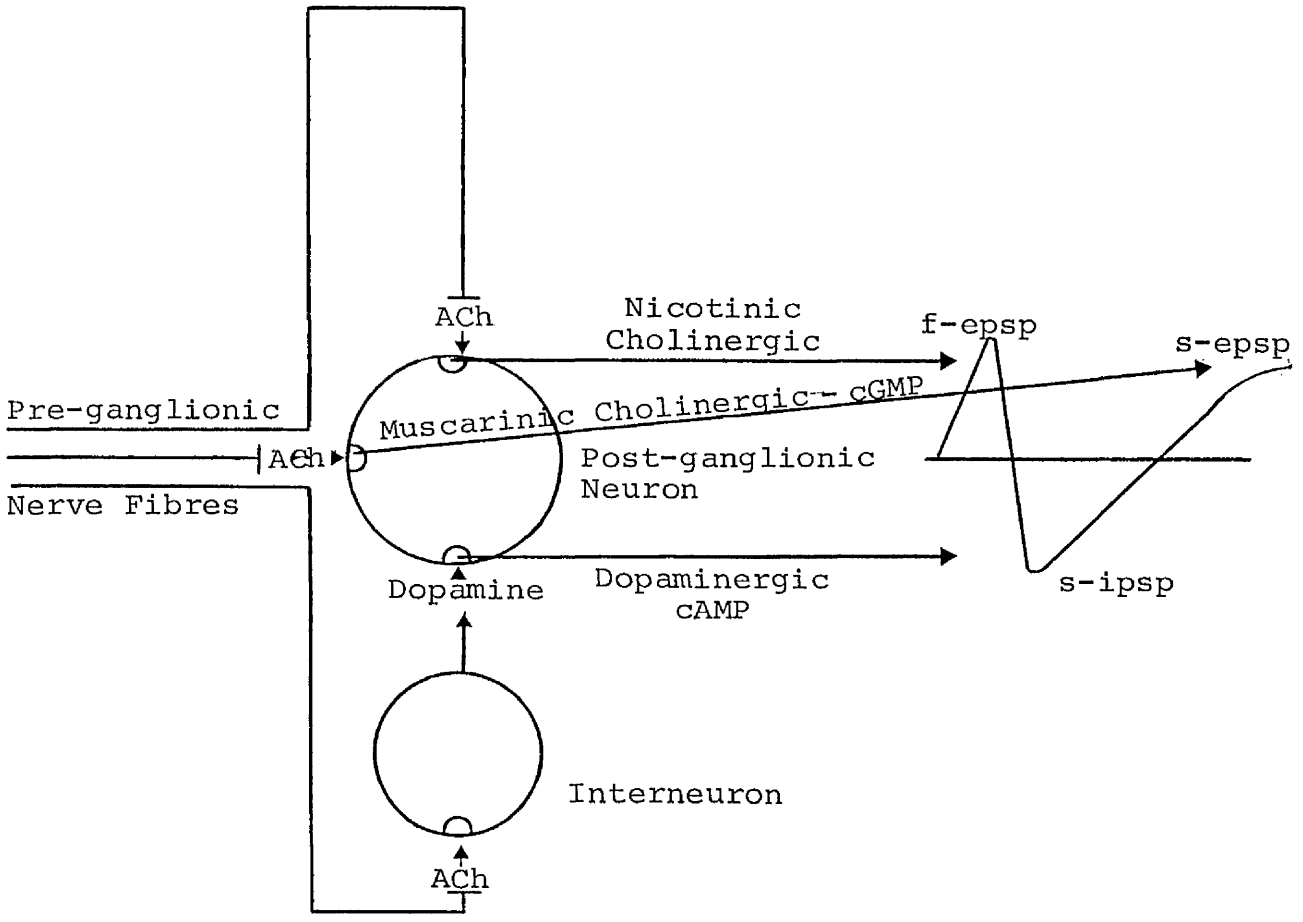
Superior cervical ganglia receive three types of innervation. Acetylcholine (ACh) released from preganglionic terminals activates nicotinic receptors in the postganglionic neuron, leading to the generation of the fast-excitatory post-synaptic potential (f-epsp). In addition, stimulation of muscarinic cholinergic receptors leads to the generation of a slow-excitatory post-synaptic potential (s-epsp), a process which may involve cyclic GMP (Kebabian et al, 1975). Acetylcholine also activates muscarinic receptors within the interneuron, leading to the release of dopamine from small intensely fluorescent (SIF) cells within the interneuron (Libet and Owman, 1974). The released dopamine activates a dopamine receptor causing the generation of a slow-inhibitory post-synaptic potential (s-ipsp), a process which seems to involve cyclic AMP (Greengard et al, 1972). A diagram of the principal synaptic connections that occur within the superior cervical ganglia is shown in Figure 2.

Dopamine applied exogenously caused a rise in cyclic AMP levels in the sympathetic ganglia of the cow (Kebabian and Greengard, 1971), rabbit (Kalix et al, 1974) and cat (Black et al, 1978).

Electrical stimulation of preganglionic fibres elicited a two-fold increase in cyclic AMP levels in rabbit superior cervical

FIGURE 2

Schematic Diagram of the Principal Synaptic Connections in the Mammalian Superior Cervical Ganglion and the Postulated Roles of Cyclic AMP and Cyclic GMP in the Generation of the Post-ganglionic Synaptic Potentials



ganglia (Greengard et al, 1972) a four-fold increase in cat (Chatzkel et al, 1974) and an eight-fold increase in the bullfrog (Weight et al, 1974). Postganglionic stimulation of rabbit superior cervical ganglia had no effect (Greengard et al, 1972). The rise in cyclic AMP in rabbit was blocked by atropine, a muscarinic cholinergic antagonist or by α-adrenergic antagonists (Kalix et al, 1974) leading to a reduction in the s-ipsp response. The rise in cyclic AMP was mimicked by the muscarinic agonist bethanechol (Kalix et al, 1974). If monobutyryl cyclic AMP was added to rabbit superior cervical ganglia, a s-ipsp response was obtained which was similar to that observed with preganglionic stimulation (McAfee and Greengard, 1972). These studies suggested that cyclic AMP mediated dopaminergic transmission in the rabbit superior cervical ganglion and that cyclic AMP through the generation of the s-ipsp regulated the membrane potential of the postganglionic neuron (Greengard, 1976).

However, a number of objections have been raised to Greengard's interpretation that the role of cyclic AMP is to mediate the s-ipsp. In bullfrog ganglia, although electrical stimulation is known to elevate cyclic GMP and cyclic AMP (Weight et al, 1974), cyclic nucleotide administration did not mimic the membrane potential associated with the generation of the respective s-epsp and s-ipsp potentials (Weight et al, 1978) suggesting that its generation was independent of cyclic nucleotides. In rabbit superior ganglia, the addition of dibutyryl cyclic AMP caused an increase in the s-epsp (Libet et al, 1975) but had no effect on the s-ipsp (Libet, 1979).

1. Model for the Involvement of Cyclic AMP in Neurotransmission

The model proposed by Greengard et al, 1972 for the possible mechanism by which cyclic AMP can mediate dopaminergic transmission in the superior cervical ganglion has been extended to chemical neurotransmission in the central nervous system (Greengard, 1976) (Figure 3).

It is proposed that:

a) the post-synaptic action of a number of neurotransmitters may be mediated through the action of cyclic AMP or cyclic GMP

b) the immediate target of cyclic AMP and cyclic GMP is the protein kinases whose action results in the phosphorylation of specific membrane proteins. Furthermore, the alteration of the degree of phosphorylation of these proteins produce the physiological effect of the neurotransmitters.

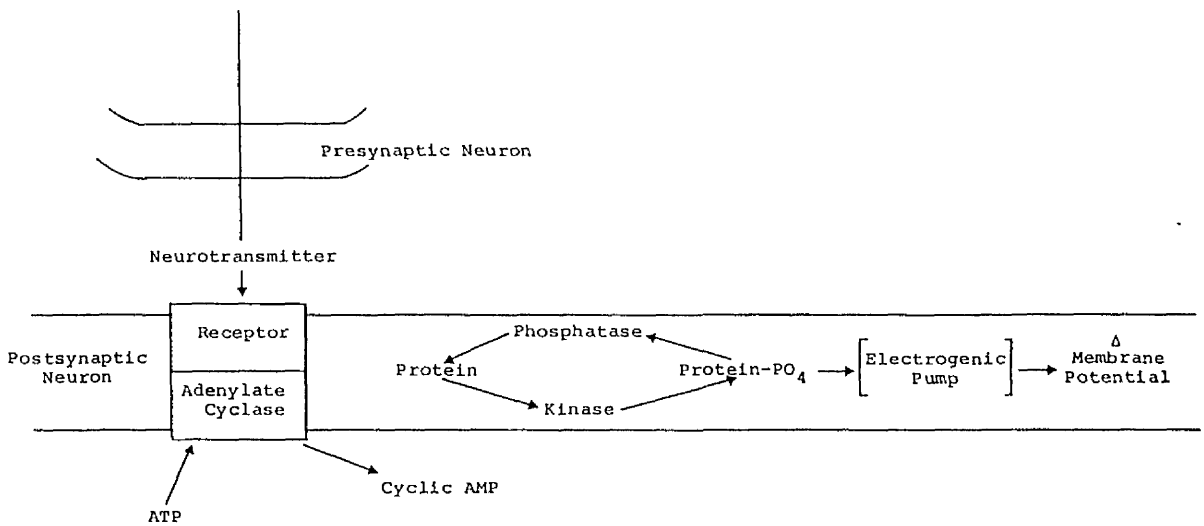
Evidence for the Model

1. Sub-cellular Localisation

Differential centrifugation has permitted the isolation of distinct populations of sub-cellular organelles from mammalian brain (Whittaker, 1968; De Roberts et al, 1965).

FIGURE 3

Proposed Role of Cyclic AMP in Generating Postsynaptic Potential



The released neurotransmitter activates an adenylate cyclase. The cyclic AMP produced activates cyclic AMP-dependent protein kinase which catalyses the phosphorylation of a substrate protein, a protein which controls the permeability of the postsynaptic membrane. Phosphorylation of this protein thus leads to a change in membrane potential. Termination occurs through hydrolysis of cyclic AMP by phosphodiesterase and dephosphorylation of the substrate protein by a phosphatase.

Nuclei, capillaries, myelin, mitochondria, nerve endings (synaptosomes) and microsomes have been identified.

The five proteins postulated in the model for the post-synaptic neurons; adenylate cyclase (De Robertis et al, 1967), protein kinase (Maeno et al, 1971), phosphodiesterase (Florendo et al, 1971), protein kinase substrate (Johnson et al, 1971) and phosphoprotein phosphatase (Maeno and Greengard, 1972) are all present in high concentrations in those sub-cellular fractions of rat brain tissue that are richest in synaptic membranes.

2. Monoamine-Sensitive Adenylate Cyclase

2.1 Vertebrate

Considerable evidence exists for the presence of noradrenaline-, dopamine- and, to a lesser extent, serotonin-stimulated adenylate cyclases in vertebrate nervous tissues. In contrast, little is known about octopamine.

Electrophysiological studies showed that noradrenaline applied iontophoretically to the cerebellum results in a hyperpolarisation and a slowing of the discharge rates of the Purkinje cells (Hoffer et al, 1971; Siggins et al, 1971a). This effect was potentiated by the phosphodiesterase inhibitors, theophylline and papaverine (Siggins et al, 1971b) and mimicked by ion-

tophoretically applied dibutyryl cyclic AMP and cyclic AMP (Hoffer et al, 1971). Fluphenazine and α-flupenthixol were potent antagonists of noradrenaline inhibition of Purkinje neurons (Freedman and Hoffer, 1975; Skolnick et al, 1976). Iontophoresis of lithium antagonised noradrenergic inhibition of rat Purkinje cells (Siggins et al, 1979). Lithium is a known antagonist of noradrenaline induced increase in cyclic AMP (Forn and Valderasas, 1971). Electrical stimulation of the locus coeruleus also caused a slowing of the firing rate of Purkinje cells (Siggins et al, 1971c; Hoffer et al, 1972) through noradrenaline projections which terminate on Purkinje cell dendrites. Using an immunofluorescence technique for the histochemical detection of cyclic AMP, it was shown that either topical application of noradrenaline to cerebellum or electrical stimulation of locus coeruleus elicited an increase in the fluorescence of Purkinje cells (Siggins et al, 1973) suggesting an increased formation of cyclic AMP. Based on studies such as these, Bloom, 1975 suggested that noradrenaline released from noradrenergic terminals originating from neurons in the locus coeruleus regulated spontaneous electrical activity in Purkinje cells by a mechanism involving a noradrenaline-sensitive accumulation of cyclic AMP within the Purkinje cell.

A noradrenaline-sensitive adenylate cyclase is present in the cerebral cortex of sheep, steer (Klainer et al, 1962), rat (Van Inwegan et al, 1975) and in rat corpus striatum (Harris, 1978). In homogenates of rat limbic forebrain, although a two fold increase in adenylate cyclase activity was observed with noradrenaline, the addition of octopamine had no effect (Horn and Philipson, 1976). In contrast to the absence of evidence for an adenylate cyclase stimulated by octopamine in vertebrate nervous tissue, considerable evidence exists for dopamine. A dopamine-sensitive adenylate cyclase is present in the mammalian caudate nucleus (Kebabian et al, 1972; Roufogalis et al, 1979), cortex, (Von Hungen and Roberts, 1973), olfactory tubercle (Horn et al, 1974; Krieger, 1980) and nucleus accumbens (Horn et al, 1974; Watling et al, 1979). The enrichment of a dopamine-sensitive adenylate cyclase in a crude synaptosome preparation (P_2) from rat striatum (Clement-Cormier and George, 1978) is consistent with its localisation in synaptic membranes.

A serotonin-sensitive adenylate cyclase was present in homogenates of several brain regions of newborn rat and guinea pig which declined during maturation (Enjalbert et al, 1977) and in slices of rat brain (Kakiuchi and Rall, 1968). Others have reported no effect of serotonin on adenylate cyclase in rat brain

homogenates (Burkard and Grey, 1968; Duffy and Powel, 1975) or in synaptosomes from rat cerebral cortex (Izumi et al, 1975).

2.2 Invertebrate

The discovery of adenylate cyclase that is stimulated by octopamine and dopamine in the thoracic ganglia (Nathanson and Greengard, 1973) and cerebral ganglia (Harmar and Horn, 1977) of the cockroach P. americana and the cerebral ganglia of M. configurata (Bodnaryk, 1979), L. migratoria (Hiripi and S-Rozsa, 1980) and S. gregaria (Kilpatrick et al, 1980) has provided additional support for a neurotransmitter function for octopamine and dopamine in invertebrates. In the cockroach, the effects of octopamine and dopamine upon adenylate cyclase activity were additive indicating the presence of separate receptors for these compounds (Nathanson and Greengard, 1973; Harmar and Horn, 1977). Although noradrenaline stimulated adenylate cyclase, the addition of noradrenaline to adenylate cyclase in the presence of octopamine and dopamine did not result in an increase in activity suggesting that noradrenaline was having its effect by binding to either octopamine or dopamine receptors. An octopamine-sensitive adenylate cyclase is present in the cerebral ganglia of the horseshoe crab Limulus polyphemus (Atkinson et al, 1977).

A serotonin-sensitive adenylate cyclase found in the thoracic ganglia of P. americana was shown to be dis-

tinct from the dopamine- and octopamine-sensitive adenylate cyclase (Nathanson and Greengard, 1973). The activation by serotonin was selectively inhibited by lysergic acid diethylamide, 2-bromolysergic acid diethylamide and, to a lesser extent, cyproheptadine, antagonists which are known to block serotonin receptors (Nathanson and Greengard, 1974). A serotonin-sensitive adenylate cyclase is present in cerebral ganglia of the locust S. gregaria (Kilpatrick et al, 1980).

3. Cyclic AMP-Dependent Phosphodiesterase

Phosphodiesterase hydrolyses cyclic AMP to 5'adenosine monophosphate and is thought to represent the only means of inactivating cyclic AMP. Phosphodiesterase activity, which was initially detected in extracts of heart, brain and liver (Sutherland and Rall, 1958) with brain having the highest activity, was subsequently shown to be widely distributed throughout the body (Butcher and Sutherland, 1962). Most mammalian tissues contain three different phosphodiesterase isoenzymes which may be controlled separately. One phosphodiesterase has a high affinity for cyclic AMP (Thompson and Appleman, 1971) whereas another has a higher affinity for cyclic GMP than cyclic AMP and may be regulated by calcium and a calcium-dependent regulatory protein (Calmodulin) (Kakiuchi and Yamazaki, 1970). The third phosphodiesterase has a low affinity for cyclic AMP and is suggested to be regulated by cyclic GMP (Beavo et al, (1971).

In liver cells, glucagon stimulated the high affinity form of phosphodiesterase while the low affinity form was unaltered (Allan and Sneyd, 1975). Calcium and calmodulin have more prominent effects on the lower affinity soluble cyclic AMP phosphodiesterase, when compared with the higher affinity membrane-bound phosphodiesterase (Thompson and Strada, 1978).

A variety of compounds including theophylline, caffeine and 3-isobutyl-1-methylxanthene have been reported to inhibit these phosphodiesterases (Levin and Weiss, 1976; Freedholm et al, 1976; Glass and Moore, 1979). However, a calcium-dependent phosphodiesterase from rat cerebellum was more strongly inhibited by trifluorperazine than the calcium-independent phosphodiesterase while theophylline was more effective as an inhibitor of the calcium independent form (Daly, 1977).

4. Receptors for Cyclic AMP

The receptors for cyclic AMP have been identified as the cyclic AMP-dependent protein kinases. Cyclic AMP activates protein kinase by binding to the regulatory sub-unit of the protein kinase which results in the dissociation of the holoenzyme into the regulatory and catalytic sub-unit [1] (Langan, 1973; Rubin and Rosen, 1975).

$$R_2C_2 + 2 \text{ cyclic AMP} \rightleftharpoons R_2 \text{ cyclic AMP} + 2C \qquad [1]$$

R is the regulatory sub-unit and C is the catalytic sub-unit. R_2C_2 is inactive.

Two forms of soluble cyclic AMP-dependent protein kinase, designated Type I and Type II have been identified which differ in their distribution (Corbin et al, 1975) and biochemical properties (Hoffman et al, 1975). Type I and Type II are also membrane bound and are present in a wide variety of tissue (Uno et al, 1976). Mammalian brain is a rich source of both soluble and particulate enzymes (Hoffman et al, 1977). In bovine cerebral cortex, 50% of the total kinase activity was localised in particulate fractions whereas only 10% of the kinase activity observed in heart and skeletal muscle was membrane bound (Rubins et al, 1979).

In brain, as in other tissue (Nimmo and Cohn, 1977), membrane and cytosol cyclic AMP-dependent protein kinases have identical catalytic sub-units but differ in their regulatory sub-units R_1 and R_2 (Walter et al, 1978). Membrane bound Type II (but not Type I) cyclic AMP-dependent protein kinase is suggested to be the enzyme which phosphorylates membrane proteins in synaptic membranes (Walter et al, 1979).

5. Phosphorylation of Membrane Proteins

Several proteins in synaptic membranes from mammalian brain can be shown to be phosphorylated in a cyclic AMP-dependent reaction (Uedo et al, 1973). The proteins termed

Ia and Ib and II (Tetsufumi and Greengard, 1977) have different tissue distribution. Proteins Ia and Ib have been found exclusively in neural tissue whereas Protein II has a wide distribution (Ueda and Greengard, 1977).

The regulation of membrane potential suggested by Greengard, 1976 is not the only proposed role for cyclic AMP in nervous tissue. For example, cyclic AMP has been implicated in the control of the enzyme tyrosine hydroxylase.

5. Tyrosine Hydroxylase Activation by Cyclic AMP

Tyrosine hydroxylase catalyses the first and rate limiting step in the biosynthesis of the catecholamines, dopamine and noradrenaline. It requires oxygen, ferrous ions and tetrahydropteridine co-factor for activity (Nagatsu et al, 1964). The observation that brain tyrosine hydroxylase activity was increased by cyclic AMP (Lloyd and Kaufman, 1975; Morgenroth et al, 1975) raised the possibility that tyrosine hydroxylase may be regulated by phosphorylation and, if so, whether the enzyme itself was phosphorylated. Incubating tyrosine hydroxylase with [^{32}P]-ATP, cyclic AMP and protein kinase and isolating the [^{32}P]-labelled enzyme Joh et al, 1978 demonstrated that the enzyme could be phosphorylated. The phosphorylation was cyclic AMP-dependent and increased the enzyme activity two fold without any change in the apparent Km for either tyrosine or 6-methyltetrahydropteridine. Other studies have suggested that under phosphorylating conditions, a reduction in the

Km for dimethyltetrahydropteridine (DMPH) was accompanied with the activation of tyrosine hydroxylase by cyclic AMP (Lloyd and Kaufman, 1975).

Control of Adenylate Cyclase

Adenylate cyclase activity, although affected by neurotransmitters or hormones, is thought to be controlled by GTP, calcium, macromolecular regulators and the availability of ATP within the cell (Daly, 1977).

The substrate for adenylate cyclase appears to be an ATP-metal complex (De Haen, 1974; Lin et al, 1975). Both magnesium-ATP and manganese-ATP, but not calcium-ATP, can act as substrates (Sutherland et al, 1962; Birnbaumer et al, 1969; Perkins, 1973) and free ATP appears to act as an inhibitor (De Haen, 1974; Lin et al, 1975).

1. Guanyl Nucleotides

The regulation of activity and hormone responsiveness of adenylate cyclase by GTP has been extensively studied in cell-free preparations and considerable evidence exists to show that adenylate cyclase requires the presence of both GTP and hormone or neurotransmitter for maximal activity (Rodbell et al, 1971, 1974; Levitzki, 1977). Guanyl nucleotides have been reported to stimulate adenylate cyclases from a wide variety of tissue (Yamamara et al, 1974; Roufogalis et al, 1976; Cassel and Selinger, 1977, 1978;

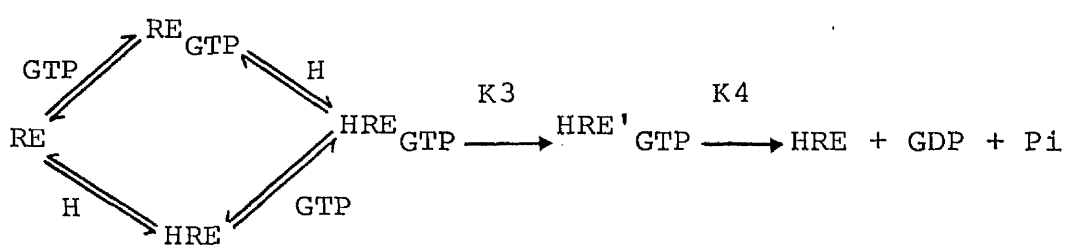
Hoffman, 1979; Johnson and Makku, 1979). In rat brain, GTP stimulated basal adenylate cyclase activity (Roufogalis et al, 1976) whereas others found it inhibitory (Clement-Cormier et al, 1975, 1978). GTP stimulated slightly adenylate cyclase from rat cerebral cortex (Van Inwegan et al, 1975).

If GTP was replaced by the non-hydrolysable analogue Gpp-(NH)p, adenylate cyclase from liver (Salomon et al, 1975) and turkey erythrocyte (Sevilla et al, 1976) was converted to a "permanently" active state. This observation suggested that GTP is hydrolysed at the guanine regulatory site resulting in the deactivation of the enzyme (Levitzki, 1977).

Various models have been proposed to explain the role of GTP in the activation of adenylate cyclase, but the two that will be discussed are:

a) the turkey erythrocyte (Levitzki, 1978)
b) the liver enzyme (Salomon et al, 1975)

a) Turkey Erythrocyte System



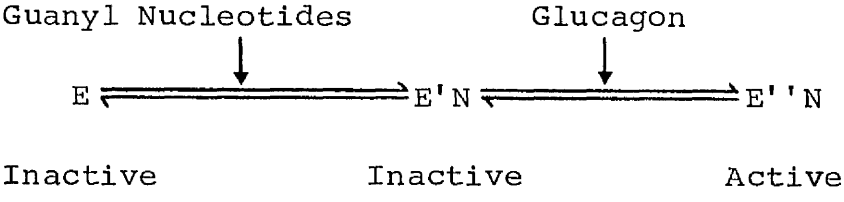
Inactive Active Inactive

R is the receptor, E the enzyme, K_3 is the rate of enzyme activation and K_4 the rate of enzyme deactivation. Only the activated form (HRE'GTP) is capable of synthesising cyclic AMP. The hydrolysis of GTP to GDP and phosphate leads to the deactivation of the enzyme.

This model is based on the observation that i) activation of adenylate cyclase required the presence of both hormone (adrenaline) and Gpp(NH)p. Gpp(NH)p alone had little effect on adenylate cyclase activity and ii) the formation of a stable complex with Gpp(NH)p and adrenaline was time dependent and could be prevented by the addition of propranolol, a β-antagonist. Once the cyclase was fully activated, the addition of propranolol did not cause an inhibition. If, however, propranolol was added during the formation of the stable complex, the resulting activity depended on the time of addition of propranolol (Sevilla et al, 1976).

The necessity for hormone to be present for Gpp(NH)p activation in turkey eruthrocyte has been questioned by the observation that turkey erythrocyte membranes pre-treated with GMP and isoproterenol, a β-antagonist, and washed will display considerable adenylate cyclase activity in the presence of Gpp(NH)p alone (Lad et al, 1980).

b) Liver Adenylate Cyclase



The basal state E, after binding of the guanyl nucleotide alters to form E'N, an enzyme state that is still inactive. E''N is the active form of the enzyme and the conversion of E'N to E''N represents a rate-limiting step. Glucagon, the stimulatory ligand, acts by accelerating the rate of isomerisation to E''N. This model is based on the observation that Gpp(NH)p alone can stimulate adenylate cyclase activity and that the activation displays a lag period which is abolished by the addition of glucagon (Salomon et al, 1975).

The receptor-dependent adenylate cyclase is composed of three basic units - the receptor, the GTP regulatory unit and the catalytic moiety which catalyses the conversion of ATP to cyclic AMP (Maguire et al, 1977). It is suggested that the guanyl nucleotide alters the "coupling" between the hormone or neurotransmitter receptor and adenylate cyclase unit and the coupling factor resides in the GTP regulatory unit (Pfeuffer and Helmreich, 1975; Pfeuffer, 1977).

2. Calcium and Calmodulin

Brain tissue contains an adenylate cyclase that is activated by low (μM) concentrations and inhibited by higher (mM) concentrations of calcium (Bradham et al, 1970; Brostrom et al, 1975; MacDonald, 1975). Calcium inhibited forms of adenylate cyclase have been reported from a wide variety of tissue (Birnbaumer, 1973; Jard and Bochaert, 1975).

A low molecular weight calcium binding protein (Calmodulin) conferred a calcium-dependent activation upon adenylate cyclase from porcine brain (Brostrom et al, 1975) and rat cerebral cortex (Brostrom et al, 1977, 1978). Calmodulin is present in a wide variety of brain tissue (Cheung et al, 1975; Gregg et al, 1976).

EGTA, a chelator which has a low affinity for magnesium compared with calcium (Holloway and Reilley, 1960; Sillin and Martel, 1964) has been considered a "specific calcium" chelator.

In rat brain slices (Schwabe and Daly, 1977) and rat cerebral cortex (Schwabe et al, 1978), the rate of accumulation of cyclic AMP elicited by noradrenaline was greatly reduced by EGTA. Following calcium depletion, the ability of glial tumour (C6) cells to accumulate cyclic AMP in response to noradrenaline was reduced by 60-70% (Brostrom et

al, 1979). Micromolar free calcium was sufficient to restore the maximum noradrenaline response.

Advantages and Structure of Insect CNS

1. Advantages

The study of the central nervous system of mammals, especially in humans is made difficult by the large number of neurons and interneural contacts involved. The human brain is suggested to contain about 10^{12} neurons, many of which have as many as 10^{5} synaptic contacts distributed over the whole neuron (Eisenstein, 1972). The CNS of insects contains relatively fewer neurons. The brain of the honey bee Apis mellifera contains approximately $8x10^{5}$ neurons and glial cells (Willhoft, 1967), whereas the fly Musca domestica contains approximately $3x10^{5}$ neurons (Strausfeld, 1975). Insect neurons are concentrated in ganglia which are connected in sequence by paired connectives. The number of ganglia varies in different species and developmental stages (Bullock and Horridge, 1965).

Decapitated insects are known to retain certain biological activities, for example, isolated thoracic ganglia of the cockroach are able to generate several types of leg movements (Horridge, 1962). Many functions such as breathing and walking can be controlled by single ganglia isolated from the head ganglion (Hoyle, 1970) giving the impression that they represent "mini" brains. There are two head ganglia, the "brain" (cerebral ganglion or supraoesophageal

ganglion) which innervates the mouth, neck muscles and salivary gland (Klemm, 1976). The brain or cerebral ganglion consists of 4 or 5 or more fused ganglia (Bullock and Horridge, 1965). In contrast with vertebrate CNS, the cell body layer in insects is situated near the surface of the ganglia. Thus the somata are isolated from the neuropile, the region of the ganglia where synaptic cells are accessible for electrophysiological and biochemical studies and can be identified from one preparation to the next. By recording the muscle tension generated at the same time as electrical activity in the penetrated neurons, the leg motor neurons in the locust metathoracic ganglia (Hoyle and Burrows, 1973) and the flight motor neurons in the locust mesothroacic ganglion (Bentley, 1970) were identified. Electrophysiological studies on the action of catecholamines on insect CNS have identified nerve cell bodies in the nerve cord of Periplaneta americana and Blabera craniifer that are excited by dopamine, noradrenaline and adrenaline (Piclon, 1974). Another advantage of the insect is that the large cell bodies can provide sufficient material to measure levels and synthesis of putative neurotransmitters. For example, the synthesis of octopamine by the dorsal unpaired median neurons (Hoyle and Barker, 1975) and the identification of endogenous octopamine, present at 0.1 pmole/cell soma in DUMETi cells (Evans and O'Shea, 1977). The single identified neuron may also determine whether specific noradrenergic neurons exist and if found, provide evidence that noradrenaline does not act as a false neurotransmitter in insects.

2. Structure

In the locust, the most anterior ganglion is the cerebral ganglion lying dorsal to the oesophagus in the head. From this ganglion, the circumoesophageal connectives pass one another, one on either side of the oesophagus to the first of a chain of ganglia lying ventrically in the haemocoel. The ganglia are joined to each other longitudinally by connectives made up of axons and supporting cells (Chapman, 1969).

When S. gregaria cerebral ganglia are cut in sections, 3 distinct regions are identified (Figures 4 and 5):

a) the sheath or neural lamella which forms a fairly permeable continuous membrane surrounding the ganglia

b) a layer of cells composed of perineural and at least three classes of neuroglia

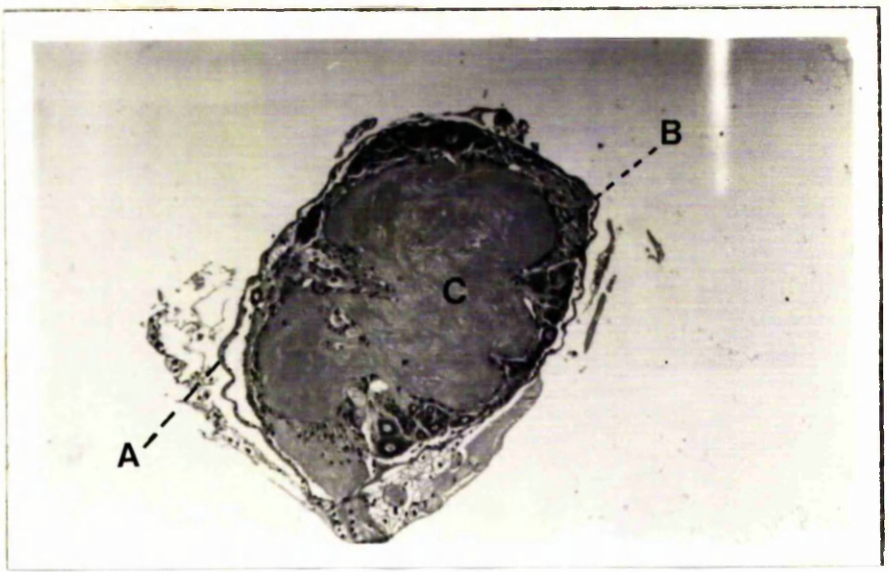
c) the neuropile, a complex of afferent interneural and efferent fibres and their supporting glial elements

Objectives of this Thesis

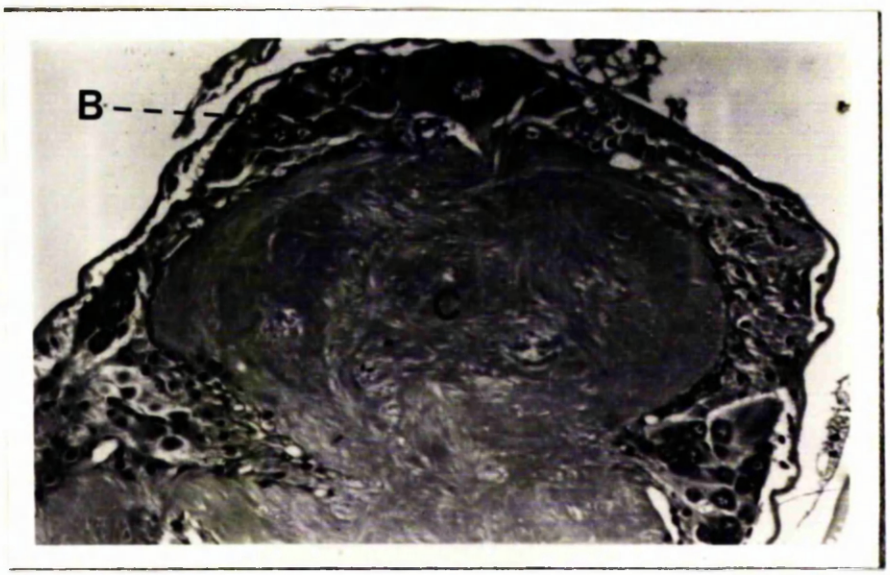
The aim of this work was to determine whether locust cerebral ganglia possessed an adenylate cyclase that was affected by the major monoamines present in S. gregaria nervous tissue. If so, to see if the response was mediated by separate receptors and to compare the properties of the insect adenylate cyclase with those from other organisms.

FIGURES 4 AND 5

Cross Sections of Cerebral Ganglion



x 48 magnification



x145 magnification

MATERIALS AND METHODS

Materials

All common laboratory reagents were of analytical grade. Fine chemicals were obtained as follows:

Adenosine-5'-triphosphate

Adenosine-5'-diphosphate

Adenosine-3'5'-cyclic phosphate

Guanosine-5'-diphosphate

DL-Octopamine hydrochloride

5,5'-Dithiobis (2-nitrobenzoic acid)

Acetylthiocholine iodide

Dihydroxyacetone phosphate

Cobra venom (Ophiophagus hannah)

Sigma Chemical Company
Kingston on Thames
Surrey, KT2 7BH, UK

2-Oxoglutaric acid, disodium salt

Dopamine hydrochloride

1,2-Di (2-aminoethoxy)-ethane-
N N N'N'tetra acetic acid (EGTA)

Guanosine-5'-triphosphate

Theophylline

Nicotinamide-adenine dinucleotide reduced, disodium (NADH)

Cyclic AMP binding protein and charcoal

Ethylenediaminetetra acetic acid, disodium salt (EDTA)

BDH Chemicals
Poole
Dorset, UK

Guanylyl-imidodiphosphate Guanylyl-[βγ-methylene]-diphosphate Adenylyl-imidodiphosphate	Boehringer (London) Lewes East Sussex, BN7 1LG, UK
Serotonin creatine sulphate	Ralph N. Emanuel Wembley, UK
Phentolamine Cyproheptadine Propranolol Chlorpromazine Phenylephrine	Generously supplied by: Dr. D. Pollock Pharmacology Department University of Glasgow
[8-^{3}H]-Adenosine-3'5'-cyclic phosphate, specific activity 30 Ci/mmol	The Radiochemical Centre Amersham Bucks., HP7 9LL, UK
Ficoll	Pharmacia (GB) Limited London
AG-1-X8, 200-400 mesh ion chloride form	Biorad Laboratories Caxton Way Watford, Herts, UK
Schistocerca gregaria	Larujon Locust Suppliers Welsh Mountain Zoo Colwyn Bay North Wales

Methods

1. Tissue Preparation

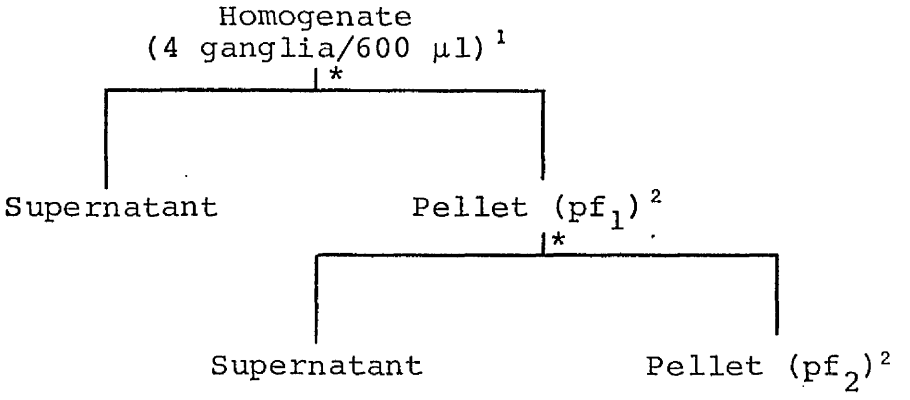
Cerebral ganglia were carefully dissected, placed in ice cold insect saline (Usherwood et al, 1965) and thoroughly washed. The ganglia were then homogenised in $6x10^{-3}$M Tris-HCl buffer (pH 7.5) containing $2x10^{-3}$M EGTA (4 ganglia/600 µl) using a glass homogeniser with a motor driven teflon pestle. With the exception of sub-cellular fractionation experiments (45 ganglia/ml), the ratio of 4 ganglia/600 µl Tris-HCl buffer was maintained throughout all experiments. Twenty microlitres of the homogenate (approximately 25 µg protein) was then assayed for adenylate cyclase activity.

1.1 Preparation of Crude Membrane Fractions (pf_1 and pf_2)

The pf_1 and pf_2 fractions were prepared as shown in Figure 6. The homogenate (4 ganglia/600 µl) was centrifuged at 9000 g for 10 minutes at $4^{o}C$, the supernatant discarded and the pellet (pf_1) was re-suspended in 600 µl $6x10^{-3}$M Tris-HCl buffer (pH 7.5) containing $2x10^{-3}$M EGTA. A pf_2 fraction was prepared by further centrifugation of a pf_1 fraction at 9000 g for 10 minutes at $4^{o}C$, discarding the supernatant, and re-suspending the pellet in 600 µl $6x10^{-3}$M Tris-HCl buffer (pH 7.5) containing $2x10^{-3}$M EGTA. Twenty microlitres of either a pf_1 or pf_2 fraction (approximately 10 µg protein) was assayed for adenylate cyclase activity.

FIGURE 6

Preparation of pf_1 and pf_2 Fractions from Cerebral Ganglia



1 Homogenised in 600 µl 6 mM Tris-HCl (pH 7.5) buffer containing 2 mM EGTA

2 Suspended in 600 µl Tris-HCl (pH 7.5) buffer containing 2 mM EGTA

* Centrifugation at 9000 g for 10 minutes at 4°C

1.2 Formation of Stable Complex

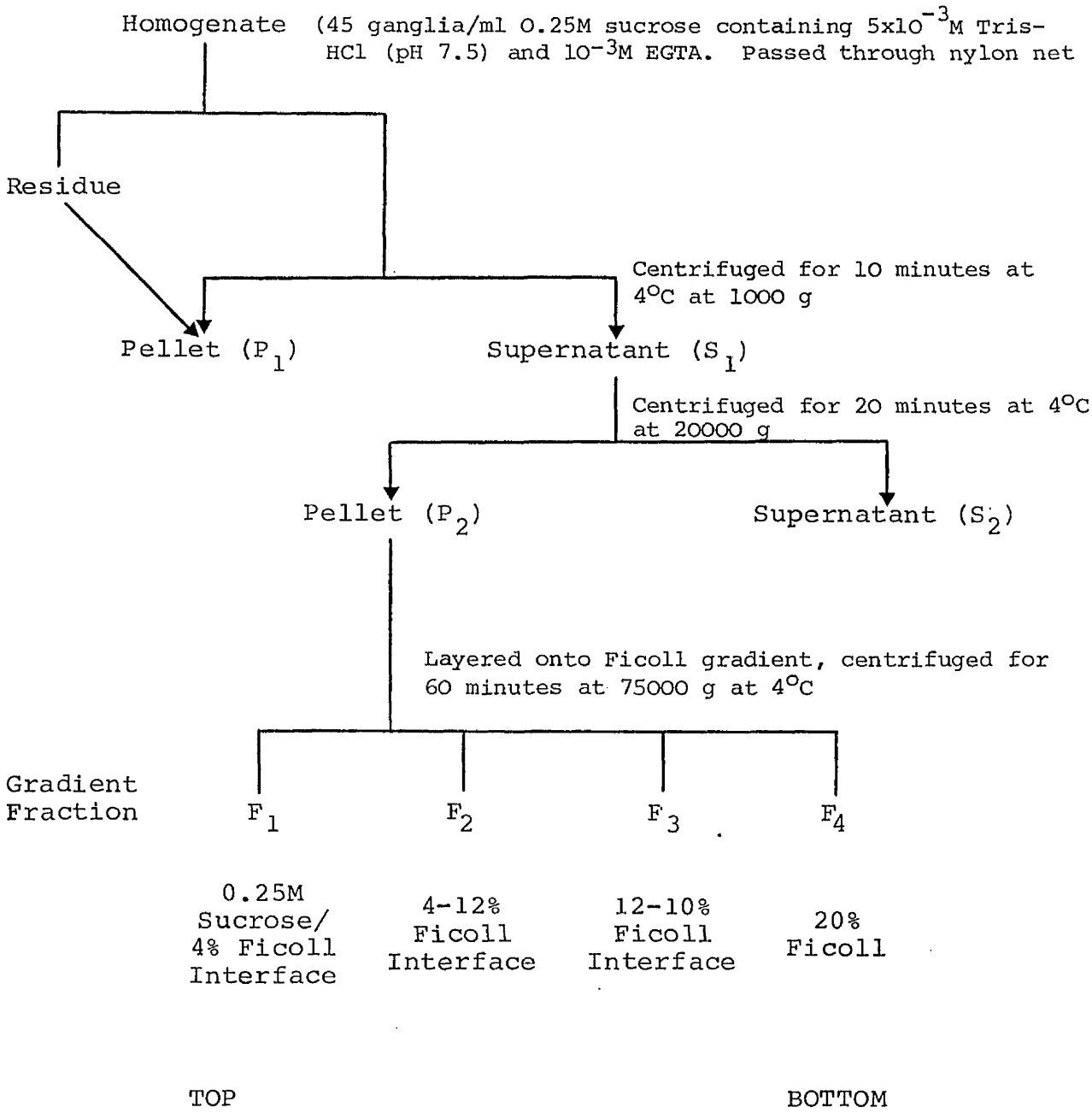
A pf_2 fraction (0.6 ml) prepared as previously described was incubated with 0.2 ml of $4x10^{-4}$M Gpp(NH)p prepared in $8x10^{-2}$M Tris-maleate buffer (pH 7.5) containing 10^{-2}M theophylline, $5x10^{-3}$M magnesium sulphate and $5x10^{-4}$M EGTA for 5 minutes at 30°C. To remove unbound Gpp(NH)p, the mixture was centrifuged twice at 9000 g for 10 minutes at 4°C and the final pellet re-suspended in 0.6 ml of $6x10^{-3}$M Tris-HCl buffer (pH 7.5) containing EGTA ($2x10^{-3}$M). Twenty microlitres of this pellet fraction was then added to the test substance as indicated and adenylate cyclase activity measured.

1.3 Sub-cellular Fractionation

Sub-cellular fractionation was carried out by a method slightly modified from that of Donnellan et al, 1976. Cerebral ganglia (45 ganglia/ml) were homogenised in 0.25M sucrose containing Tris-HCl ($5x10^{-3}$M) (pH 7.5) and EGTA (10^{-3}M). After filtration through a nylon net (159 μm aperture) to remove gross particulate material, the homogenate was centrifuged at 1000 g for 10 minutes and the pellet combined with the gross particulate material (P_1). The supernatant (S_1) was centrifuged at 20000 g for 20 minutes to give a crude mitochondrial pellet (P_2) and a supernatant (S_2). The P_2 pellet was suspended in 1 ml 0.25M sucrose (pH

FIGURE 7

Sub-cellular Fractionation of Cerebral Ganglia



7.5) and layered onto a discontinuous gradient composed of 4 ml layers of 4, 12 and 20% (w/v) Ficoll containing 0.25M sucrose (pH 7.5). After centrifugation on a swing out rotor for 1 hour at 75000 g at $4^{o}C$, the indicated fractions (Figure 7) were collected. Material sedimenting through the 20% Ficoll layer was suspended in 0.25M sucrose (pH 7.5).

2. Measurement of Adenosine-3'5'-Cyclic Phosphate

Cyclic AMP was measured by the method of Tovey et al, 1974. All additions to the assay were prepared in a $5x10^{-2}M$ Tris-HCl buffer (pH 7.5) containing EDTA ($4x10^{-3}M$), with the exception of unlabelled cyclic AMP standards which were dissolved in $8x10^{-2}M$ Tris-maleate buffer (pH 7.5) containing theophylline ($10^{-2}M$), magnesium sulphate (2 or 5x $10^{-3}M$) and EGTA ($5x10^{-4}M$).

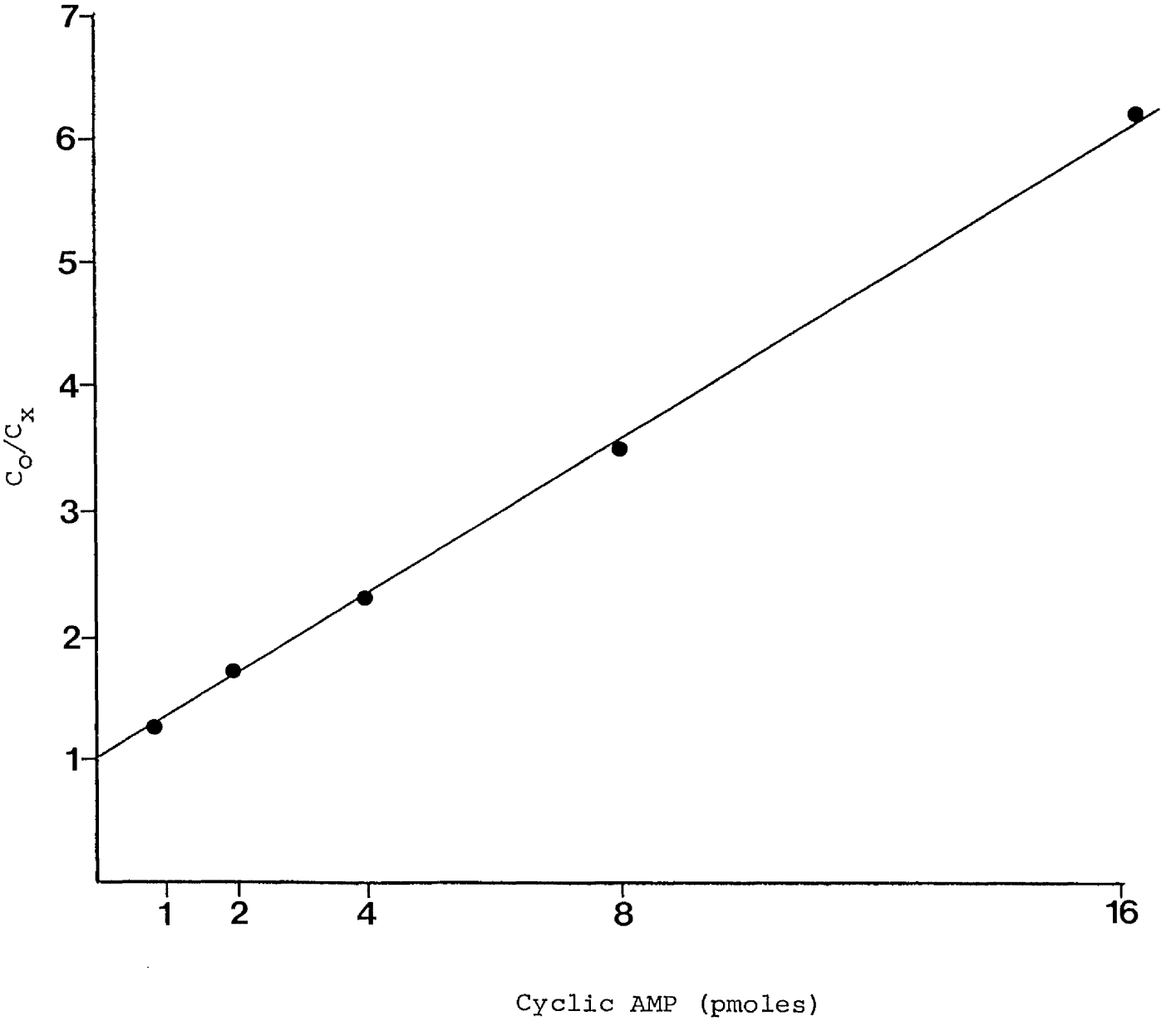
Fifty microlitres of the samples were added to Eppendorf tubes containing tritiated cyclic AMP (50 µl, 0.5 µCi/ml) and mixed before the addition of binding protein (100 µl), diluted 15 fold with Tris-HCl ($5x10^{-2}M$, pH 7.5) containing EDTA ($4x10^{-3}M$). After mixing the contents, the tubes were incubated for 2 hours in an ice bath. To remove the free [^{3}H]-cyclic AMP from the bound [^{3}H]-cyclic AMP, 100 µl of a charcoal suspension (0.1% w/v prepared in Tris-HCl buffer [$5x10^{-2}M$], pH 7.5 containing EDTA [$4x10^{-3}M$] and 1% BSA) was added to each tube, mixed and then centrifuged at 10000 g for 1 minute at room temperature in an Eppendorf bench

centrifuge. Two hundred microlitres of the supernatant, containing the bound [^{3}H]-cyclic AMP was added to 3 ml of a toluene-Triton X-100 scintillant prepared by adding 500 ml Triton-X-100 to 1 litre of toluene containing 19.8 g PPO and 1.98 g POPOP. Distilled water (0.2 ml) was then added to each vial and mixed to obtain a clear solution. The radioactivity was determined by liquid scintillation counting. To prepare a standard curve and to measure the charcoal blank, Eppendorf tubes containing the following were included in each measurement of cyclic AMP. The standard curve contained 50 µl unlabelled cyclic AMP (1-16 pmoles/assay), 50 µl [^{3}H]-cyclic AMP and 100 µl binding protein. The charcoal blank was prepared by adding 50 µl [^{3}H]-cyclic AMP to 150 µl Tris-HCl buffer (5×10^{-2}M, pH 7.5) containing EDTA (4×10^{-3}M). One hundred microlitres of the charcoal suspension were added to each tube and assayed as described previously. The radioactivity determined in 200 µl of the blank supernatant was subtracted from the values observed from the standard curve and unknown samples.

The radioactivity bound in the absence of unlabelled cyclic AMP was designated C_o from which ratio C_o/C_x was calculated for each concentration of unlabelled cyclic AMP. A linear relationship was obtained by plotting C_o/C_x against pmoles of standard cyclic AMP (Figure 8). The amount of cyclic AMP in each sample was then determined from the corresponding C_o/C_x value. A new standard curve was included in all measurements of cyclic AMP and each standard determined in duplicate.

FIGURE 8

A Typical Standard Curve for the Measurement of Cyclic AMP



Each determination was carried out in duplicate

3. Enzyme Assays

3.1 Adenylate Cyclase

Adenylate cyclase activity was measured in an assay system similar to that described by Harmar and Horn, 1977 containing as a final concentration, GTP (10^{-4}M), ATP ($5x10^{-4}$M), test substance where indicated and tissue preparation in a final volume of 80 µl. All additions to the cyclase assay (with the exception of tissue preparation) were prepared in an incubation buffer such that the final concentration in 80 µl was $8x10^{-2}$M Tris-maleate (pH 7.5), 10^{-2}M theophylline, $2x10^{-3}$M or $5x10^{-3}$M magnesium sulphate and $5x10^{-4}$M EGTA. After preliminary incubation of the assay system in the absence of ATP for 5 minutes at 30°C, ATP was added to start the reaction. The samples were incubated for 3 minutes at 30°C and the reaction terminated by boiling for 3 minutes. The contents of each Eppendorf tube were then centrifuged for 5 minutes at 10000 g in an Eppendorf bench centrifuge to remove denatured protein and 50 µl aliquots of the supernatant assayed for cyclic AMP.

For some of the experiments with Gpp(NH)p, the procedure was modified by initiating the reaction by the addition of the tissue preparation.

Adenylate cyclase activity was calculated from the following formula:

$$(\text{pmoles/min/ml}) = \frac{\text{pmoles cyclic AMP}}{50\ \mu l} \times \frac{\text{total volume of assay}}{\text{incubation time}} \times \frac{1}{\text{tissue volume added to assay}}$$

$$= \text{pmoles cyclic AMP } \frac{0.08}{0.050} \times \frac{1}{3} \times \frac{1}{0.02}$$

$$= \text{pmoles cyclic AMP} \times 26.6$$

The specific activity was therefore obtained by dividing the value obtained by the appropriate mg protein/ml.

3.2 Cyclic AMP Phosphodiesterase

Cyclic AMP phosphodiesterase activity was measured by a modification of the method of Thompson and Appleman, 1971. The volume of the assay mixture was 0.4 ml which contained, as a final concentration, 5×10^{-3}M magnesium sulphate, 4×10^{-2}M Tris-HCl (pH 8.0), 3.75×10^{-3}M 2-mercaptoethanol, 0.1 μCi [^{3}H]-cyclic AMP, 10^{-4}M cyclic AMP and 0.1 ml of a pf_1 preparation (approximately 50 μg protein). The reaction was initiated by the addition of the pf_1 fraction to the mixture also equilibrated to 30°C. After incubation for 5 minutes at 30°C, the reaction was terminated by boiling for 45 seconds followed by cooling in an ice bath.

5'-Adenosine-monophosphate, the product formed by cyclic AMP phosphodiesterase, was hydrolysed to adenosine by the addition of 5×10^{-5} g of snake venom (Ophiaphagus hannah). The tubes were mixed and incubated for 10 minutes at 30°C in a shaking water bath.

To terminate the reaction, the samples were immersed in an ice bath before the addition of 1 ml methanol. The entire contents of each tube were transferred to AG-1-X8 columns. The columns were then washed with 1 ml methanol and the eluate added to 10 ml of a toluene scintillant prepared by dissolving 19.8 g PPO and 1.98 g POPOP in 2.5 litres of toluene. The radioactivity in the eluate was determined by liquid scintillation counting.

Cyclic AMP phosphodiesterase specific activity was calculated as indicated:

$$\text{specific activity} = \frac{\text{cpm (measured - blank)}}{\text{cpm (maximum) blank}} \times \frac{\text{pmol substrate}}{\text{0-4 ml}} \times \frac{1}{\text{time}} \times \frac{1}{\text{mg protein added}}$$

3.3 Glutamate Dehydrogenase (L-glutamate: NAD Oxidoreductase E.C.1.4.1.3)

Glutamate dehydrogenase activity was measured by a modification of the method of Donnellan et al, 1974. The sub-cellular fractions (0.2 ml) were added to an assay containing as final concentration 5×10^{-2}M Tris-HCl (pH 7.9), 10^{-1}M ammonium acetate, 0.1% (w/v) Triton-X-100, 2.2×10^{-4}M NADH, 10^{-3}M ADP and 5×10^{-3}M sodium 2-oxoglutarate in a final volume of 0.5 ml. Glutamate dehydrogenase activity was proportional to the decrease in absorbance at 340 nm due to the oxidation of NADH. The endogenous oxidation of NADH by the tissue was followed for 10 minutes in 1 cm micro-

cuvette before the addition of 2-oxoglutarate and the change in absorbance ΔE measured for a further 10 minutes. The absorbance change due to glutamate dehydrogenase was calculated by subtracting the endogenous ΔE from that obtained in the presence of 2-oxoglutarate. Thus, from the molar extinction coefficient for NADH (6220 litres/mol/cm), the activity was calculated from the following equation:

$$\text{nmol/min/ml} = \Delta E/\text{min} \times \frac{1}{\text{extinction coefficient}} \times \frac{\text{assay volume}}{\text{light path}} \times \frac{1}{\text{volume of tissue added}}$$

$$= \Delta E/\text{min} \times \frac{1}{6220} \times \frac{0.5}{1} \times \frac{1}{0.20}$$

$$= \Delta E/\text{min} \times 402$$

The specific activity was calculated by dividing the activity by the appropriate mg protein/ml.

3.4 Glycerol-3-Phosphate Dehydrogenase (L.3-Glycerophosphate: NAD Oxidoreductase E.C.1.1.1.8)

Glycerol-3-phosphate dehydrogenase activity was measured by a method slightly modified from that of Donnellan et al, 1974. Sub-cellular fractions (0.1 ml) were added to an assay containing, as a final concentration 5×10^{-2}M potassium dihydrogen phosphate (pH 6.9), 0.1% (w/v) Triton-X-100, 2.5×10^{-4}M NADH and 2.5×10^{-3}M dihydroxyacetone phosphate in a final volume of 0.4 ml. The change in absorbance (ΔE) as a result of endogenous NADH oxidation was followed for 10 minutes in a 1 cm micro-cuvette at 340 nm before the ΔE, in the presence of dihydroxyacetone phosphate was measured for a further 10 minutes. Thus, the change in absorbance as a result of glycerol-3-phosphate dehydrogenase activity was calculated by subtracting the endogenous ΔE from that observed in the presence of dihydroxy-

acetone phosphate. From the molar extinction coefficient for NADH (6220 litres/mol/cm), the activity was calculated from the equation shown below.

$$\text{nmol/min/ml} = \Delta E/\text{min} \times \frac{1}{\text{extinction coefficient}} \times \frac{\text{assay volume}}{\text{light path}} \times \frac{1}{\text{volume of tissue added}}$$

$$= \Delta E/\text{min} \times \frac{1}{6220} \times \frac{0.4}{1} \times \frac{1}{0.1}$$

$$= \Delta E/\text{min} \times 643.1$$

The specific activity was therefore determined by dividing the activity by the appropriate mg protein/ml.

3.5 Acetylcholinesterase (E.C.3.1.1.7)

Acetylcholinesterase activity was measured by the method of Ellman et al, 1961. Acetylcholinesterase hydrolyses acetylthiocholine into acetate and thiocholine. The liberated thiocholine reacts with 5,5'-dithiobis(2-nitrobenzoic acid) to form thionitrobenzoic acid and the colour intensity of this product measured at 405 nm.

Sub-cellular fractions (0.1 ml) were incubated with, as a final concentration, 2×10^{-2}M sodium orthophosphate (pH 7.7), 6×10^{-3}M acetylthiocholine iodide and 2.5×10^{-4}M 5,5'-dithiobis(2-nitrobenzoic acid) in a final volume of 2.1 ml in a 1 cm cuvette. The time taken (seconds) for an absorbance change corresponding to 0.1 units was measured. From the molar ex-

tinction coefficient for thionitrobenzoic acid (13300 litres/mol/cm), the activity was calculated from the equation shown below.

$$\text{nmoles/min/ml} = \frac{\Delta E}{\text{extinction coefficient}} \times \frac{\text{assay volume}}{\text{light path}} \times \frac{1}{\text{sample volume}} \times \frac{60}{\text{time taken for}\Delta E\ (S)}$$

$$= \frac{0.1}{13300} \times \frac{2.1}{1} \times \frac{1}{0.1} \times \frac{60}{\text{time (S)}}$$

$$= \frac{9473.7}{\text{time (S)}}$$

The specific activity was calculated by dividing by the appropriate mg protein/ml.

4. Protein

Protein concentration was determined as described by Bailley, 1962, a modification of the method of Lowry et al, 1951. Bovine serum albumin was used as the standard protein and a typical protein standard curve is shown in Figure 9.

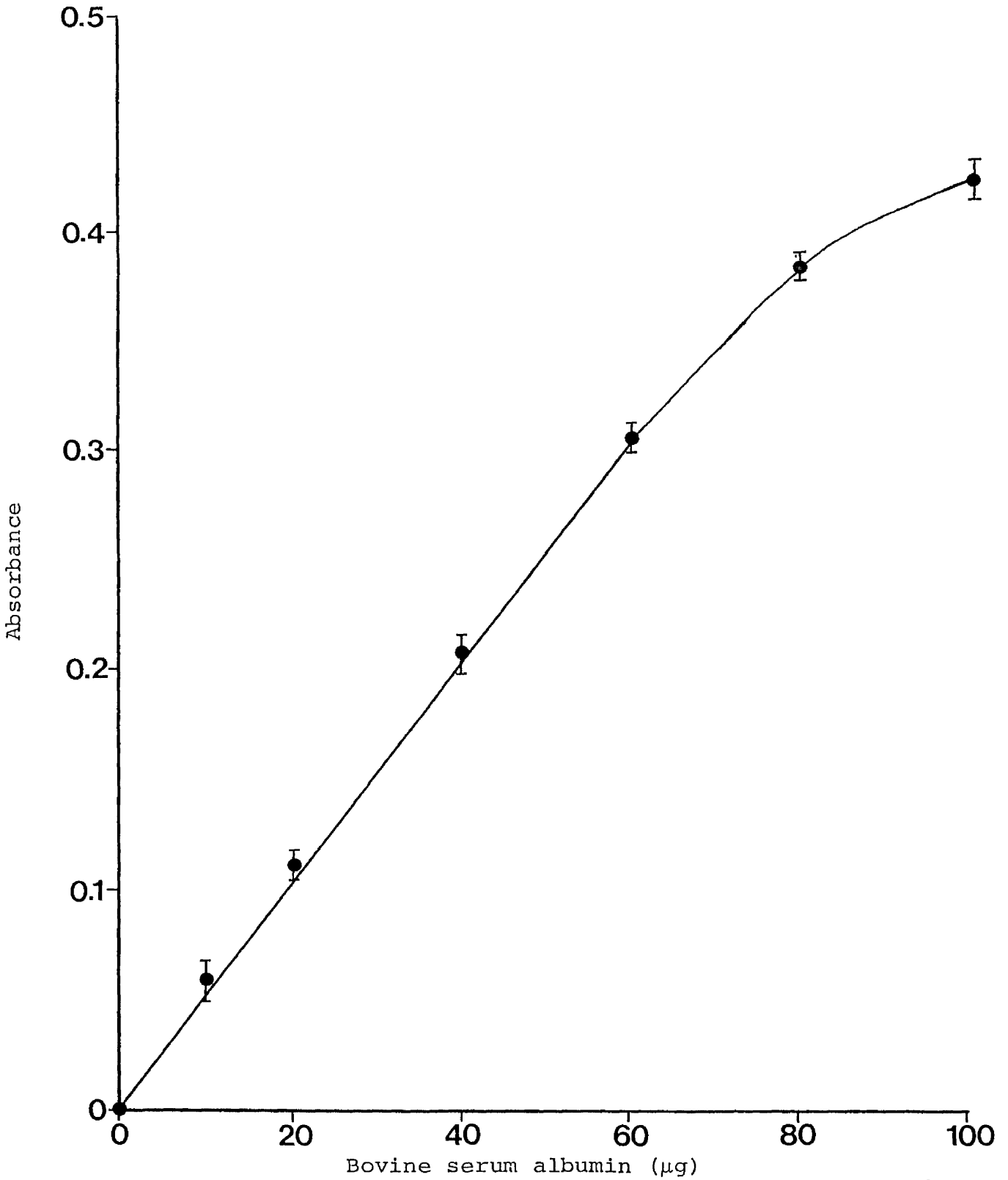
5. Mounting and Staining Cerebral Ganglia

5.1 Embedding and Sectioning (Chayen and Miles, 1954)

Cerebral ganglia were dehydrated by immersion in increasing concentrations of alcohol, transferred to an absolute alcohol-xylene mixture (1:1, v/v) for 30

FIGURE 9

Standard Curve for the Measurement of Protein



Each point was carried out in triplicate and is presented as the mean ± standard error.

minutes, followed by xylene alone for a further 30 minutes. The ganglia were removed and added to paraffin wax maintained at 55-66°C. The samples were frozen and the blocks cut with a microtome for staining.

5.2 Staining

The sections were stained with haematoxylin-eosin as described by Gurr, 1973. The slides were rehydrated by immersion in decreasing concentrations of alcohol and then stained in concentrated haematoxylin for 5 minutes. After rinsing in tap water, the slides were counter-stained in eosin for 1 minute, blotted, rinsed in water before dehydrating in alcohol and finally immersed in xylene for 5 minutes. The cerebral ganglia sections were then photographed and the results are shown in Figures 4 and 5.

6. Preparation of AG-1-X8 Columns

AG-1-X8 was washed thoroughly with 0.4M hydrochloric acid, water, 0.5M sodium hydroxide, water, 0.5M hydrochloric acid and water repeatedly to pH 5.0. The resin was then allowed to settle for at least 45 minutes before the supernatant was decanted and 4 volumes of water added to 1 volume of resin. A 1 ml portion was then pipetted into Pasteur pipettes plugged at the bottom with non-absorbent cotton wool. The efficiency of retention of cyclic AMP was

measured by the addition of 0.1 μCi [^{3}H]-cyclic AMP prepared in 10^{-2}M Tris-HCl (pH 8.0) buffer containing 3.75x 10^{-3}M 2-mercaptoethanol. The eluate was collected and the radioactivity counted. Under the experimental conditions, [^{3}H]-cyclic AMP represented only 1% of the total counts added to the column.

7. Statistics

The Student's "t" test was employed for all statistical analysis of the data in this thesis. For paired observations, the "t" statistic was calculated to test the null hypothesis ($U_A = U_B$) at the 95% confidence level where $U_A = U_B$ indicates that Sample A is similar to Sample B. If the "t" statistic calculated for any paired samples fell outside the accepted range (is greater than), then the null hypothesis was rejected in favour of accepting that the samples were different i.e. ($U_A \neq U_B$).

8. % Additivity

The percentage additivity for the activation of adenylate cyclase by monoamines was calculated from the equation shown below.

$$\% \text{ Additivity} = \frac{\text{activity observed experimentally with monoamines}}{\text{theoretical sum of individual monoamine activities}} \times 100$$

9. % Inhibition

The percentage inhibition of the monoamine-stimulated adenylate cyclase by phentolamine, cyproheptadine and chlorpromazine was calculated as follows:

$$\% \text{ Inhibition} = 100 - 100 \times \left(\frac{\text{activity in the presence of drug and monoamine} - \text{activity in the presence of drug alone}}{\text{activity in the presence of monoamine} \quad \text{activity in the absence of drug or monoamine}} \right)$$

RESULTS

Studies on Crude Homogenate

Preliminary studies showed that homogenates of cerebral ganglia contain an adenylate cyclase stimulated by octopamine (10^{-4}M) (Figure 10). In three experiments, the mean % stimulation of adenylate cyclase activity by octopamine alone was 315 $\pm$ 50 of basal activity. In the absence of octopamine, the small stimulation (~130%) by GTP was constant over the range 10^{-3}M - 10^{-7}M. In contrast, the addition of octopamine (10^{-4}M) resulted in maximum adenylate cyclase activity at GTP (10^{-4}M). In three experiments, the addition of GTP (10^{-4}M) and octopamine (10^{-4}M) resulted in a mean % stimulation of 430 $\pm$ 62.

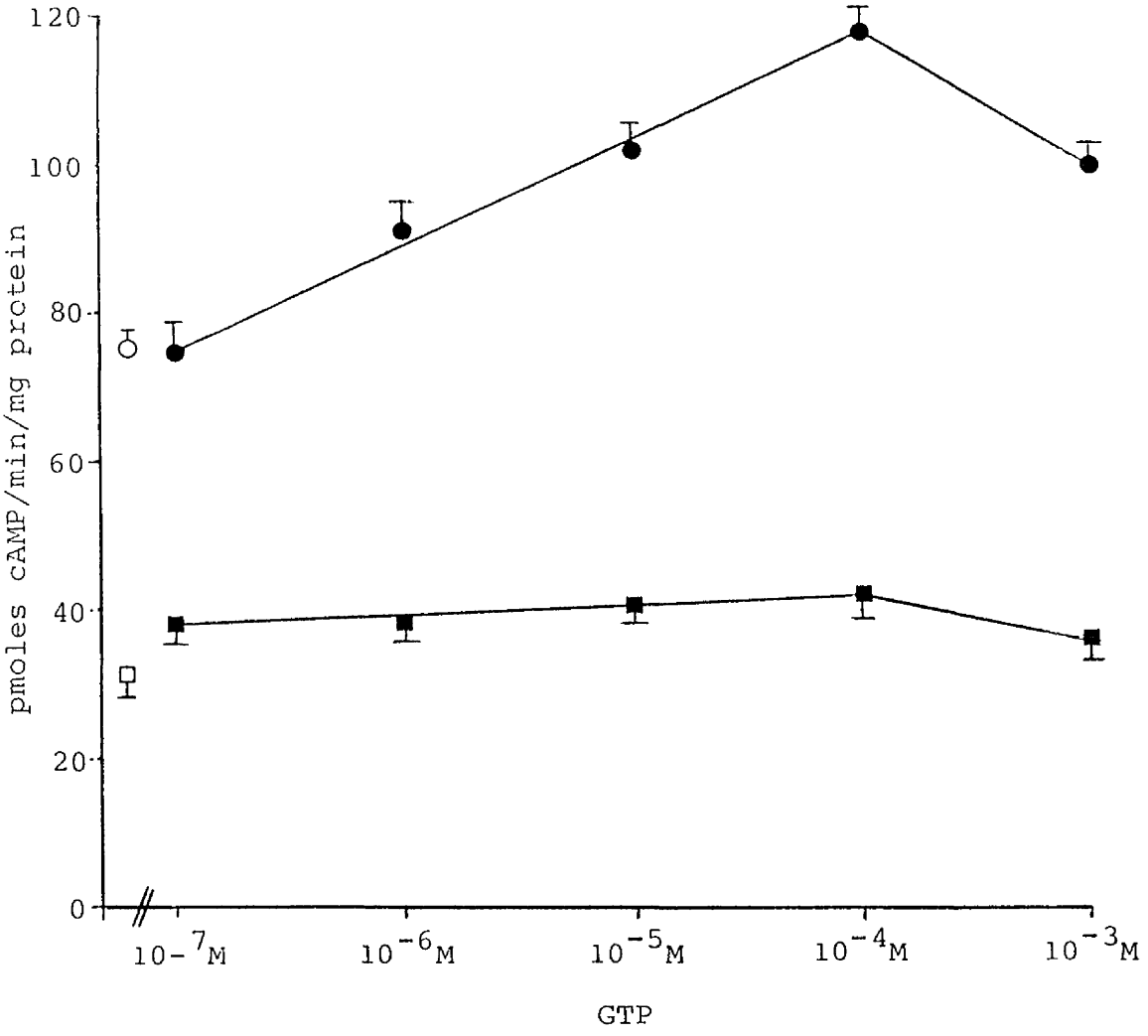
In cockroach cerebral ganglia (Harmar and Horn, 1977), the addition of octopamine (0.3×10^{-3}M) only stimulated adenylate cyclase activity to 112% of basal activity although a 378% increase was observed when both octopamine and GTP (10^{-4}M) were added to the preparation.

1. Effect of Monoamines

Figure 11 shows that adenylate cyclase activity is increased by dopamine and serotonin. From the dose response curves of activity against concentration of monoamines, maximum activity in the presence of GTP (10^{-4}M) occurred at dopamine (10^{-5}M) and serotonin (10^{-5}M). In five homogenate preparations, the mean maximal % stimulation by dopamine and serotonin was 188 $\pm$ 23 and 132 $\pm$ 14 respectively.

FIGURE 10

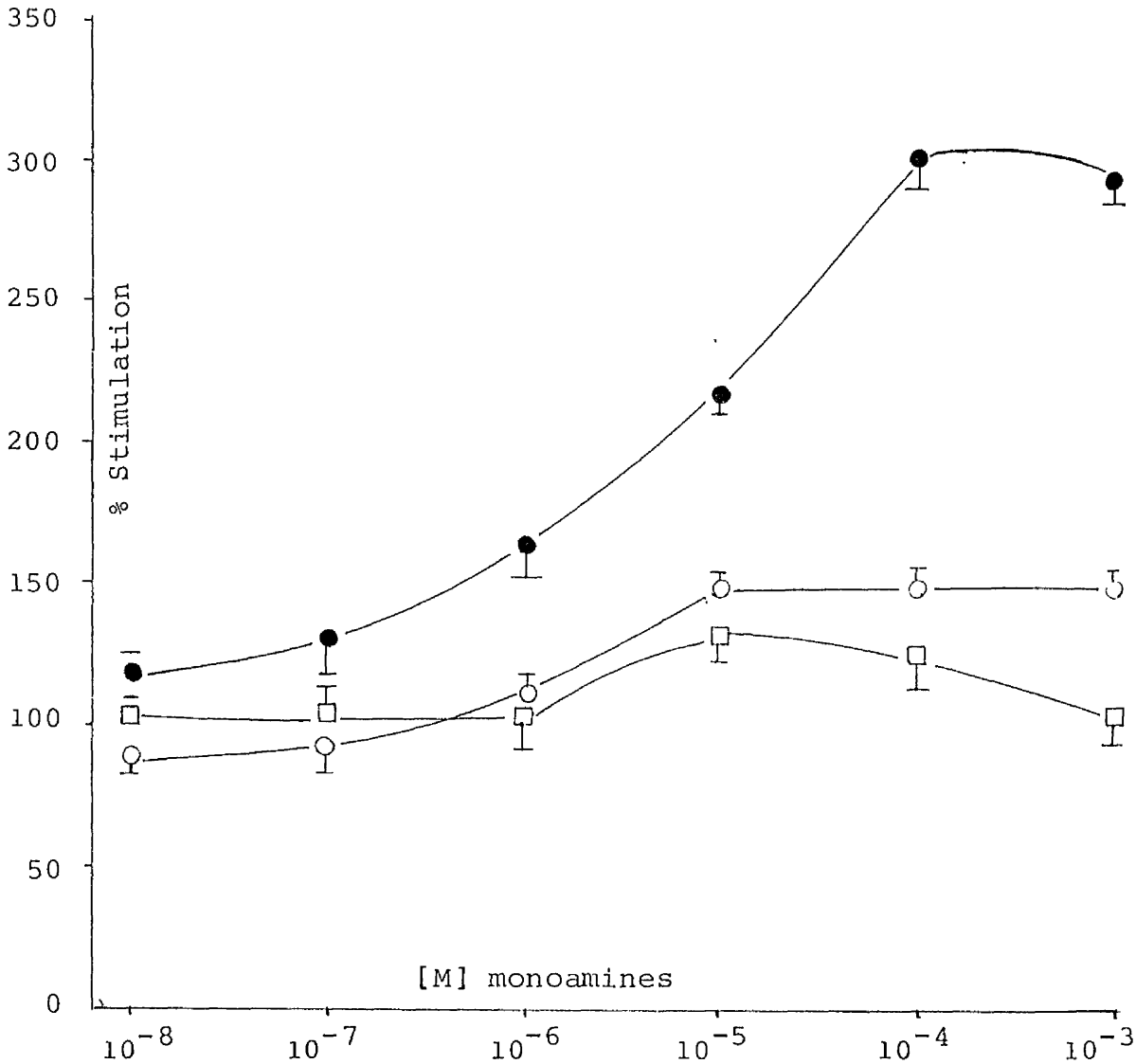
Effect of GTP on Basal and Octopamine-Stimulated Adenylate Cyclase



A homogenate of cerebral ganglia was assayed for adenylate cyclase activity in the presence (●) and absence (■) of octopamine (10^{-4}M) and increasing concentrations of GTP. (□) represents the activity observed in the absence of GTP and octopamine (basal activity) and (O) the activity observed with octopamine alone. Each point represents the mean ± standard error of three separate incubations from one homogenate preparation.

FIGURE 11

Effect of Octopamine, Dopamine and Serotonin
on Adenylate Cyclase Activity



A crude homogenate of cerebral ganglia was assayed for adenylate cyclase activity in the presence of 10^{-4}M GTP and increasing concentrations of octopamine (●), dopamine (O) and serotonin (□). Results are expressed as a percentage of the adenylate cyclase activity observed in the presence of GTP alone (34±3 pmoles cAMP/min/mg protein). Points represent the mean ± standard error of three separate incubations.

In the presence of GTP (10^{-4}M) and octopamine (10^{-4}M), adenylate cyclase activity was proportional under all conditions to a protein level of up to at least 1.6 mg protein/homogenate (Figure 12). In all subsequent experiments, the initial homogenate protein level was approximately 0.8 mg protein/600 µl.

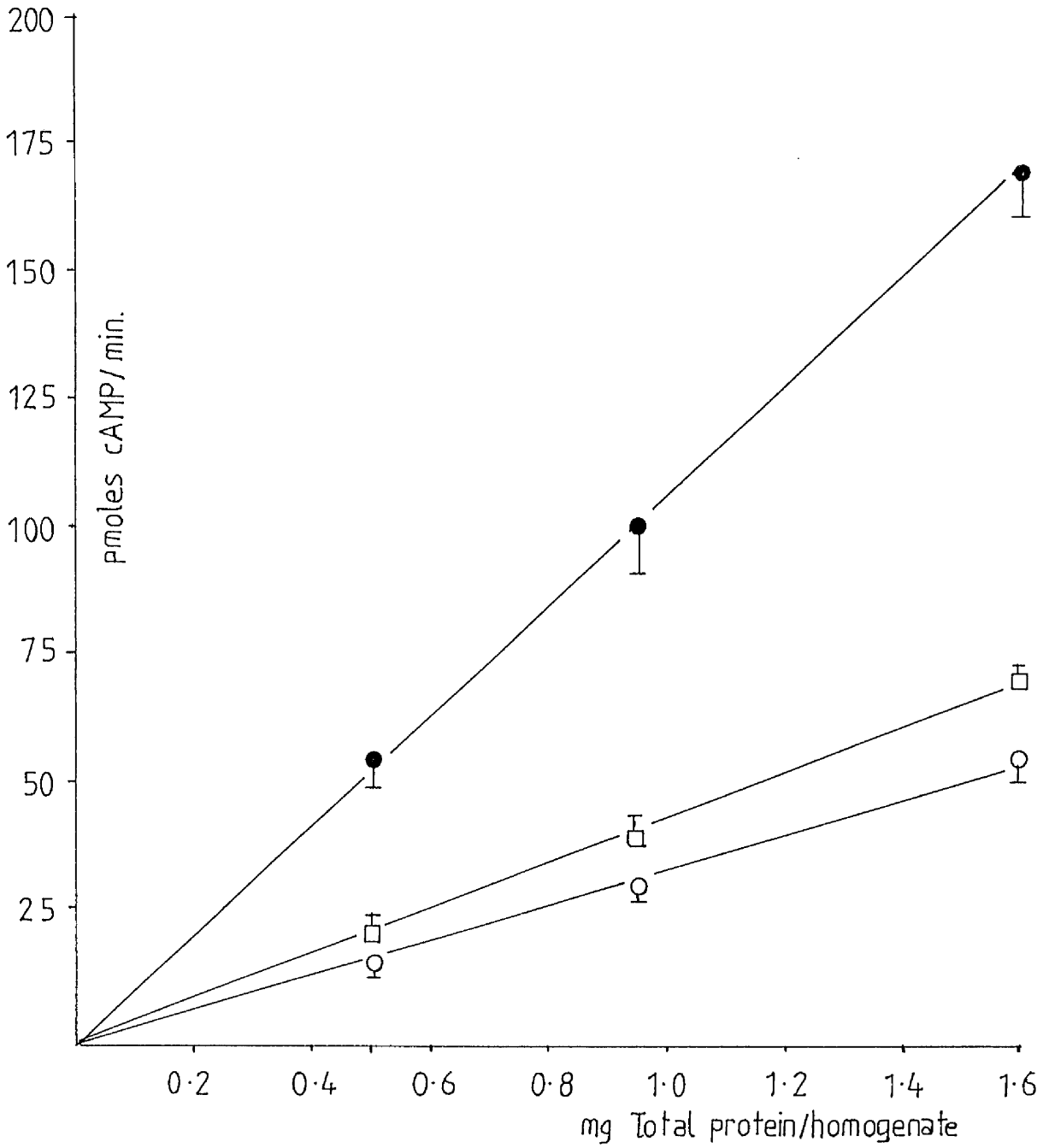
The observation that appreciable stimulation of adenylate cyclase activity was elicited by octopamine in the absence of GTP implied that GTP or some other activator may already be present in the crude homogenate. The homogenate was centrifuged at 9000 g for 10 minutes at 4°C to try and remove the endogenous activator(s).

Studies on Washed Pellet Fraction (pf_1)

The effect of washing once with homogenisation medium on the octopamine-stimulated adenylate cyclase is shown in Table 4. Washing caused a 50% decrease in basal activity and a 43% and 32% reduction in the activity observed with GTP alone when compared to the corresponding activities in the homogenate. The addition of octopamine (10^{-4}M) to the pellet fraction in two experiments only resulted in 26% and 34% of the activity observed in the homogenate being regained. In contrast in the presence of both octopamine (10^{-4}M) and GTP (10^{-4}M), the % recovery was increased to 78% and 79% suggesting that the pellet fraction contained appreciable amounts of the original adenylate cyclase and that the reduction in adenylate cyclase activity observed

FIGURE 12

Effect of Homogenate protein levels on
Adenylate Cyclase Activity



The effect of increasing homogenate protein level on adenylate cyclase was determined in the presence of buffer (O), GTP (10^{-4}M) (□) and GTP (10^{-4}M) + octopamine (10^{-4}M) (●). Each point represents the mean ± standard error of three separate incubations.

TABLE 4

The Effect of Washing on Octopamine-Stimulated Adenylate Cyclase

Fraction	Volume (µl)	Total Protein (mg)	Buffer	GTP (10^{-4}M)	Octopamine (10^{-4}M)	GTP (10^{-4}M) + octopamine (10^{-4}M)	Buffer	GTP (10^{-4}M)	Octopamine (10^{-4}M)	GTP (10^{-4}M) + octopamine (10^{-4}M)
			pmoles/min/600 µl				pmoles/min/mg protein			
Homogenate	600	0.75	19.9 ± 2.3	30.6 ± 3.1	62.3 ± 3.0	128.5 ± 12.4	26.5 ± 3.1	40.8 ± 4.1	83.1 ± 4.0	171.3 ± 16.5
	600	0.79	30.9 ± 2.7	39.9 ± 1.8	70.8 ± 2.6	108.2 ± 2.3	39.0 ± 3.4	50.5 ± 2.3	89.6 ± 3.3	137.1 ± 2.9
Pellet (pf_1)	600	0.34	10.2 ± 1.0	17.5 ± 1.3	16.4 ± 1.3	100.3 ± 1.2	30.0 ± 3.0	51.5 ± 3.9	48.2 ± 3.9	295.1 ± 3.5
	600	0.40	15.3 ± 3.7	27.2 ± 3.2	24.0 ± 2.8	85.3 ± 5.7	38.3 ± 9.3	68.0 ± 8.0	60.0 ± 7.0	213.3 ± 14.3
Supernatant	600	0.34	1.3 ± 0.2	2.2 ± 0.4	2.6 ± 0.4	7.5 ± 0.4	3.8 ± 0.5	6.5 ± 1.2	7.6 ± 1.2	22.2 ± 1.3
	600	0.37	6.8 ± 2.1	7.9 ± 0.9	9.2 ± 1.6	9.4 ± 2.2	18.4 ± 5.7	21.4 ± 2.4	24.9 ± 4.3	25.4 ± 5.9
% Recovery in pf_1	-	45	51	57	26	78	-	-	-	-
	-	51	50	68	34	79	-	-	-	-

The fractions were prepared as described in Materials and Methods and assayed in the presence of GTP and octopamine. Each value represents the mean ± standard error of three separate incubations.

with octopamine alone was due to the removal of endogenous GTP or another nucleotide.

Comparison of the specific activities of each fraction revealed that washing had resulted in a slight purification of adenylate cyclase (Table 4). In the presence of GTP and octopamine, an increase of 72% and 56% above the activity observed in the homogenate was observed in the pellet fraction.

The synergistic action of GTP and octopamine on adenylate cyclase activity, although present in the homogenate was more pronounced in the pellet fraction.

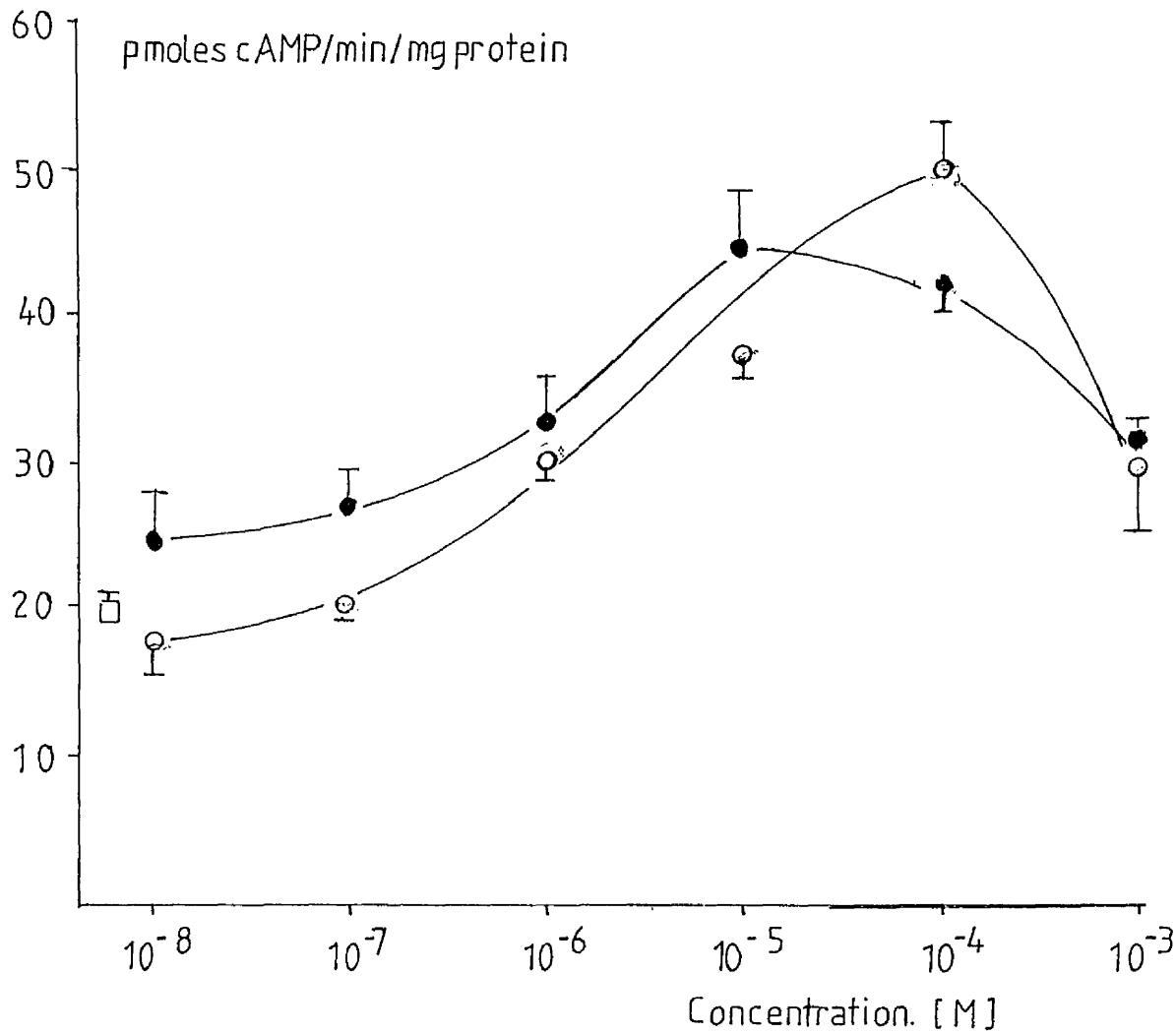
1. Effect of GTP and Octopamine Concentrations on Adenylate Cyclase

If increasing concentrations of GTP and octopamine were added to the re-suspended pellet, the largest increase in adenylate cyclase activity occurred at GTP (10^{-4}M) and octopamine (10^{-4}M - 10^{-5}M) (Figure 13).

The effect of varying the GTP concentration at fixed levels of octopamine is shown in Figure 14. As the octopamine concentration was raised, an increase in the maximum stimulation was observed. However, at 10^{-4}M and 10^{-5}M octopamine, the concentration of GTP necessary to give maximum stimulation was broadened

FIGURE 13

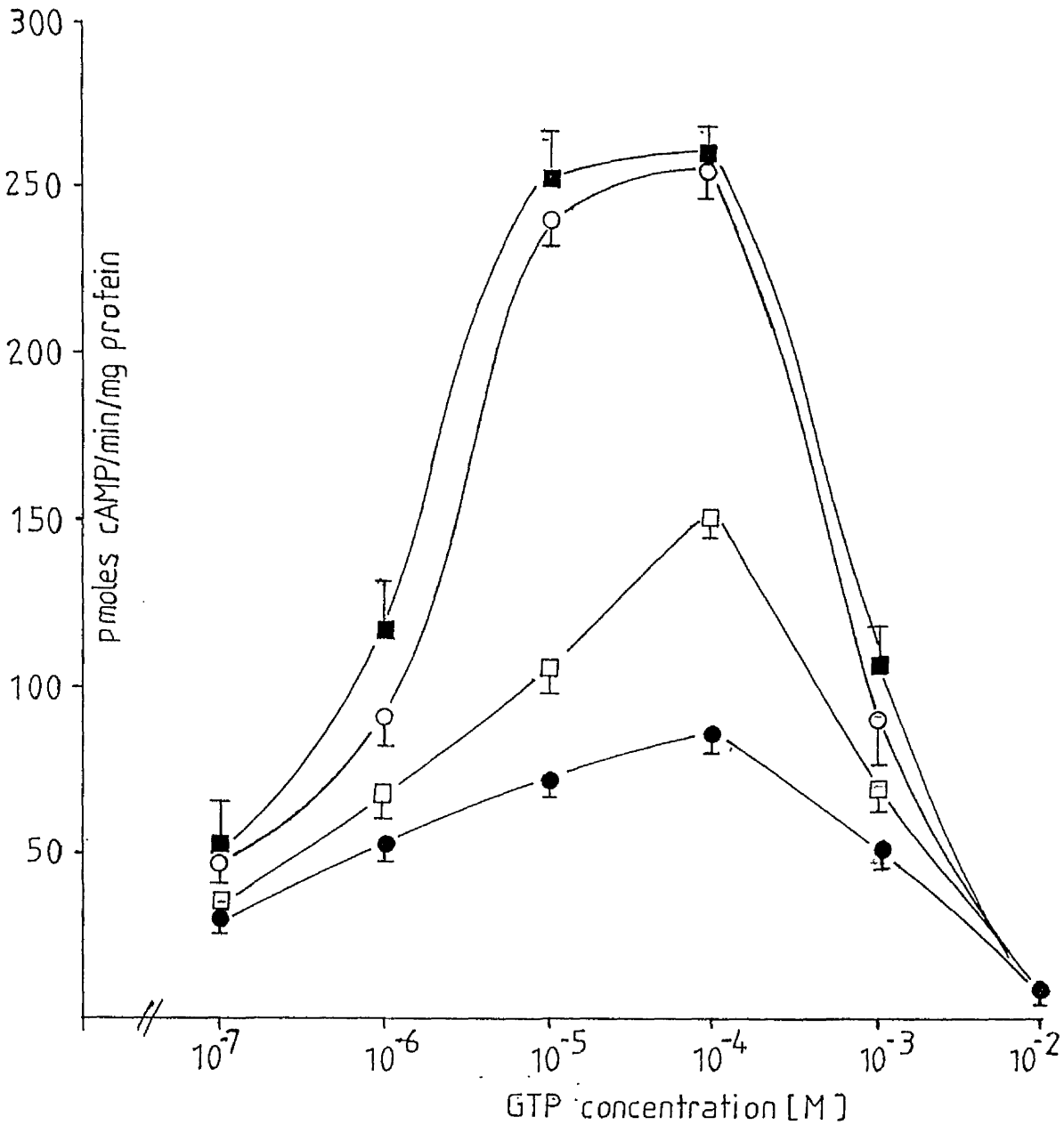
GTP and Octopamine Activation of Adenylate Cyclase



A washed pellet fraction (pf_1) prepared as described in Materials and Methods was assayed for adenylate cyclase activity in the presence of increasing concentrations of GTP (O) and octopamine (●). (□) is the activity observed with buffer alone (basal activity). Each point represents the mean ± standard error of three separate incubations.

FIGURE 14

Effect of GTP on Octopamine-Stimulated Adenylate Cyclase



A pellet fraction (pf_1) was assayed for adenylate cyclase activity in the presence of increasing concentrations of GTP and with buffer (●) and buffer + octopamine (10^{-5}M) (□), octopamine (10^{-4}M) (■) and octopamine (10^{-3}M) (○). Each point represents the mean ± standard error of three separate incubations.

to 10^{-4}M - 10^{-5}M. At all concentrations of octopamine, any increase in GTP above 10^{-4}M resulted in inhibition of adenylate cyclase activity.

2. Effect of Monoamines on Adenylate Cyclase

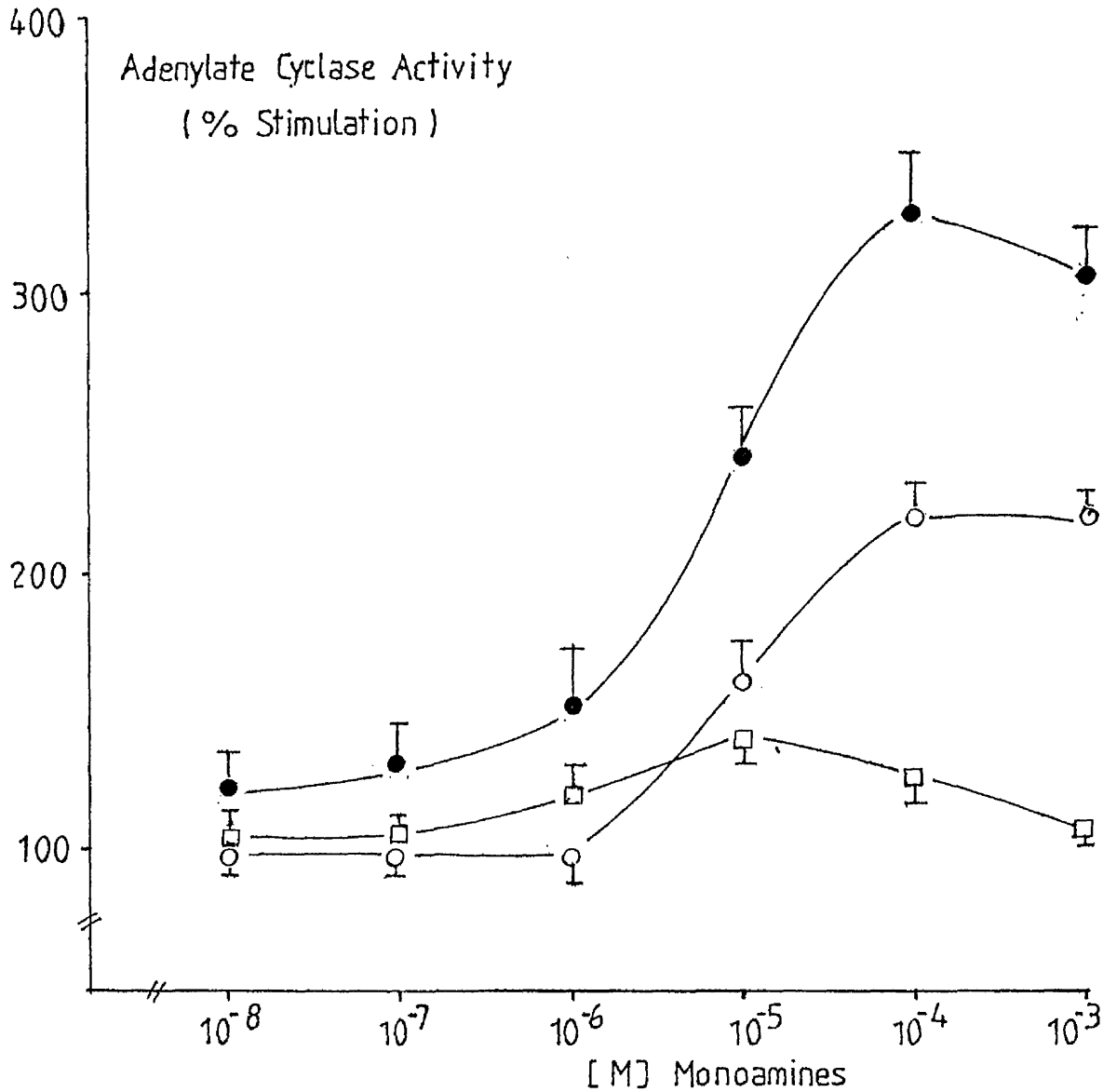
The stimulation of adenylate cyclase activity by octopamine, dopamine and serotonin is shown in Figure 15. The maximum level of activity was variable, although the response to octopamine was consistently greater than dopamine and dopamine greater than serotonin. In five experiments, the mean % maximal stimulation above that observed with GTP alone was 386 $\pm$ 52 for octopamine (10^{-4}M), 216 $\pm$ 37 for dopamine (10^{-4}M) and 141 $\pm$ 8.8 for serotonin (10^{-5}M). The concentrations that produced half maximal activation (Ka) for octopamine (8 μM) and dopamine (10 μM) were similar. However, the Ka value for serotonin (1 μM) was approximately 10-fold less than either octopamine or dopamine.

3. Magnesium/ATP Ratio

Adenylate cyclase activity was also affected by the magnesium/ATP ratio (Figure 16). If increasing concentrations of magnesium were added to 5 x 10^{-4}M ATP and 10^{-4}M GTP, a steady increase in activity was observed up to a magnesium/ATP ratio of four for up to 10^{-4}M octopamine. Any subsequent increase in the

FIGURE 15

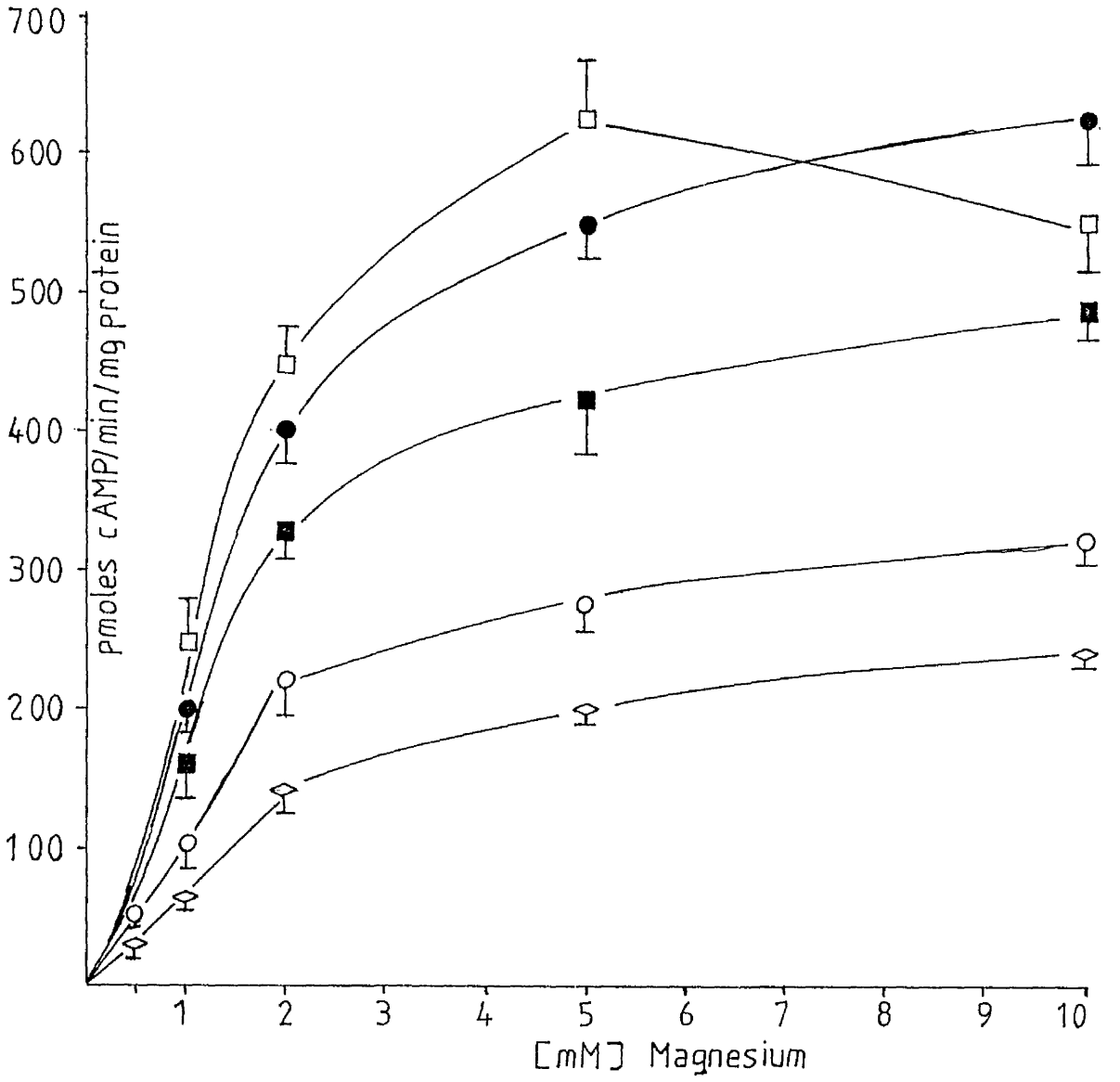
Effect of Octopamine, Dopamine and Serotonin
on Adenylate Cyclase Activity



A pellet fraction (pf_1) was assayed for adenylate cyclase activity as described in Materials and Methods in the presence of GTP (10^{-4}M) and increasing concentrations of octopamine (●), dopamine (O) and serotonin (□). Results are expressed as a percentage (%) of adenylate cyclase activity observed in the presence of GTP alone (88.4 ± 18.5 pmoles cAMP/min/mg protein). Each point represents the mean ± standard error of three separate incubations.

FIGURE 16

Magnesium/ATP Ratio and Adenylate Cyclase Activity



A pellet fraction (pf_1) was assayed for adenylate cyclase activity in the presence of 5 x 10^{-4}M ATP, increasing concentrations of magnesium and with buffer (◇) and buffer + octopamine (10^{-6}M) (○), octopamine (10^{-5}M) (■), octopamine (10^{-4}M) (●) and octopamine (10^{-3}M) (□). Each point represents the mean ± standard error of three separate incubations.

magnesium/ATP ratio had marginal effect on activity. In the presence of octopamine (10^{-3}M), a shift in the optimum magnesium/ATP ratio for maximum activity to 10 was observed. Higher concentrations of magnesium led to an inhibition of adenylate cyclase activity. All subsequent assays were incubated with 5×10^{-3}M magnesium.

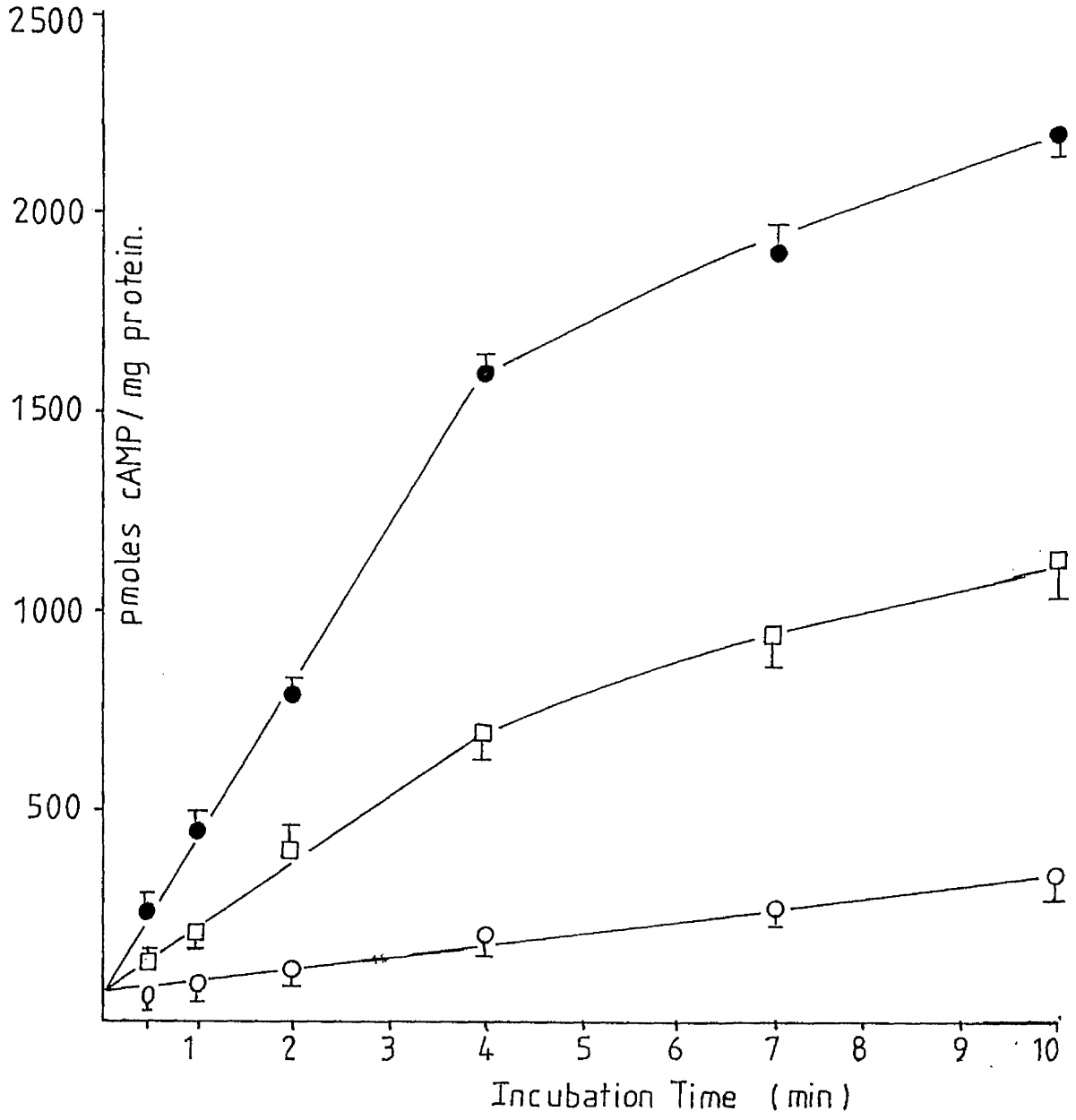
4. Effect of Time and Temperature on Adenylate Cyclase

Adenylate cyclase activity in the presence of GTP (10^{-4}M) and GTP (10^{-4}M) plus octopamine (10^{-4}M) was linear for up to 4 minutes (Figure 17). The effect of decreasing the octopamine (Figure 18) and dopamine (Figure 19) concentrations from 10^{-4}M - 10^{-6}M was to reduce the rate of cyclic AMP production. At these concentrations, octopamine- and dopamine-stimulated adenylate cyclase activity was linear for 4 minutes.

In the presence of octopamine (10^{-4}M) and GTP (10^{-4}M), the optimum temperature for adenylate cyclase activation was between 35°C and 40°C (Figure 20). If the temperature was increased above 40°C, inhibition of adenylate cyclase activity was observed. Boiling the pellet fraction for 3 minutes before commencement of the assay resulted in no adenylate cyclase activity being detected. The maximum stimulation of adenylate cyclase by GTP (10^{-4}M) and octopamine (10^{-4}M) from a pellet fraction, kept at 0°C, declined by 25% over two

FIGURE 17

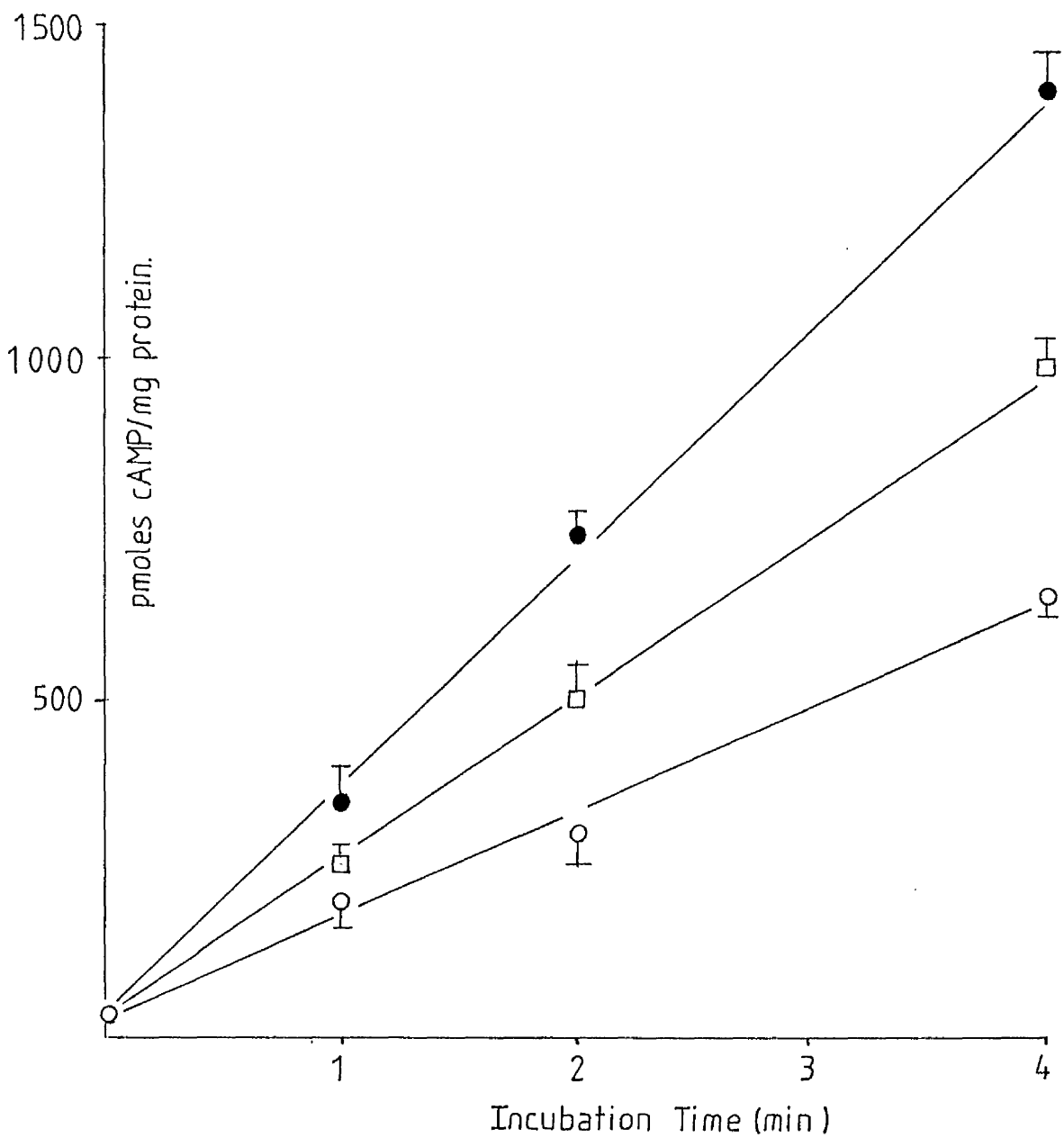
Time Course of Adenylate Cyclase Activation



A pellet fraction (pf_1) was assayed for adenylate cyclase activity at various times in the presence of buffer (O), buffer + GTP (10^{-4}M) (□) and GTP (10^{-4}M) + octopamine (10^{-4}M (●). Each point represents the mean ± standard error of three separate incubations.

FIGURE 18

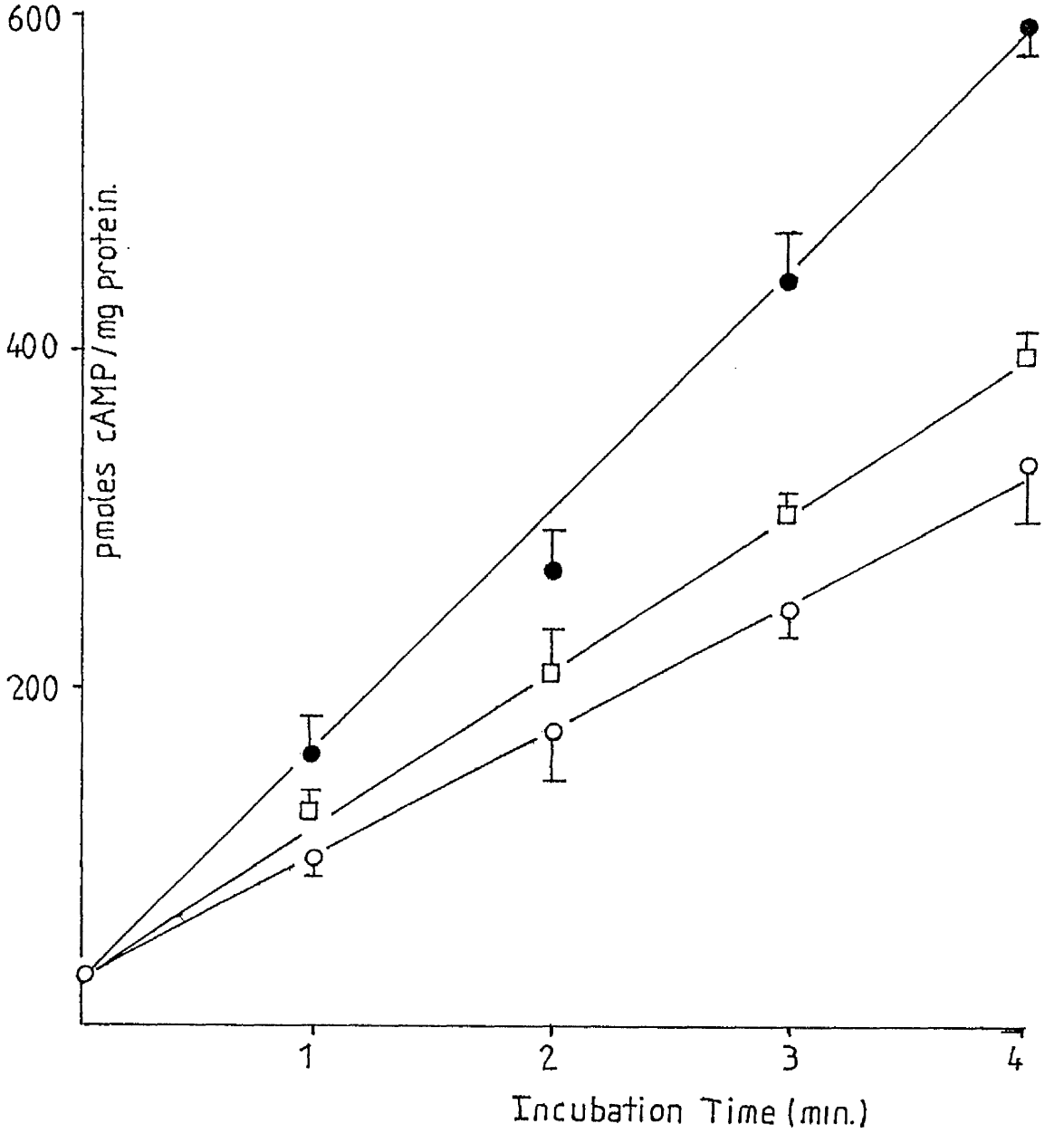
Octopamine Time Course



A pellet fraction (pf_1) was assayed for adenylate cyclase activity in the presence of GTP (10^{-4}M) and octopamine (10^{-6}M) (O), octopamine (10^{-5}M) (□) and octopamine (10^{-4}M) (●) at various times. Each point represents the mean ± standard error of three separate incubations.

FIGURE 19

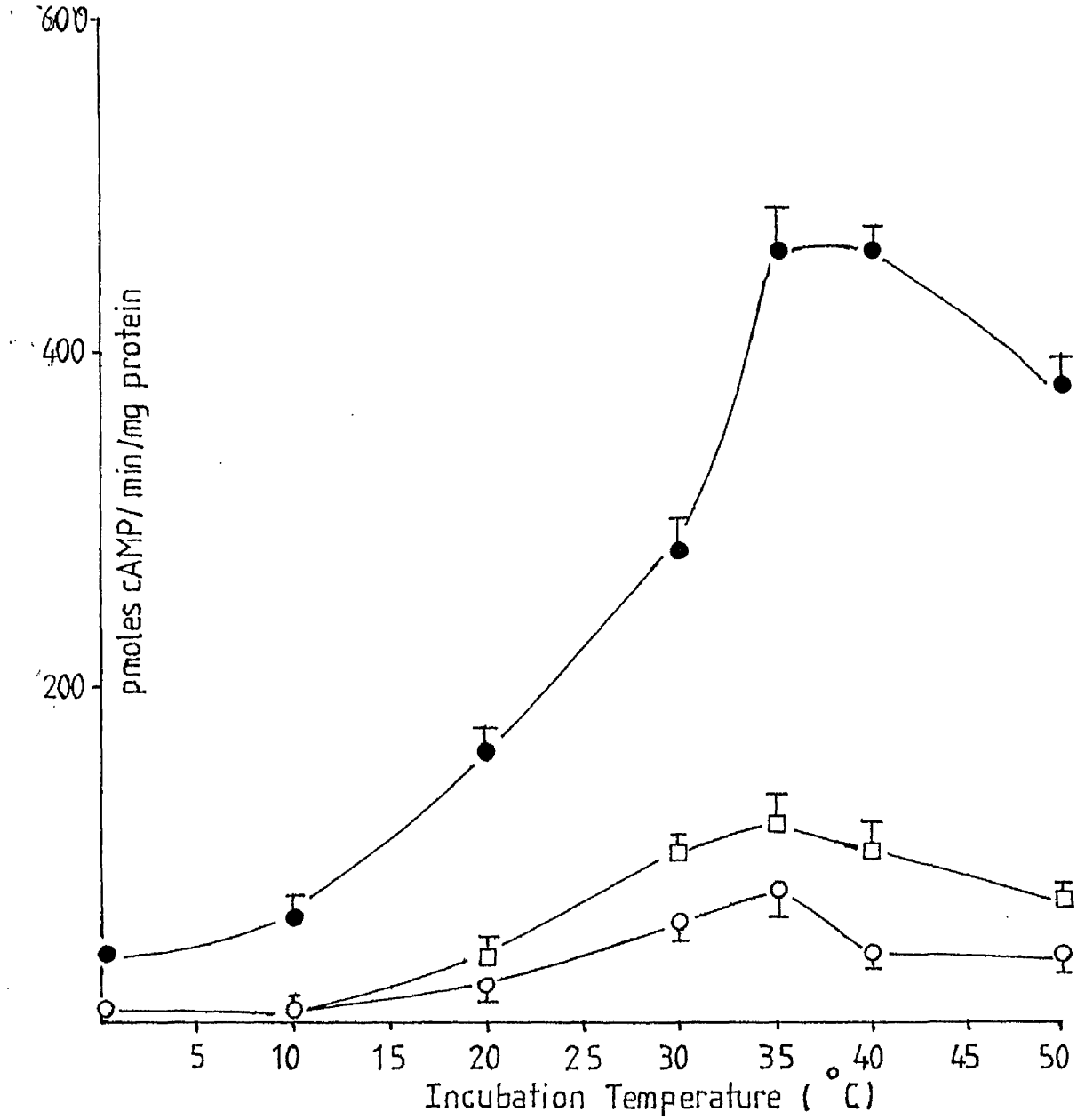
Dopamine Time Course



A pellet fraction (pf_1) was assayed for adenylate cyclase activity in the presence of GTP (10^{-4}M) and dopamine (10^{-6}M) (○), dopamine (10^{-5}M) (□) and dopamine (10^{-4}M) (●) at various times. Each point represents the mean ± standard error of three separate incubations.

FIGURE 20

Effect of Temperature on Adenylate Cyclase Activity



A pellet fraction (pf_1) was assayed for adenylate cyclase activity at increasing temperatures in the presence of buffer (O) and buffer + GTP (10^{-4}M) (□) and GTP (10^{-4}M) + octopamine (10^{-4}M) (●). Each point represents the mean ± standard error of three separate incubations.

hours (Figure 21) suggesting that the pellet fraction was relatively stable.

5. Sub-cellular Localisation of Octopamine-Stimulated Adenylate Cyclase

To study the sub-cellular distribution of octopamine-stimulated adenylate cyclase, cerebral ganglia were homogenised in 0.25M sucrose containing 5 mM Tris-HCl and 1 mM EGTA (pH 7.5) and centrifuged as described in Materials and Methods to give a crude mitochondrial pellet (P_2). This fraction was further centrifuged on Ficoll density gradients to produce three bands, F_1, F_2, F_3 and a pellet F_4.

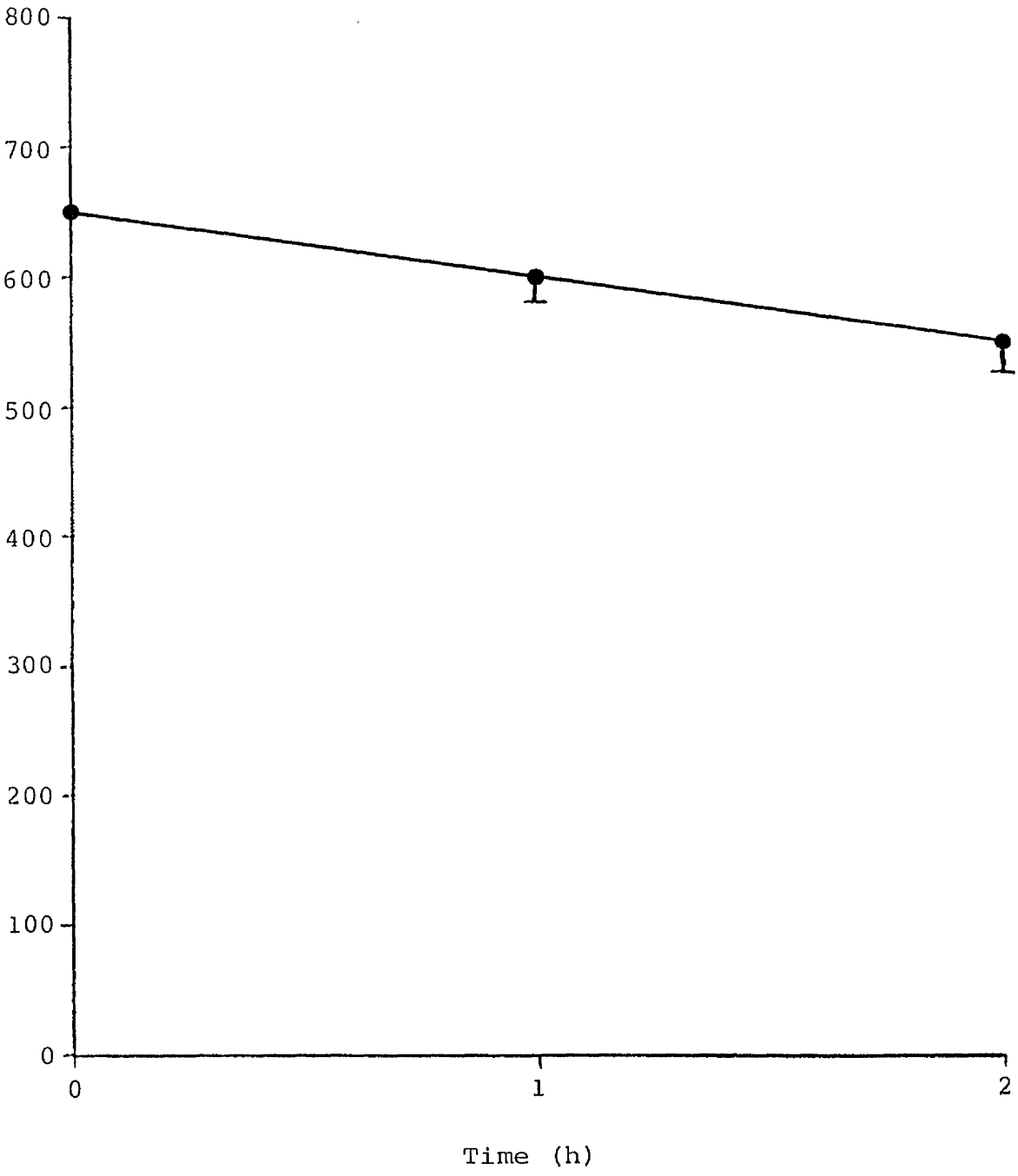
To characterise the fractions obtained, three enzyme activities were measured: glycerol-3-phosphate dehydrogenase (GPDH) a soluble enzyme (Hatefi and Stiggal, 1970), glutamate dehydrogenase (GDH) a mitochondrial enzyme (Smith et al, 1975) and acetylcholine esterase (AChE) an enzyme suggested to be associated with cholinergic membranes (Bachelard, 1974). AChE is not an unequivocal marker for cholinergic neurons as it has been reported in glial cells of insect central nervous system (Smith and Treherne, 1965).

Preliminary studies on homogenates of cerebral ganglia indicated that GDH activity was affected by ADP (Figure 22) and of the ADP concentrations assayed,

FIGURE 21

Stability of Adenylate Cyclase

A pellet fraction (pf_1) was assayed for adenylate cyclase activity in the presence (●) of 10^{-4}M GTP + 10^{-4}M octopamine, placed on ice and the activity re-measured at 1 and 2 hours. Each point represents the mean ± standard error of three separate incubations.



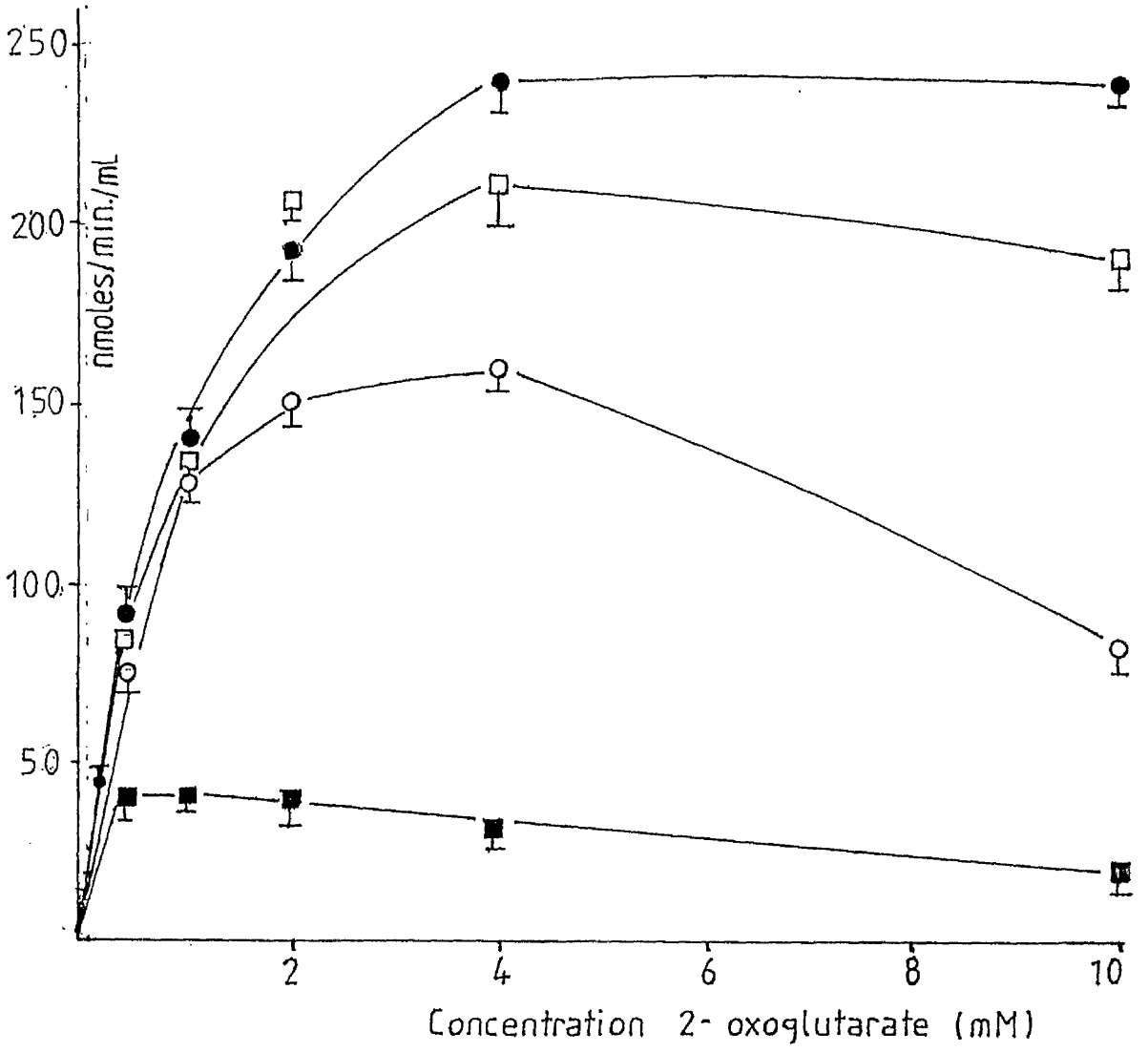
maximum activity occurred at 1 mM. At this concentration, the apparent Km of 1.1 mM for oxoglutarate (Figure 23) was similar to the Km (2 mM) observed for GDH in the flight muscle of the fleshfly Sarcophaga barbata (Donnellan et al, 1974). With GPDH, an apparent Km of 0.2 mM for dihydroxyacetone phosphate was determined (Figure 24). The apparent Km values for both enzymes were considerably less than the concentrations of oxoglutarate (5 mM) and dihydroxyacetone phosphate (2.5 mM) used in the assay.

The activities of the enzymes in the P_1, P_2 and S_2 fractions are shown in Table 5. Octopamine-stimulated adenylate cyclase, GDH and AChE were enriched in the crude mitochondrial fraction (P_2). In two experiments, the P_2 fraction accounted for 79% and 96% of the octopamine-stimulated adenylate cyclase observed in the homogenate. GPDH was not clearly confined to the soluble fractions.

Table 6 shows the enzyme activities in the four fractions produced by centrifuging the P_2 fraction on Ficoll gradients. GDPH, the soluble enzyme, was enriched in the F_1 fraction located at the top of the gradient whereas GDH enrichment occurred in F_4 at the bottom. However, a slight GDH enrichment was also observed in F_1. AChE was localised in F_1 and F_2. The F_2 fraction was reported to be the fraction where nerve terminals are located (Donnellan et al, 1976).

FIGURE 22

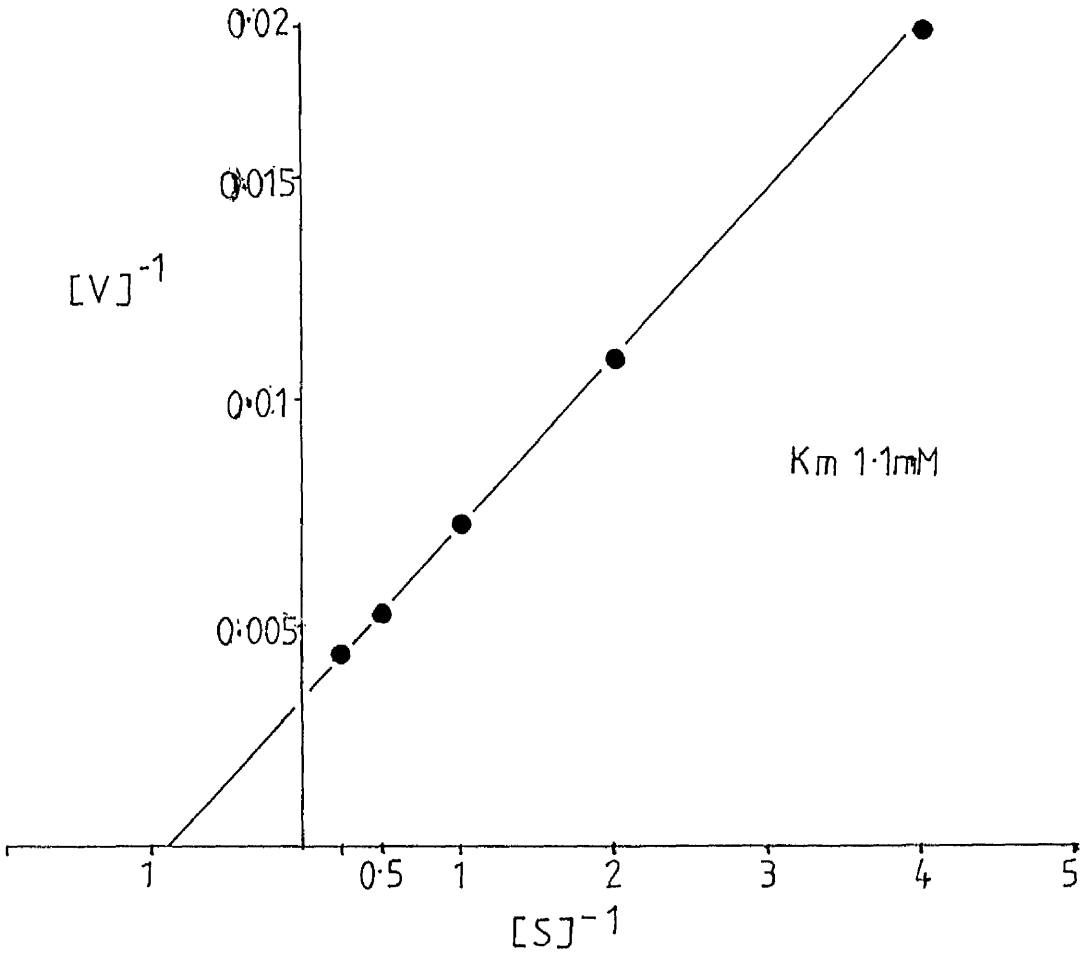
Effect of ADP on Glutamate Dehydrogenase Activity



Glutamate dehydrogenase activity was measured in a homogenate of cerebral ganglia in the presence of increasing concentrations of 2-oxoglutarate and with 0 (■), 2.5 x 10^{-4}M) (○), 5 x 10^{-4}M (□) and ADP (10^{-3}M) (●). Each point represents the mean ± standard error of three separate incubations. The homogenate contained 10.0 mg protein/ml.

FIGURE 23

Glutamate Dehydrogenase



A reciprocal plot of glutamate dehydrogenase activity $[V]^{-1}$ versus concentration of 2-oxoglutarate, $[S]^{-1}$ in the presence of ADP (10^{-3}M). The activity was measured in nmoles/min/ml and the substrate in mM.

FIGURE 24

Glycerophosphate Dehydrogenase

A homogenate of cerebral ganglia was assayed for glycerophosphate dehydrogenase activity in the presence of increasing concentrations of dihydroxy acetone phosphate. The results are shown as a reciprocal plot of [V], the activity observed measured in nmoles/min/ml against [S] the concentration of dihydroxy acetone phosphate (mM). The homogenate contained 10.0 mg protein/ml.

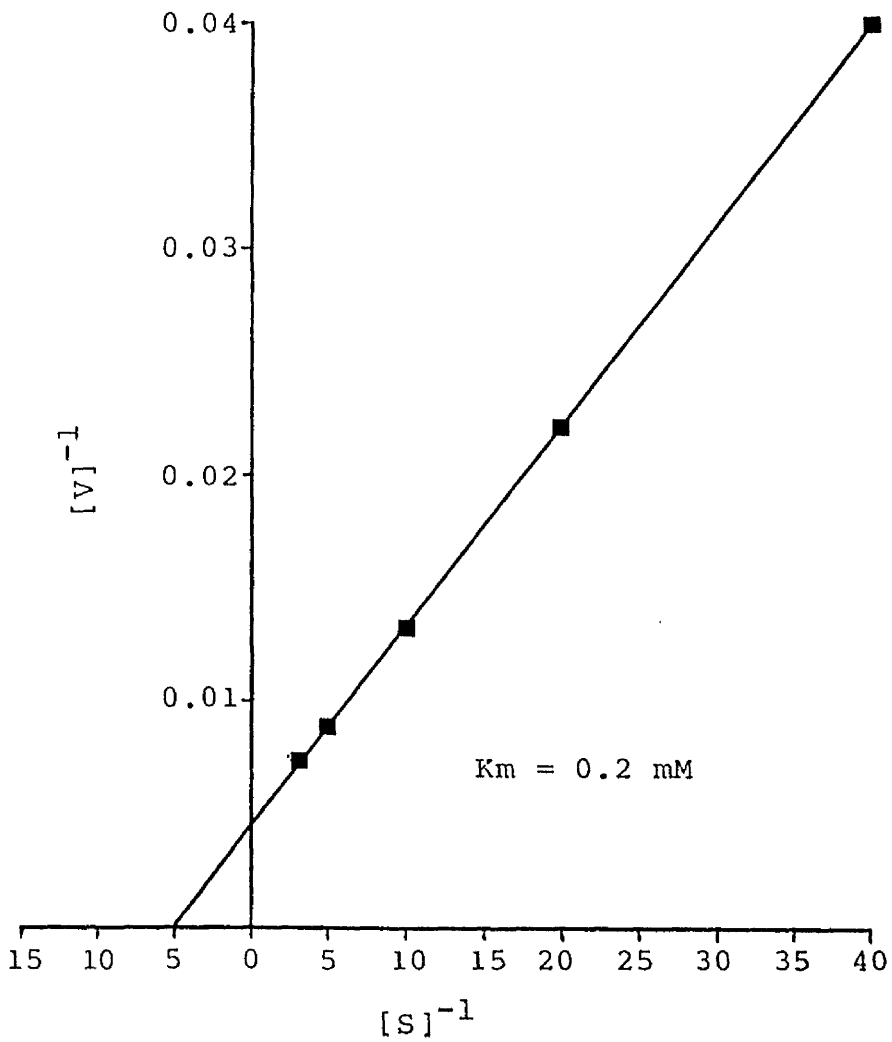


TABLE 5

Sub-Cellular Fractionation of Locust Cerebral Ganglia (P_1, P_2, S_2)

Fraction	mg protein/ml	AchE		GPDH		GDH		Adenylate Cyclase	
		nmoles/min/ml	nmoles/min/mg protein	nmoles/min/ml	nmoles/min/mg protein	nmoles/min/ml	nmoles/min/mg protein	pmoles/min/ml	pmoles/min/mg protein
Homogenate	17.2	430.6 471.3	25.0 27.4	385.8 360.1	22.4 20.9	402.0 482.4	23.4 28.0	853.0 899.1	49.6 52.3
	15.8	604.7	38.3	463.0	29.3	289.4	18.3	602.9 512.6	38.2 32.4
P_1	5.4	32.2 32.4	6.0 6.0	51.5 40.0	9.5 7.4	32.2 28.1	6.0 5.2	58.2 87.3	10.8 16.2
	2.5	39.5	15.8	22.5	9.0	16.1	6.4	22.7 20.3	9.1 8.1
P_2	7.0	278.6 294.2	39.8 42.0	160.7 130.7	23.0 18.7	301.5 321.6	43.1 45.9	628.8 646.1	89.9 92.3
	8.0	394.7	49.3	154.3	19.3	168.8	21.1	316.5 308.5	39.6 38.6
S_2	2.2	73.4 74.1	33.4 33.7	111.9 120.1	50.9 54.6	16.1 24.1	7.3 11.0	129.5 137.3	58.9 62.4
	2.9	65.8	22.7	90.0	31.0	20.1	6.9	106.1 105.8	36.6 36.5
% Recovery	85	87	-	82	-	82	-	96	-
	85	83	-	58	-	71	-	79	-

The sub-cellular fractions prepared as described in Materials and Methods were assayed for enzymic activity. The results presented are for two separate experiments and indicate the individual values obtained.

TABLE 6

Sub-Cellular Fractionation of Locust Cerebral Ganglia (F_1, F_2, F_3, F_4)

Fraction	mg Protein/ml	AchE		GPDH		GDH		Adenylate Cyclase	
		nmoles/min/ml	pmoles/min/mg protein	nmoles/min/ml	pmoles/min/mg protein	nmoles/min/ml	pmoles/min/mg protein	nmoles/min/ml	pmoles/min/mg protein
P_2	7.0	278.6 294.2	39.8 42.8	160.7 130.7	23.0 18.7	301.5 321.6	43.1 45.9	628. 646.1	89.8 92.3
	8.0	394.7	49.3	15.43	19.3	168.8	21.1	316.5 308.5	39.6 38.6
F_1	0.5	31.6 38.7	63.2 77.4	45.1 40.4	90.2 80.8	44.2 36.2	88.4 72.4	26.2 26.7	52.4 53.4
	0.6	31.6	52.7	38.6	64.3	30.2	50.3	21.6 12.1	36.0 20.2
F_2	0.9	84.6 83.1	94.0 92.3	30.3 26.1	33.7 29.0	34.1 28.1	37.9 31.2	266.5 273.1	296.1 303.4
	1.0	75.2	75.2	19.3	19.3	22.1	22.1	79.3 74.5	79.3 74.5
F_3	0.9	30.3 31.0	33.7 34.4	19.3 20.0	21.4 22.2	29.8 28.1	33.1 31.2	85.4 79.6	94.9 88.4
	1.2	48.3	40.3	16.1	13.4	22.1	18.4	16.1 12.8	13.4 10.7
F_4	1.5	46.4 47.4	30.9 31.6	34.7 31.7	23.1 21.1	144.7 168.8	96.5 112.5	100.7 112.1	67.8 74.7
	1.6	72.8	45.5	18.1	11.3	70.3	43.9	14.9 13.6	9.3 8.5
% Recovery	54	68	-	85	-	83	-	78	-
	55	58	-	60	-	86	-	78	-

The sub-cellular fractions prepared as described in Materials and Methods were assayed for enzymic activity. The results presented are for two separate experiments and indicate the individual values obtained.

The high activity in F_1 suggests that some solubilisation had occurred during the gradient procedure.

6. Effect of Octopamine and Theophylline on Phosphodiesterase

Cerebral ganglia contain an active phosphodiesterase which readily hydrolyses cyclic AMP to adenosine-5'-monophosphate. To obtain linear rates of hydrolysis of cyclic AMP, unlabelled cyclic AMP (10^{-4}M) was included in the assay. Theophylline (10 mM) present in all measurements of adenylate cyclase activity inhibits the rate of cyclic AMP hydrolysis by phosphodiesterase (Figure 25). Phosphodiesterase activity was independent of octopamine and GTP. At octopamine concentrations of 10^{-3}M to 10^{-5}M, little difference in activity in the presence of or absence of GTP (10^{-4}M) was observed (Table 7).

Evidence for Separate Receptors for Octopamine, Dopamine and Serotonin

The preliminary results show that S. gregaria cerebral ganglia contain an adenylate cyclase stimulated by octopamine, dopamine and serotonin in the presence of GTP. To investigate distinguish between the possibility that separate receptors contributed to the cyclase activation, the effect of adding the monoamines simultaneously and the effect of monoamine antagonists on adenylate cyclase activity was studied.

FIGURE 25

Time Course for Phosphodiesterase

A pellet fraction (pf_1) was assayed as described in Materials and Methods for phosphodiesterase activity in the absence (●) and presence (■) of theophylline (10^{-2}M). Each point represents the mean ± standard error of three separate incubations

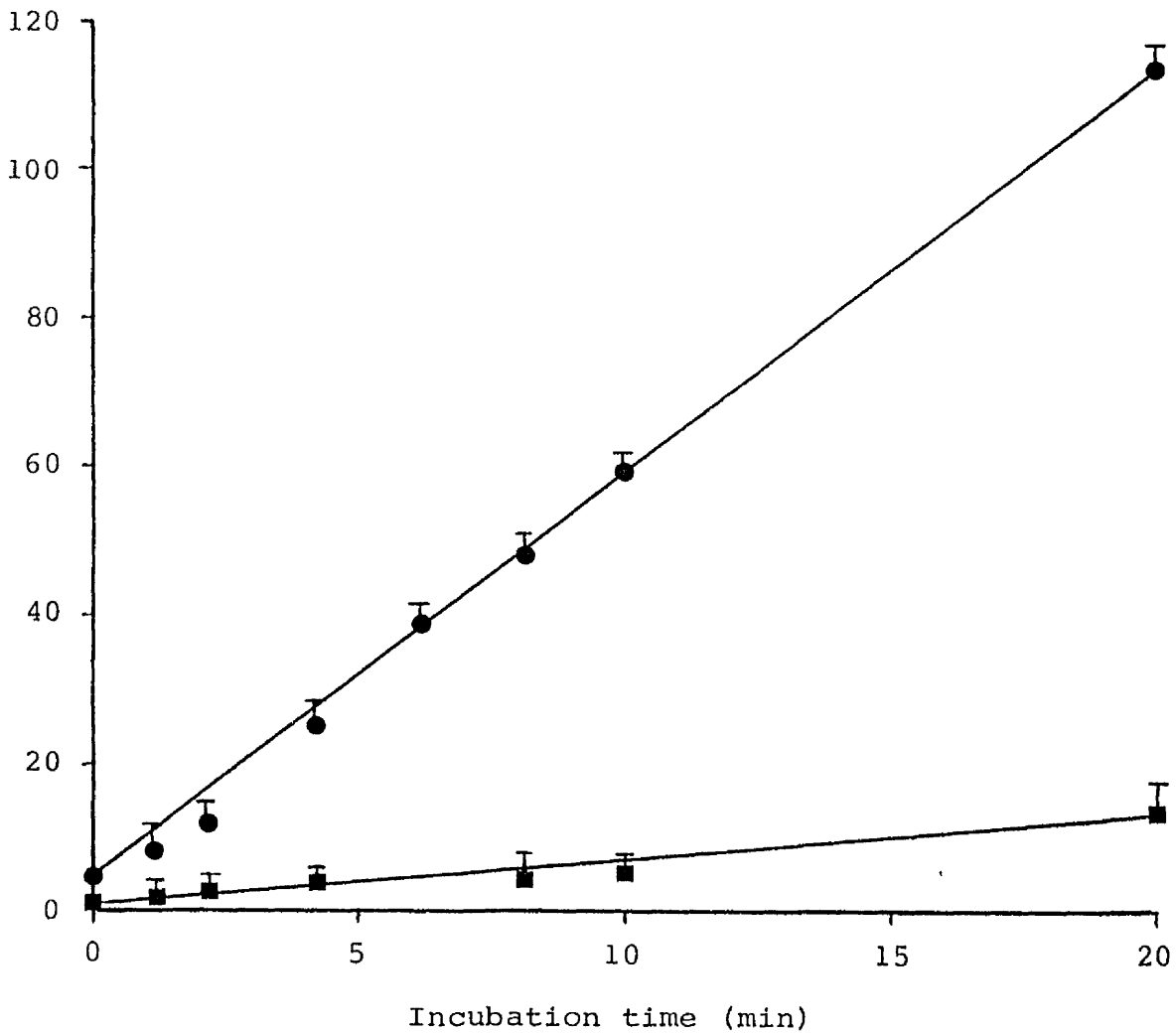


TABLE 7

Effect of GTP + Octopamine on Phosphodiesterase

	Phosphodiesterase Activity			
	pmoles/min/mg protein			
Additions	Octopamine (10^{-3}M)	Octopamine (10^{-4}M)	Octopamine (10^{-5}M)	Buffer
Buffer	4.0* ±0.07	4.12* ±0.24	4.12* ±0.16	3.9* ±0.6
GTP (0.1 mM)	3.95* ±0.01	3.9* ±0.07	4.02* ±0.11	4.0* ±0.04

* Difference between values not statistically significant

A pellet fraction (pf_1) was assayed for phosphodiesterase activity in the presence of octopamine (10^{-3}M, 10^{-4}M and 10^{-5}M) and in the absence and presence of GTP (10^{-4}M). Each value represents the mean ± standard error of three separate incubations.

1. Additive Effects of Octopamine, Dopamine and Serotonin

The addition of dopamine (10^{-4}M) or serotonin (10^{-5}M) to octopamine (10^{-4}M) resulted in a greater activation of adenylate cyclase than obtained with octopamine alone (Table 8). Assuming complete additivity, then the theoretical activity for any combination can be determined. The % additivity can be calculated by comparing the experimental value with the theoretical. In three separate experiments, the % additivity for the addition of dopamine to octopamine was 93, 92 and 105, for serotonin to dopamine 89, 90 and 213 and serotonin to octopamine 116, 115 and 96 suggesting the existence of separate receptors for octopamine, dopamine and serotonin.

2. Use of Antagonists

Phentolamine, an α-antagonist, propranolol, a β-antagonist, chlorpromazine, a dopaminergic antagonist, cyproheptadine, a serotonin and histamine antagonist and phenylephrine, an α-agonist were tested for their effect on the octopamine-, dopamine- and serotonin-stimulated cyclase in re-suspended pellets of locust cerebral ganglia. Maximal activity was defined as the stimulation obtained with monoamines alone and 100% inhibition when the activity in the presence of antagonists was reduced to the activity observed with GTP (10^{-4}M) in the absence of

TABLE 8

Additive Effects of Monoamines on Adenylate Cyclase Activity in a pf_2 Preparation

Additions	Increase in Adenylate Cyclase Activity pmoles/min/mg protein					
	Experiment 1	% Additivity	Experiment 2	% Additivity	Experiment 3	% Additivity
Octopamine	280.5 ± 2.6	-	244.4 ± 1.3	-	250.0 ± 5.2	-
Dopamine	108.0 ± 4.6	-	86.4 ± 1.4	-	40.6 ± 8.9	-
Serotonin	41.0 ± 0.7	-	32.8 ± 0.6	-	24.2 ± 0.9	-
Octopamine + Dopamine	361.7 ± 10.0	93	289.0 ± 8.0	92	297.6 ± 7.9	105
Octopamine + Serotonin	372.0 ± 22.0	115	297.6 ± 18.1	116	268.2 ± 2.6	96
Dopamine + Serotonin	133.0 ± 9.7	89	106.4 ± 7.8	90	112.4 ± 7.3	213
Octopamine + Dopamine + Serotonin	400.5 ± 8.6	93	320.4 ± 6.9	93	277.2 ± 1.9	90

Adenylate cyclase activity was measured as described in Materials and Methods in the presence and absence of octopamine (10^{-4}M), dopamine (10^{-4}M) and serotonin (10^{-5}M) in an assay containing GTP (10^{-4}M). These amine concentrations elicited maximal increase in adenylate cyclase activity when added alone. All values are mean ± standard error of three separate incubations and represent the absolute increase in activity above, 106.7 ± 1.6, 85.4 ± 3.3, 126.0 ± 4.1, observed respectively with GTP alone in Experiments 1, 2 and 3. The % additivity was calculated as described in Materials and Methods.

monoamines. The I_{50} value is the concentration of antagonist that produces 50% inhibition.

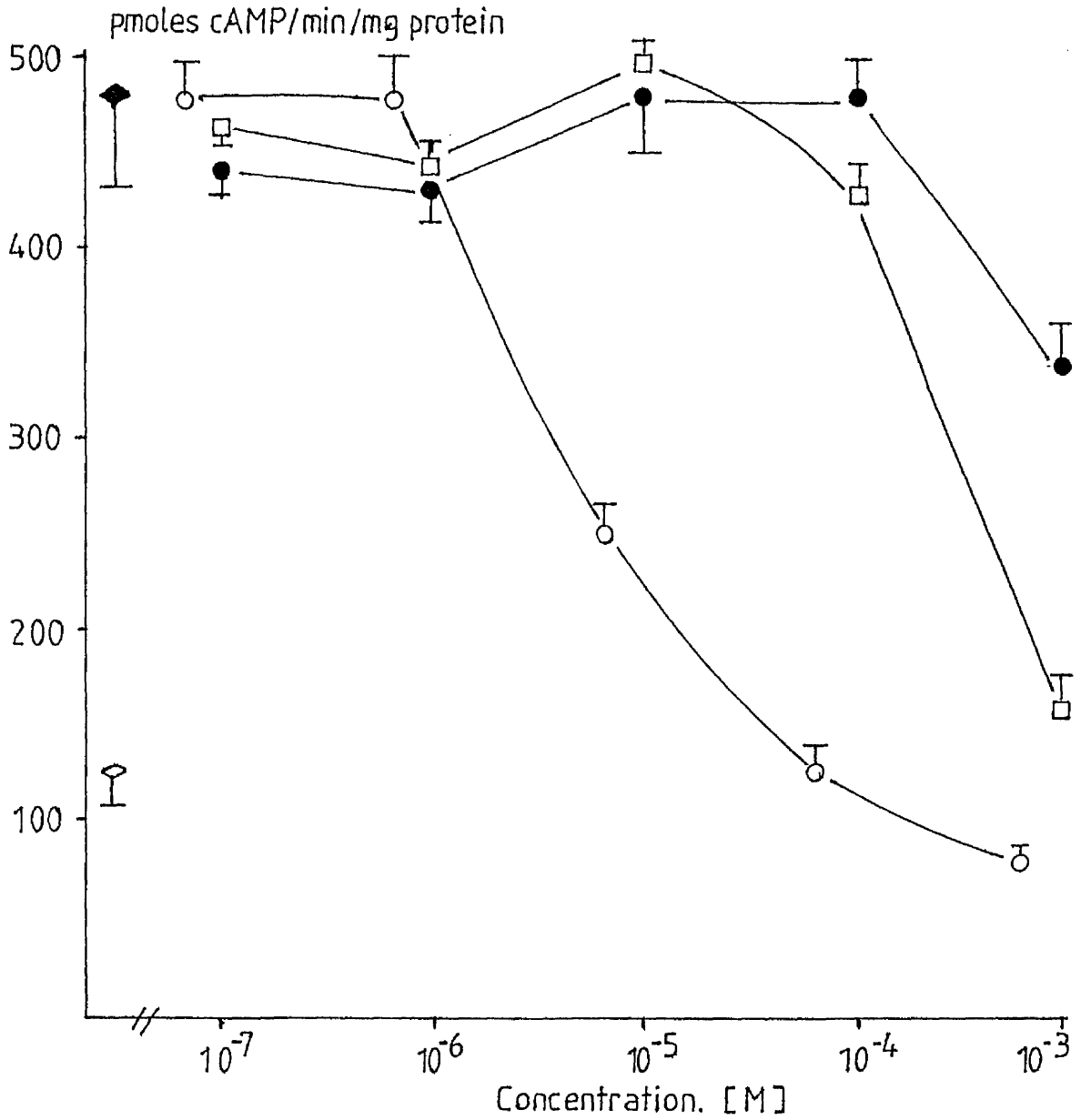
Phentolamine was more potent than propranolol in the inhibition of the octopamine-stimulated adenylate cyclase (Figure 26). In two experiments, the I_{50} values for phentolamine were approximately 4 and 6 x 10^{-6}M and, for propranolol, 1 and 0.8 x 10^{-4}M. Inhibition of the octopamine stimulation by phenylephrine was observed at concentrations greater than 10^{-4}M.

In contrast with the situation for the octopamine-stimulated adenylate cyclase, phentolamine and propranolol were relatively ineffective as antagonists of the dopamine response (Figure 27). In two experiments, the I_{50} value for phentolamine (10^{-4}M and 0.8 x 10^{-4}M) and propranolol (10^{-4}M and 0.9 x 10^{-4}M) were similar. The most potent dopamine antagonist was cyproheptadine with an I_{50} of approximately 4 x 10^{-6}M. Phenylephrine however gave a more complex pattern. In two experiments, phenylephrine (10^{-5}M) had no effect on dopamine stimulation whereas inhibition at 10^{-7}M (40%, 30%) and 10^{-4}M (35%, 20%) was observed.

The small stimulation by serotonin made the interpretation of the effect of antagonists difficult. Phentolamine and propranolol appear to have little effect on the serotonin-stimulated adenylate cyclase (Figure 28). Cyproheptadine (10^{-6}M) inhibited serotonin

FIGURE 26

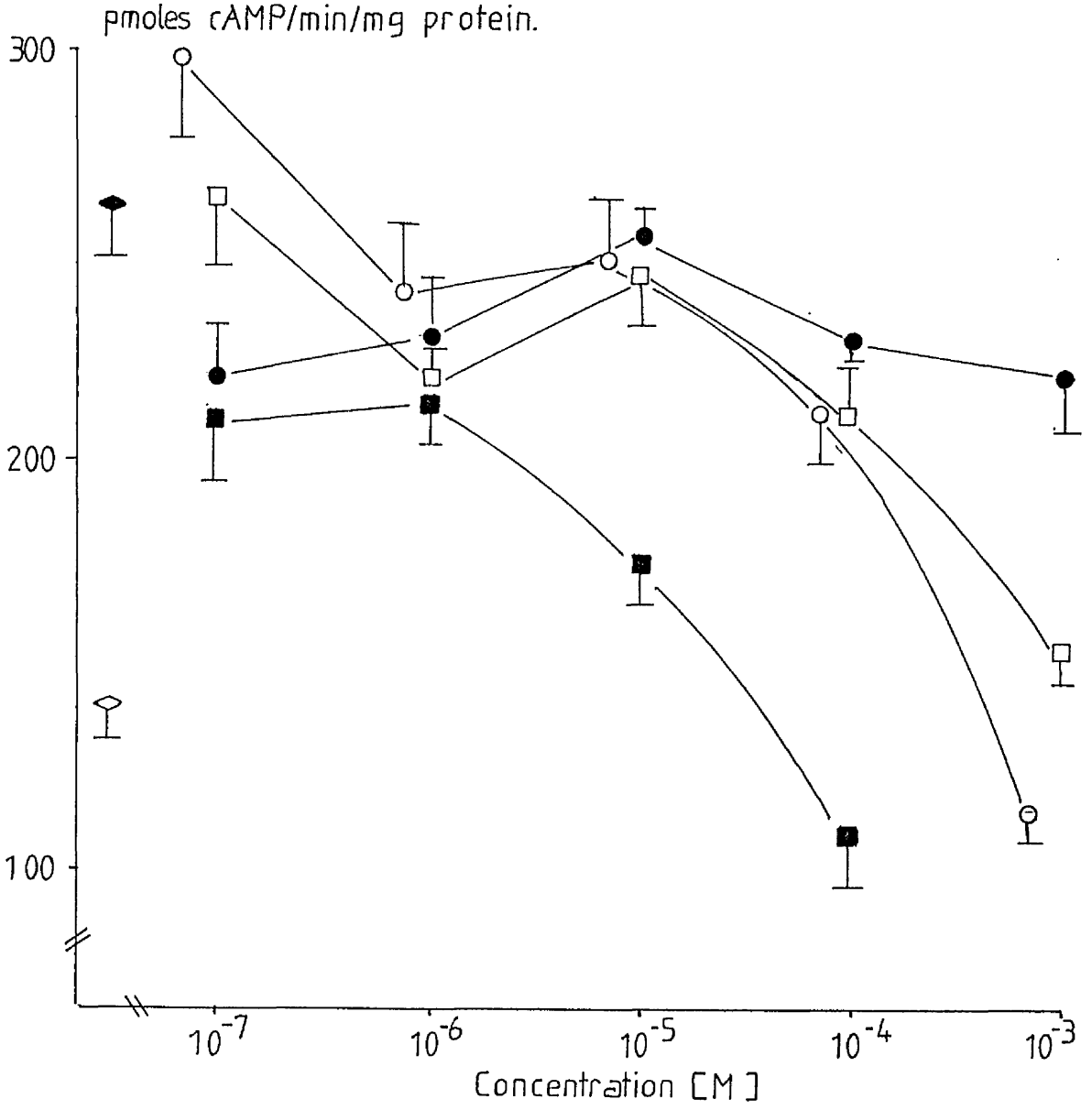
Antagonism of Octopamine-Stimulated Adenylate Cyclase



A washed pellet fraction (pf_1) was assayed for adenylate cyclase activity in the presence of 10^{-4}M GTP + 10^{-4}M octopamine and in the absence and presence of increasing concentrations of (O) phentolamine, (□) propranolol and (●) phenylephrine. (◆) is the activity (480 ± 50 pmol/min/mg protein) observed with GTP + octopamine alone and (◇) the activity (125 ± 18 pmol/min/mg protein) with GTP alone. Each point represents the mean ± standard error of three separate incubations.

FIGURE 27

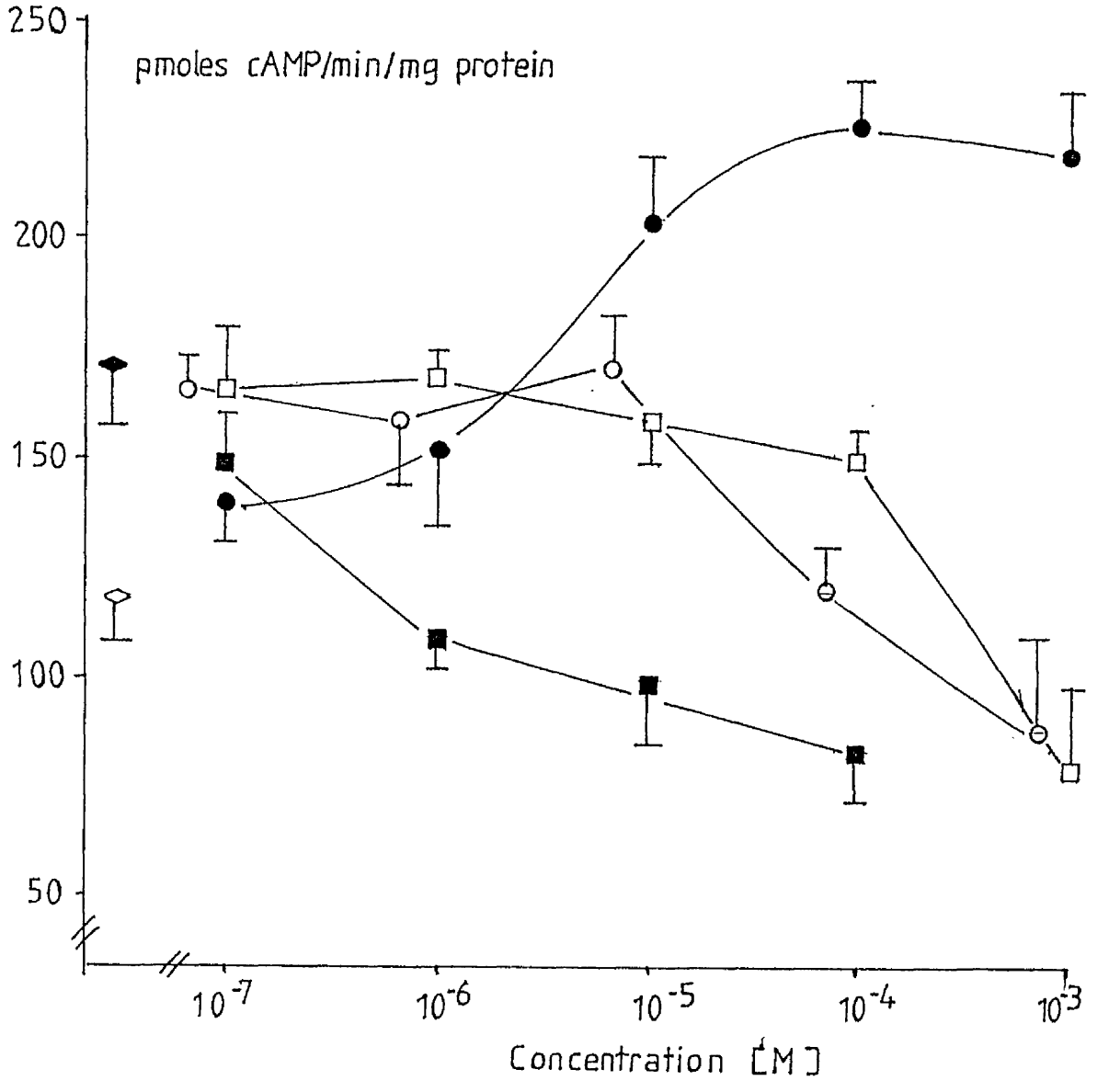
Antagonism of Dopamine-Stimulated Adenylate Cyclase



A pellet fraction (pf_1) was assayed for adenylate cyclase activity in the presence of 10^{-4}M GTP + 10^{-4}M dopamine and in the absence and presence of increasing concentrations of (O) phentolamine, (□) propranolol, (●) phenylephrine and (■) cyproheptadine. (◆) is the activity (260 ± 15 pmol/min/mg protein) observed with GTP + dopamine alone and (◇) the activity (140 ± 10 pmol/min/mg protein) with GTP alone. Each point represents the mean ± standard error of three separate incubations.

FIGURE 28

Antagonism of Serotonin-Stimulated Adenylate Cyclase



A pellet fraction (pf_1) was assayed for adenylate cyclase activity in the presence of 10^{-4}M GTP + 10^{-5}M serotonin and in the absence and presence of increasing concentrations of (O) phentolamine, (□) propranolol, (●) phenylephrine and (■) cyproheptadine. (◆) is the activity <170 ± 14 pmol/min/mg protein) observed with GTP + serotonin alone and (◇) the activity (120 ± 10 pmol/min/mg protein) with GTP alone. Each point represents the mean ± standard error of three separate incubations.

stimulation by 100%. Higher concentrations of cyproheptadine decreased the activity below that observed with GTP alone making it difficult to determine I_{50} values. Phenylephrine at concentrations greater than 10^{-5}M increased adenylate cyclase activity above that observed with serotonin. In two experiments, a 1.2 and 1.3 fold increase was observed with phenylephrine (10^{-4}M). At this concentration, the stimulation was completely additive (Table 9).

Selective concentrations of phentolamine, cyproheptadine and chlorpromazine were added to an octopamine-, dopamine- and serotonin-stimulated adenylate cyclase from the same preparation. Table 10 shows that phentolamine (8 μM) allows the preferential inhibition of the octopamine response while having little effect on the dopamine-stimulated adenylate cyclase. The effect on the serotonin response was inconsistent. In contrast, chlorpromazine (10 μM) inhibited the dopamine-stimulated adenylate cyclase leaving the octopamine response relatively unaffected suggesting that separate receptors exist for octopamine and dopamine. Cyproheptadine was equally effective at inhibiting the octopamine- and dopamine-stimulated adenylate cyclase but resulted in a relatively greater inhibition of serotonin-stimulated adenylate cyclase. This, together with the additive effects of serotonin and phenylephrine (Table 9) suggested that a separate serotonin receptor, distinct from that of octopamine and dopamine may exist in locust cerebral ganglia.

TABLE 9

Additive Effects of Serotonin and Phenylephrine upon Adenylate Cyclase Activity in Pellet Fractions of Locust Cerebral Ganglia

Additions	Increase in adenylate cyclase activity (pmoles/min/mg protein)
Serotonin (10^{-5}M)	40.7 ± 2.5
Phenylephrine (10^{-4}M)	44.9 ± 5.0
Serotonin + Phenylephrine	91.7 ± 10.0

Adenylate cyclase activity was measured in the presence of GTP (10^{-4}M), serotonin (10^{-5}M) and phenylephrine (10^{-4}M). This serotonin concentration elicited maximal increase in adenylate cyclase activity when added alone. All values are means ± standard errors for three separate incubations and represent the absolute increase in adenylate cyclase activity above that (128.8 pmoles/min/mg protein) observed in the absence of serotonin or phenylephrine.

TABLE 10

Selective Inhibition of Adenylate Cyclase

Phentolamine (8 μM)

pmoles cAMP/min/mg protein						
Buffer	Octopamine (10^{-4}M)	% Inhibition	Dopamine (10^{-4}M)	% Inhibition	Serotonin (10^{-5}M)	% Inhibition
154.5 ± 22.7	713.0 ± 23.3	-	422.0 ± 29.1	-	238.0 ± 10.2	-
128.8 ± 14.2	634.8 ± 14.0	-	361.0 ± 10.1	-	208.5 ± 11.1	-
173.1 ± 24.1	340.9 ± 24.1	70	381.2 ± 12.1	22	204.7 ± 26.7	62
130.1 ± 10.0	261.1 ± 27.3	74	352.0 ± 20.0	5	218.7 ± 12.7	0

Cyproheptadine (10 μM)

pmoles cAMP/min/mg protein						
Buffer	Octopamine (10^{-4}M)	% Inhibition	Dopamine (10^{-4}M)	% Inhibition	Serotonin (10^{-5}M)	% Inhibition
152.1 ± 18.1	722.9 ± 69.2	-	322.4 ± 8.3	-	240.0 ± 3.6	-
164.1 ± 18.9	597.1 ± 43.2	-	438.3 ± 9.0	-	190.1 ± 2.5	-
163.7 ± 17.2	678.8 ± 10.0	17	311.3 ± 10.3	13	180.0 ± 2.5	82
129.5 ± 11.3	432.8 ± 36.4	30	380.8 ± 9.2	8	136.8 ± 6.0	72

Chlorpromazine (10 μM)

pmoles cAMP/min/mg protein						
Buffer	Octopamine (10^{-4}M)	% Inhibition	Dopamine (10^{-4}M)	% Inhibition	Serotonin (10^{-5}M)	% Inhibition
117.4 ± 1.8	710.8 ± 48.2	-	513.2 ± 14.6	-	169.7 ± 9.3	-
140.7 ± 13.6	700.0 ± 52.1	-	526.0 ± 9.8	-	ND	-
121.4 ± 1.8	683.9 ± 64.4	5	176.3 ± 21.5	86	133.3 ± 13.7	78
143.7 ± 3.7	655.0 ± 35.1	8	260.9 ± 6.6	70	ND	-

ylate cyclase activity stimulated in the presence of GTP (10^{-4}M), octopamine (10^{-4}M), dopa- (10^{-4}M) and serotonin (10^{-5}M) was measured in a washed pellet fraction (pf_1) in the absence presence of phentolamine (8 μM), cyproheptadine (10 μM) and chlorpromazine (10 μM). All es are the mean ± standard error of three separate incubations and the table shows the re- s of two separate experiments. The % inhibition was calculated as described in Materials Methods. ND = not determined.

Role of Guanyl Nucleotides in the Activation of Adenylate Cyclase

Locust adenylate cyclase is similar to adenylate cyclase from other organisms in its requirement for GTP for activation in the presence of monoamines. To determine to what extent S. gregaria adenylate cyclase is similar to mammalian and avian adenylate cyclase, the effect of two non-hydrolysable analogues, Gpp(NH)p and Gpp(CH_2)p on adenylate cyclase was studied.

1. Preliminary Studies

In a washed pellet fraction and in the absence of monoamines, Gpp(NH)p and Gpp(CH_2)p stimulated adenylate cyclase activity maximally at 10^{-4}M (Table 11). The effect of this concentration of Gpp(NH)p and Gpp(CH_2)p on adenylate cyclase was compared with GTP (Table 12). The stimulation of cyclase activity, above that observed with GTP alone, by Gpp(NH)p (10^{-4}M) (10 fold) was greater than the 4 fold increase by Gpp(CH_2)p (10^{-4}M). The addition of octopamine to the guanyl nucleotides, although causing a 3 fold stimulation of cyclase activity with GTP had no effect on Gpp(NH)p. With Gpp(CH_2)p, however, a small but consistent increase was observed (Table 12). In two experiments, the % increase by octopamine above that obtained with Gpp(CH_2)p alone was 24 and 36. The addition of dopamine or serotonin did not result in an increase in cyclase activity with Gpp(NH)p

TABLE 11

Effect of $Gpp(CH_2)p$ and Gpp(NH)p on Adenylate Cyclase

Additions	pmoles/cAMP/min/mg protein	
	$Gpp(CH_2)p$	Gpp(NH)p
10^{-3}M	434.8 ± 9.4 410.0 ± 20.1	1030.0 ± 107.0 1236.6 ± 56.0
10^{-4}M	618.6 ± 55.8 534.9 ± 9.7	1386.0 ± 150.0 1360.8 ± 30.0
10^{-5}M	353.1 ± 13.2 ND	1091.3 ± 103.5 1049.6 ± 41.0

ND = Not determined

A washed pellet fraction (pf_1) was assayed for adenylate cyclase activity in the presence of increasing concentrations of $Gpp(CH_2)p$ and Gpp(NH)p. Each value represents the mean ± standard error of three separate incubations and the results shown are for two separate experiments.

TABLE 12

Effect of Guanyl Nucleotides on Adenylate Cyclase

Additions	pmoles, cAMP/min/mg protein		
	10^{-4}M GTP	10^{-4}M Gpp(CH$_2$)p	10^{-4}M Gpp(NH)p
Buffer	138.2 ± 14.6 194.8 ± 4.4	519.6 ± 45.9 582.0 ± 20.0	1386.1 ± 260.3 1139.2 ± 58.0
Octopamine (10^{-4}M)	431.6 ± 32.7 562.8 ± 38.6	643.1 ± 76.4 791.0 ± 100.0	1279.4 ± 63.9 1226.0 ± 85.0
Dopamine (10^{-4}M)	341.1 ± 45.3 360.9 ± 24.0	583.2 ± 35.2 640.1 ± 20.0	1064.2 ± 118.0 1110.3 ± 98.0
Serotonin (10^{-5}M)	207.6 ± 7.5 277.1 ± 20.3	569.7 ± 27.9 620.5 ± 19.0	1047.5 ± 144.1 1054.6 ± 100.1

A washed pellet fraction (pf_1) was assayed for adenylate cyclase activity, stimulated by octopamine (10^{-4}M), dopamine (10^{-4}M) and serotonin (10^{-5}M) in the presence of GTP (10^{-4}M), Gpp(CH$_2$)p (10^{-4}M) and Gpp(NH)p (10^{-4}M). Each value represents the mean ± standard error of three separate incubations and the results shown are for two separate experiments.

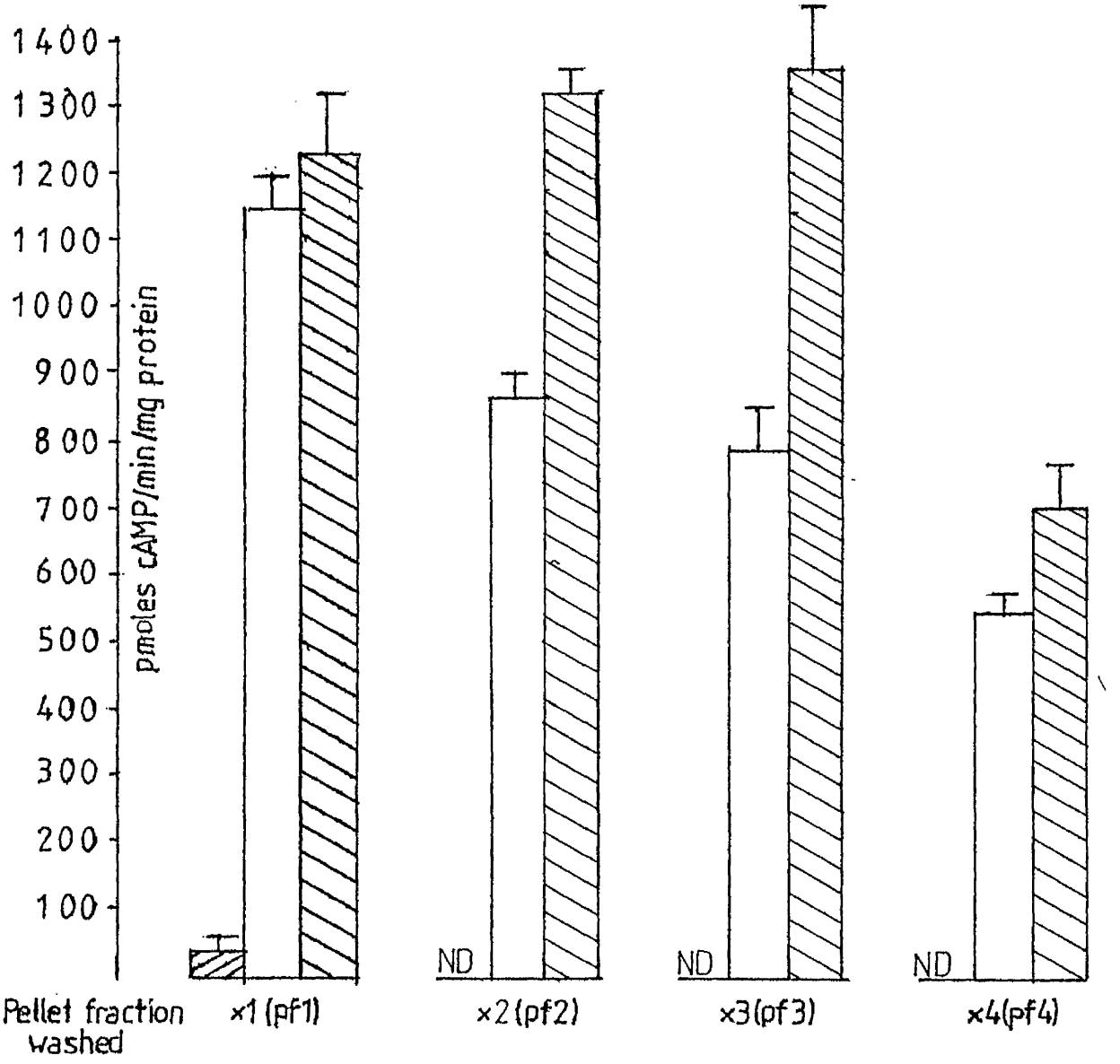
The substantial activation of adenylate cyclase activity by Gpp(NH)p suggested that endogenous octopamine or an activator may be present in the preparation. The pellet fraction was further washed with homogenisation medium to try and recover the octopamine effect. Washing exposed an octopamine-sensitive component (Figure 29) which was inhibited by phentolamine (80 μM) (Figure 30). Phentolamine however had no effect on the residual Gpp(NH)p activity.

2. Time Course

If the pellet fraction was incubated with Gpp(NH)p (10^{-4}M) or Gpp(NH)p + octopamine (10^{-4}M) for 5 minutes at 30°C and the reaction started by addition of ATP, adenylate cyclase activity was linear for three minutes (Figure 31). Salomon et al, 1975 observed that the addition of Gpp(NH)p to hepatic adenylate cyclase resulted in a lag in activity which was completely abolished by the addition of glucagon. In the locust, pre-incubation of the enzyme with Gpp(NH)p may have been responsible for the failure to detect a lag with Gpp(NH)p activation. The experiment was repeated but with the reaction initiated by the addition of a pf_2 fraction, equilibrated to 30°C, to a reaction cocktail containing ATP and Gpp(NH)p (10^{-4}M) or Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M) maintained at 30°C. Adenylate cyclase activity was linear for 3 minutes (Figure 32) but no lag with Gpp(NH)p acti-

FIGURE 29

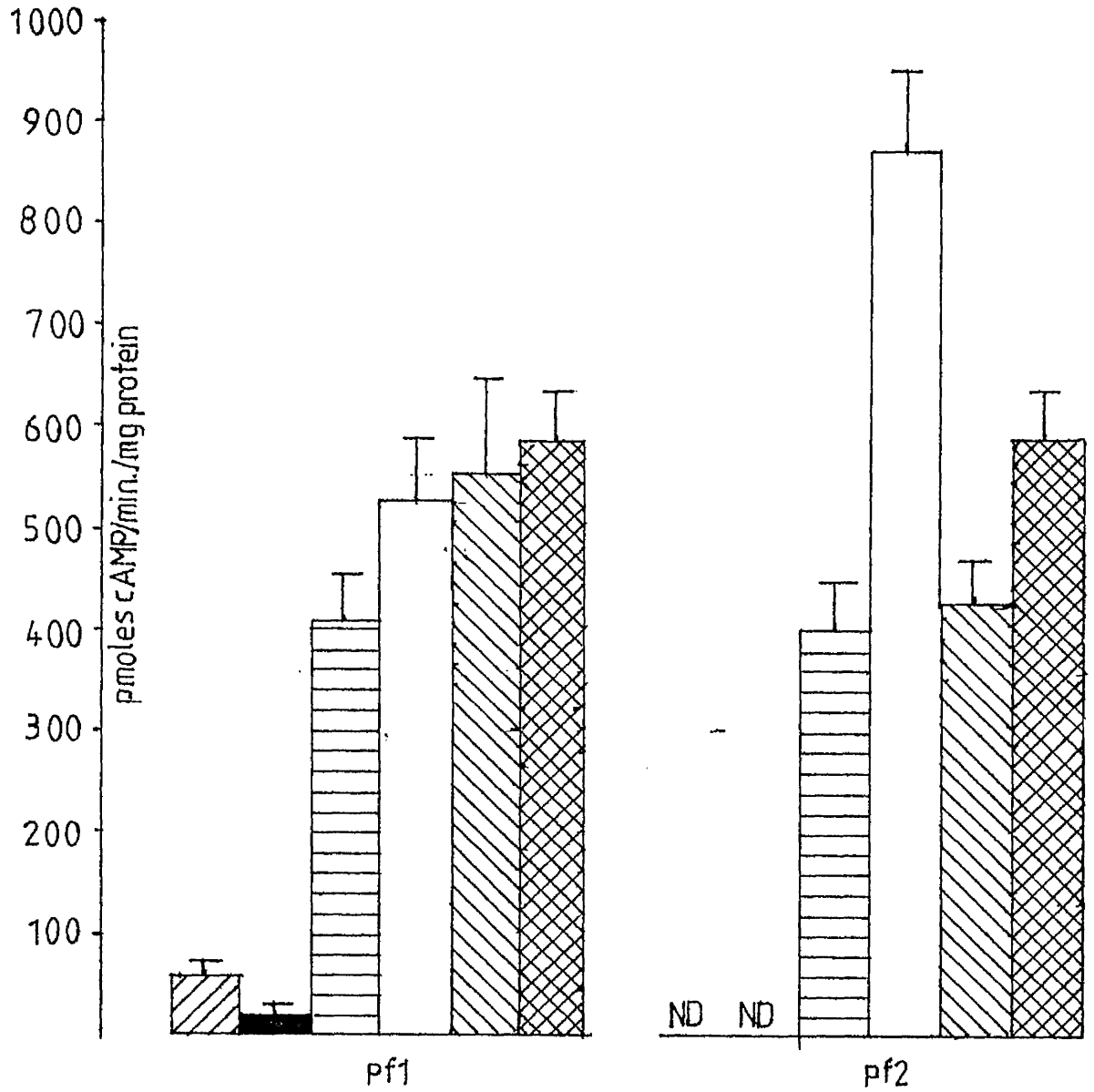
Effect of Washing on Gpp(NH)p Activation



A pellet fraction (pf_1) prepared by centrifugation of a crude homogenate at 9000 g for 10 minutes at $4^{\circ}C$ was further washed and each preparation assayed for adenylate cyclase activity in the presence of (▨) buffer and buffer + (□) 10^{-4}M Gpp(NH)p and (▧) 10^{-4}M Gpp(NH)p + 10^{-4}M octopamine. Each point represents the mean ± standard error of three separate incubations. ND = no activity detected.

FIGURE 30

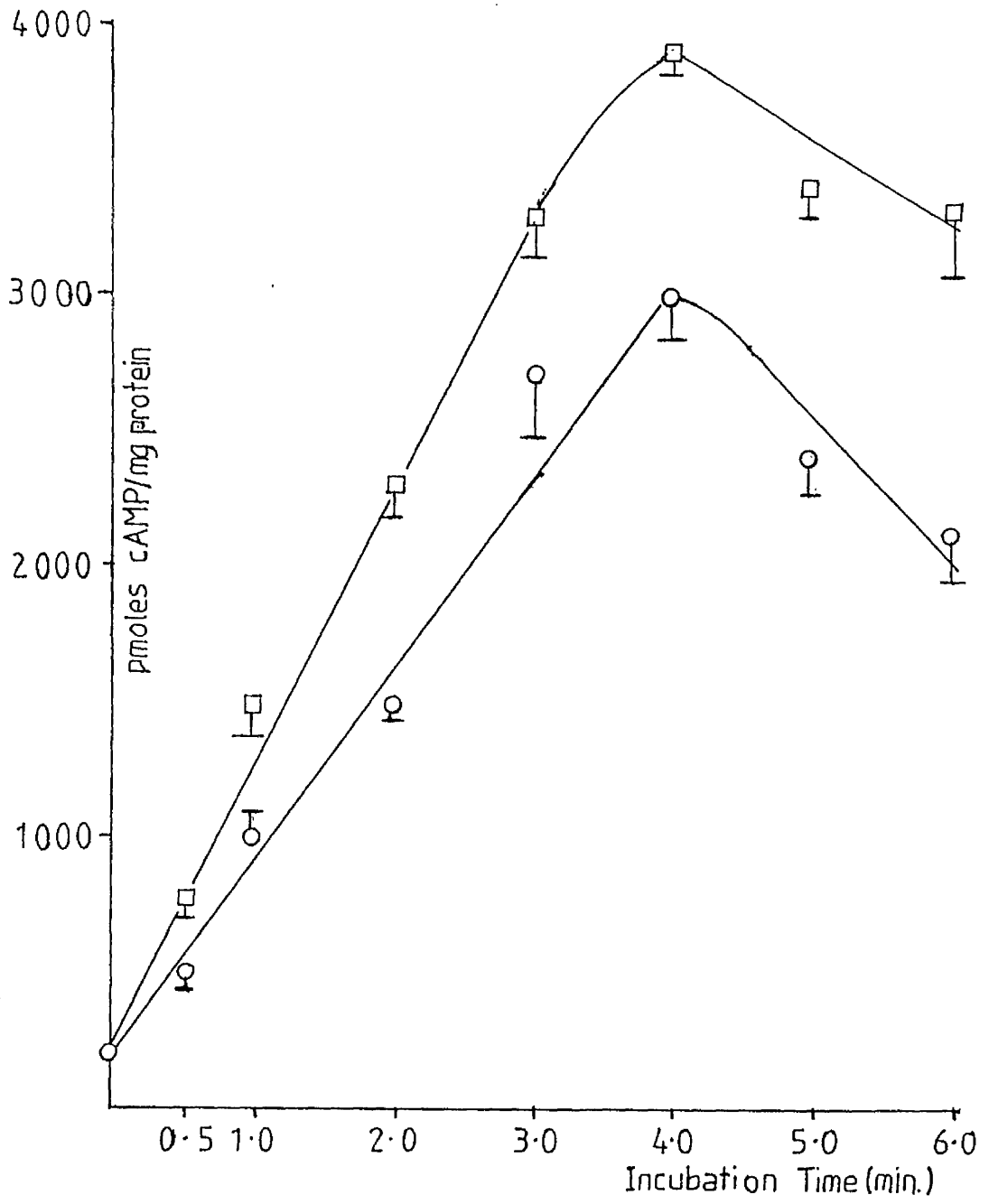
Effect of Washing and Phentolamine on Gpp(NH)p Stimulation



A pf_1 and pf_2 preparation prepared as described in Materials and Methods were assayed for adenylate cyclase activity in the presence of (▨) buffer and buffer + (■) 8×10^{-5}M phentolamine, (▤) 10^{-4}M Gpp(NH)p, (□) 10^{-4}M Gpp(NH)p + 10^{-4}M octopamine, (▧) 10^{-4}M Gpp(NH)p + 8×10^{-5}M phentolamine and (▩) 10^{-4}M Gpp(NH)p + 8×10^{-5}M phentolamine + 10^{-4}M octopamine. Each value represents the mean ± standard error of three separate incubations. ND = no activity detected.

FIGURE 31

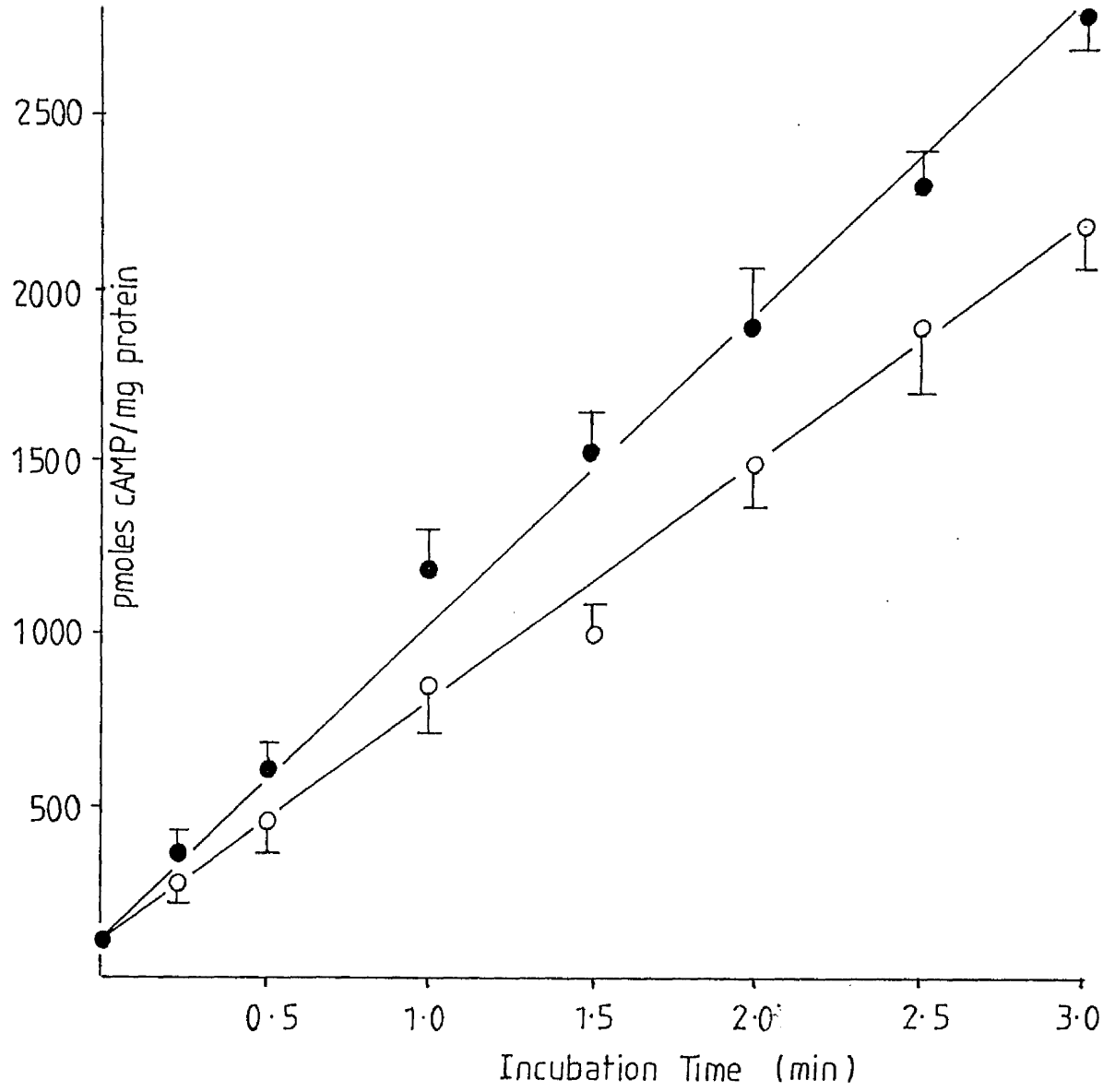
Time Course for Gpp(NH)p and Octopamine Activation of Adenylate Cyclase



A pf_2 preparation prepared as described in Materials and Methods was pre-incubated for 5 minutes at 30°C with (O) 10^{-4}M Gpp(NH)p and (□) 10^{-4}M Gpp(NH)p + 10^{-4}M octopamine and assayed for adenylate cyclase activity at various incubation times. Each point represents the mean ± standard error of three separate incubations.

FIGURE 32

Time Course for Gpp(NH)p and Octopamine Activation of Adenylate Cyclase



A pellet fraction washed twice (pf_2) was assayed for adenylate cyclase activity at various times. The reaction was initiated by the addition of the pellet fraction to a cocktail containing 5×10^{-4}M ATP and either (O) 10^{-4}M Gpp(NH)p or (●) 10^{-4}M Gpp(NH)p + 10^{-4}M octopamine. Each point represents the mean ± standard error of three separate incubations.

vation was detected. This method was adopted for all subsequent assays involving Gpp(NH)p. In addition, the incubation time was reduced to 2 minutes.

3. Ka for Gpp(NH)p Activation

From a Linweaver-Burke plot of activity against concentration of Gpp(NH)p, an apparent Ka of 2.2×10^{-6}M was determined for adenylate cyclase in a pf_2 preparation (Figure 33). In three experiments, the Ka value determined was 2×10^{-6}M, 2.2×10^{-6}M and 7×10^{-6}M.

4. Formation of Stable Complex

Pre-incubation of a pf_2 fraction with Gpp(NH)p (10^{-4}M) for 5 minutes at 30°C followed by washing twice with homogenisation medium resulted in an adenylate cyclase whose activity, when assayed in the presence or absence of Gpp(NH)p was similar (Table 13). If the stable complex was added to GTP (10^{-4}M) + octopamine (10^{-4}M), substantial inhibition of the activity when compared with the relatively small effect of GTP or octopamine alone was observed (Table 14).

If pre-incubation was carried out with GTP (10^{-4}M) and washed twice with homogenisation medium, inhibition of the subsequent Gpp(NH)p stimulation was observed (Table 13) although the activity was regained in the presence of Gpp(NH)p + octopamine.

FIGURE 33

A pellet fraction washed twice (pf_2) was assayed for adenylate cyclase activity in the presence of increasing concentrations of Gpp(NH)p. The results are shown as a reciprocal plot of [V], the activity observed measured in pmoles/min against [S], the concentration of Gpp(NH)p (μM). Each value was determined in triplicate and is expressed as activity/tube containing 10.0 μg protein.

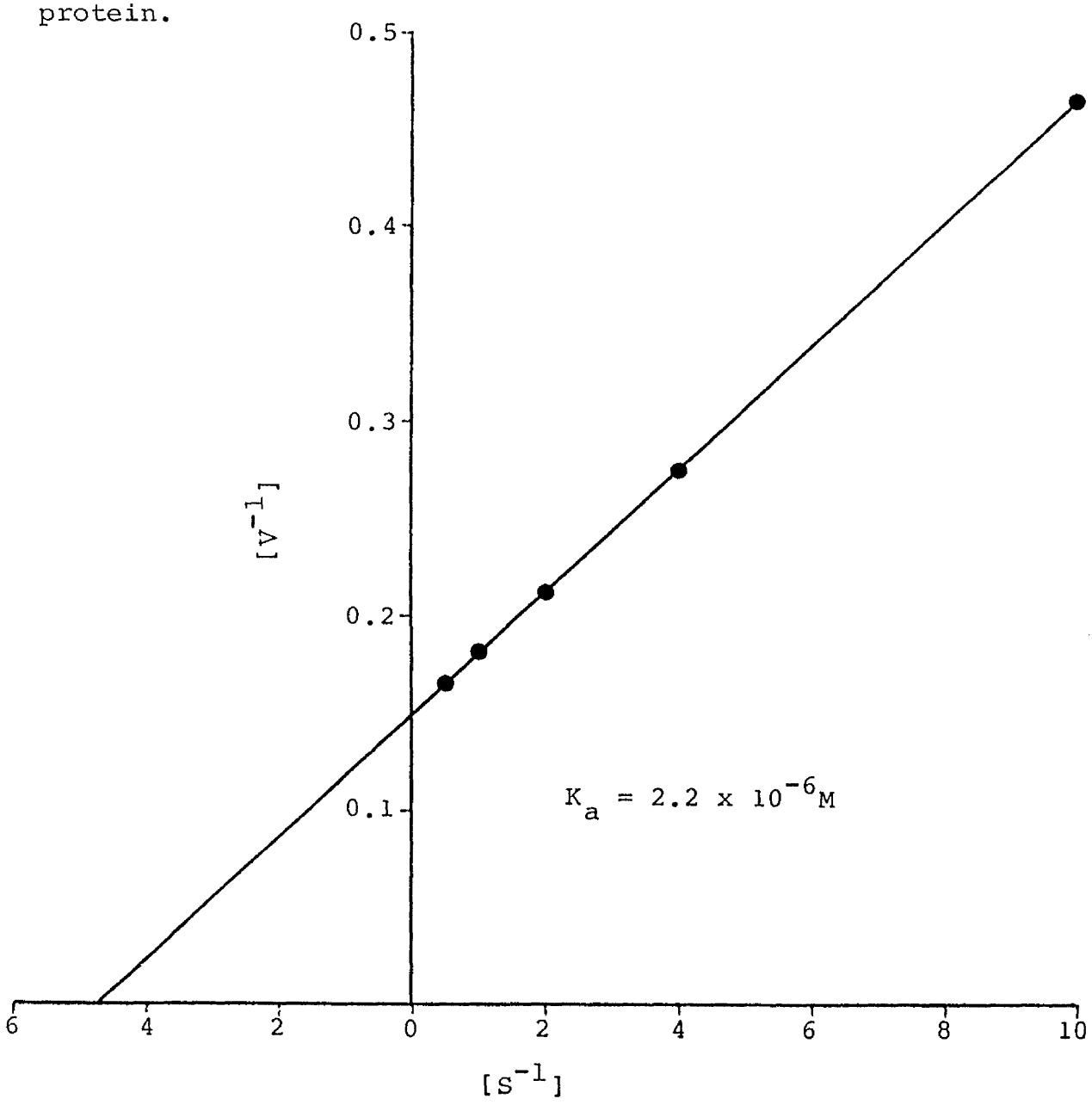


TABLE 13

Formation of Stable Complex

Pre-incubation conditions	Adenylate cyclase activities (pmol/min/mg protein) in the presence of		
	Buffer	Gpp(NH)p (10^{-4}M)	Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M)
No addition	NA NA	618.9 ± 12.7 744.9 ± 22.0	881.5 ± 25.3 1180.0 ± 74.6
GTP (10^{-4}M)	NA NA	[1]415.5 ± 17.5 [3]518.2 ± 54.3	[2]886.8 ± 28.9 [2]1043.9 ± 55.1
Gpp(NH)p (10^{-4}M)	839.2 ± 68.5 885.5 ± 50.0	[1]894.4 ± 22.5 [4]1021.6 ± 48.4	[2]891.5 ± 48.9 [2]1074.5 ± 26.9

NA = Not detected

The superscript numbers indicate the significance of pre-incubation with GTP (10^{-4}M) and Gpp(NH)p (10^{-4}M):

[1] = $p > 0.001$
[2] = not significant
[3] = $0.002 < p < 0.01$
[4] = $0.005 < p < 0.001$

A pf_2 fraction was pre-incubated with GTP (10^{-4}M) and Gpp(NH)p (10^{-4}M) for 5 minutes at 30°C, washed twice with homogenising medium at 9000 g for 10 minutes and assayed for adenylate cyclase activity in the presence of Gpp(NH)p (10^{-4}M) and Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M). Each value represents the mean ± standard error of three separate incubations and the results shown are for two separate experiments.

TABLE 14

Reversal of Stable Complex

Experiment	Stable Complex Activity	Additions to stable complex		
		GTP (10^{-4}M)	Octopamine (10^{-4}M)	GTP (10^{-4}M) + octopamine (10^{-4}M)
	pmol/min/mg protein	pmol/min/mg protein	pmol/min/mg protein	pmol/min/mg protein
Experiment 1	651.0 ± 21.3	[1]587.9 ± 28.9	[2]538.4 ± 10.1	[3]269.3 ± 15.3
Experiment 2	660.6 ± 27.3	[4]518.2 ± 13.3	[4]536.1 ± 17.7	[3]258.9 ± 10.1

The superscript numbers indicate the significance of the additions to the stable complex:

[1] = Not significant
[2] = $0.01 < p < 0.005$
[3] = $p > 0.001$
[4] = $0.025 < p < 0.02$

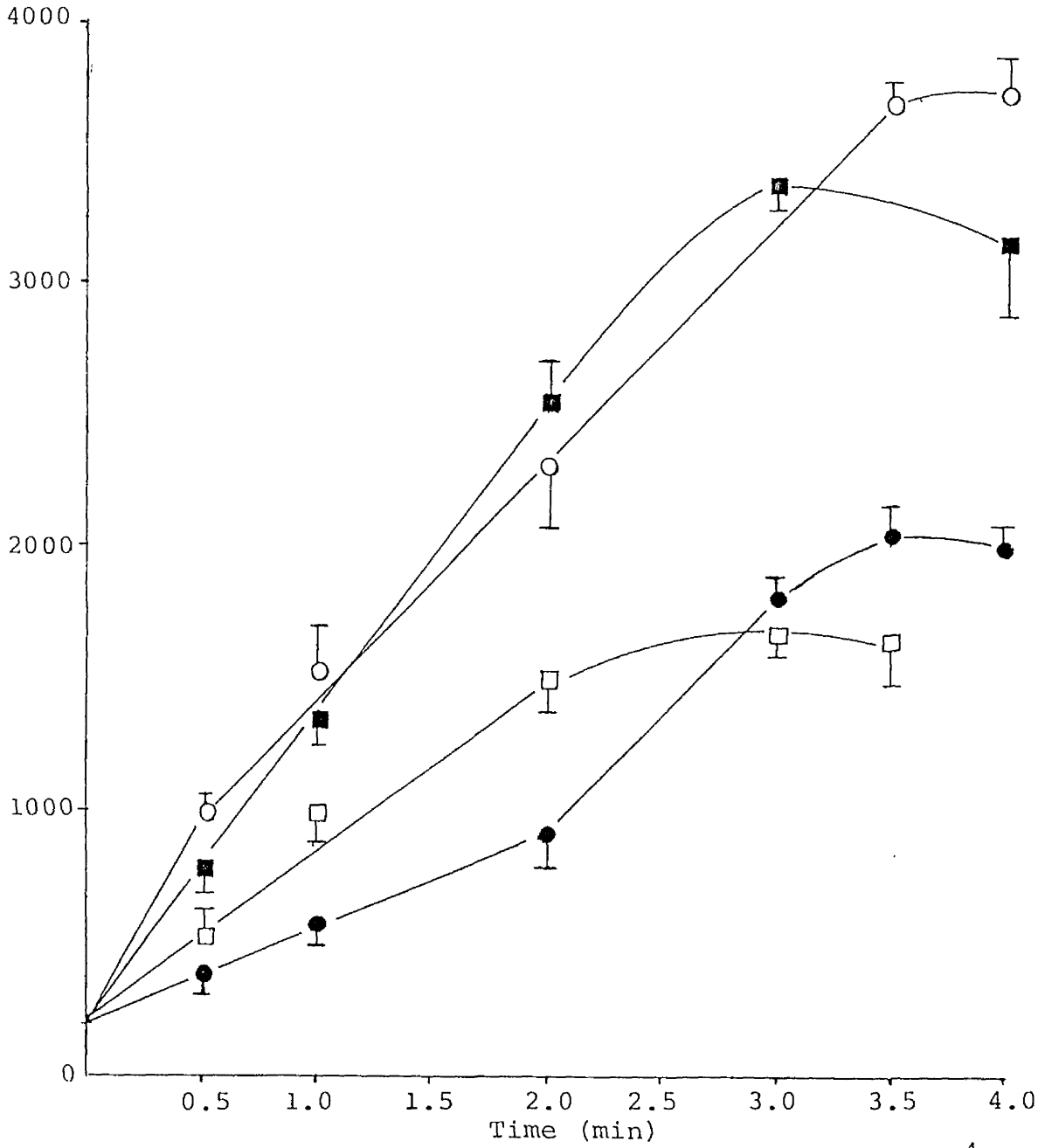
The stable complex prepared by incubating a pf_2 fraction with Gpp(NH)p (10^{-4}M) for five minutes at 30°C and washing twice was assayed for adenylate cyclase activity in the presence of GTP (10^{-4}M), octopamine (10^{-4}M) and GTP (10^{-4}M) + octopamine (10^{-4}M). Each value represents the mean ± standard error of three separate incubations and the results shown are for two separate experiments.

Figure 34 shows the time course for the inhibition of Gpp(NH)p activation observed in an enzyme preparation pre-incubated with GTP. If pre-incubation was carried out in the absence of GTP and washed, the subsequent stimulation by Gpp(NH)p was linear for 2 minutes. In contrast, pre-incubation with GTP under identical conditions gave rise to a biphasic time course. Although linear for 2 minutes, the activity was less than the preparation pre-incubated in the absence of GTP. A marked increase in the rate of cyclic AMP production was observed after 2 minutes before a final decrease in activity above 3 minutes. The addition of Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M) to this preparation resulted in a biphasic activation. The activity after an initial "burst" was followed by a constant slower rate.

A possible explanation for the lag observed, in a preparation pre-incubated with GTP, is that GTP is hydrolysed to GDP which remains tightly bound to the enzyme even after washing. The lag represents the time taken for Gpp(NH)p to replace GDP. Figure 35 shows the effect of pre-incubation of a pf_2 fraction with increasing concentrations of GDP, washing twice with homogenisation medium and measuring the activity in the presence of Gpp(NH)p (10^{-4}M). In two experiments, incubating with GDP (10^{-3}M and 10^{-4}M) produced a lag with subsequent Gpp(NH)p activation.

FIGURE 34

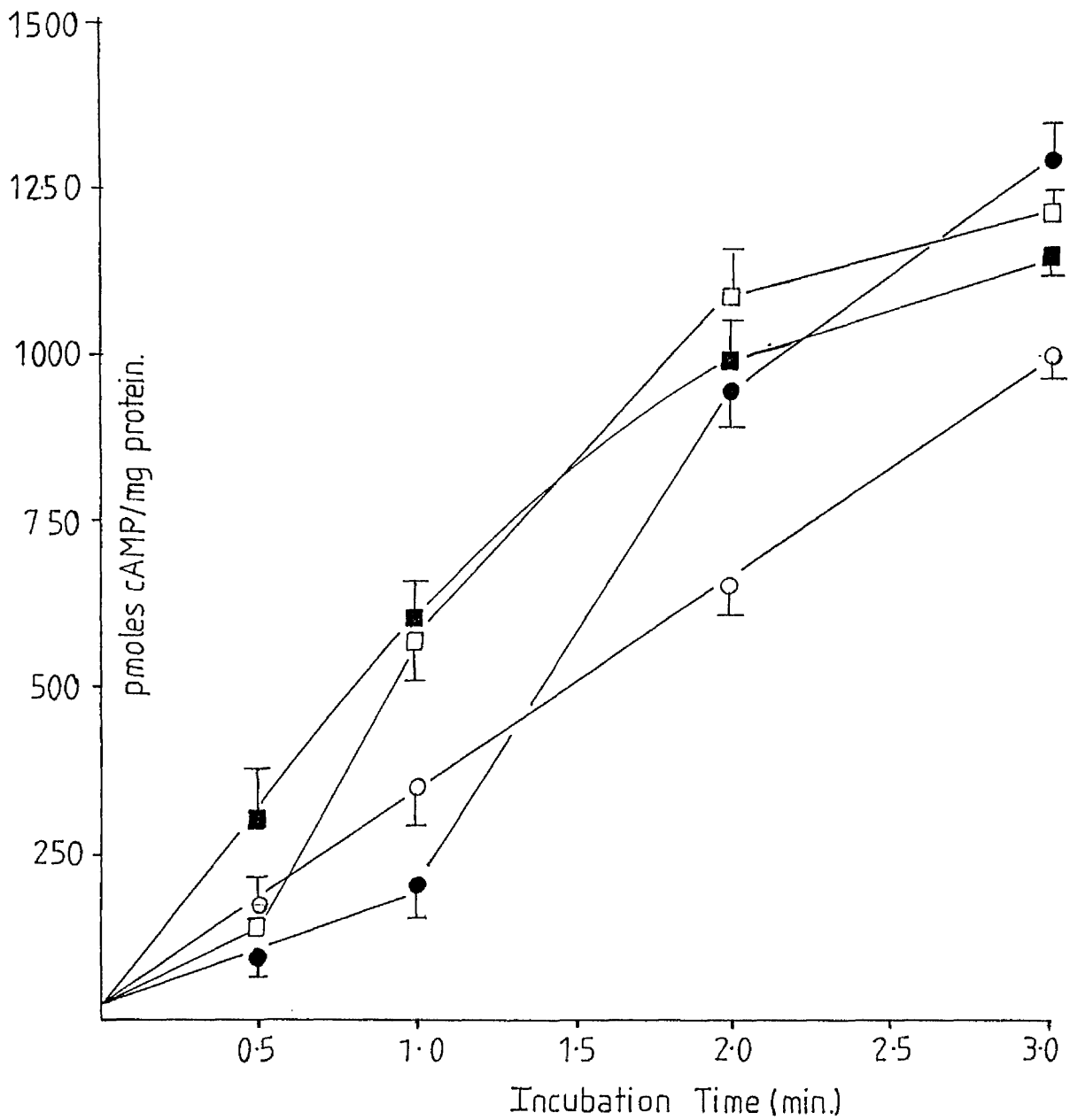
Effect of Pre-Incubation with GTP on
Time Course of Gpp(NH)p Stimulation



A pf_2 preparation pre-incubated with buffer + GTP (10^{-4}M) for 5 minutes at 30°C was washed twice at 9000 g for 10 minutes at 4°C. To the preparation pre-incubated with buffer, adenylate cyclase activity was measured in the presence of Gpp(NH)p (10^{-4}M) (□) and Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M) (■) and for pre-incubation with GTP (10^{-4}M), activity was measured with Gpp(NH)p (10^{-4}M) (●) and Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M) (○). Each point represents the mean ± standard error of three separate incubations.

FIGURE 35

Effect of Pre-Incubation with GDP on Time Course of Gpp(NH)p Stimulation



A pellet fraction (pf_2) was incubated with (O) buffer and buffer + (●) 10^{-3}M GDP, (□) 10^{-4}M GDP and (■) 10^{-5}M GDP for 5 minutes at 30°C washed as described in Materials and Methods and assayed for adenylate cyclase activity in the presence of 10^{-4}M Gpp(NH)p. Each point represents the mean ± standard error of three separate incubations.

5. Effect of GDP on Adenylate Cyclase

GDP inhibited the Gpp(NH)p and Gpp(NH)p + octopamine-stimulated adenylate cyclase at 10^{-3}M and 10^{-4}M (Table 15). In two experiments, a % inhibition of 96 and 95 for Gpp(NH)p (10^{-4}M) and 75 and 83 for Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M) was observed with GDP (10^{-3}M). The concentration of GDP that was responsible for 50% inhibition of both responses was approximately 10^{-4}M. No inhibition was observed with either 10^{-5}M or 10^{-6}M GDP.

The addition of GDP (10^{-4}M) to the preparation resulted in a consistent stimulation of adenylate cyclase above the basal level. In two experiments, the % stimulation by GDP (10^{-4}M) was 283 and 186 values which were similar to the stimulation observed with GTP (10^{-4}M) (270 and 150).

Interpretation of the effect of GDP on adenylate cyclase activity is complicated by the suggestion that ATP can act as a substrate for the conversion of GDP to GTP (McSwigan et al, 1980). ATP was replaced by adenylylimidodiphosphate, a structural analogue of ATP containing nitrogen substituted for oxygen before the terminal phosphate. This nucleotide was only slowly hydrolysed by rat liver membranes and acted as a substrate for adenylate cyclase (Rodbell et al, 1971). In the presence of App(NH)p (10^{-3}M), the

TABLE 15

Effect of GDP on Gpp(NH)p Stimulation

Additions	Buffer	Gpp(NH)p	Gpp(NH)p + octopamine	% Inhibition of Gpp(NH)p response	% Inhibition of Gpp(NH)p + octopamine response
Buffer	21.5 ± 3.6 22.8 ± 0.2	425.4 ± 49.4 465.4 ± 10.0	544.9 ± 38.5 523.6 ± 6.2	0 0	0 0
GDP (10^{-3}M)	29.8 ± 3.8 21.0 ± 0.9	44.2 ± 1.6 44.8 ± 4.3	162.2 ± 12.5 104.6 ±	96 95	75 83
GDP (10^{-4}M)	61.0 ± 1.55 30.6 ± 2.6	266.1 ± 30.6 220.0 ± 3.6	349.9 ± 7.4 280.1 ± 12.5	50 57	43 50
GDP (10^{-5}M)	ND ND	429.1 ± 4.6 460.5 ± 6.1	498.8 ± 22.9 510.1 ± 7.7	- -	- -
GDP (10^{-6}M)	ND ND	466.3 ± 26.3 460.0 ± 20.1	627.2 ± 88.8 515.1 ± 8.4	- -	- -
GTP (10^{-4}M)	58.2 ± 12.3 33.5 ± 1.00	ND ND	ND ND	- -	- -

A washed pellet fraction (pf_2) was assayed for adenylate cyclase activity with Gpp(NH)p (10^{-4}M) and Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M) and in the absence and presence of increasing concentrations of GDP. The % inhibition was calculated as described in Materials and Methods. Each value represents the mean + standard error of three separate incubations and the results shown are for two separate experiments. ND = Not determined.

addition of GDP (10^{-3}M) and GDP (10^{-4}M) inhibited the Gpp(NH)p and Gpp(NH)p + octopamine-stimulated adenylate cyclase (Table 16). In contrast to ATP as substrate for adenylate cyclase, no stimulation was observed with GDP (10^{-4}M). However, substantially lower activities and % stimulations were also observed.

In the presence of App(NH)p, the adenylate cyclase sensitive to octopamine and GTP was still present (Table 17). In two experiments, the addition of octopamine to GTP (10^{-4}M) resulted in a % increase of 75 and 187 above that observed with GTP alone. In contrast, the addition of octopamine to GDP (10^{-4}M) resulted in only a 17% and 5% increase in activity, an increase that was less than that observed with octopamine alone. The small stimulation of adenylate cyclase by octopamine and GDP suggests that GDP may prevent the coupling between adenylate cyclase and the receptor, when App(NH)p is used as substrate for adenylate cyclase.

TABLE 16

Effect of GDP on Gpp(NH)p Stimulation with App(NH)p as Substrate for Adenylate Cyclase

Additions	Buffer	Gpp(NH)p (10^{-4}M)	Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M)	% Inhibition Gpp(NH)p	% Inhibition Gpp(NH)p + octopamine
Buffer	19.2 ± 2.3	37.5 ± 0.6	50.5 ± 2.0	-	-
GDP (10^{-3}M)	11.6 ± 0.2	18.1 ± 1.3	22.2 ± 2.7	64	66
GDP (10^{-4}M)	14.4 ± 0.9	24.8 ± 1.2	31.0 ± 2.7	43	47
GDP (10^{-5}M)	16.9 ± 1.2	38.4 ± 3.5	41.7 ± 4.1	0	20
GDP (10^{-6}M)	19.3 ± 1.1	42.8 ± 1.6	43.9 ± 6.9	0	20

A washed pellet fraction (pf_2) was assayed for adenylate cyclase activity as described in Materials and Methods. ATP was replaced with App(NH)p (10^{-3}M) and the activity measured in the presence of Gpp(NH)p (10^{-4}M) + octopamine (10^{-4}M) and in the absence and presence of increasing concentrations of GDP. Each value represents the mean ± standard error of three separate incubations.

TABLE 17

Effectiveness of Guanylnucleotides in Restoring Octopamine Sensitivity when App(NH)p is Substrate

Guanyl Nucleotide added	Adenylate cyclase activity (pmol/min/mg protein)		Increase due to octopamine % increase
	No addition	+ Octopamine (10^{-4}M)	
None	19.2 ± 1.4	[1]24.3 ± 0.1	26
	21.2 ± 0.5	[1]27.4 ± 1.8	28
GDP (10^{-4}M)	14.4 ± 0.5	[2]16.9 ± 0.6	17
	13.7 ± 3.4	[5]14.5 ± 0.6	5
GTP (10^{-4}M)	15.8 ± 0.7	[3]27.7 ± 1.8	75
	11.7 ± 2.1	[4]33.6 ± 7.2	187

The superscript numbers indicate the significance of the octopamine stimulation:

[1] = $0.05 < p < 0.025$
[2] = $0.02 < p < 0.01$
[3] = $0.005 < p < 0.001$
[4] = $p > 0.001$
[5] = not significant

Adenylate cyclase activity stimulated by octopamine (10^{-4}M) was assayed with App(NH)p (10^{-3}M) and in the presence of GDP (10^{-4}M) and GTP (10^{-4}M) in a pellet fraction washed twice (pf_2). Each value represents the mean ± standard error of three separate incubations and the results shown are for two separate experiments.

DISCUSSION

Monoamine-Stimulated Adenylate Cyclase

Several studies have now shown that invertebrate nervous tissue contains adenylate cyclases, stimulated by monoamines (Nathanson and Greengard, 1973; Harmar and Horn, 1977; Robertson and Osborne, 1979). This thesis has studied the monoamine-sensitive adenylate cyclase of locust nervous tissue and the results will now be discussed in relation to work on adenylate cyclases in invertebrate and vertebrate tissue. The discussion, however, will not be confined to the CNS since most vertebrate work has been carried out on either the rat liver or turkey erythrocyte enzyme.

The present work clearly demonstrates that S. gregaria cerebral ganglia contains an adenylate cyclase that is stimulated by octopamine, dopamine and serotonin. The locust adenylate cyclase is similar to the enzyme in cockroach thoracic ganglia (Nathanson and Greengard, 1973) since dopamine was approximately half as effective as octopamine. Furthermore, although both enzymes were activated by serotonin, the magnitude of the maximal stimulation (170%) in the cockroach (Nathanson and Greengard, 1973) and locust (132 $\pm$ 14%) was small when compared with the 400% and 430 $\pm$ 62% observed respectively for octopamine. This, together with the failure of Harmar and Horn, 1977 to detect an effect of serotonin on adenylate cyclase from cockroach cerebral ganglia at concentrations as high as 1 mM, suggests that serotonin has only a minor effect on this enzyme

in insects. In contrast to insects, the activation of adenylate cyclase by serotonin in lobster skeletal muscle (Battelle and Kravitz, 1978) and the cerebral ganglion of the earthworm Lumbricus terrestris (Robertson and Osborne, 1979) was greater than that due to octopamine.

Cockroach thoracic ganglia adenylate cyclase responded to octopamine concentrations as low as 0.03 μM, was maximally stimulated to 30 μM and had a Ka of approximately 1.5 μM (Nathanson and Greengard, 1973). This Ka value was similar to the Ka (8 μM) observed in the locust (Figure 33).

The effect of chemical modification of octopamine on the stimulation of adenylate cyclase from cockroach cerebral ganglia has been studied (Harmar and Horn, 1977). The naturally occurring D(-) isomer of octopamine was approximately 200 times more potent than the L(+) isomer in activating adenylate cyclase. The presence of a β-hydroxyl group was essential for activity and the absence of a p-hydroxyl group reduced the effectiveness of activation, although other groups (e.g. fluoro) could replace the p-hydroxyl group. The presence of a m-hydroxyl group and a m-methyl group reduced activity. Alpha methylation of octopamine resulted in a 60 fold decrease in potency whereas N-methylation resulted in only a slight reduction in potency. The stimulation of adenylate cyclase by octopamine in this study and in cockroach thoracic (Nathanson and Greengard, 1975) and cerebral ganglia (Harmar and Horn, 1977) was achieved using DL-octopamine. Thus, the concentration observed for stimulation of adenylate cyclase is an over-estimation by a factor

of 2. In this thesis, the effect of chemical modification of octopamine was not examined.

1. Characterisation of Octopamine Receptors

There was no obvious similarity between the structures of the various drugs found to be potent antagonists of the octopamine-stimulated adenylate cyclase in cockroach cerebral ganglia (Harmar and Horn, 1977). Thus, the most effective octopamine antagonists were cyproheptadine (a potent histamine and serotonin antagonist), phentolamine (an imidazoline with potent α-adrenergic blocking activity) and promethazine (a phenothiazine with histamine blocking activity). The observation in the cockroach (Nathanson, 1976) and locust (Figure 24) that phentolamine was a potent inhibitor of octopamine-stimulated adenylate cyclase whereas propranolol, a β-antagonist, was relatively ineffective, suggested that octopamine was acting through α-receptors. However, the finding that the α-adrenergic agonist phenylephrine was only a relatively weak activator of adenylate cyclase (Table 9) indicates that the octopamine receptor may have characteristics different from those of classical α-adrenergic receptors. A detailed pharmacological investigation in insects may provide evidence for the existence of multiple α-receptors similar to those observed in vertebrates (Snyder and Goodman, 1980).

2. Evidence for Separate Receptors

2.1 Additive Experiments

The effects of octopamine, dopamine and serotonin on the stimulation of cyclic AMP formation in the locust cerebral ganglia were completely additive which suggests that each monoamine acts via separate receptors. This is in agreement with reports that the stimulation of adenylate cyclase by these amines was additive in cockroach thoracic ganglia (Nathanson and Greengard, 1973) and additive with octopamine and dopamine in cockroach cerebral ganglia (Harmar and Horn (1977). However, stimulation by noradrenaline was not additive in either cockroach thoracic (Nathanson and Greengard, 1973) or cerebral ganglia (Harmar and Horn, 1977) which suggested the absence of a separate receptor. Furthermore, in cockroach thoracic ganglia (Nathanson and Greengard, 1973), the Ka for noradrenaline (30 μM) was considerably greater than that observed for octopamine (1.5 μM). Thus, the stimulatory effect of noradrenaline on adenylate cyclase activity was attributed to a partial activation of the receptor sites for octopamine, dopamine or serotonin. The effect of noradrenaline on locust adenylate cyclase was not examined. Additivity was also reported for serotonin and octopamine in the cerebral ganglia of Lumbricus terrestris (Robertson and Osborne, 1979) although no dopamine-stimulated

adenylate cyclase could be detected. The failure to detect a dopamine-sensitive adenylate cyclase in the cerebral ganglia of L. terrestris, a tissue known to contain dopamine (Gardner and Cashin, 1975) suggests that not all the actions of dopamine (and likewise octopamine and serotonin) necessarily involve an alteration of cyclic AMP levels. Thus, in invertebrates, receptors, analogous to the vertebrate dopamine D_1 and D_2 receptors (Kebabian and Calne, 1979) may exist. In the moth Mamestra configurata, dopamine-stimulated adenylate cyclase activity increased up to a concentration of 3 μM, remained relatively constant to 10 μM and then rose again to a maximum at 100 μM (Bodnaryk, 1979). The addition of 100 μM dopamine to a saturating concentration of octopamine (40 μM) did not result in additivity. If, however, the dopamine concentration was reduced to 3 μM, additivity was observed. It was suggested that for dopamine concentrations up to 10 μM, the stimulation of activity was attributed to specific activation of dopamine-sensitive adenylate cyclase, whereas the further increase in activity between 10 and 100 μM was due to the effect of dopamine on the octopamine-sensitive enzyme. In contrast to M. configurata, dopamine stimulation of adenylate cyclase in the locust resulted in only one maximal level of activation (Figure 15).

2.2 Use of Agonists and Antagonists

Studies with agonists and antagonists have provided additional evidence for separate receptors in insect nervous tissue. For example, in cockroach thoracic ganglia, a lower concentration of phentolamine (K_i = 0.4 μM) was required to inhibit octopamine-stimulated adenylate cyclase than was required for dopamine (K_i = 5 μM) or serotonin (K_i = 6 μM) (Nathanson, 1976). Phentolamine (8 μM) added to locust adenylate cyclase inhibited the octopamine- or dopamine-stimulated adenylate cyclase by 70% and 22% respectively. Further evidence that phentolamine has little effect on dopamine receptors was provided by the observation that in rat nucleus accumbens, phentolamine failed to antagonise the dopamine stimulation (Watling et al, 1979) whereas fluphenazine and flupenthixol were both potent antagonists. In cockroach brain, flupenthixol, although an antagonist of the octopamine-stimulated adenylate cyclase (Harmar and Horn, 1977), was a more potent antagonist of the dopamine-stimulated adenylate cyclase.

Chlorpromazine (Miller and Iversen, 1974) and haloperidol (Kebabian et al, 1972; Nakahara, 1978) inhibit dopamine-sensitive adenylate cyclase in rat brain and in canine caudate nucleus (Sano et al, 1979). In cockroach thoracic ganglia, haloperidol inhibited both dopamine (K_i = 0.2 μM) and serotonin (K_i = 0.5 μM)

stimulation at lower concentrations than that required for octopamine (K_i = 10 μM) (Nathanson, 1976). In the locust, chlorpromazine (10 μM) selectively inhibited dopamine stimulation but had negligible effect on octopamine stimulation (Table 10). This finding, together with the selective inhibition of octopamine-stimulated adenylate cyclase by phentolamine, suggests the existence of separate receptors for octopamine and dopamine.

LSD, BOL and, to a lesser extent, cyproheptadine, were highly potent antagonists of serotonin stimulation of adenylate cyclase in cockroach thoracic ganglia (Nathanson and Greengard, 1974). The small stimulation of locust adenylate cyclase by serotonin made interpretation of the effect of antagonists difficult. However, cyproheptadine (10 μM) although inhibiting octopamine (30%, 17%), dopamine (13%, 8%) and serotonin (82%, 72%) stimulation appeared to cause a greater % inhibition of the serotonin stimulation (Table 10) suggesting the possibility of a separate serotonin receptor.

In conclusion, the preferential inhibition of octopamine and dopamine-stimulated cyclase by phentolamine (8 μM) and chlorpromazine (10 μM) respectively and the inhibition of serotonin response by cyproheptadine (10 μM) together with the ability of the monoamines to stimulate additively in the presence of

saturating concentrations, suggest that activation of locust adenylate cyclase by monoamines occurs via separate receptors.

Role of Neurotransmitters/Hormones and Guanyl Nucleotides in the Regulation of Adenylate Cyclase

Investigations on adenylate cyclase from a variety of sources, suggest that regulation of this enzyme is complex involving at least three macromolecular components; the catalytic moiety separate from the receptor, and a guanine nucleotide binding protein (MacGuire et al, 1977). Furthermore, it has been suggested that more than one guanine nucleotide site is involved in regulation of hormonal stimulation of the enzyme; one regulating the catalytic unit and another the activity of the receptor (Iyengar et al, 1979). Thus for the rat liver enzyme, guanine nucleotides influence the coupling of the nucleotide binding protein with the catalytic moiety and also the binding of glucagon to the receptor. The requirement for guanine nucleotides in the activation of locust adenylate cyclase was studied to provide further comparison between the locust enzyme and adenylate cyclase(s) from a variety of sources. Before discussing the results obtained with guanine nucleotides in relation to current views on the regulation of adenylate cyclase and the involvement of GTP-ase, the similar effects of guanine nucleotides on locust and other adenylate cyclases will be described.

1. Activation by Guanine Nucleotides

The present work clearly demonstrates that guanine nucleotides have a pronounced effect on S. gregaria adenylate cyclase. Furthermore, the finding that in the presence of GTP, activation of locust adenylate cyclase by the stimulatory ligands was synergistic, indicates that the locust adenylate cyclase is similar to the enzyme present in rat liver (Rodbell et al, 1971), striatum (Chen et al, 1980) and turkey erythrocyte (Sevilla et al, 1976). This synergism was greatly increased by washing, suggesting that GTP could replace the removed endogenous activator(s) and hence supports the idea that agonist activation of adenylate cyclase depends upon guanine nucleotides (Rodbell et al, 1979). Further evidence that GTP is an endogenous activator of adenylate cyclase was provided by the finding that repeated washing of rabbit cerebellum (Cote et al, 1980) and rat striatum (Chen et al, 1980) abolished the isoproterenol and dopamine sensitivities of their respective cyclases. Neurotransmitter sensitivity was restored by the addition of GTP, isolated from the soluble fraction.

In order to study in more detail the role of GTP in the activation of adenylate cyclase, the non-hydrolysable GTP analogues Gpp(NH)p, Gpp(CH_2)p and GTP(γ)S have been used (Schramm and Rodbell, 1975; Kaslow et al, 1978; Svobedo et al, 1978; Birnbaumer, et al, 1980). This study shows clearly that Gpp(NH)p is a potent activator of locust adenylate cyclase and that Gpp(NH)p is more effective than

$Gpp(CH_2)p$. This order of effectiveness of the guanine nucleotides i.e. $Gpp(NH)p > Gpp(CH_2)p > GTP$ is similar to that observed in rat liver (Birnbaumer et al, 1980). The stimulation of locust adenylate cyclase by Gpp(NH)p was not dependent upon the addition of either octopamine, dopamine or serotonin. This suggested that an endogenous stimulatory ligand(s) may be bound to the receptors. Indeed, further washing of the membrane fraction did reveal an octopamine-dependent activation of adenylate cyclase (Figure 29). However, the residual activity observed with Gpp(NH)p alone is unlikely to be due to endogenous octopamine as it was not affected by $8x10^{-5}M$ phentolamine, a concentration which reduced greatly the octopamine-dependent activation of adenylate cyclase. Therefore, in locust cerebral ganglia, two pools of adenylate cyclase, one dependent on octopamine and Gpp(NH)p and another dependent only on Gpp(NH)p may exist. In rat hepatic membranes, Gpp(NH)p activated adenylate cyclase in the absence of glucagon (Salomon et al, 1974) whereas in turkey erythrocyte, activation by Gpp(NH)p was not observed (Sevilla et al, 1976). In pigeon erythrocyte membranes, pre-treatment with isoproterenol and GMP was a necessary step prior to the solubilisation of a nucleotide regulatory protein involved in adenylate cyclase activation (Pfeuffer and Helmreich, 1975) suggesting that the lack of effect of Gpp(NH)p may be related to tightly bound endogenous nucleotides. Pre-treatment of turkey erythrocyte membranes with isoproterenol and GMP resulted in a substantial activation by Gpp(NH)p alone (Lad et al, 1980).

Thus, the results with Gpp(NH)p would suggest that the locust enzyme is similar to the enzyme in liver and turkey erythrocyte membranes cleared of endogenous nucleotides.

2. Formation of Stable Complex

Adenylate cyclase incubated with Gpp(NH)p or GTP(γ)S retains its activity even after extensive washing (Schramm and Rodbell, 1975; Lefkowitz and Caron, 1975; Northup and Mansour, 1978). This implies that a stable enzyme is formed, possibly due to tight binding of the nucleotides. In contrast, Gpp(CH_2)p failed to form a stable complex in the liver fluke Fasciola hepatica (Northup and Mansour, 1978) which suggests that Gpp(CH_2)p dissociates more rapidly from the guanine nucleotide site than either Gpp(NH)p or GTP(γ)S. This interpretation is also consistent with the finding that Gpp(NH)p was more effective than Gpp(CH_2)p in activating locust adenylate cyclase.

Gpp(NH)p activation of locust adenylate cyclase was resistant to washing. Moreover, the washed enzyme, preactivated with Gpp(NH)p was susceptible only to a small further activation by Gpp(NH)p or Gpp(NH)p + octopamine. This indicated that full activation by the nucleotide had occurred and that a negligible amount of bound Gpp(NH)p had been removed by washing. Thus, locust adenylate cyclase is similar to the enzyme in pancreas (Svoboda et al, 1978), liver (Birnhaumer et al, 1980, frog erythrocyte (Schramm and Rodbell, 1975) and Fasciola hepatica (Northup and Mansour, 1978).

3. Role of GDP in Adenylate Cyclase Regulation

The finding that Gpp(NH)p was more efficient than GTP in activating adenylate cyclase from a variety of sources suggests that hydrolysis of GTP is important in the regulation of adenylate cyclase. The results obtained in this study will now be discussed together with the evidence for GDP termination of cyclic AMP synthesis.

3.1 Catecholamine-Stimulated GTP-ase

The failure of GTP to form a stable enzyme complex (Cautrecasas et al, 1975; Schramm and Rodbell, 1975) has led to the suggestion that GTP is hydrolysed at the guanine nucleotide site and that the GDP formed inhibits the enzyme (Cassel and Selinger, 1976). The observation that GDP competitively inhibited Gpp(NH)p activation of liver adenylate cyclase (Salomon et al, 1975; Lin et al, 1978) supports this hypothesis and suggests that a GTP-ase may be important in the regulation of adenylate cyclase. Cassel and Selinger, 1977 while studying the catecholamine-activated adenylate cyclase in turkey erythrocyte membranes observed a catecholamine-stimulated GTP-ase whose activity was blocked upon the addition of GTP(γ)S and catecholamines. This suggested that the hydrolysis of GTP takes place at the regulatory guanine site. On the assumption that the hydrolysis of GTP to GDP + Pi inhibits adenylate cyclase

activity, Cassel and Selinger, 1977 proposed the following model to show how a hormone can increase adenylate cyclase activity and enhance the hydrolysis of GTP at the regulatory site (Figure 36).

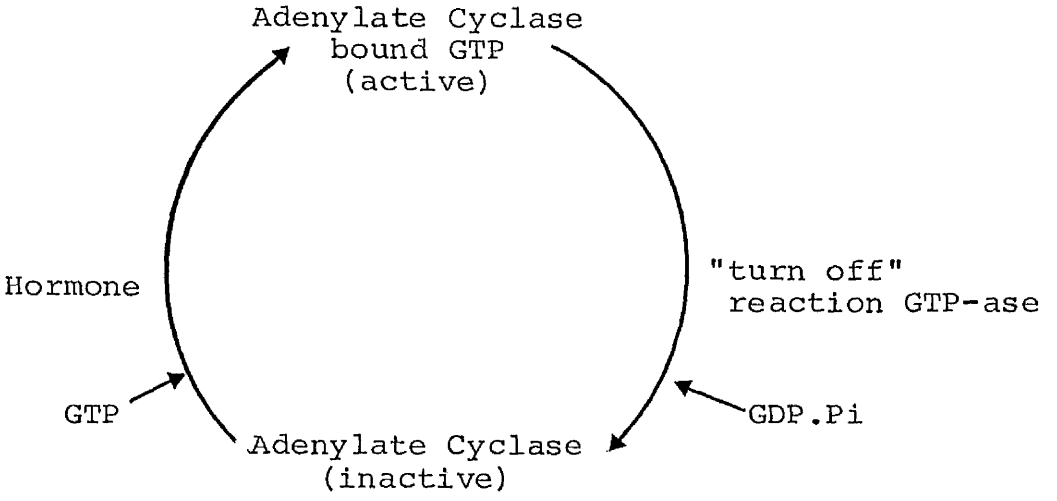


FIGURE 36

Model for Regulation of Adenylate Cyclase
(Cassel and Selinger, 1977)

Adenylate cyclase in the presence of hormone and GTP oscillates constantly between inactive and active states. The function, therefore, of the hormone is to enhance the formation of an active adenylate cyclase - GTP complex. Thus, persistent activation of adenylate cyclase is observed with Gpp(NH)p and GTP(γ)S as these analogues are not hydrolysed by the GTP-ase.

3.2 Cholera Toxin

Further evidence to support a role for a GTP-ase in the control of adenylate cyclase has been provided by studies involving cholera toxin. This toxin, produced by Vibrio cholerae has its diarrhoeic effects by activating small intestinal adenylate cyclase (Schafer et al, 1970; Sharp and Hynie, 1971). Cholera toxin has been observed to stimulate adenylate cyclase from a wide variety of intact cells and tissue (Finkelstein, 1973). The effectiveness of cholera toxin in stimulating adenylate cyclase appears to be due to the fact that the primary recognition site on the cell for the toxin is the ubiquitous GM_1 ganglioside in plasma membranes (Cautrecasas, 1973a, 1973b, 1973c, 1973d; Moss et al, 1976). An understanding of the mechanism of action of cholera toxin was gained when it was observed that the guanine binding protein was ADP-ribosylated (Cassel and Pfeuffer, 1978). This modification inhibited GTP-ase hydrolysis and, therefore, by preventing the formation of GDP maintained the enzyme in an active configuration. Furthermore, following cholera toxin treatment of turkey erythrocytes (Cassel and Selinger, 1977b), mouse neuroblastoma (Levison and Blume, 1977) and rat liver (Birnbaumer et al, 1980) adenylate cyclase, GTP activation resembled that observed with Gpp(NH)p in untreated membranes suggesting that GTP-ase action was important in the regulation of adenylate cyclase.

3.3 Effect of GDP on Adenylate Cyclase

The observation that GDP was equally effective as GTP in potentiating hormone or neurotransmitter stimulation of adenylate cyclase from rat liver (Kimura and Nagata, 1977), rat striatum (Chen et al, 1980; McSwigan et al, 1980) and rabbit cortex (Cote et al, 1980) suggested that an ATP-dependent transphosphorylase reaction resulting in the formation of GTP from GDP was present. The result that GDP (10^{-4}M) was equally as effective as GTP (10^{-4}M) in increasing locust basal adenylate cyclase activity is consistent with this explanation. If ATP was replaced by App(NH)p, a substrate for adenylate cyclase but not for the transphosphorylase reaction, activation of locust adenylate cyclase by octopamine and GTP was observed. However, in the presence of GDP, octopamine-stimulated adenylate cyclase activity was considerably reduced. Thus, locust adenylate cyclase is similar to the enzyme in rat striatum (Chen et al, 1980; McSwigan et al, 1980) and rabbit cortex (Cote et al, 1980) in that, in the presence of App(NH)p, GDP can prevent subsequent stimulation by agonists.

When App(NH)p was used as a substrate for locust adenylate cyclase, an approximate 10 fold reduction in activity was observed. Similarly, adenylate cyclase activity in the presence of App(NH)p at pH 7.5 was approximately 50% of that observed with ATP in

plasma membranes from rat fat pads (Rodbell, 1975). The difference in activity with ATP and App(NH)p was suggested to be due to a pH optima difference. Thus, at pH 7.5, the proportion of protonated App(NH)p is greater than protonated ATP. Furthermore, protonated ATP and App(NH)p were shown to be potent inhibitors of rat liver adenylate cyclase. The difference in activity between locust and rat fat pad adenylate cyclase in the presence of App(NH)p may be due to a lower K_i for protonated App(NH)p with the locust adenylate cyclase compared with the fat pad enzyme.

Further evidence for an involvement of GDP in the regulation of locust adenylate cyclase was provided by the finding that GDP inhibited Gpp(NH)p or Gpp(NH)p + octopamine activation irrespective of whether ATP or App(NH)p was used as the substrate. GDP inhibition of Gpp(NH)p activation has also been observed in mouse neuroblastoma cells (Levison and Blume, 1977). Although GDP clearly has an effect on Gpp(NH)p activation of locust adenylate cyclase, its mode of action remains speculative i.e. GDP may function as an inhibitor by "uncoupling" the guanine nucleotide protein and catalytic unit or simply prevent the binding of Gpp(NH)p. However, the inhibitory actions of GDP together with the failure of GDP to potentiate fully the activation of adenylate cyclase by the stimulatory ligand suggests that GDP may function by "uncoupling" the enzyme-guanine protein complex.

Hormone-Guanyl Nucleotide Regulation of Adenylate Cyclase

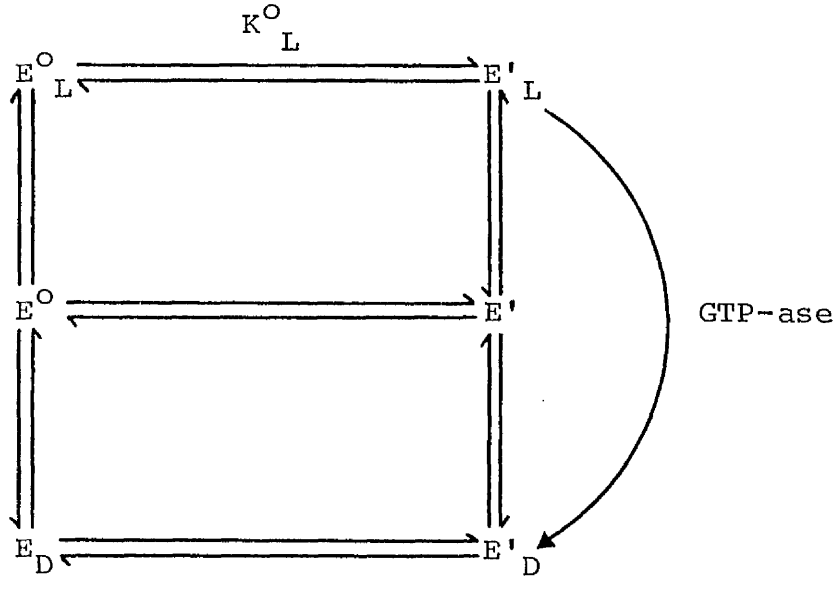
Blume and Foster, 1976 suggested that the guanine nucleotide may be the primary enzyme regulator for neuroblastoma cells and that prostaglandin E_1, the stimulatory ligand, may be considered to be a "secondary" cyclase regulator which acts on the primary regulatory mechanism. The observation that locust adenylate cyclase was activated substantially by Gpp(NH)p alone and that octopamine had a marginal stimulatory effect in the absence of guanyl nucleotides is consistent with this suggestion. This idea will now be discussed in relation to current views on guanyl nucleotide interaction and hormone-mediated stimulation of adenylate cyclase. In particular, the results of this study will be compared with two models proposed to account for guanyl nucleotide hormone interaction in rat liver (Birnbaumer et al, 1980) and mouse neuroblastoma cells (Blume and Foster, 1976).

1. Rat Liver Adenylate Cyclase

Time course experiments showed that a lag could be observed in the activation of rat liver adenylate cyclase, even in the presence of saturating concentrations of Gpp(NH)p (Salomon et al, 1975). This lag was abolished by the addition of glucagon. Thus, it was suggested that the hormone accelerates the slow guanyl nucleotide induced isomerisation of an inactive to active form of the enzyme. This together with the following observations led Birnbaumer et al, 1980 to propose a two state model for the regulation of adenylate cyclase (Figure 37).

FIGURE 37

Adenylate Cyclase Regulation in Rat Liver
(Birnbaumer et al, 1980; Iyengar et al, 1980)



where: E = enzyme
L = GTP
D = GDP
K^{O}_{L} = Isomerisation rate

The model assumes that adenylate cyclase exists in two states, E^{O} inactive and E' active and that the equilibrium of the reaction is displaced towards E^{O}. This is a result of the minimal activity observed in the absence of nucleotides. In the presence of GTP or Gpp(NH)p, $(L)E^{O}_{L}$ is displaced towards E'_{L} thus accounting for the activation by guanyl nucleotides. GTP but not Gpp(NH)p can be removed not only by dissociation but also through the action of the GTP-ase as suggested by Cassel and Selinger, 1976, 1977. The formation of GDP (D) displaces the equilibrium of E^{O}_{D} and E'_{D} towards the inactive E^{O}_{D} state. This is

consistent with competitive inhibition by GDP of enzyme activation with Gpp(NH)p (Salomon et al, 1975; Lin et al, 1978). Evidence for an adenylate cyclase species, active in the absence of guanine nucleotides, was suggested by the finding that with turkey erythrocyte adenylate cyclase treated with GMP and isoproterenol, the initial rate of the GDP-free enzyme was high even in the absence of subsequent hormone or guanyl nucleotides (Abramowitz et al, 1980). Iyengar et al, 1980 extended this model to account for hormone-receptor mediated stimulation of adenylate cyclase suggesting that the hormone functions by accelerating the isomerisation rate (K^{o}_{L}).

The addition of Gpp(NH)p (10^{-4}M) stimulated locust adenylate cyclase without a lag being observed in time course experiments (Figure 31). Furthermore, this was not due to the method chosen for measuring cyclic AMP activity i.e. pre-incubation of the tissue with Gpp(NH)p for 5 minutes before initiating the reaction with ATP. If the assay was altered, such that the reaction was started by the addition of the tissue, a lag was not observed. Therefore, if a transition state exists, its interconversion must be very rapid.

2. Neuroblastoma Adenylate Cyclase

Blume and Foster, 1976 proposed that for neuroblastoma cells, the enzyme GDP complex has to dissociate into the free enzyme plus GDP before GTP of Gpp(NH)p can interact

with adenylate cyclase to form an active complex. Thus, unlike the previous model, the dissociation of GDP has been suggested to be the rate limiting step in the normal turnover cycle of the enzyme (Figure 38).

FIGURE 38

Regulation of Neuroblastoma Adenylate Cyclase
(Blume and Foster, 1976)

A further difference between this model and the previous one is that the function of the hormone is to facilitate the removal of GDP from the guanine binding site. On the assumption that most of the "unstimulated" enzyme population is in the E_{GDP} state, shifting the equilibrium $E_{GDP} \rightarrow E_O$ towards E_o by adenylate cyclase activators would make more of the enzyme available for binding of GTP and therefore increase the amount of active neuroblastoma adenylate cyclase.

Further evidence for the hormone being responsible for the removal of tightly bound GDP was implied by the observation that turkey erythrocyte membranes exposed to [^{3}H]-

GTP and then washed, retained a small amount of radioactivity identified as GDP. The rate of dissociation of [^{3}H]-GDP was stimulated by hormone suggesting that bound [^{3}H]-GDP was converted by the GTP-ase to [^{3}H]-GDP and retained until stimulated to exchange by hormone (Cassel and Selinger, 1978).

As previously described, incubation of locust adenylate cyclase with Gpp(NH)p (10^{-4}M) led to the formation of a stable enzyme which retained its activity even after extensive washing. The subsequent addition to the stable complex of either GTP or octopamine resulted in only a small reduction in adenylate cyclase activity. In contrast, in the presence of both GTP and octopamine, a substantial reduction in activity was observed implying that octopamine was necessary to induce the exchange of Gpp(NH)p by GTP before reversal of the stable complex could be achieved.

Pre-incubation of locust adenylate cyclase with GTP followed by thorough washing resulted in the failure of the enzyme to be activated fully by the subsequent addition of Gpp(NH)p. Examination of the time course for the experiment revealed that a lag occurred with the Gpp(NH)p activation. Furthermore, this lag was abolished by the addition of octopamine initially suggesting that a slow isomerisation step similar to that observed with the rat liver enzyme occurs in the activation of locust adenylate cyclase by Gpp(NH)p. However, it seems more likely that

the lag represents the time taken for Gpp(NH)p to remove the tightly bound nucleotide (presumably GDP) from the guanine nucleotide site. Support for this interpretation is based on the following:

a) The addition of Gpp(NH)p to an enzyme preparation pre-incubated in the absence of GTP i.e. with buffer, stimulated adenylate cyclase without a lag being observed in time course experiments.

b) Pre-incubation of locust adenylate cyclase with 10^{-3}M and 10^{-4}M GDP followed by washing to remove the free nucleotide resulted in a lag in Gpp(NH)p activation.

c) GDP (10^{-3}M and 10^{-4}M) inhibited Gpp(NH)p and Gpp(NH)p + octopamine stimulated adenylate cyclase.

The observation that octopamine could abolish the lag suggests that whatever nucleotide was present at the guanine binding site, octopamine induces its exchange with Gpp(NH)p. Thus, the results presented are consistent with the model proposed by Blume and Foster, 1976 and suggest that locust adenylate cyclase is similar to the enzyme in mouse neuroblastoma cells.

The suggestion that GDP dissociation is the rate limiting step in the normal turnover cycle of adenylate cyclase has recently been questioned. If correct, it predicts that the rates of nucleotide activation of any adenylate cyclase should

be independent of the nucleotide added. However, in adenylate cyclase from rat liver (Birnbaumer et al, 1980) and turkey erythrocyte cleared of endogenous GDP by isoproterenol plus GMP (Abramowitz et al, 1980), the rate of enzyme activation varied with each guanyl nucleotide. Thus, GTP(γ)S > Gpp(NH)p > Gpp(CH_2)p > GTP. In addition, the rate of activation of locust adenylate cyclase in the presence of Gpp(NH)p was greater than that observed with GTP. Therefore, this finding suggests that, although the results presented are consistent with the model proposed by Blume and Foster, 1976, they do not provide conclusive proof for it.

Future Studies

Several questions are raised as a result of this study on adenylate cyclase from locust cerebral ganglia:

1. The lag observed after pre-incubation was interpreted as the result of the failure of Gpp(NH)p to replace GDP, formed by the hydrolysis of GTP by a GTP-ase. However, no evidence is presented in this study for the occurrence of a GTP-ase associated with locust adenylate cyclase. Thus, repeating the work of Cassel and Selinger, 1977 and 1978, whereby the fate of [^{3}H]-GTP is followed may provide evidence for a GTP-ase in the locust.

2. Although guanine nucleotides regulate adenylate cyclase activity, the molecular mechanism by which this occurs

remains unclear. However, studies involving isolated guanine regulatory protein and adenylate cyclase from pigeon erythrocyte have indicated that activation requires the association of the two components (Pfeuffer, 1979). In contrast, evidence for a guanine binding protein-adenylate cyclase dissociation in the activation process has been suggested from x-ray inactivation studies on rat liver adenylate cyclase. Thus, from measuring the size of the complex before and after activation of adenylate cyclase Martin et al, 1979 proposed the following model (Figure 40).

In the presence of Gpp(NH)p alone, the catalytic unit of adenylate cyclase (C) dissociates from the guanine regulatory component (G). The free catalytic unit is then fully activated. The dissociation of G from C is unaffected by the independent glucagon receptor (R) which in the absence of hormone is not coupled to the catalytic unit or to the guanine binding component. Thus, the effect of Gpp(NH)p is essentially irreversible and complete. In contrast, the effect of GTP is reversible in that an equilibrium is established between the activated, dissociated state (C) and the inactive associated state (CG). In the presence of glucagon, the receptor (R) associates with the CG complex to form a ternary complex (CGR) which dissociates to release a fully activated catalytic unit. Thus, the role of the hormone is to facilitate the action of GTP in causing the release of the free catalytic unit. This model, with only a slight modification, will also account for the results obtained in

liver and neuroblastoma cells and, therefore, is consistent with the rat liver and neuroblastoma models previously described. If the release of the free activated catalytic unit is time-dependent, then hormone acceleration of Step 2 is consistent with the rat liver model. Equally, if GDP is not released by recombination of the catalytic unit and the guanine binding protein Step 3, but is dependent on a hormone-receptor interaction, Step 1, then this model is applicable to neuroblastoma cells.

Adenylate cyclase modelling is aimed at developing a single model which is applicable to all adenylate cyclases (Birnbaumer et al, 1980; Iyengar et al, 1980). Furthermore, the model should not require a new assumption each time a new enzyme is found. At present, there is still debate as to whether the function of the hormone is to facilitate the release of inhibitory GDP from the nucleotide binding site or to facilitate a slow isomerisation of an inactive to active form of the enzyme. Thus, it would appear that a single model must await the results of further research.

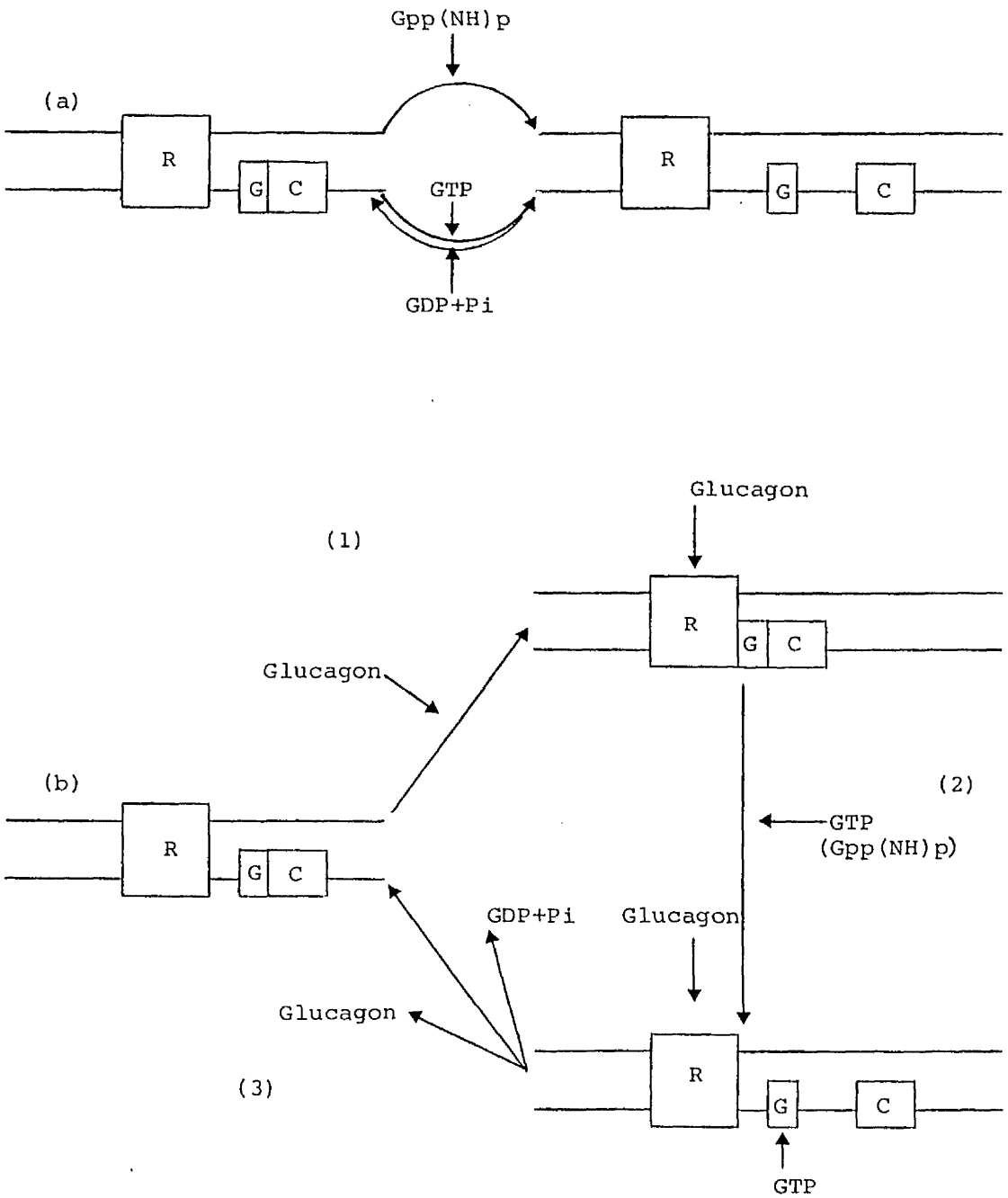
3. Finally, although an octopamine-, dopamine- and serotonin-sensitive adenylate cyclase exists in locust cerebral ganglia, not all the physiological responses to these enzymes necessarily involve an alteration of adenylate cyclase activity. A dopamine-sensitive adenylate cyclase has been identified in mammalian substantia nigra (Kebabian and Saavedra, 1976) and striatum (Kebabian et al,

1972). However, the enzyme would appear to occur outwith "dopaminergic" neurons. Lesions in the nigra and striatum induced by 6-hydroxydopamine (which selectively destroys dopaminergic neurons) do not alter the activity of dopamine-sensitive adenylate cyclase within either the nigra (Kebabian and Saavedra, 1976) or striatum (Krueger et al, 1976). Furthermore, injection of 6-hydroxydopamine into rat substantia nigra (Quik et al, 1979) depleted the binding of the dopaminergic antagonist spiroperidol but failed to reduce the dopamine-sensitive adenylate cyclase. Thus, an indication of whether the dopamine-sensitive adenylate cyclase in locust is associated with dopaminergic neurons is possible from studies using 6-hydroxydopamine.

The identification of the factors involved in the activation of locust adenylate cyclase was a prerequisite before commencement of studies on cyclic AMP induced phosphorylation of membrane proteins could begin. Thus, the finding that locust adenylate cyclase was stimulated by octopamine, dopamine and serotonin will lead to studies designed to determine whether these monoamines also induce phosphorylation of membrane proteins. Furthermore, the observation that the octopamine-sensitive adenylate cyclase (Table 6) was enriched in a fraction (F_2) previously shown to contain a high proportion of cholinergic nerve endings (Donnellan et al, 1976) and presumably other types of nerve endings will allow the phosphorylation of a possible nerve ending fraction to be studied.

FIGURE 40

Activation of Adenylate Cyclase by
Glucagon and Guanine Nucleotides
(Martin et al, 1979)



The figure describes the model proposed for alteration in the aggregation state of adenylate cyclase during activation by guanine nucleotides (1) or by guanine nucleotides in the presence of glucagon (b). The glucagon receptor is represented by R, the catalytic unit by C and the GTP binding unit by G.

At present, however, little is known about the functional role of cyclic AMP in monoaminergic neurotransmission in insects and it would appear that a definitive demonstration of a cyclic AMP involvement must await the results of further studies on identified aminergic neurons.

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