CYCLIC AMP IN THE NORMAL STATE AND DEPRESSIVE ILLNESS

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ABSTRACT

Two methods for the measurement of cyclic AMP, enzymic radioisotopic displacement and protein binding (saturation) assay, were established.

Vitamins B_{12} and C were found, in *in vitro* studies, to be more effective inhibitors of cyclic AMP phosphodiesterase than theophylline.

Plasma and urinary levels of cyclic AMP were found to exhibit a diurnal variation in normal volunteers. The majority gave a plasma pattern with a maximum at 24.00h and a minimum at 16.00h. The minority plasma pattern showed a peak at 12.00h. The urinary excretion gave a pattern in the majority with a maximum between 04.00 - 08.00h and a minimum between 12.00 - 16.00h. The minority showed a peak urine value between 12.00 - 16.00h and a trough between 24.00 - 04.00h.

A further pattern was noted in menstrual cycle with a mid-cycle peak. This was absent in males, and was lost in secondary amenorrhoea, pregnancy and after prolonged ingestion of oral contraceptives. The amplitude was more pronounced in pre-menstrual tension syndrome, suggesting the possibility of overswing.

Muscular activity produced a transient increase in plasma cyclic AMP, but this was not reflected in the urinary level. Limited studies on dietary change showed no significant influence.

Patients suffering from endogenous depression showed low plasma and urinary levels of cyclic AMP. On clinical improvement urinary levels returned to the normal range. The urinary excretion of cyclic AMP in endogenous depression showed a peak between 08.00 - 12.00 h. On clinical improvement the pattern became bimodal with peaks between 04.00 - 08.00h and 16.00 - 20.00h. The reactive depression group showed a bimodal pattern (peaks between 24.00 - 04.00h and 12.00 - 16.00h) both before and after clinical improvement.

Electro-convulsive therapy caused a marked rise in both plasma and urinary levels of cyclic AMP on the day of treatment. The plasma concentration doubled and remained elevated for up to 90 min after electrical stimulation. Patients who did not respond to E.C.T. in terms of cyclic AMP increase also showed no clinical improvement.

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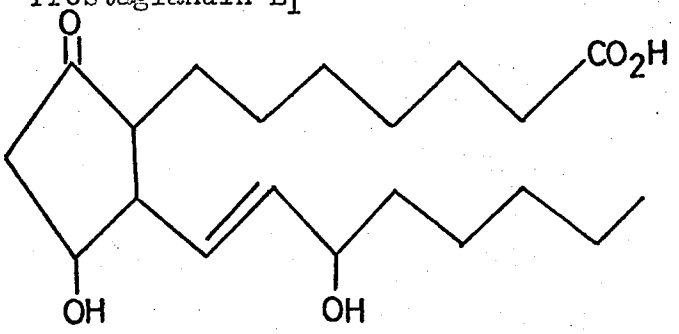
CONTENTS

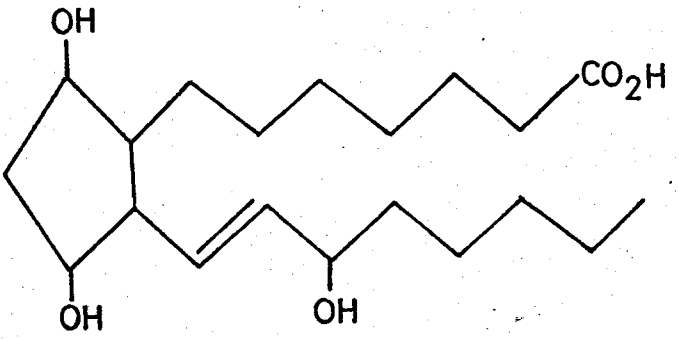
ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ADH	Antidiuretic hormone (Vasopressin)
ADP	Adenosine 5' diphosphate
5' AMP	Adenosine 5' monophosphate
ATP	Adenosine 5' triphosphate
ATPase	Adenosine triphosphatase
db-cyclic AMP	$N^6,O^{2'}$-Dibutyryl adenosine 3'5' cyclic monophosphate
db-cyclic GMP	$N^6,O^{2'}$-Dibutyryl guanosine 3'5' cyclic monophosphate
CNS	Central nervous system
c.p.m.	Counts per minute
C.S.F.	Cerebrospinal fluid
Cyclic AMP	Adenosine 3'5' cyclic monophosphate
Cyclic CMP	Cytidine 3'5' cyclic monophosphate
Cyclic GMP	Guanosine 3'5' cyclic monophosphate
Cyclic IMP	Inosine 3'5' cyclic monophosphate
Cyclic UMP	Uridine 3'5' cyclic monophosphate
d.p.m.	Disintegrations per minute
E.C.T.	Electro-convulsive therapy
E. coli	Escherichia coli
EDTA	Ethylene diamine tetra-acetic acid
FSH	Follicle stimulating hormone
GTP	Guanosine 5' triphosphate

G-1-P	Glucose - 1 - phosphate
G-6-P	Glucose - 6 - phosphate
5-HT	5 - Hydroxytryptamine (Serotonin)
5-HIAA	5 - Hydroxyindoleacetic acid
LH	Luteinising hormone
mRNA	Messenger ribonucleic acid
MSH	Melanocyte stimulating hormone
PGE_1	Prostaglandin E_1



PGF_1	Prostaglandin F_{1a}



PPi	Inorganic pyrophosphate
PTH	Parathyroid hormone
r.p.m.	Revolutions per minute
TRH	Thyroid releasing hormone
TSH	Thyroid stimulating hormone

PUBLICATIONS

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Hamadah, K., Holmes, H., Stokes, M.L., Hartman, G.C. & Parke, D.V. (1974) Biochem. Soc. Trans. 2, 461 - 462.

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CHAPTER I

INTRODUCTION

1.1. Cyclic AMP

A heat stable factor mediating the glycogenolytic effect of adrenaline and glucagon in the liver, was discovered by Rall et al. (1957). This was later identified (Sutherland and Rall, 1957; Lipkin et al., 1959) as adenosine 3',5' cyclic monophosphate, commonly referred to as cyclic AMP (Figure 1.1.). This nucleotide occurs throughout the animal kingdom (Robison et al., 1971), in micro-organisms (Perlman and Pastan, 1971; Pastan, 1972; Ashworth, 1974) and in plant tissues (Newton, 1974; Po-Chao Lin, 1974).

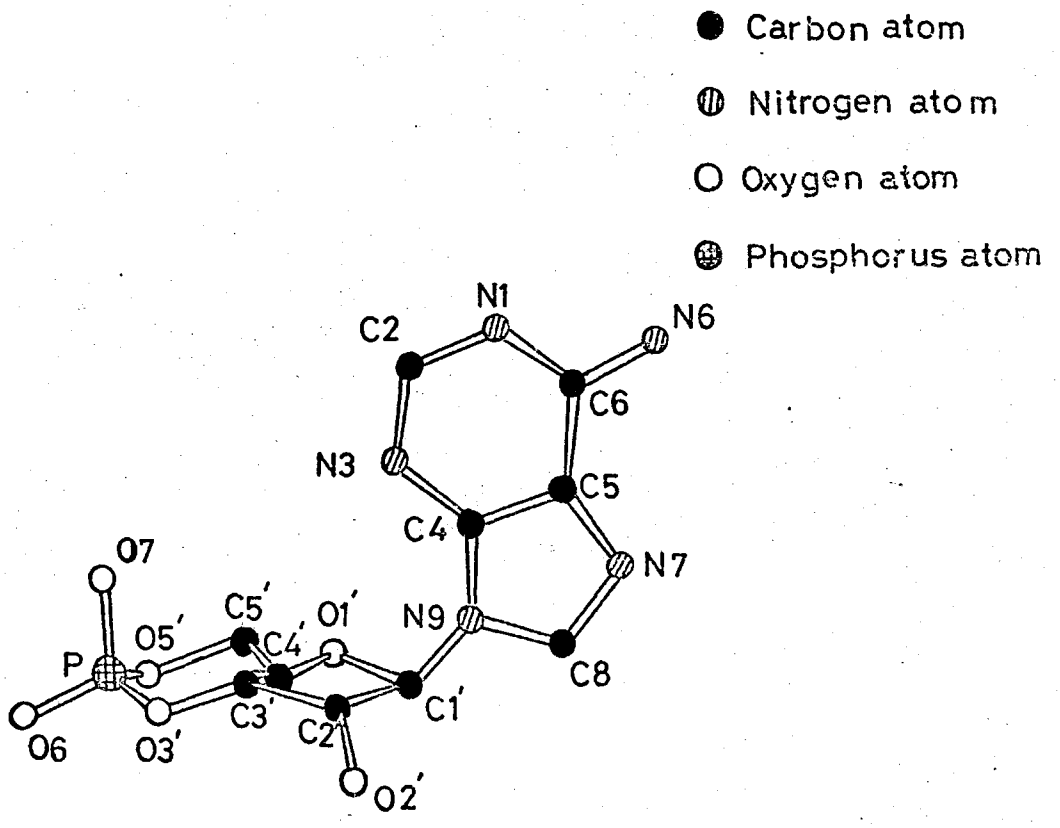


Figure 1.1. One of the two conformations for cyclic AMP in the crystalline state (Watenpaugh et al., 1968).

1.2. Biogenesis of Cyclic AMP

Figure 1.2. illustrates the reaction by which cyclic AMP is produced from ATP under the catalytic influence of adenyl cyclase (Rall and Sutherland, 1962). This enzyme system is found in the plasma membrane of vertebrates (Rodbell, 1972; Bitensky & Gorman, 1972), and requires a divalent cation (Mg^{++} or Mn^{++}) as co-factor (Robison et al., 1971). The apparent K_m value ranges from 0.08 - 0.50 mM depending upon the tissue preparation (Hardman et al., 1971a).

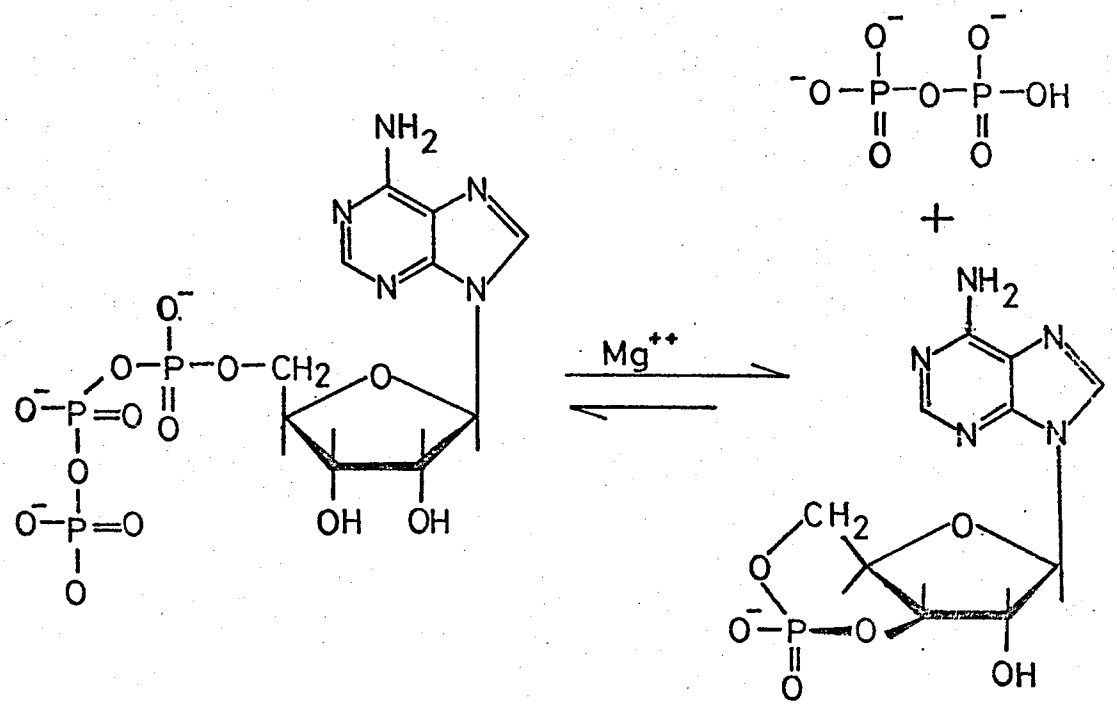


Figure 1.2. The adenyl cyclase reaction.

The adenyl cyclase reaction is reversible under certain conditions (Greengard et al., 1972) indicating that cyclic AMP is itself an energy rich molecule. Measurement of the free energy of hydrolysis of cyclic AMP to AMP gives a value of 48 kJ/mol as compared with the value of 36 kJ/mol for the hydrolysis of ATP to ADP.

1.2.1. Distribution of Adenyl Cyclase

Adenyl cyclase has been found in all animals so far studied as well as in micro-organisms and in plants (Table 1.1.).

Adenyl cyclase activity has been detected in a multiplicity of mammalian tissues and some of these are listed in Table 1.2. together with hormones which affect the enzyme activity. The enzyme appears to be absent from the erythrocytes of some mammals (Sutherland *et al.*, 1962), but is present in the nucleated red blood cells of birds and frogs (Davoren & Sutherland, 1963; Rosen & Rosen, 1969). The brain, and especially the grey matter, contains the highest adenyl cyclase activity of the mammalian tissues studied (Robison *et al.*, 1970); this activity probably having a post-synaptic location (McIlwain, 1971).

1.2.2. Properties of Adenyl Cyclase

Adenyl cyclase preparations from a variety of tissues have lipoprotein characteristics, show lability and are sensitive to certain hormones (Robison *et al.*, 1970). This last aspect is of key importance and seems to develop at different times for different tissues (Schmidt *et al.*, 1970; Palmer *et al.*, 1972b). Catecholamines (beta component) and a number of polypeptide hormones (excluding insulin and melatonin), activate the adenyl cyclase system leading to an increase in the intracellular concentration of cyclic AMP in the target tissues (Table 1.3.). This

Table 1.1. Species distribution of adenyl cyclase

Mammals	Robison, Butcher & Sutherland (1971).
Birds	Langslow (1971); Riddick, Kregenow & Orloff (1971).
Amphibians	Rosen (1971); Bockaert, Roy & Jard (1972).
Fish	Menon & Smith (1971).
Insects	Sutherland, Rall and Menon (1962).
Segmented worms	Sutherland, Rall and Menon (1962).
Liver fluke	Sutherland, Rall and Menon (1962).
Bacteria	Pastan (1972).
Slime molds	Ashworth (1974).
Plants	Newton (1974).

aspect will be discussed later (1.4.) in greater detail.

The prostaglandins, a group of complex lipids with hormonal activities (Horton, 1972), can increase cyclic AMP in a variety of tissues (e.g. heart, thyroid, platelets) through activation of adenyl cyclase, with PGE compounds being more effective than PGF (Shio et al., 1971; Harwood et al., 1972). This action does not appear to involve specific receptors on the plasma membrane. Prostaglandins may, however, cause a decrease in cyclic AMP levels possibly by interfering with ATP binding to adenyl cyclase. Such inhibitory effects are encountered in lipolysis, in vasopressin-induced water movement in renal tubules, in dopamine-induced hyperpolarisation of postganglionic neurones in superior cervical ganglia (McAfee & Greengard, 1972) and in noradrenaline-induced inhibition of the spontaneous discharge of cerebellar Purkinje cells (Horton, 1972). Again the PGE compounds show more potent inhibition than the PGF series. Currently it is not possible to predict whether the effects of prostaglandins on a particular tissue will be to inhibit or to stimulate cyclic AMP formation (Shio et al., 1971).

Fluoride can markedly stimulate adenyl cyclase activity in a variety of preparations from multicellular organisms (Rall & Sutherland, 1958) but not in bacteria (Tao & Lipman, 1969). In the developing rat brain fluoride has little influence for the first 5 days postpartum, then stimulation

Table 1.2. Distribution and Hormonal Sensitivity of Mammalian Adenyl Cyclase

Tissue	Hormone affecting Adenyl Cyclase	Reference
Liver	Glucagon and adrenaline	Park and Exton (1972).
Skeletal muscle	Adrenaline	Mayer and Stull (1971).
Cardiac muscle	Catecholamines	Robison et al. (1971); Levey (1971a).
	Glucagon	Mayer et al. (1970); Levey (1971b).
	Triiodothyronine	Levey (1971c).
Kidney	Vasopressin	Major and Kilpatrick (1972).
	Parathyroid	Melson et al. (1970); Marcus et al. (1971).
Bone	Parathyroid	Chase et al. (1969); Aurbach et al. (1972).
	Calcitonin	Murad et al. (1970).
Brain	Catecholamines	Rall (1972); Skolnick et al. (1973).
Adrenal	ACTH	Grahame-Smith et al. (1967); Albano and Brown (1974).
Corpus Luteum	LH and prostaglandins	March (1970a,b).
Ovary	LH	Dorrington and Baggett (1969).
Testis	LH and FSH	Hollinger (1970); Kuehl et al. (1970).
Thyroid	TSH	Major and Kilpatrick (1972).
	Prostaglandins	Field et al. (1969).
Parotid	Catecholamines	Schramm and Naim (1970); Selinger and Schramm (1971).
Pineal	Catecholamines	Weiss and Strada (1972).

Table 1.2. (Contd.)

Tissue	Hormone affecting Adenyl Cyclase	Reference
Lung	Adrenaline	Klainer et al. (1962); Kaliner et al. (1971).
Spleen	Adrenaline	Klainer et al. (1962).
Adipose	Adrenaline, Glucagon TSH, ACTH, LH, Secretin	Rodbell (1972).
Brown Adipose	Catecholamines	Butcher and Baird (1969).
Platelets	Prostaglandins	Cole et al. (1971); Harwood et al. (1972).
Leucocytes	Catecholamines and Prostaglandins	Scott (1970); Bourne and Melson (1971).
Erythrocytes	None demonstrated	Sheppard and Burghardt (1969).
Uterus	Catecholamines	Triner et al. (1971).
Pancreas	None demonstrated	Cohen and Bitensky (1969).
Anterior pituitary	Hypothalamic releasing factors (e.g. TRH)	Steiner et al. (1970); Bowers and Robison (1971).
Vascular smooth muscle	None demonstrated	Klainer et al. (1962).
Hair follicles	Dihydrotestosterone	Adachi and Kano (1970).

becomes apparent reaching a maximum after a further 10 to 15 days (Schmidt et al., 1970). Fluoride stimulation only occurs with homogenized tissues (i.e. broken cells) and not with intact cells (Oye & Sutherland, 1966). It is probable that fluoride bypasses the hormone receptor and acts directly on the catalytic side of the cyclase (Drummond et al., 1971). This direct action being possible only when the plasma membrane is disrupted (Rodbell, 1972).

Studies on cations indicate that Mn^{++} can replace Mg^{++} in some preparations (Birnbaumer et al., 1969). Ca^{++} is inhibitory in general (Rasmussen, 1970) although it may be required for optimal cyclase activity in adrenal cortex (Bar & Hechter, 1969) and brain (Bradham et al., 1970). Cu^{++} and Zn^{++} are strongly inhibitory and Co^{++} causes some inhibition (Birnbaumer et al., 1969), whereas Na^{+} and K^{+} have little effect in homogenates. In brain slices (Sattin & Rall, 1967) and rat diaphragm muscle (Lundholm et al., 1967) an increase in the ratio of K^{+} to Na^{+} can lead to an increase in cyclic AMP level. Rodbell (1972) has discussed the implications of a changing ionic microenvironment on the adenyl cyclase system and has reported (Rodbell et al., 1971) a need for the presence of GTP to achieve glucagon activation of liver plasma membrane adenyl cyclase. This allosteric modulation by GTP has also been reported for the prostaglandin activation of adenyl cyclase in human platelet membranes (Harwood et al., 1973; Krishna et al., 1972), and the oxytocin stimulation of adenyl cyclase in frog bladder

Table 1.3. Some Hormones That Mediate Their Action Through Cyclic AMP

Hormone	Tissue	Effect of Hormone	
		On tissue cyclic AMP	On Adenyl Cyclase
Catecholamines (beta component)	Liver	Increase	Stimulation
	Fat	Increase	Stimulation
	Muscle	Increase	Stimulation
	Heart	Increase	Stimulation
	Parotid gland	Increase	Stimulation
	Uterus	Increase	Stimulation
	Leukocytes	Unsettled	Stimulation
	Cerebellum	Increase	Stimulation
Glucagon	Liver	Increase	Stimulation
	Fat	Increase	Stimulation
	Heart	Increase	Stimulation
	Pancreatic islets	Increase	Unsettled
ACTH	Adrenal Cortex	Increase	Stimulation
	Fat	Increase	Stimulation
Vasopressin	Toad Bladder	Increase	Unsettled
	Kidney	Increase	Stimulation
	Pituitary gland	Increase	Unsettled
Luteinizing hormone	Corpus luteum	Increase	Stimulation
	Ovary	Increase	Stimulation
	Testis	Increase	Stimulation
FSH	Testis	Unsettled	Stimulation
Melanocyte stimulating hormone	Frog skin	Increase	Unsettled

Table 1.3. (Contd.)

Hormone	Tissue	Effect of Hormone	
		On tissue cyclic AMP	On Adenyl Cyclase
Parathyroid hormone	Kidney	Increase	Stimulation
	Bone	Increase	Stimulation
Calcitonin	Kidney	Increase	Stimulation
	Bone	Increase	Unsettled
Thyrotropin releasing factor	Pituitary gland	Increase	Unsettled
Insulin	Fat	Decreased	Unsettled
	Liver	Decreased	Unsettled
Melatonin	Frog skin	Decreased	Unsettled
Catecholamines (alpha component)	Pancreatic islets	Decreased	Unsettled
	Platelets	Decreased	Unsettled
	Frog skin	Decreased	Unsettled

epithelial cells (Bockaert et al., 1972). More recently the suggestion has been made that cyclic AMP can modify adenyl cyclase activity by a kinase-dependent phosphorylation (Constantopoulos & Najjar, 1973) and in brain a low molecular weight activator of adenyl cyclase has been reported (Kauffman & Johnson, 1973).

1.3. Breakdown of Cyclic AMP - Phosphodiesterase

Cyclic 3'5' - nucleotide phosphodiesterase was first demonstrated by Sutherland and Rall (1958). It is found mainly in the soluble fraction of the cell and catalyses the hydrolysis of cyclic AMP to 5'-AMP (Figure 1.3.).

Phosphodiesterase(s) is widely distributed in nature including bacteria (Ide, 1971), slime molds (Ashworth, 1974) and plants.

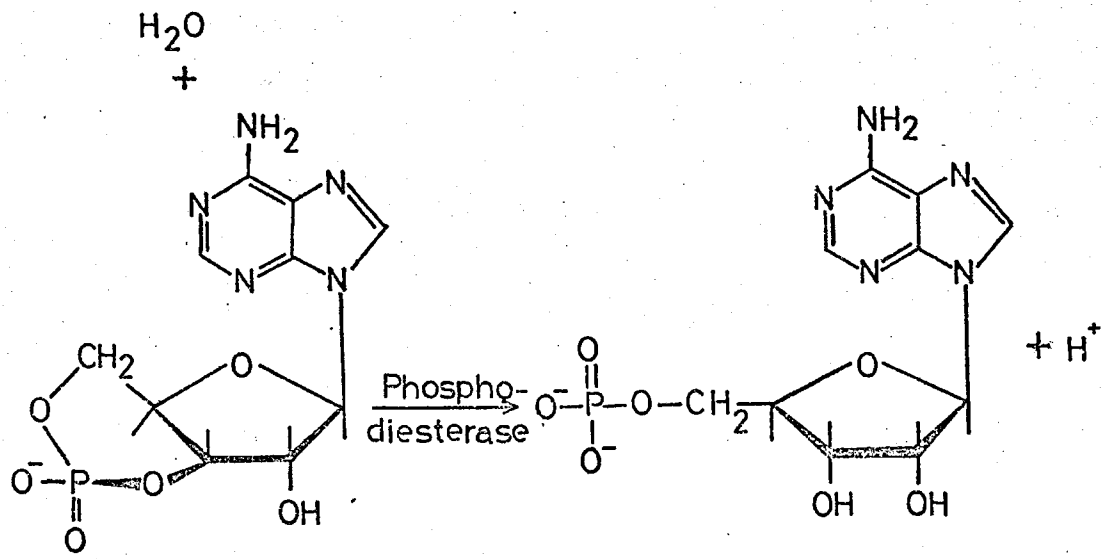


Figure 1.3. The reaction catalysed by phosphodiesterase.

A table (1.4.) showing the range of values in dog tissues has been adopted from Butcher and Sutherland (1962).

Table 1.4. Tissue distribution of Mammalian phosphodiesterase (dog)

Tissue	µmol cyclic AMP converted to 5' AMP per min per g fresh weight (30°C)
Brain cortex	41
Brain stem	15
Adrenal	12
Kidney medulla	7
Kidney cortex	6
Small intestine (whole)	6
Cardiac ventricle	5
Spleen	4
Liver	4
Pancreas	4
Cardiac auricle	4
Cerebellum	3
Lung	3
Testis	2.5
Sartorius muscle	2
Aorta	1.5
Femoral artery	1
Diaphragm muscle	1
Femoral nerve	0.5
Erythrocytes	0

Gel filtration, electrophoresis and kinetic studies have indicated the presence of isoenzymes (Thompson & Appleman, 1971; Russell et al., 1973). Rat brain, kidney and fat pad supernatants contain in the mol.wt. region 400,000 a phosphodiesterase with a higher affinity for cyclic GMP (K_m of 1×10^{-5}M) than cyclic AMP (K_m of 1×10^{-4}M). In the mol.wt. region 200,000 from the same tissues an enzyme can be located which does not catalyse cyclic GMP hydrolysis but has a K_m for cyclic AMP of 5×10^{-6}M. Liver contains detectable levels of the high mol.wt. enzyme only. It is possible that the lower mol.wt. form is associated with the membrane bound adenyl cyclase in an organised control system. The high mol.wt. form may be associated with the apparently soluble guanyl cyclase (Tata, 1973). Other reports of tissues containing multiple forms of phosphodiesterase include cerebrum (Fertel et al., 1973), gastric mucosa (Sung et al., 1972), human platelets (Pichard et al., 1973) and testis (Catt et al., 1972).

Phosphodiesterase requires divalent cations for maximal activity. For the bovine brain preparation, Mg^{++} and Mn^{++} (1.0 mM), Co^{++} (0.1 mM) and Zn (0.01 mM) are effective, Ca^{++} and Ba^{++} are ineffective, while Cu^{++} and Hg^{++} are inhibitory. In the absence of added cations about 30% activity is found, which is suppressed by the addition of 1 mM EDTA.

A number of substances have been found to alter phosphodiesterase activity. Cheung (1970) reported that

nucleotide triphosphates are inhibitors at a concentration of 3 mM with ATP the most-effective (75%). Cyclic GMP was found to inhibit the action of phosphodiesterase in heart, and activate it in liver (Beavo et al., 1970; Hardman, 1972). There is some evidence that triiodothyronine (Mandel & Kuehl, 1967) cyclic AMP (De Lorenzo et al., 1973) and db cyclic AMP (Pastan, 1972) have an inhibitory effect on phosphodiesterase activity. A calcium dependent protein activator of phosphodiesterase has been found in brain (Kakiuchi et al., 1972) and heart (Teo et al., 1973). Cyclic AMP modulates the activator-enzyme interaction by enhancing the affinity of the enzyme for the activator. There would seem to be no equivalent in mammalian tissue to the potent protein inhibitor secreted by slime mold (Ashworth, 1974).

Methyl xanthines and especially theophylline have been the most widely used inhibitors of phosphodiesterase (Stanfield et al., 1971), although recently some much more potent compounds have been available (e.g. ICI 63,197 and Squibb SQ 20,009). A wide range of drugs have been checked for their inhibitory activity (Usunov & Weiss, 1971; Weinryb et al., 1972), and these include the tricyclic antidepressants (Beer et al., 1972) and Δ^9 -tetrahydrocannabinol (Moffat et al., 1972). Activation of cyclic nucleotide phosphodiesterase may be achieved by imidazole, nicotinic acid (Abdulla & Hamadah, 1970), imidazole acetic acid (Roberts and Simonsen, 1970) and ammonium ions (Nair, 1966). It has been suggested that the therapeutic

effects of a number of the drugs quoted above may be mediated by the cyclic AMP system (Beer et al., 1972).

1.4. The Second Messenger Hypothesis

The presence of cyclic AMP in most animal cells and the fact that many hormones increase the intracellular concentration of cyclic AMP in their target tissues (Table 1.3.), led to the development of the "second messenger" concept of hormone action (Sutherland et al., 1968). This is represented in figure 1.4. Rodbell (1972) has suggested that the adenyl cyclase system is composed of a regulatory sub-unit on the outer surface of the plasma membrane and a catalytic sub-unit with its active centre directed towards the interior of the cell. He proposed a discriminator (i.e. hormone receptor) - transducer - amplifier (adenyl cyclase) model.

To account for hormone-tissue selectivity, the regulatory sub-unit is made up of receptors specific for different hormones. Rodbell (1972) has reviewed evidence for a single discriminator in the majority of target tissues - see for example the work of Reik et al. (1970) on liver parenchyma and reticulo-endothelial cells. In the fat cell, however, there would seem to be several receptors affecting a single adenyl cyclase (Birnbaumer & Rodbell, 1969). The receptor is protein in character and phospholipids may be involved in the linking of it to the appropriate hormone (Rodbell et al., 1968). Rodbell (1971) and Levey (1973) provided evidence for the involvement of phospholipids in the coupling mechanism (transduction) of

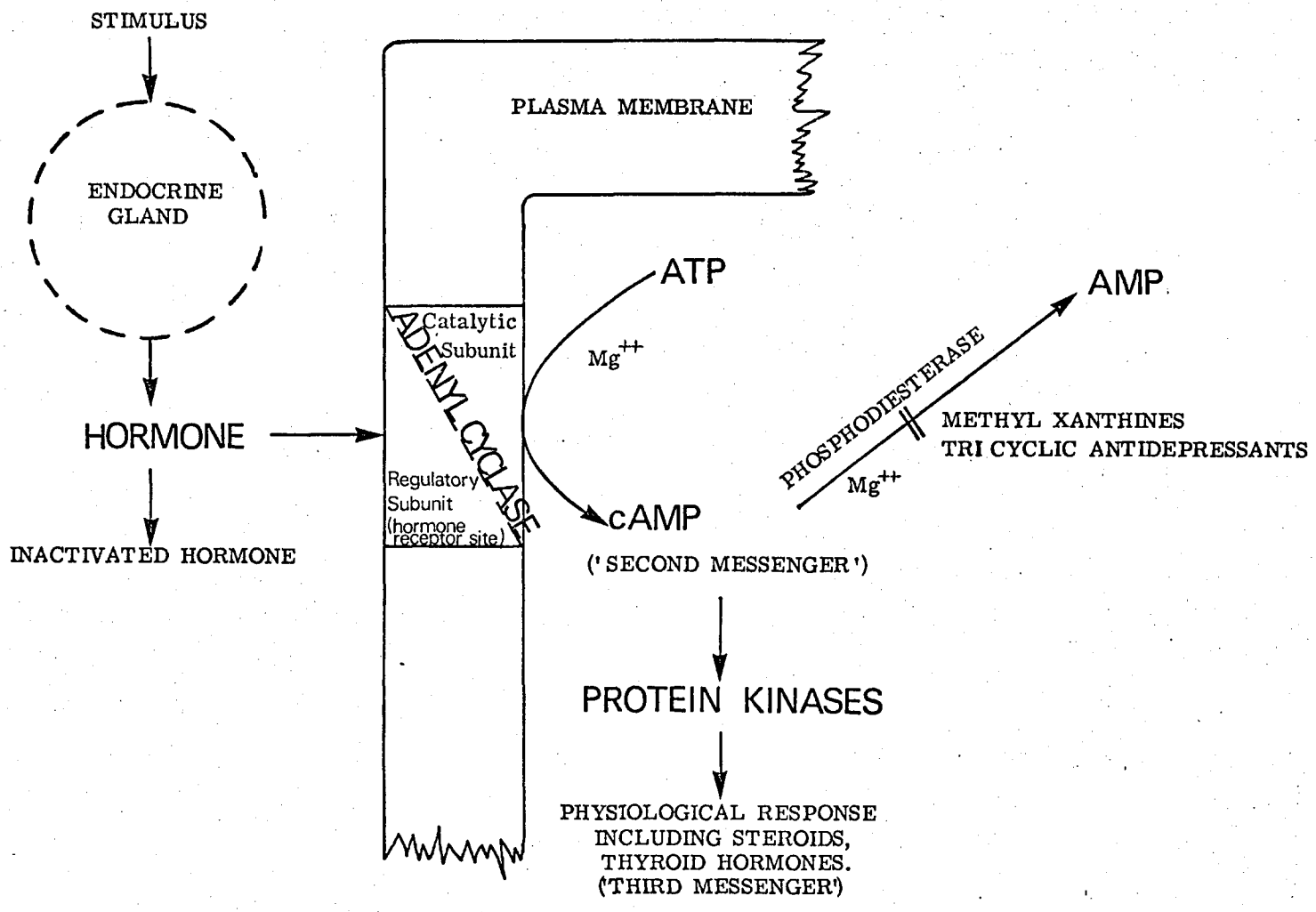


FIGURE 1.4: SCHEMATIC REPRESENTATION OF THE SECOND MESSENGER CONCEPT.

discriminator to cyclase. Storm and Dolginow (1973) have shown that receptor interaction at the extracellular surface produces a conformational change in the cyclase, and thus an increase in its activity, at the intracellular surface.

The localization of the enzyme in the cell membrane is also thought to regulate its activity. Disruption of membrane function in brain by homogenization (Krishna et al., 1971) leads to a loss of hormonal sensitivity while the enzyme becomes fully active catalytically. Similar observations have been made with the use of digitonin in retina (Bitensky & Gorman, 1972) and in hepatomas where normal membrane function is absent (Chayoth et al., 1972). Adenyl cyclase is thought, therefore, to be intrinsically active (Bitensky & Gorman, 1972) and under physiological conditions this activity is regulated by membrane components which exercise inhibitory constraint. This constraint is decreased by appropriate hormonal interaction or by a change in membrane organisation such as may occur in neoplasia.

The switch-on, switch-off mechanism for the cyclase may involve co-operative interactions between free and bound receptors on the membrane as discussed by de Meyts et al. (1973).

1.5. Mechanism of Action of Cyclic AMP

The discovery of a cyclic AMP-dependent protein kinase in rabbit skeletal muscle (Walsh et al., 1968), lead to the

hypothesis that the diverse actions of cyclic AMP may be mediated through regulation of the activity of a family of protein kinases (Kuo and Greengard, 1969a, b). The latter authors demonstrated a tenfold rise in protein kinase activity by the presence of 5 μM cyclic AMP in a variety of mammalian tissues. There is now evidence for the wide distribution of cyclic GMP-dependent protein kinases in the animal kingdom (Kuo & Greengard, 1970). Even though the two groups of enzymes have been found in the same tissue they act independently of each other (Greengard and Kuo, 1970; Greengard, 1971). Protein phosphokinase may employ ATP or GTP as phosphorylator and there is evidence for ATP inhibiting the GTP requiring enzyme (Davis & Lazarus, 1974). Thus the ratio of ATP to GTP concentration in the membrane may have a regulatory function (Tata, 1973).

It has been suggested that the cyclic AMP stimulated protein kinase system is composed of two sub-units, a regulatory sub-unit to which cyclic AMP binds and a catalytic sub-unit, which is inhibited by the regulatory sub-unit (Gill & Garren, 1971). When cyclic AMP binds to the regulatory sub-unit the catalytic sub-unit becomes freely active catalysing the phosphorylation of its substrate, thus resulting in the physiological response. Walsh & Ashby (1973) have extended this scheme and introduced the concept of an inhibitor protein which binds to the catalytic sub-unit after dissociation of the holoenzyme into its respective sub-units. It has been suggested that inhibitor protein and regulatory sub-unit

modulate catalytic sub-unit activity by an identical mechanism.

The identification of natural substrates for the protein kinases in various tissues is at present under investigation. However, a limited number of substrates are at present known, e.g. phosphorylase kinase and glycogen synthetase in skeletal muscle (Pastan & Perlman, 1971), lipase in adipose tissue (Huttunen et al., 1970; Soderling et al., 1973), histones and protamine in liver (Langan, 1968), brain (Miyamoto et al., 1969) and testis (Garbers et al., 1973). High cyclic AMP levels were found in seminal fluid (Broadus et al., 1971) and spermatozoa contain an active cyclic AMP-dependent protein kinase (Hoskins et al., 1972) which may be involved with mobility (Tash & Marin, 1973) and fertilization (Morton & Albagli, 1973). The activity of this kinase is affected by oestrogens and androgens (Bernard & Wasserman, 1972; Ichii et al., 1973). Evidence for the involvement of cyclic AMP in the stimulation of protein phosphorylation has been adduced for a variety of tissues including liver membrane (Shlatz and Marinetti, 1972), muscle troponin (Pratje and Hulmeyer, 1972), retina (Kuhn et al., 1973), erythrocyte membrane (Rubin and Rosen, 1973), pancreatic islet tissue (Montague and Howell, 1972), heart (La Raia et al., 1973) and brain (Weller and Rodnight, 1970; Johnson et al., 1971a)

Some hormones, such as insulin, may lower the level of cyclic AMP, but there is no clear role for cyclic AMP activated

protein kinase in such cases. The mode of action of the adenohypophyseal lactogenic hormone - the protein prolactin - has been investigated by Majumder and Turkington (1971). They suggest that the action concerns the increased production of protein kinase rather than its activation by cyclic AMP. This kinase rather than cyclic AMP is the rate limiting material in the control of the rate of casein production. There have been no reports on the role of cyclic AMP in the luteotrophic and steroid secretory effects of prolactin in ovarian tissue (Major and Kilpatrick, 1972). Prolactin may enhance the intrinsic capability of a tissue (e.g. prostrate) to bind steroids (Farnsworth, 1972).

Steroid hormones also seem to bypass cyclic AMP by entering within the cell rather than through an intermediary. Szego (1972) has suggested, however, that cyclic AMP may be involved in the transport of oestrogen from the cell surface into the nucleus. Steroids stimulate protein synthesis and effects have been reported both at the level of transcription and translation (Bitensky & Gorman, 1972). At the transcriptional level the steroid may bind to a receptor protein which is involved in activating RNA polymerase prior to the function of RNA on the DNA template. This is akin to the sigma or initiation factor in E. coli. It is also akin to a proposed mode of action of cyclic AMP in bacteria (Pastan, 1972). Thus cyclic AMP receptor protein in E. coli stimulates transcription in the Lac operon probably by binding to DNA enabling RNA polymerase to bind and initiate

transcription (Anderson et al., 1971). Langan (1971) has proposed a role for cyclic AMP at the transcriptional level in eukaryotes, possibly via the stimulation of histone phosphorylation. Glucagon or dibutyryl cyclic AMP can cause a marked rise in tyrosine transaminase activity in liver. This rise is inhibited by actinomycin D (Langan, 1971). At the translational level Gill and Garren (1970) suggested that a cyclic AMP-dependent protein kinase phosphorylates ribosomal protein. This is supported by the findings of Chuah and Oliver (1971) that cyclic AMP is involved in the release of tyrosine transaminase from polysomes.

1.6. Extracellular Levels

In addition to the rates of formation and degradation of cyclic AMP the rate at which it is extruded from the cell may be a factor in the regulation of its internal concentration. Very little is known about how this extrusion process works but at least one cell type - the avian erythrocyte (Davoren and Sutherland, 1963), appears able to pump cyclic AMP out against a concentration gradient and E. coli may have a similar capability (Makman and Sutherland, 1965). Endogenous cyclic AMP can function extracellularly in promoting aggregation and differentiation of the cellular slime mold (Bonner et al., 1972; Ashworth, 1974). Studies on cells in tissue culture indicate that exogenous cyclic AMP (usually $N^6,O^{2'}$ - dibutyryl cyclic AMP, which is more lipophilic and thus crosses membranes

more readily) can increase contact inhibition of human fibroblasts and thus decrease mobility and growth (Johnson et al., 1972; Pastan, 1972; Burger et al., 1972). Cyclic AMP and cyclic GMP may be involved in cell division (Hadden et al., 1972). Extracellular cyclic AMP has been reported both to stimulate (Ishizuka et al., 1971) and inhibit (Bosing-Schneider & Kolb, 1973) the induction of antibody synthesis. Watson et al., (1973) provided further evidence which suggests that cyclic AMP and cyclic GMP mediate the intracellular signals which determine whether antigen-sensitive cells follow an inductive (antibody synthesis) or non-inductive (paralytic) pathway. Cyclic AMP alone is postulated to mediate paralysis whereas both cyclic AMP and cyclic GMP present in an optimum ratio are required for induction. Cyclic AMP has been shown to suppress the tumorigenicity of virus-transformed skin cells (Reddi & Constantinides, 1972). It has been suggested by Korinek et al., (1973) that the antitumor activities of cyclic AMP are associated with an increase in DNA-dependent RNA polymerase activity.

In mammalian body fluids the highest level is found in seminal plasma (3-5 x 10^{-5}M; Gray, 1970). In blood plasma the level is much lower (1-3 x 10^{-8}M; Broadus et al., 1971). Normally humans excrete 2-9 μmol of cyclic AMP in a 24 h urine. The rat excretes more cyclic AMP relative to body weight than does man, the former achieving 350-800 pmol/min/kg

whereas the latter is in the range 20-90 pmol/min/kg. Extreme fluctuations in urine volume cause little if any change in the total amount of the nucleotide excreted (Hardman et al., 1969; Owen & Moffat , 1973). Cyclic AMP and cyclic GMP are the only nucleotides so far found in urine; cyclic pyrimidine nucleotides are either absent from urine or too low to measure (Broadus et al., 1971). The mechanism of renal plasma clearance of cyclic AMP and cyclic GMP and the source(s) of these compounds in the urine were investigated by Broadus et al. (1970a,b). The renal clearance of inulin, intravenously administered tritiated-cyclic nucleotides and endogenous cyclic nucleotides were measured in normal humans. The clearances of inulin and $[^3H]$ - cyclic AMP were found to be equal, thus indicating that the mechanism of renal clearance of plasma cyclic AMP was simple glomerular filtration. The clearance of endogenous cyclic AMP, however, exceeded both inulin and $[^3H]$ - cyclic AMP clearances, suggesting that the kidney adds a variable quantity of cyclic AMP to that filtered from plasma. Thus, in humans, under basal conditions approximately 50% of the urinary cyclic AMP is derived by glomerular filtration of plasma while the other 50% is nephrogenous. In contrast to cyclic AMP, urinary cyclic GMP appears to be derived from plasma by simple glomerular filtration. Cyclic AMP has also been found in cerebrospinal fluid ($0.5 - 3.0 \times 10^{-8}$M, Steiner et al., 1970a; Broadus et al., 1971), gastric juice (2×10^{-8}M, Robison et al., 1971), amniotic fluid (25×10^{-9}M, Broadus

et al., 1971), bile (actual level not reported, Levine et al., 1969) and milk (8 - 80 x 10^{-7}M, Kobata et al., 1961). It has also been measured in extracellular media from perfused liver (Hardman et al., 1971a) and adrenals (Albano & Brown, 1974).

The plasma cyclic nucleotide represents a dynamic pool in a steady state (Broadus et al., 1971). The half-time for the disappearance from plasma of injected radioactive cyclic AMP is about 2 min in rat (Chase & Aurbach, 1967), rather longer in man (about 30 min, Broadus et al., 1971), however, Barling et al., (1974) have reported 14-16 min half-life after a pulse of endogenous cyclic AMP caused by PTH infusion. About 15% of the injected labelled nucleotide appears in the urine in the short term (Chase & Aurbach, 1967), indicating that extrarenal factors account for the removal of most of the material from plasma. Metabolism of the nucleotide by the formed elements of blood may account for part of this extra-renal removal, but in vitro studies suggest that this is too slow to be a major factor (Broadus et al., 1970a,b). The apparent volume of distribution of the labelled cyclic AMP is significantly greater than the extracellular space (Broadus et al., 1970a). Under appropriate hormonal stimulation both the liver and kidney are capable of adding cyclic AMP to plasma (Broadus et al., 1971), but the sources of the normal plasma level of this nucleotide are far from clear. In their chapter in Annual Review of Physiology (1971) Hardman, Robison and Sutherland conclude "Unfortunately, too little is known about

the sources and hormones responsible for the normal levels of cyclic nucleotides in plasma and urine to permit more than speculative conclusions regarding abnormal levels. Studies are badly needed".

1.7. Role of Cyclic AMP and Cyclic GMP in Mammals

Most of the known functions of cyclic AMP are given in table 1.5. Besides mediating the actions of several hormones (table 1.3.) cyclic AMP regulates the release of most of the others (e.g. steroid hormones). Sometimes a hormone is both released by cyclic AMP and then acts via the nucleotide, e.g. ACTH, TSH and possibly glucagon (Chesney & Scofield, 1969). Some hormones released by cyclic AMP do not act directly via the nucleotide, e.g. steroids. Pathological conditions involving a fault in the control of cyclic AMP production (e.g. pseudo-hypoparathyroidism) and function, are described later (Section 1.10). Evidence implicating cyclic AMP in the control of the immune response (Section 1.6), in the regulation of visual processes (Bitensky et al., 1971; Miller, 1973), possibly in the control of balding in males (Adachi & Kano, 1970), the control of some of the effects of ions and especially Ca^{++} (Lundholm et al., 1967; Namm et al., 1968) and other nucleotides (Sattin & Rall, 1970) is now available.

Guanyl cyclase, the enzyme responsible for cyclic GMP formation is found in all mammalian tissues with the exception of whole blood (White & Aurbach, 1969; Hardman & Sutherland, 1969), the highest levels in rat tissue being in the lung and

Table 1.5. Relationships between the metabolic actions of hormones and cyclic AMP

Hormone	Principal Responses
Adrenaline	Glycogenolysis (liver, muscle, heart)
	Heart force
	Rate of heart contraction
	Stimulation of enzyme secretion
	Lipolysis in fat cells
	Insulin secretion
	Melanophore stimulation
Noradrenaline	Discharge frequency of Purkinje cells (brain)
	Acetyl-choline release (nerve)
	Melatonin synthesis (pineal)
Glucagon	Glycogenolysis (liver, heart)
	Heart force
	Rate of heart contraction
	Gluconeogenesis
	Enzyme synthesis
	Ketogenesis
	Urea and K^+ transport
	Lipolysis (adipose tissue)
ACTH	Steroid synthesis (adrenal)
	Adrenal growth
	Phosphorylase activation
	Protein kinase activation
	Lipolysis (adipose tissue)

Table 1.5. (Contd)

Hormone	Principal Responses
LH	Steroid synthesis (corpus luteum)
	Glucose uptake
	Glycogenolysis
FSH	Ovarian amino acid uptake
TSH	Thyroid growth and cell division
	Uptake of I^-
	Organification of I^- and thyroid hormone production
	Release of thyroid hormones
	Phosphorylase activity
	Glucose uptake
Growth hormone	Lipolysis
	Glucose metabolism in adipose tissue
Vasopressin	H_2O transport
	Urea transport
	Na^+ transport
Oxytocin	Enzyme secretion
MSH	Melanophore expansion (frog skin)
Parathyroid hormone	Ca^{2+} uptake (intestine)
	Mobilization of skeletal Ca^{2+}
	Protein synthesis in bone
	Gluconeogenesis (kidney)
	Phosphaturia (renal cortex)
	Ca^{2+} resorption (bone)
Pancreozymin	Amylase secretion

Table 1.5. (Contd.)

Hormone	Principal Responses
Secretin	Pancreatic secretion
Gastrin	Lipolysis
Testosterone	Enzymes of seminal vesicles
Progesterone	Oviduct protein (avidin) synthesis
Oestrogen	Uterus (enzyme synthesis)
	Pineal activity
Thyroid hormones	Metabolic rate
	Heart rate
	Lipolysis
	Spermatozoal activity

Reproduced from Major and Kilpatrick (1972).

small intestine (Gray et al., 1970).

Initially cyclic GMP was considered to mimic cyclic AMP; on these lines it seemed relatively unimportant in that it had lower potency in, for example, a cell free system and its endogenous level in plasma and urine is an order of magnitude lower than cyclic AMP (Hardman et al., 1971b). It is now realised that cyclic GMP formation is under a separate control mechanism from cyclic AMP (Kuo et al., 1971; Steiner et al., 1972; Ferrendelli et al., 1972; Thompson et al., 1973). The two cyclic nucleotides may show reciprocity in their intracellular levels (Illiano et al., 1973) but this does not always follow (Ferrendelli et al., 1973). It is suggested that these two nucleotides antagonise the intracellular effects of each other, the so-called 'Yin-Yang' hypothesis (Estensen et al., 1973). This concept of mutual antagonism is supported by studies on cell growth and differentiation (Hadden et al., 1972; Boring et al., 1972; Vorhees et al., 1973; Kram & Tomkins, 1973). It has also been suggested that the nature of the primary messenger interaction with the receptor site of "purine nucleotide cyclase" can select the nucleotide specificity (i.e. either ATP or GTP as substrate) of this enzyme (Illiano et al., 1973). This 'induced specificity' could explain the reciprocal relationship between the intracellular levels of the two cyclic nucleotides.

Cyclic GMP-dependent protein kinases have been demonstrated in tissues from lobster and other arthropoda

(Kuo & Greengard, 1970; Kuo et al., 1971) and in bovine (Greengard & Kuo, 1970; Kuo et al., 1971) and rat cerebellum (Hoffman & Sold, 1972). Physiological roles for such kinases have not yet been proposed; however, the demonstration that they do occur in nature would suggest that a unitary hypothesis for cyclic GMP action, similar to that proposed for cyclic AMP by Greengard et al. (1972) is feasible, protein phosphorylation being the primary event.

Cyclic GMP has been shown to be involved in the initiation of DNA synthesis in haemopoietic stem cells (Byron, 1973), the regulation of DNA synthesis in lymphocytes (Hadden et al., 1972), the mediation of lymphocyte transformation (Estensen et al., 1973; Illiano et al., 1973), the regulation of the pleiotypic effects of cyclic AMP at the microtubular level (Kram & Tomkins, 1973), the mechanism of cholinergic transmission (George et al., 1970; Eichhorn et al., 1974), the immune response (Watson et al., 1973), and possibly in photoreceptor function (Goridis & Virmaux, 1974).

1.8. Regulation of Cyclic AMP Levels in Brain Tissue and its Role in Neurotransmission

Phosphodiesterase(s) (Table 1.4) and adenyl cyclase show a much higher activity in the brain than in other mammalian organs (Robison et al., 1971). The activity of adenyl cyclase is some 20 times higher than that found in other mammalian tissues, most of it being associated with the grey matter (Weiss & Kidman, 1969). De Robertis et al. (1967) suggested a presynaptic location for adenyl cyclase, whereas

Weiss & Costa (1967) proposed a postsynaptic location for the enzyme. Most of the brain phosphodiesterase is associated with the microsomal and synaptosomal fractions (Cheung & Salaganicoff, 1966; De Robertis et al., 1967; Johnson et al., 1973).

The level of cyclic AMP in any tissue is a balance between the activities of these enzymes, yet in brain, despite the fact that the total activity of phosphodiesterase is some 100 times greater than that of adenyl cyclase, this tissue still has the highest content of cyclic AMP in mammalian tissue (Ebadi et al., 1971). This is due to the control mechanisms governing the activities of the two enzymes (Rasmussen et al., 1972; Bitensky & Gorman, 1972a) and their distinct subcellular localisations (De Robertis et al., 1967; Greengard et al., 1972).

Daly et al., (1972) proposed a model for a cyclic AMP-generating system in brain (Figure 1.5). Basically the system consists of a small compartmentalized pool of adenine nucleotides which serve through the action of adenyl cyclase as precursors of cyclic AMP, which in turn evokes a physiological response or re-enters the adenine nucleotide pool via the action of phosphodiesterase. The activity of the adenyl cyclase is governed by receptors which may either control independent pools or may interact with one pool of adenine nucleotides. Incubation of brain tissues with $[^{14}C]$-adenine provides evidence that such small compartmentalized pools of adenine nucleotides do exist and serve as precursors for cyclic AMP (Shimizu et al., 1970; Daly et al., 1972).

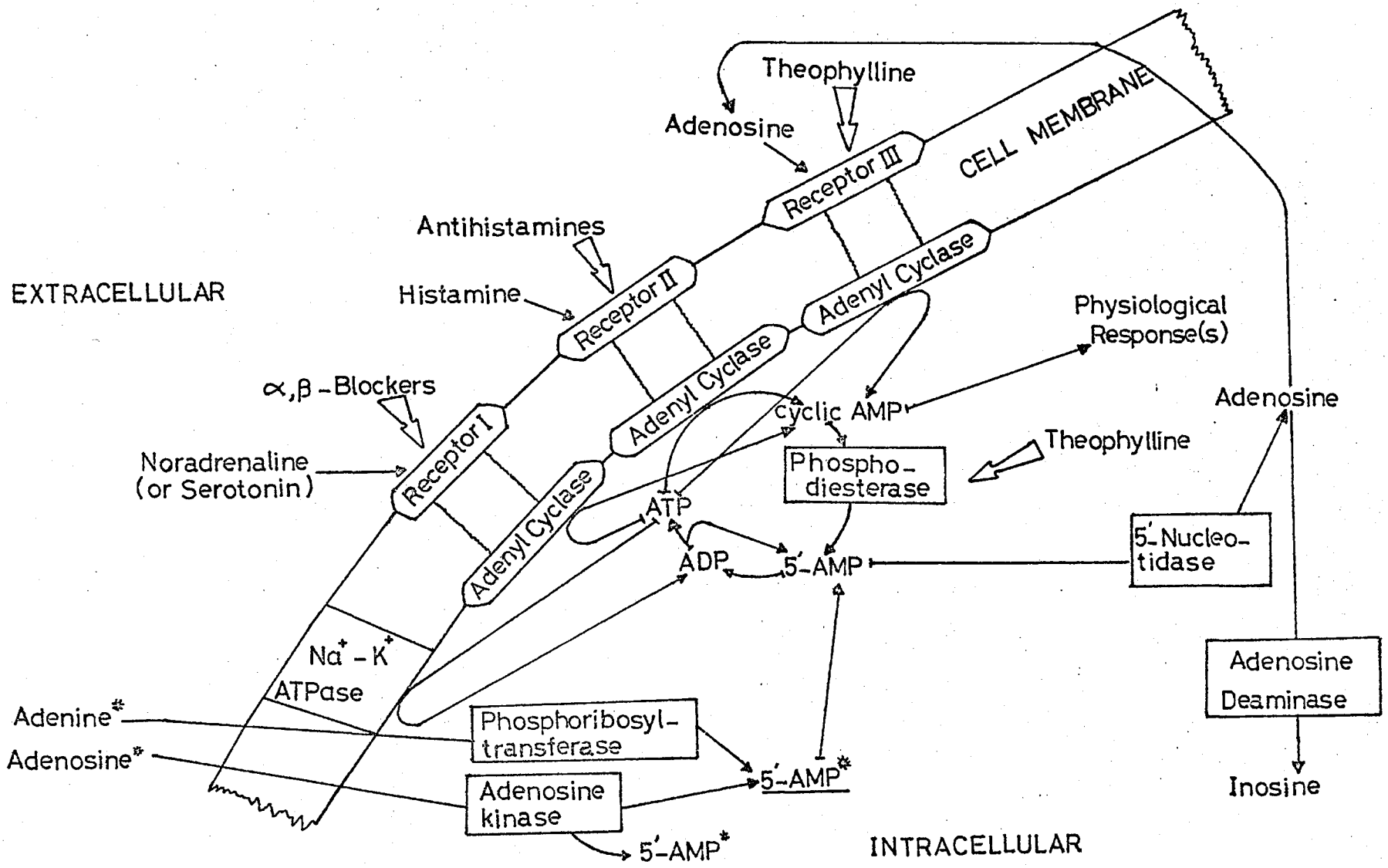


Figure 1.5: Hypothetical model for a cyclic AMP-generating system present in guinea pig cerebral cortex. Stimulatory activity is indicated by thin arrows directed at receptors I, II and III. Inhibitory activity is indicated by thick arrows. (Daly et al 1972)

Under control conditions cyclic AMP accounts for less than 0.3% of the pool, which consists primarily of ATP and to a lesser extent ADP and 5' AMP. However, under stimulatory conditions as much as 40% of the labelled adenine nucleotides in the pool could be converted into cyclic AMP in guinea pig cerebral cortical slices and 78% in human cerebral slices (Shimizu et al., 1970; Kodama et al., 1973; McIlwain, 1974). The compartmentalized pools are remarkably stable and do not mix with the remaining 90% or so of adenine nucleotides in the brain tissue. Thus the endogenous levels of cyclic AMP after maximal stimulation represents less than 10% of the total adenine nucleotides in brain tissue.

Both adenine and adenosine can serve as precursors of cyclic AMP in brain tissue (McIlwain, 1974) with adenine showing a much higher incorporation into the cyclic AMP pool than the nucleoside. The incorporation of adenine involves phosphoribosyl transferase activity which has been shown to be associated to a large extent with the cyclic AMP precursor pools of cerebral cortical tissues (Schimizu & Daly, 1972); for adenosine, a kinase is required which is less specifically associated with cyclic AMP precursor pools. Kuroda and McIlwain (1973) have confirmed by subcellular fractionation studies different distributions of label after pre-incubation of cerebral tissue with $[^{14}C]$ - adenine and $[^{3}H]$-adenosine.

The cyclic AMP-generating system responds to a number of substances with an enhanced accumulation of cyclic AMP.

Several biogenic amines can be shown to modulate this system in cerebral cortical slices. Adrenaline and noradrenaline are effective presumably by interaction with α - and β - receptors (the analogue isoproterenol which specifically interacts with β - receptors is much less effective); dopamine is inactive. Histamine (and the terminal N - and N,N - dimethyl derivatives) is markedly active whereas the ring N - methyl derivative, a natural metabolite, is inactive. Serotonin elicits a similar response to the active catecholamines. There is evidence for adenosine as a regulatory amine as well as a precursor for adenine nucleotides; this regulatory side is blocked by 5'-deoxyadenosine and 3'-deoxyadenosine - the latter blocking the stimulatory effect of both adenosine and histamine. The relative magnitude of effects evoked by these amines with regard to accumulation of cyclic AMP varies greatly from species to species and from one brain region to another (Huang & Daly, 1972; Daly et al., 1972; Skolnick et al., 1973; Schultz & Daly, 1973). In addition depolarizing agents (Schimizu et al., 1970; Huang et al., 1972), electrical stimulation (Kakiuchi et al., 1969), tricyclic drugs such as the antidepressants imipramine, desipramine, protryptyline and the tranquillizer chlorpromazine (Kodama et al., 1971; Huang & Daly, 1972), have been demonstrated to enhance accumulation of cyclic AMP in cerebral slices. These are postulated to exert their action on the cyclic

AMP-generating system via release of adenosine, which then interacts with an adenosine-sensitive regulatory unit for adenyl cyclase (Huang & Daly, 1972).

Radioautography of guinea pig cerebral cortical slices which were labelled by incubation with $[^{14}C]$ -adenine indicated that a major portion of the radioactive nucleotides in the slice are associated with synaptic structures. No radioactivity is associated with cell bodies, axons or glial cells (Daly et al., 1972). Thus the precursor pools appear to be associated with synaptic structures. This is further supported by evidence that adenyl cyclase (McIlwain, 1974) and the catabolic enzyme being immediately adjacent to the synaptic membrane (Florendo et al., 1971) are associated with postsynaptic nerve endings. The above evidence suggests a possible role of cyclic AMP in regulating physiological responses in the hypothetical model of figure 1.5. This is further supported by the finding of a parallel distribution of cyclic AMP stimulated protein kinases (Johnson et al., 1971a, 1972a). Haas et al., (1972) have also suggested that cyclic AMP might play a role in the maturation of nervous tissue and nerve regeneration. In fact the protein nerve growth factor may act via the stimulation of adenyl cyclase.

Greengard et al. (1972) proposed a hypothesis for the role of cyclic AMP in the physiology of synaptic transmission in sympathetic ganglia (figure 1.6). According to his hypothesis, activity in the presynaptic fibres leads to depolarization (excitation) of postganglionic neurons with

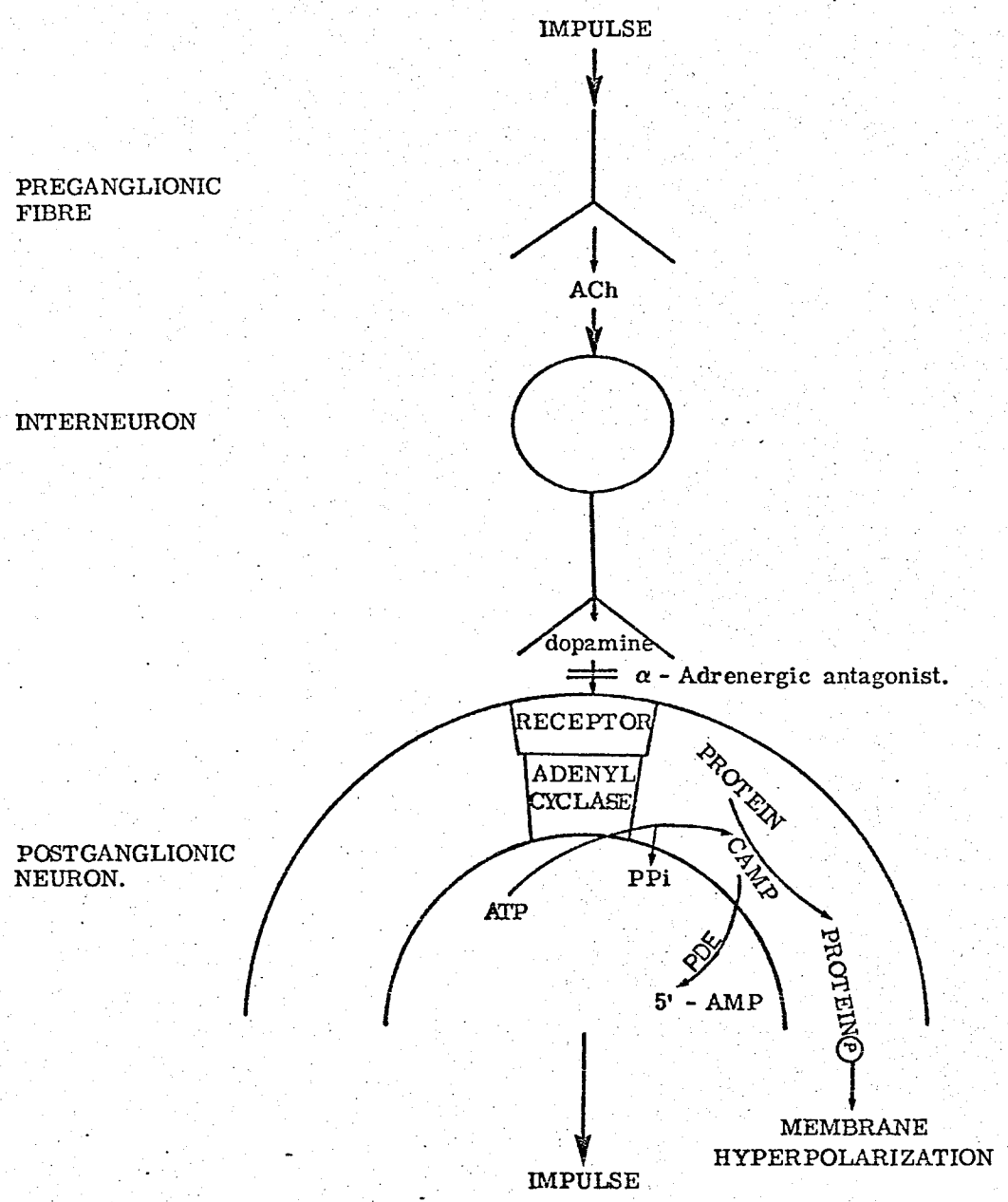


FIGURE 1.6: MODEL FOR SYNAPTIC TRANSMISSION (Greengard , 1972.)

the generation of impulses in the postganglionic axons. Activity in the presynaptic fibres leads to acetylcholine mediated excitation of dopamine-containing interneurons resulting in the release of dopamine (Keen & McLean, 1972; Greengard et al., 1972) which then activates (muscarinic adrenergic mechanism; Schorderet et al., 1972) adenyl cyclase in the plasma membrane of the postganglionic neurons and, thereby, causes the accumulation of cyclic AMP in these neurons. The newly formed cyclic AMP activates a protein kinase leading to the phosphorylation of a protein constituent of the plasma membrane (Weller & Rodnight, 1970, 1973). This causes an alteration in the movement of ions across the membrane, resulting in hyperpolarization. Thus cyclic AMP mediates dopaminergic transmission and, thereby, modulates cholinergic transmission in the ganglion. Greengard's hypothesis is supported by measurements of synaptic potentials and changes in resting membrane potentials of superior cervical ganglia (McAfee & Greengard, 1972). Both dopamine and db-cyclic AMP cause a hyperpolarization of post ganglionic neurons, which is inhibited by PGE_1. Db-cyclic GMP causes a depolarization of postganglionic neurons suggesting that cyclic GMP mediates synaptic transmission of muscarinic cholinergic synapses.

Adrenergic transmission is also believed to be mediated by cyclic AMP. Gilman and Nirenberg (1971) found that noradrenaline or isoproterenol caused a marked rise in cyclic AMP in glial tumor cells in tissue culture. Hoffer et al.,

(1972) provided evidence for a noradrenaline synapse in the mammalian cerebellum and suggested that the effect of noradrenaline on the postsynaptic Purkinje cells is mediated by cyclic AMP. These investigators have observed that noradrenaline or cyclic AMP causes hyperpolarization in Purkinje cells and this is potentiated by phosphodiesterase inhibitors. However, Lake and Jordan (1974) were unable to mimic the strongly depressant action of noradrenaline on spontaneously firing Purkinje cells by the microiontophoretic application of cyclic AMP. They have therefore challenged the hypothesis that cyclic AMP is the second messenger for noradrenaline in the cerebellum. Yarborough et al., (1974) present data indicating that the inhibitory effects of biogenic amines involve changes in calcium ions flux, and Rasmussen et al., (1972) have suggested that calcium serves to couple hormone-receptor interactions to physiological effects in a variety of peripheral systems.

Breckenridge et al., (1967) and Goldberg and Singer (1969) proposed that cyclic AMP mediates the presynaptic action of adrenaline on the nerve endings in the skeletal neuromuscular junction by potentiating the release of acetylcholine. Both db-cyclic AMP and methylxanthines mimic the effects of catecholamines on end plate potentials. However, Greengard et al. (1972) and Ginsborg and Hirst, (1972) have results opposed to the role of cyclic AMP in neuromuscular transmission.

Injection of catecholamines into the cerebral ventricles of cannulated rats increased the content of cyclic AMP *in vivo* (Buckard, 1972). Gessa *et al.*, (1970) injected db-cyclic AMP into various regions of the brain of cannulated rats and cats and elicited specific responses (Table 1.6). Pre-treatment with reserpine, which destroys noradrenaline stores in nerve cells or with chlorpromazine, which blocks adrenergic receptor sites, did not prevent this cyclic AMP response indicating a postsynaptic site of action.

1.9. Cyclic GMP and the Nervous System

The situation with regard to cyclic AMP and neurotransmission is far from clear and cyclic GMP studies are even more in their infancy. The highest concentrations of cyclic GMP in rat brain occur in the cerebellum (Steiner *et al.*, 1972). Early observations in the rat heart and brain showed that acetylcholine and oxotremorine which increases myocardial acetylcholine content, produced marked increases in the cyclic GMP content of the tissues (George *et al.*, 1970; Ferrendelli *et al.*, 1970); thus cyclic GMP may be involved in cholinergic transmission (Hardman *et al.*, 1971). Lee *et al.*, (1972) and Kebabian (1972) found that the increase in cyclic GMP elicited by acetylcholine was muscarinic in nature. McAfee and Greengard (1972) using the lipid soluble db-cyclic GMP showed that this nucleotide mimicked the physiological effects of acetyl-choline in superior cervical ganglia, giving further support to the role for cyclic GMP in cholinergic transmission.

Table 1.6. Behavioural and vegetative effects observed after the injection of db-cyclic AMP in the cat brain

Site of Injection	Effects
Cisterna magna	Scratching, convulsions (miosis, mydriasis, salivation, defecation, micturition).
Lateral ventricle	Psychomotor stimulation, convulsions.
Third ventricle	Psychomotor stimulation, convulsions.
Hypothalamus (anterior)	Psychomotor stimulation, convulsions, adrenergic effects.
Hypothalamus (posterior)	Psychomotor stimulation, convulsions, cholinergic effects.
Formatio reticularis mesencephali	Catatonia, psychomotor stimulation, contraversive turning of the head.
Amygdala	Contraversive circling movement, Jacksonian convulsions, convulsions, rage.
Caudate nucleus	No effects.
Putamen	No effects.
Pallidum	No effects.
Cerebellum (fastigial nucleus)	Non-rapid eye movement sleep.

Reproduced from Gessa et al., (1970).

In cerebellar slices from rat (Kuo et al., 1972) the acetylcholine dependent increase in cyclic GMP, was matched by a decrease in cyclic AMP content, while noradrenaline-induced increase in cerebellar cyclic AMP content was found to decrease cyclic GMP levels.

A study in mouse cerebellar slices on drugs (e.g. amphetamine) which affect non aminergic transmitter mechanisms and motor activity in vivo (Ferrendelli et al., 1972), showed that increases in the cyclic GMP levels in vitro could be correlated with the pharmacological actions of these drugs.

Ferrendelli et al., (1973) have found that the depolarising agents veratridine and ouabain and elevated concentrations of K^+ caused marked increases in the cyclic GMP content of mouse cerebellar slices, reaching about thirty times the basal levels. Atropine has no effect on these increases and acetylcholine does not mimic them, indicating that the cholinergic mechanism is not involved. Also in contrast to the cholinergic-mediated increases in cerebellar cyclic GMP content these depolarising agents increase the cyclic AMP content. The depolarisation induced increase in cyclic GMP level involves calcium flux and synaptic transmission (Blaustein, 1971).

These preliminary investigations of the effect of depolarising agents and acetylcholine on mouse and rat cerebellar cyclic GMP content would suggest that the complexity of these responses may be similar to those observed with cyclic AMP in guinea pig neocortex (Daly et al., 1972); however, in view of the paucity of data available at the moment this observation

is somewhat speculative. It is increasingly clear that the content of cyclic AMP and cyclic GMP in nervous tissue are to a certain extent under separate control mechanisms (Kuo et al., 1972) but whether the "Yin-Yang" hypothesis (Estensen et al., 1973) is applicable to nervous tissue remains to be seen.

1.10. Cyclic AMP in Clinical conditions

Cyclic AMP has been implicated in a variety of clinical conditions with perhaps the most detailed studies being made on pseudohypoparathyroidism, a rare hereditary disease in which parathyroid hormone appears incapable of stimulating cyclic AMP formation in the target tissues in vivo (Chase et al., 1969a; Marcus et al., 1971; Murad and Pak, 1972). It would seem that the lesion lies in the receptor rather than in the catalytic centre. Other disorders in which cyclic AMP has been implicated include bronchial asthma (Szentivanyi, 1968; Alston et al., 1974), allergic skin reactions (Burton & Graves, 1972), diabetes insipidus (Fichman & Brooker, 1972), diabetes mellitus (Cerasi and Luft, 1970), and thyrotoxicosis (Bitensky & Gorman, 1972).

Some bacterial infections may also involve cyclic AMP, and an especially interesting example is cholera. The toxin produces an apparently irreversible increase in adenyl cyclase activity, leading to prolonged high levels of cyclic AMP in intestinal epithelia; this in turn leads to the debilitating loss of fluids and electrolytes characteristic of this disease (Kimberg et al., 1971;

Bennett et al. 1975). Tumors and transformed cells have intracellular levels of cyclic AMP significantly lower than corresponding normal cells (Sheppard, 1972) and db-cyclic AMP reduces the rate of growth of transformed cells (Johnson et al., 1972). Burger et al., (1972) proposed that a reduced level of cyclic AMP may trigger the mitotic cycle and Korinek et al., (1973) suggested that cell division control involves a change in cell surface which in turn requires mRNA synthesis induced by cyclic AMP. Finally cyclic AMP has been implicated in affective disorders (mania and depression).

1.11. Cyclic AMP and its Correlation with affective disorders

Abdulla and Hamadah (1970) showed that 24-hour cyclic AMP levels were high in manic and low in depressed patients (table 1.7). These authors put forward the hypothesis that cyclic AMP is the driver rather than the passenger in affective disorders, i.e. depressive illness is due to a severe fall in cyclic AMP concentration in the cells of all tissues including those of the central nervous system. Evidence supporting this hypothesis includes the demonstration that chlorpromazine and tricyclic antidepressants inhibit phosphodiesterase activity in vitro, thus increasing cyclic AMP concentration (Abdulla and Hamadah, 1970; Ramsden, 1970). Kodama et al., (1971) reported that the tricyclic antidepressants stimulated cyclic AMP formation in brain slices.

Paul et al., (1970) in a study on 47 patients divided

Table 1.7: Urinary Cyclic AMP Excretion in Patients with Affective Disorders before and after Treatment

Patient	Age	Diagnosis	Previous Attacks	Treatment: Antidepressants	Treatment: ECT	Treatment: Tranquillisers	Clinical rating 1st collection	Progress rating (2nd collection)	Urinary cyclic AMP (nmol/24h) 1st collection	Urinary cyclic AMP (nmol/24h) 2nd collection
1	68	D	2	Amitriptyline 150 mg			Depression ++	Recovered	796	2808
2*	46	M-D	3			Chlorpromazine 300 mg Haloperidol 4.5 mg	Hypomania	Depression	5440	738
3	28	M-D	2	Nortriptyline 150 mg		Chlorpromazine 300 mg	Depression +++	Recovered	560	1716
4	56	D	1		4		Depression +++	Improved	254	636
5	64	M-D	6	Nortriptyline 150 mg		Chlorpromazine 300 mg	Depression +++	Hypomania	998	6410
6	55	M-D	16	Imipramine 150 mg		Haloperidol 9 mg	Depression ++	Recovered	0	762
7	71	D		Amitriptyline 75 mg			Depression +++	Improved	558	644
8	35	D	2	Nortriptyline 75 mg		Chlordiazepoxide 30 mg	Depression ++	No change	324	326
9	47	D	2	Imipramine 75 mg	7	Diazepam 15 mg	Depression +++	No change	642	336
10	44	M-D	7	Imipramine 200 mg	4		Depression +++	Hypomania	270	3340
11	54	D	5	Protriptyline 30 mg	3	Chlorpromazine 150 mg	Depression +++	Recovered	324	2910
12	80	D	5	Imipramine 75 mg		Stelazine 300 mg	Depression +++	Recovered	420	872
13	54	M-D	7			Haloperidol 4.5 mg	Hypomania	Improved	3560	2220
14	65	D	3	Amitriptyline 150 mg		Chlordiazepoxide 15 mg	Depression ++	Recovered	1104	4828
15	58	D	1	Amitriptyline 75 mg			Depression ++	Improved	326	628
16	64	D	0	Nortriptyline 150 mg			Depression +++	Improved	408	960
17	23	D	0	Nortriptyline 150 mg			Depression ++	Improved	762	1462
18	43	M-D	16	Nortriptyline 75 mg	6	Chlorpromazine 300 mg	Depression +++	Improved	670	1226
19	38	M-D	6			Haloperidol 9 mg	Mania	Improved	15116	7598
20	29	M-D	4	Nortriptyline 150 mg			Depression ++	Recovered	622	1960
22	54	D	2	Imipramine 150 mg	1		Depression ++		520	
23	66	D	1	Protriptyline 30 mg			Depression ++		1088	
24	62	D	1	Protriptyline 30 mg	5		Depression ++		530	
25	67	D	2	Protriptyline 30 mg	6	Chlorpromazine 120 mg	Depression +++		376	
26	20	M-D	0			Chlorpromazine 400 mg	Hypomania		3286	

* The patient was readmitted 3 months later with moderate depression after a short period of normal mood and urinary cyclic AMP output of 600 nmol per 24 hours.

A urine analysis done 3 weeks after the first collection when the patient was severely depressed showed cyclic AMP output of 212 nmol per 24 hours.

D = Depressive. M-D = Manic-depressive.

Normal range (18 female members of nursing staff) was 1,000-3,400 nmol/24h.

them into four groups on the basis of their 24-hourly cyclic AMP excretion. Their results are summarised in table 1.8.

Table 1.8.

Group	n	Urinary cyclic AMP µmol/24h ± SEM
Normal	10	5.64 ± 0.68
Psychotic depression	7	3.64 ± 0.19
Neurotic depression	25	6.70 ± 0.37
Manic	5	9.94 ± 1.88

No correlation between age, sex and cyclic AMP excretion was found. Paul et al., (1971a) reported a marked elevation in urinary cyclic AMP at the time of the switch from depression into mania.

It has been suggested that changes in urinary cyclic AMP reflect exercise rather than the clinical status of depression or mania (Eccleston et al., 1970). However, Paul et al., (1971b) found that there was no significant difference in cyclic AMP excretion between 10 hyperkinetic children and an age-matched control group. They also reported that physically active and inactive depressed patients show similar (low) levels of cyclic AMP. No significant difference in urinary cyclic AMP levels were found in the psychotic phases of two patients with periodic

catatonia, one of whom exhibited profound stupor, whereas the other exhibited violent and destructive episodes (Perry et al., 1973). Prolonged physical activity in normal controls was found either to have no effect (Paul et al., 1971b; Owen, 1975), or cause only an insignificant increase in cyclic AMP excretion (Williams et al., 1972). However, Heath et al., (1973) reported a rise in plasma and urinary cyclic AMP after exercise (see Chapter 3).

Brown et al., (1972) studied four patients - 1A, manic-depressive receiving amitriptyline, chlorpromazine or haloperidol and ECT (4); 1B, manic-depressive as 1A but no ECT but chloramphenicol for a urinary infection; 1C, manic receiving phenothiazines and lithium carbonate; 1D (the only male) receiving lithium carbonate after an episode of schizophrenic excitement. There was no obvious correlation between cyclic AMP and mood for this diverse group. Patient 1C showed a 70% decrease in cyclic AMP excretion coincident with the commencement of lithium therapy. Jenner et al., (1972) also reported a failure to obtain a correlation between cyclic AMP excretion and 'mood' in a "number of depressed and manic-depressive patients". However, they found a marked correlation in a patient with a regular 48-hour cycle of 'mood'. They suggested that cyclic AMP changes were secondary to ADH variation and thus reflected pituitary or hypothalamic function. Lithium carbonate was effective in the control of this patient. Dousa and Hechter, (1970) implied that the

therapeutic effect of lithium in the treatment of mania may be due to inhibition of cyclic AMP formation. Paul et al., (1971b) reported that lithium treatment of manic and manic depressive patients can cause changes in urinary cyclic AMP, which followed the direction of clinical change; and L-dopa administration to depressed patients is paralleled by dose related increases in urinary cyclic AMP.

Hansen (1972) has reported that cyclic AMP in blood is significantly lower in a group of depressed patients compared with controls. He suggests, however, that this is a reflection of changes in ATP concentration. His measurement procedure was individualistic and this is indicated by normal blood levels of cyclic AMP reported as 2.3 μg/ml (3 orders of magnitude higher than other workers). Robison et al., (1970a) found no correlation between clinical condition and cyclic AMP level in cerebrospinal fluid pooled from a manic group, or a depressed group, or a 'neurological' control group. Cramer et al., (1972, 1973) supported this lack of correlation in a study of 'baseline' cerebrospinal fluid samples. However, administration of 'probenecid' led them to conclude that the "turnover" of cyclic AMP in brain is significantly greater in the manic group than in the depressed or the 'neurological' control group.

1.12. Affective Disorders

1.12.1. Definition

The term "affective disorders" is used for a group of mental diseases with a primary disturbance of affect (emotional-mental status) from which all other symptoms seem more or less directly derived (Slater & Roth, 1969). The most important characteristics of the affective disorders are: anxiety and irritability; periodicity, i.e. elevation and depression of mood alternating with free intervals in which there is a complete return to normality; and the capacity for recovering from a single attack without impairment of mental integrity.

There are two major subgroups of primary affective disorders; the unipolar, or depressive disease and the bipolar or manic-depressive disease (in which periodic attacks of mania occur). This classification is based on genetic* (Cadoret et al., 1970; Perris, 1971) and general background. It includes evaluation of personality and temperament (Slater and Roth, 1969) and emotional status post-partum (Baker et al., 1971). Response to drugs and other forms of treatment (Murphy et al., 1971; Goodwin et al., 1972; Buchsbaum et al., 1971) and biochemical findings (Dunner et al., 1971, 1972; Murphy & Weiss, 1972). Mania and involutional melancholia (depressions of later life) are also included in the affective group of mental illnesses, whereas mental

*Also see Becket (1974).

conditions, in which the affective disturbances are mainly secondary alterations, are excluded.

Unipolar affective disorders are further subdivided into endogenous (or psychotic) depression and reactive (or neurotic) depression (Slater & Roth, 1969). Many psychiatrists regard the two conditions as extremes of the same disease state (Rosenthal & Gudeman, 1967a,b). However, the presence of qualitative differences, differential response to treatment and distinct clinical features provide evidence for the existence of two types of illness. Slater and Roth (1969) stated that the clinical features, which significantly correlate with the diagnosis of endogenous depression are: early morning wakening, depression worse in the morning, qualitative change in affect, marked degree of psychomotor retardation, duration of symptoms of one year or less, age 40 years or above, depression regarded as being of considerable depth, failure of concentration, weight loss of 3 kg or more and a history of previous attacks. On the other hand the clinical features characteristic of reactive depression are: responsiveness of depression to environmental change (i.e. attacks occur in response to stress), self-pity and inadequacy, hysterical features, initial insomnia, depression worse in the evening, sudden onset, irritability, hypochondriasis, obsessionality and variability of illness. As might be expected, there is an overlap in the symptoms of endogenous and reactive forms of depression.

1.12.2. Incidence - Basic Personality - Physical Constitution - Heredity

Estimations of the incidence of mental illnesses have been made from samples of the general population of several countries. The tendency in recent years is to find larger numbers of people suffering from affective diseases. At present the life-time expectation of developing such an illness in Western Europe is as high as 16% in women and 8% in men (Coppen, 1973). Ethnic variations have also been noted, for example, affective disorders were found to be commoner in people of Jewish stock and in the Hutterites of America (Slater & Roth, 1969). There is also some evidence that affective disturbances are more frequent in professional people than in the general population (Helgason, 1964). The distribution of onset of manic-depressive reaction by age in the United Kingdom is shown in figure 1.7.

Kretschmer, (1936) demonstrated an affinity between affective illnesses, basic personality and body type. The pyknic or ectomorphic physique (short, fat) is associated with cycloid (changeable) temperament and with an increased risk of manic-depressive psychosis. The endocrine system, with its evident influence on bodily habits and its subjection to a biological periodicity has been held responsible by some for the fluctuation of mood in affective diseases (Slater & Roth, 1969). The menstrual cycle provides a further perturbation and thus may explain

an increased risk in pre-menopausal females (figure 1.7), as may the stress of pregnancy and its aftermath.

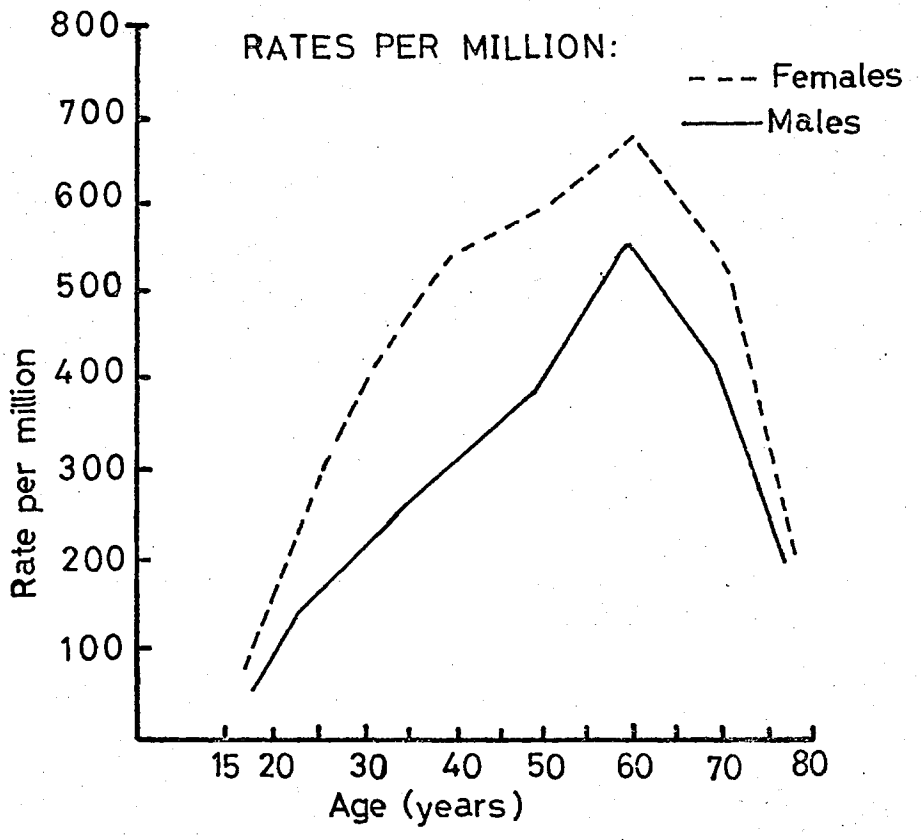


Figure 1.7. First admissions for manic-depressive reaction (from Slater & Roth, 1969).

Heredity is believed to play an important part in the aetiology of affective disorders (Winokur et al., 1971; Dorzab et al., 1971; Gershon et al., 1971; Baker et al., 1972; Bunney et al., 1972b; Nies et al., 1973). The following lines of evidence provide support for this view: there is an increased history of depression among the family members of depressed patients compared to the general

population; there is an increased concordance of monozygotic (75%) over dizygotic twins (38%). The involvement of a single autosomal dominant gene has been suggested (Hopkinson, 1964; Winokur & Clayton, 1967). More recently evidence has been built up for a polygenic, rather than dominant gene, transmission of the depressive trait (Baker et al., 1972; Perris, 1971). However, mania could be attributable to a dominant gene (Slater & Roth, 1969).

1.13. Biochemical aspects of Affective Disorders

Affective disorders can now be described as truly psychosomatic conditions. The view that biochemical changes are of primary importance in their aetiology, is at present widely accepted, though social and psychological factors are also of great importance. The mode of interaction of these environmental events with the biochemistry of the nervous system to produce the syndromes of depression and mania is far from clear. Two major areas of research have involved cations (especially Na^+, K^+) and biogenic amines.

1.13.1. Cations and Water

Early work on electrolyte and body fluids balance in affective disorders produced controversial results. Retention of sodium during depression and diuresis of sodium during mania were reported by Strom-Olsen et al., (1958) and Crammer (1959). However, Russell (1960) could

not detect any change in water, sodium or potassium balance during clinical improvement from depressive illness.

The introduction of isotope-dilution techniques enabled estimates to be made of the distribution of electrolytes - the relative concentrations of sodium and potassium in cells and in the extracellular space. The most consistent finding was an increase in residual sodium (mostly intracellular sodium plus a small amount of exchangeable bone sodium) both in depression and mania, the increase amounting to about 50% of the normal in the former, and to the highly significant 200% in the latter. The values returned to the normal range on recovery (Coppen, 1970; Singh, 1970; Baer et al., 1970).

However, Glen and Bellinger (1973) found the intracellular concentration of erythrocyte sodium to be within normal limits for both manic and depressed patients, and Mendel et al. (1971) reported low sodium concentrations in erythrocytes from manics. Extracellular sodium was found to be within the normal range during either a depressed or a manic phase, but on recovery there was a significant reduction in its concentration (Coppen et al., 1966). The decrease was more marked in mania.

The values for total body potassium, intracellular potassium and the intra-/extracellular ratio for K^+, obtained from depressive and manic patients were reported to be consistently lower than normal both during illness

and on recovery (Coppen, 1970; Singh, 1970). Baer et al., (1970), however, could not show any measurable change in body potassium with recovery, thus confirming earlier findings of Gibbons (1960). Furthermore, body K^+ in patients was not found to differ significantly from that of a control population (Baer et al., 1970; Glen & Bellinger, 1973).

Sodium is extruded from the cell by means of the sodium pump, which is associated with Na^+, K^+-stimulated ATPase. It has been suggested that the cause of the increased intracellular sodium, in affective disorders, could reside in a genetically inherited enzymatic membrane defect which reduces the ability of the membrane to remove sodium from the cell (Singh, 1970; Bunney et al., 1972a; Dick et al., 1972; 1974; Naylor et al., 1973; Glen and Reading, 1973). Glen et al., (1968, 1969) reported that active transport of Na^+ across the duct walls of the parotid gland is significantly diminished in depressed patients compared to healthy individuals. Maizels (1968) and Glen et al., (1972) have shown that Li^+ can stimulate Na^+ efflux from the erythrocyte. Calcium and magnesium ions are known to be involved in membrane transport systems and Li^+ treatment of affective disorders has also been found to increase the plasma concentration of these ions (Mellerup et al., 1973; Lyttkens et al., 1973).

The distribution of body water has also been investigated. An increase in the total body water and

extracellular water, was observed during recovery from depression (Coppen, 1970), whereas no significant change was found on recovery from a manic episode (Singh, 1970).

Studies on the brains of suicides by Shaw et al., (1969) indicated that electrolyte changes could occur in the CNS. Support for this was provided by Kjeldsen et al., (1973) who showed that Li^+ decreases Na^+ and K^+ concentrations in brain-cortex slices. The significance of electrolyte changes in mental disorders is controversial. However, alterations in the balance of electrolytes in the central nervous system could result in a reduced rate of protein synthesis (Coppen, 1970) and changes in the transport of biogenic amines, amino acids and sugars (Bunney et al., 1972b).

It has been shown by many investigators that lithium salts are effective both in the treatment of affective disorders (Johnson et al., 1971; Prien et al., 1972; Coppen, 1973; Samuel & Gottesfeld, 1973), and in their prophylaxis (Baastrup et al., 1970; Coppen et al., 1971; Hullin et al., 1972; Prien et al., 1973; Coppen, 1973). Although a considerable volume of basic biological information has been obtained on the effect of lithium cations, the site and the mode of its action in patients suffering from an affective illness is still unknown. Theories of action include interference with the electrolyte balance in body fluids and tissues (Murphy and Bunney, 1971; Kjeldsen et al., 1973; Jenner, 1973; Shaw, 1973; Samuel & Gottesfeld, 1973); change in the metabolism of biogenic

amines and other neurotransmitters (Colburn et al., 1967; Katz & Kopin, 1970; Shaw et al., 1972; Gershon, 1972; Shaw, 1973; Samuel & Gottesfeld, 1973; Friedman and Gershon, 1973; Gottesfeld et al., 1971;); change in hormonal levels (Platman et al., 1970; Sachar et al., 1970; Murphy et al., 1970; Krulik, 1971; Noyes et al., 1971; Mannisto et al., 1971; Barrow et al., 1971; Temple et al., 1971) or energy supply in the CNS (De Feudis, 1971, 1972; Mellerup et al., 1970, 1973; Shopsin et al., 1972; Schou, 1973; Samuel & Gottesfeld, 1973).

In comparison with the many studies of effects of Li^+ on nerve endings relatively little work has been carried out on its possible interference with conditions in the synaptic cleft and at the postsynaptic receptor site. It has been suggested that Li^+ is bound by the mucopolysaccharides of the synaptic cleft and thus may produce significant alterations in transmission properties (Schou, 1973). There is evidence that the cyclic AMP system is involved in the mechanism underlying postsynaptic receptor response (Greengard et al., 1972). Lithium inhibits adenyl cyclase activity, in vitro, in rabbit cerebral cortex and rat hypothalamus (Dousa & Hechter, 1970; Forn & Valdecasas, 1971; Palmer et al., 1972a) but stimulates adenyl cyclase activity in glial tissue (Schimmer, 1971). In humans 24-hour urinary excretion of cyclic AMP has been found to increase in mania and decrease in depression (section 1.11). Paul et al., (1971b) reported that lithium

treatment of manic and manic-depressive patients can cause changes in urinary cyclic AMP which parallel the direction of clinical change. Jenner et al., (1972) have reported that the mood change in a patient who showed a regular switch (48-hour cycle) from mania to depression was paralleled by changes in urinary ADH and urinary cyclic AMP. The peptide hormone and the cyclic nucleotide both decreased in depression and increased in mania. Lithium was very effective as a prophylactic agent in this patient. When the lithium salt was replaced by a placebo the 48-hour rhythm of illness returned (Jenner, 1973).

Lithium inhibition of adenyl cyclase system has been reported in thyroid (Burke, 1970; Williams et al., 1971), kidney (Dousa & Hechter, 1970; Forrest et al., 1971; Harris & Jenner, 1971, 1972; Jenner, 1973; Wraae et al., 1972; Geisler et al., 1972), fat cells (Birnbaumer et al., 1969), ovary (Smith et al., 1971) and toad bladder (Rotenberg et al., 1971; Harris & Jenner, 1972).

1.13.2. Endocrine Functions

Endocrine studies in affective disorders have concentrated on adrenocortical and thyroid activity.

Adrenocortical Activity

Depression was long ago noted to be prevalent in patients diagnosed as having Cushing's syndrome (Glaser, 1953; Spillane, 1951) whereas euphoria occurred in patients receiving exogenous corticosteroids (Glaser, 1953; Lidz

et al., 1952). In more recent studies patients suffering from depressive illness were shown to have high morning plasma cortisol levels which fell on recovery (Bridges & Jones, 1966; Coppen, 1970; Carpenter & Bunney, 1971a). There is evidence that the hypothalamic-pituitary control mechanism in adrenocortical activity is normal in depressed patients (Carpenter & Bunney, 1971a; Shopsin & Gershon, 1971). However, Carpenter and Bunney, (1971a) suggested that the cortisol production rate and the metabolic clearance rate are proportionately elevated during a depressed phase, thus maintaining normal plasma concentration. On recovery the clearance rate continues to be elevated whereas the production rate falls, resulting in significantly lower plasma concentrations. During a manic phase normal morning and evening plasma levels were obtained but the evening values fell on recovery (Carpenter & Bunney, 1971b).

Few other steroids have been investigated in affective disorders. The plasma levels of 11-hydroxycorticosteroids were slightly higher in depression than in normal controls or recovered patients (Brooksbank and Coppen, 1967). The urinary excretion of 17-hydroxycorticosteroids was found to be elevated in depression (Ellman & Blacker, 1969), but normal in mania (Carpenter & Bunney, 1971b). Ferguson et al., (1964) reported low urinary levels of 11-deoxy and 17-oxosteroid excretion in depressive illness in female patients who returned to normal on recovery. These findings could not be confirmed for males by Coppen et al., (1967) but they

reported decreased urinary excretion of oestrone and oestradiol during depression. Finally, Goodwin et al., (1968) reported a decrease in the excretion rate of aldósterone in the depressive phase and an increase in the manic phase. The weight of evidence suggests an increase in adrenocortical activity in depressive illness. It seems probable that this is a "passenger" of the disorder not the "driver".

Thyroid activity

Investigations on the thyroid have shown no abnormalities or change in affective disorders (Coppen, 1967). However, recent studies have shown that the administration of the peptide thyrotropin releasing hormone (TRH) to patients suffering from depressive illness may effect a rapid clinical improvement albeit of short duration (Kastin et al., 1972; Prange & Wilson, 1972; Van der Vis Melsen & Wiener, 1972; Tiwary et al., 1972; Plotnikoff et al., 1971, 1972). Kastin et al., (1972), Prange et al., (1972) and Van der Vis Melsen and Wiener (1972) reported that the thyrotropin (TSH) response to TRH is diminished in depressed patients thus suggesting an abnormality in the hypothalamic/pituitary axis in some cases of depression. However, in a later report, Prange and his colleagues (1973) could not provide conclusive support for their earlier finding. Van der Vis Melsen and Wiener (1972) provided evidence that the therapeutic effects

of TRH are not mediated by the pituitary-thyroid axis. These findings support the concept that peptide hormones found in the hypothalamus may have a direct effect on the brain irrespective of their actions in releasing pituitary hormones (Plotnikoff et al., 1971, 1972). Henriques, (1972) suggested that besides its direct action on the brain, the antidepressant effects of TRH may also be brought about by an unknown quick acting substance secreted by the thyroid gland.

1.13.3. Biogenic Amines

An increasing number of publications implicate the biogenic amines (catecholamines and indolamines) in the aetiology of affective disorders.

Catecholamines

Bunney and Davis (1965) and Schildkrout (1965) put forward the "catecholamine hypothesis of affect", which states that depression is associated with low functional brain amines (noradrenaline and/or dopamine), while mania is associated with a high brain amine level. Low homovanillic acid levels have been reported in the CSF of depressive patients (Papeschi & McClure, 1971). Similarly, van Praag & Korf (1973) using the probenecid technique found a diminished dopamine turnover in the central nervous system of patients suffering from depression. However, Bunney et al., (1967) reported elevated urinary noradrenaline in psychotically depressed patients but not in those with

neurotic depression. Perez-Reyes (1969) found the opposite pattern, i.e. urinary noradrenaline to be high in neurotic but not psychotic depression. Plasma concentrations of adrenaline and noradrenaline from depressed patients not on drug treatment were found to be significantly higher than in normal controls. Furthermore, the plasma catecholamines concentration decreased in consort with clinical improvement (Wyatt et al., 1971). In patients suffering from depressive illness, high concentrations of CSF noradrenaline (Deneker et al., 1966a) and normal CSF homovanillic acid levels (Mendels et al., 1972) have been reported. Also, normal concentrations of noradrenaline have been found in brains of depressed patients who have committed suicide (Bourne & Israels, 1968; Pare, 1969). The specific inhibitor of dopamine and noradrenaline synthesis, α - methyl - p - tyrosine, when administered to manic patients was found to decrease catecholamine synthesis both centrally and peripherally and to cause some clinical improvement (Bunney et al., 1972a); however, a catecholamine depletion in the brain by as much as 80%, resulted in a swing to depression in only a small minority of patients (Wyatt et al., 1971). Clearly this is a most controversial region.

Indolamines

The "indolamine hypothesis of depression", postulates a correlation between depression and a shortage of

functionally active 5-hydroxytryptamine (serotonin, 5-HT) in brain (Coppen, 1967; Van Praag, 1970). Thus a decrease in 5-HT and 5-hydroxy-indolacetic acid (5-HIAA), the principal metabolite of 5-HT (figure 1.8), has been found in the brains of patients who had suffered from severe depression and had committed suicide (Shaw et al., 1967; Bourne et al., 1968; Carpenter et al., 1969; Pare et al., 1969). The CSF concentration of 5-HIAA has been shown to be lower than normal in depressed and manic patients (Deneker et al., 1966b; Coppen et al., 1972a). Furthermore, the accumulation of 5-HIAA in CSF after probenecid administration, which blocks its egress from the CSF, is less in depressed patients than in non-depressed controls (Tamarkin et al., 1970; van Praag & Korf, 1971; van Praag & Korf, 1973). This finding was assumed to be indicative of a low turnover of 5-HT in brain tissues. Contradictions to the indolamine hypothesis are again not lacking. Normal CSF levels of 5-HIAA have been reported both in depression (Mendels et al., 1972) and mania (Ashcroft et al., 1966). Studies on controls indicate a wide range of 5-HIAA values in normality (van Praag & Korf, 1973) and this scatter could provide an explanation for the contradictory findings.

It has been suggested (Lapin & Oxenkrug, 1969; Curzon, 1969) that the reduction in levels of 5-HT and 5-HIAA and tryptamine in depression could result from channelling tryptophan metabolism through the kynurenine

pathway (figure 1.8). However, Frazer et al., (1973) could not confirm this suggestion despite reports by Coppen et al., (1972a) that excretion rates of tryptamine are reduced during the depressive period but return to normal on recovery.

Coppen et al., (1971a) found that the CSF concentration of tryptophan, the precursor of 5-HT, is significantly lower in patients compared to a control group of neurological patients. Thus low tryptophan levels in lumbar CSF may account for the reduced brain 5-HT and 5-HIAA levels. In a more recent investigation Coppen et al., (1972b) reported no significant difference in total plasma tryptophan between the depressive patients and the control group, whereas the concentration of plasma free-tryptophan was significantly lower in the depressive group than in controls. Consequently they suggested that the reduced plasma free-tryptophan levels may be the cause of the reduction in CSF tryptophan, although it is possible that there may also be an abnormality in its transport from blood to brain and CSF.

Ashcroft and his group (1972) have reformulated the "amine hypothesis for the aetiology of affective illness" as follows: "The activities of amine-mediated synapses in brain are modified in affective illness either as a result of altered input into the neuronal systems from other centres or as a result of altered sensitivity of the postsynaptic receptors. It is the balance between transmitter availability at the receptor and receptor sensitivity which

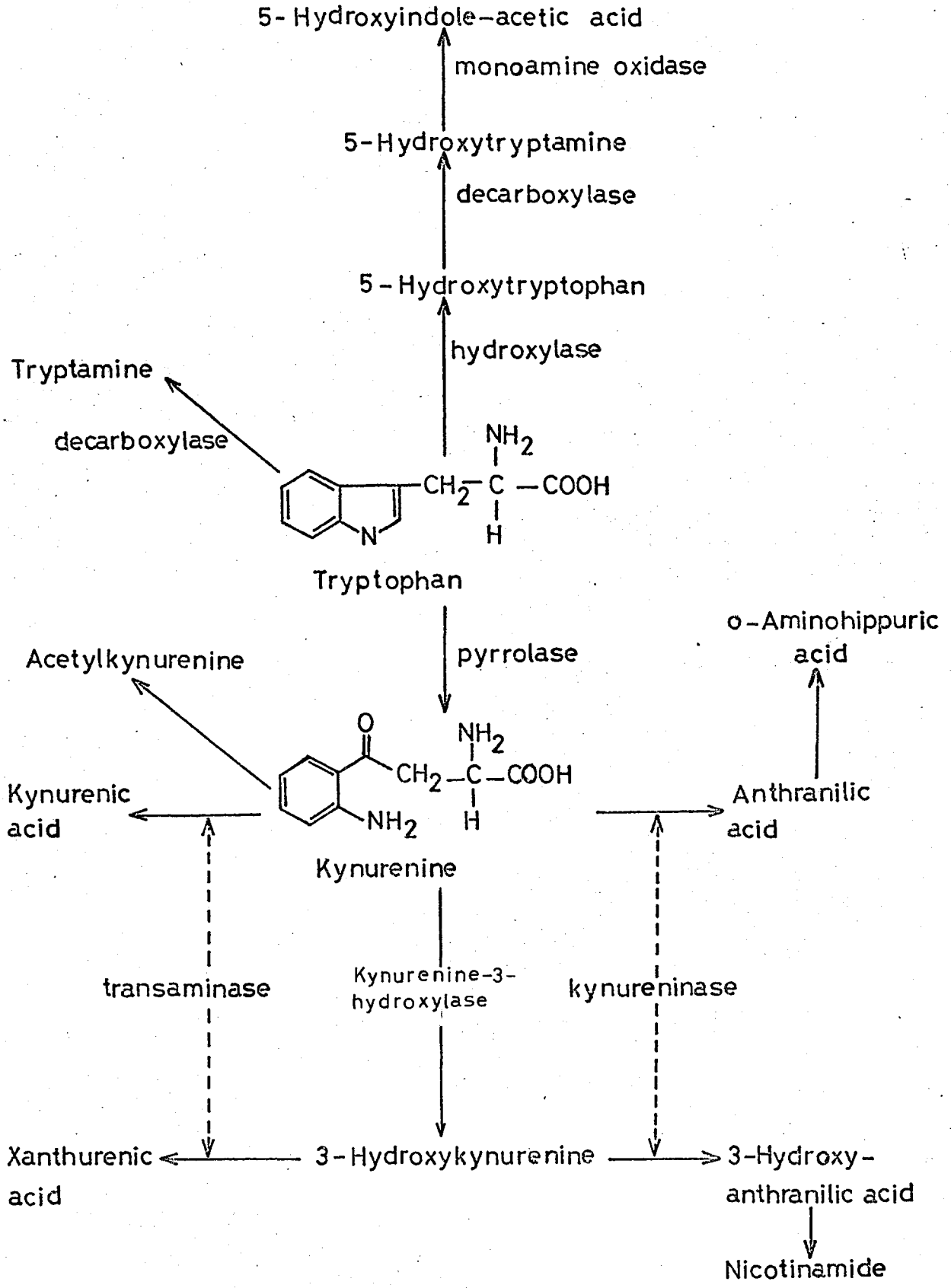


Figure 1.8: Pathways of tryptophan metabolism.

determines the functional state of the systems. One or more of the amine-mediated systems may be implicated and the pattern of involvement of the systems will be important in determining the characteristic pattern of behaviour and autonomic components comprising the clinical picture. Functional recovery of these systems in depression will follow either a rise in receptor sensitivity or a rise in transmitter output or both. True functional restoration of the systems will, however, occur only when both transmitter output and receptor sensitivity and the reactivity of the system to incoming stimuli are all restored to normal."

Coppen et al., (1972b) suggested that affective disturbance is associated with an imbalance rather than a simple depletion of amines. A cholinergic-adrenergic hypothesis of mania and depression has been postulated by Janowsky and his group (1972). According to this hypothesis, a given affective state may represent a balance between central cholinergic and adrenergic neurotransmitter activity in those areas of the brain which regulate affect, with depression being a disease of cholinergic dominance and mania being an illness of adrenergic dominance. Support for this hypothesis comes from animal experiments demonstrating antagonistic cholinergic and adrenergic central behavioural effects. Furthermore, reserpine, a drug which causes depression, has central cholinomimetric properties. Conversely, tricyclic antidepressants have

central anticholinergic properties. In man, physostigmine and other centrally acting cholinomimetric agents which increase central acetylcholine levels (e.g. cholinesterase - inhibitors such as the fluorophosphate insecticides) counteract mania and cause depression in some individuals (Janowsky, et al. 1973).

The biochemical hypotheses applied to affective disorders are numerous and sometimes controversial. There is an urgent need for a unifying concept.

CHAPTER II

Materials and Methods

2.1: INTRODUCTION

Several methods have been used for the estimation of cyclic AMP in extracellular fluids (Table 2.1). For our studies 24-hour urinary and plasma cyclic AMP levels were determined by the enzymic radioisotopic displacement technique of Brooker *et al.* (1968) and later by the saturation assay of Brown *et al.* (1971).

Table 2.1: Cyclic AMP Levels in Extracellular Fluids of Normal Humans

Amount of cyclic AMP	n	Fluid	Method Used	Reference
7.1 ± 0.3 μmol/24h (4.7 - 9.0)	12	Urine	Enzymic radioisotopic displacement	This study
2.3 ± 0.1 μmol/24h (1.4 - 3.2)	18	Urine	Enzymic radioisotopic displacement	Abdulla and Hamadah (1970)
5.6 ± 0.7 μmol/24h	10	Urine	Enzymic radioisotopic displacement	Paul et al. (1970)
1.1 - 2.0 μmol/24h	3	Urine	Enzymic radioisotopic displacement	Eccleston et al. (1970)
2.2 ± 0.1 μmol/24h (1.5 - 3.8)	35	Urine	Saturation assay (A)	This study
3.6 ± 0.4 μmol/24h (1.7 - 6.0)	16	Urine	Saturation assay (A)	Brown et al. (1972)
3.2 ± 0.2 μmol/24h or 2.5 ± 0.1 μmol/g creatinine	12	Urine	Saturation assay (M)	Murad & Pak (1972)
3.3 ± 0.2 μmol/24h	10	Urine	Saturation assay (A)	Jenner et al. (1972)
2.7 - 5.6 μmol/24h	5	Urine	Saturation assay (A)	Owen and Moffat (1973)
2.2 ± 0.8 μg/mg creatinine	25	Urine	Saturation assay (A)	Barlow & Wilkinson (1973)
6.5 ± 0.3 μmol/24h	15	Urine	Saturation assay (M)	Neelon et al. (1973)
2.7 ± 0.5 μmol/g creatinine (2.3 - 3.5)	6	Urine	High Pressure anion exchange chromatography	Fichman and Brooker (1972)
4.2 - 6.1 μmol/24h	5	Urine	Activation of phosphorylase	Takahashi et al. (1966)
2.0 - 7.0 μmol/24h	10	Urine	Cyclic — AMP → ADP → A (↓ Pi) Colorimetric estimation of Pi	Butcher and Sutherland (1962)
1.1 - 8.6 μmol/24h	11	Urine	Cyclic AMP → → ATP then radioactive phosphate exchange reaction	Chase et al. (1969a)

Continuation of Table 2.1:

Amount of cyclic AMP	n	Fluid	Method Used	Reference
1.8 - 9.0 μmol/24h	81	Urine	Cyclic AMP→→ATP→ADP (Pi) Colorimetric estimation of Pi	Broadus et al. (1970)
3.2 ± 0.2 μmol/24h	21	Urine	Cyclic AMP→→ATP; G-6-P; [1 - ^{14}C]-glucose → $^{14}CO_2$ Scintillation counting of trapped $^{14}CO_2$	Taylor et al. (1970)
15.6 ± 2.5 pmol/ml	20	Plasma	Enzymic radioisotopic displacement	This study
10 - 25 pmol/ml		Plasma	Cyclic AMP→→ATP→ADP (Pi) Colorimetric estimation of Pi	Broadus et al. (1970)
25.9 ± 2.1 pmol/ml		Plasma	Cyclic AMP → Phosphorylase (I) → Phosphorylase (A); Glycogen ← G-1-P Colorimetric estimation of Glycogen	Murad et al. (1969)
15.0 pmol/ml		Plasma	Radioimmunoassay	Steiner et al. (1970a)
22.3 ± 2.2 pmol/ml	31	Plasma	Saturation assay (A)	This study
12.8 ± 3.5 pmol/ml	12	Plasma	Saturation assay (A)	Barling et al. (1974)
5 - 25 pmol/ml		Plasma	Radioimmunoassay	Heath et al. (1973)
15.8 ± 2.8 pmol/ml	7	Plasma	Saturation assay (A)	Latner and Prudhoe (1973)
8.0 - 16.0 pmol/ml	10	Plasma	Saturation assay (A)	Rabinowitz & Katz (1973)
6.9 ± 0.6 nmol/ml	17	Whole Blood	TLC chromatography and fluorimetry	Hansen (1972)

Continuation of Table 2.1:

Amount of cyclic AMP	n	Fluid	Method Used	Reference
31.1 ± 3.5 pmol/ml		Cerebro-spinal fluid	Phosphorylase (I) ↓ Phosphorylase (A); G-1-P → (↓) Glycogen; Colorimetric estimation of Glycogen	Murad et al. (1969)
5 - 22 pmol/ml		Cerebro-spinal fluid	Cyclic AMP → → ATP → ADP (↓ Pi); Colorimetric estimation of Pi	Broadus et al. (1970)
15 - 25 pmol/ml		Cerebro-spinal fluid	Radioimmunoassay	Steiner et al. (1970a)
11.0 ± 1.0 pmol/ml	15	Cerebro-spinal fluid	Saturation assay (M)	Cramer et al. (1972)
11.0 1.1 pmol/ml		Cerebro-spinal fluid	Saturation assay (M)	Cramer et al. (1973)
25 pmol/ml		Amniotic fluid	Cyclic AMP → → ATP → ADP (↓ Pi); Colorimetric estimation of Pi	Broadus et al. (1971)
30 - 50 nmol/ml		Semen	Cyclic AMP → → ATP → ADP (↓ Pi); Colorimetric estimation of Pi	Broadus et al. (1971)
0.8 nmol/ml		Milk; colostrum	Ion exchange chromatography followed by paper chromatography and infra-red spectroscopy	Kobata et al. (1961)
20 pmol/ml		Gastric juice	-	Robison et al. (1971)

(A) Adrenal cortical binding protein (Brown et al., 1971)

(M) Bovine muscle binding protein (Gilman 1970)

2.2: MATERIALS

[8 - ^{3}H] Cyclic AMP (6.5 Ci/mmol and later 27.5 Ci/mmol) was purchased from the Radiochemical Centre (Amersham). Cyclic AMP, cyclic GMP, cyclic IMP, cyclic UMP, cyclic CMP, ATP, ADP, 5' AMP, adenosine, snake venom (Crotalus atrox) bovine albumin (Cohn fraction V), 2-mercaptoethanol, anion-exchange resin 'Dowex - 1' chloride form (2% cross linked, dry mesh 200-400) and cation-exchange resin 'Dowex 50W', hydrogen form (8% cross linked, dry mesh 100-200), were obtained from Sigma Chemical Co.

Before use both the anion-exchange and the cation-exchange resins were washed about ten times with N NaOH to remove alkali soluble impurities, then repeatedly with water, then with N HCl and finally with water to neutrality.

The scintillation chemicals were obtained from Nuclear Enterprises. Low potassium grade 1 scintillation vials were used throughout. All other chemicals used were ANALAR grade with the exception of ammonium sulphate which was enzyme grade.

Charcoal Norit GSX grade was purchased from Norit Clydesdale Co. Ltd. Lithium-heparin tubes were obtained from Abbot Laboratories Ltd. and stored at 0-4^{o}C until use.

2.3: GENERAL METHODS

2.3.1 Collection of Specimens:

(a) Urine

At the beginning of each collection period participants were asked to empty their bladders, this urine was discarded and timing started. 24-Hour urine samples were collected and pooled over 1 ml of chloroform (to inhibit bacterial growth), whereas 4-hourly urine specimens were pooled over 0.5 ml of chloroform. The pooled urinary output was thoroughly mixed, the volume measured, and a 10 ml sample pipetted into a "Universal" container which was stored at -20°C until analysis. Creatinine estimations (g/24h) were used to check the completeness of urine collection. Individuals with suspect urine volumes and abnormal creatinine values were excluded from this study. Where possible more than one urine specimen was collected from each subject.

(b) Plasma

Blood (10 ml) was obtained by venepuncture (ante-cubital fossa) transferred into lithium heparin tubes, and gently mixed, then immediately centrifuged (MSE 'Minor') for 2 minutes at top speed (about 3,000 rpm). The plasma was carefully removed using a pasteur pipette and divided into two equal portions. One portion was snap-frozen (solid CO_2-cellosolve at -78°C), the other was diluted by mixing with an equal volume of 50 mM Tris buffer, pH 7.4, containing 8 mM theophylline and 6 mM 2-mercaptoethanol

and then snap-frozen. The aim was to achieve a frozen sample within 5-7 minutes of venepuncture. The samples were stored at - 20°C until determination of cyclic AMP.

2.3.2 Creatinine Assay:

Creatinine determinations were carried out by the alkaline-picrate method of Bonsnes and Taussky (1945). Urine was diluted a hundred-fold with distilled water. To duplicate 3 ml samples of this diluted urine were added 1 ml of 0.04 M picric acid, followed by 1 ml of 0.75 M NaOH. After mixing and standing at room temperature for at least 15 minutes the extinction was measured at 500 nm. Standards and duplicate blanks (3 ml water) were run in parallel. Figure 2.1 shows a standard curve for creatinine. The values of unknown samples were obtained by reference to the standard curve.

The normal daily excretion of creatinine ranges from 0.8 -2.0 g/24h, being as a rule nearer to the higher limit in men and to the lower limit in women. Under normal conditions the 24-hour creatinine excretion for an individual is more or less constant.

2.3.3 Protein Estimation:

The Lowry technique (Lowry et al., 1951) was used for protein measurements. Suitable dilutions of the protein preparations (0.5 ml containing less than 200 µg protein) were mixed with 0.5 ml of N NaOH and left at room temperature to achieve a clear solution. This was mixed with 6 ml of freshly prepared copper reagent (1 litre of 2% Na_2CO_3 mixed

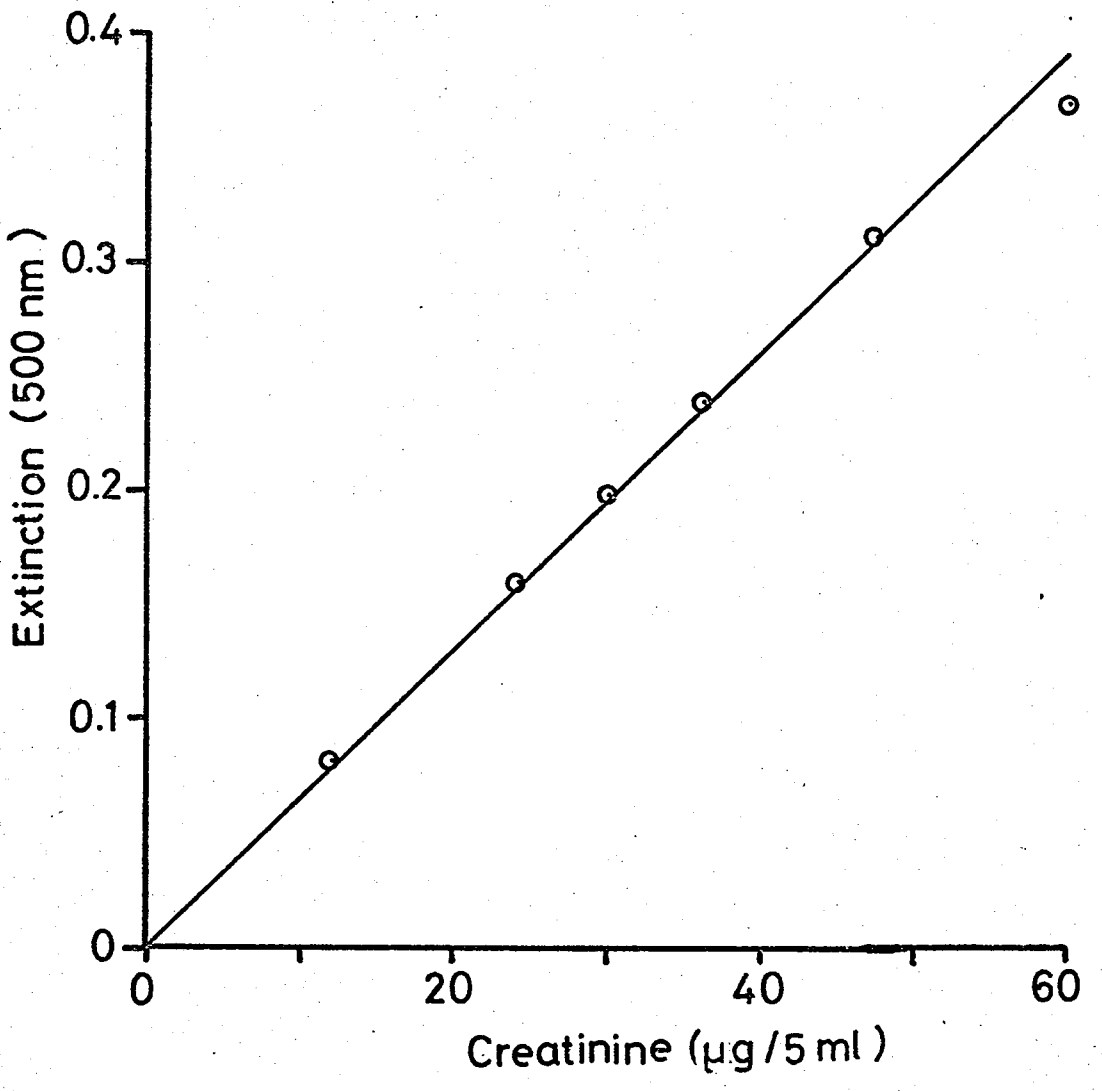


Figure 2.1: Standard curve for creatinine.

with 20 ml of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% K-tartrate) and left to stand for at least 10 minutes. Folin-Ciocalteu reagent (0.5 ml, diluted to normal acidity) was added and immediately mixed (i.e. tube by tube). The extinction was read after at least 30 minutes at room temperature in the region of 720 nm. Standards (Figure 2.2) and duplicate reagent blanks were run in parallel.

2.3.4 Radioactive Counting Procedures:

The scintillant used throughout this project had the following composition:-

Dioxan System (Bruno and Christian, 1961):

Toluene	700	ml
1,4-Dioxan	2,100	ml
2-Ethoxyethanol (cellosolve)	2,100	ml
1% 2,5-Diphenyloxazole (PPO)	49	g
0.05% 1,4-bis-[2-(4-methyl-5 phenyloxazole)]benzene (dimethyl POPOP)	2.45	g
8% Naphthalene	329	g

This system could take up to 20% water.

All radioactivity measurements were made using either a Packard Tri-Carb 3320 or Packard Tri-Carb 2425 automatic spectrometer at approximately 4°C. Settings (window 40-1000; gain 55%) were adjusted to achieve optimum efficiency which, for tritium, was of the order 30%. The conversion of cpm to dpm was achieved by internal standardisation; 50 µl of tritiated toluene (4,000 dpm) was added to already monitored vials which were then recounted.

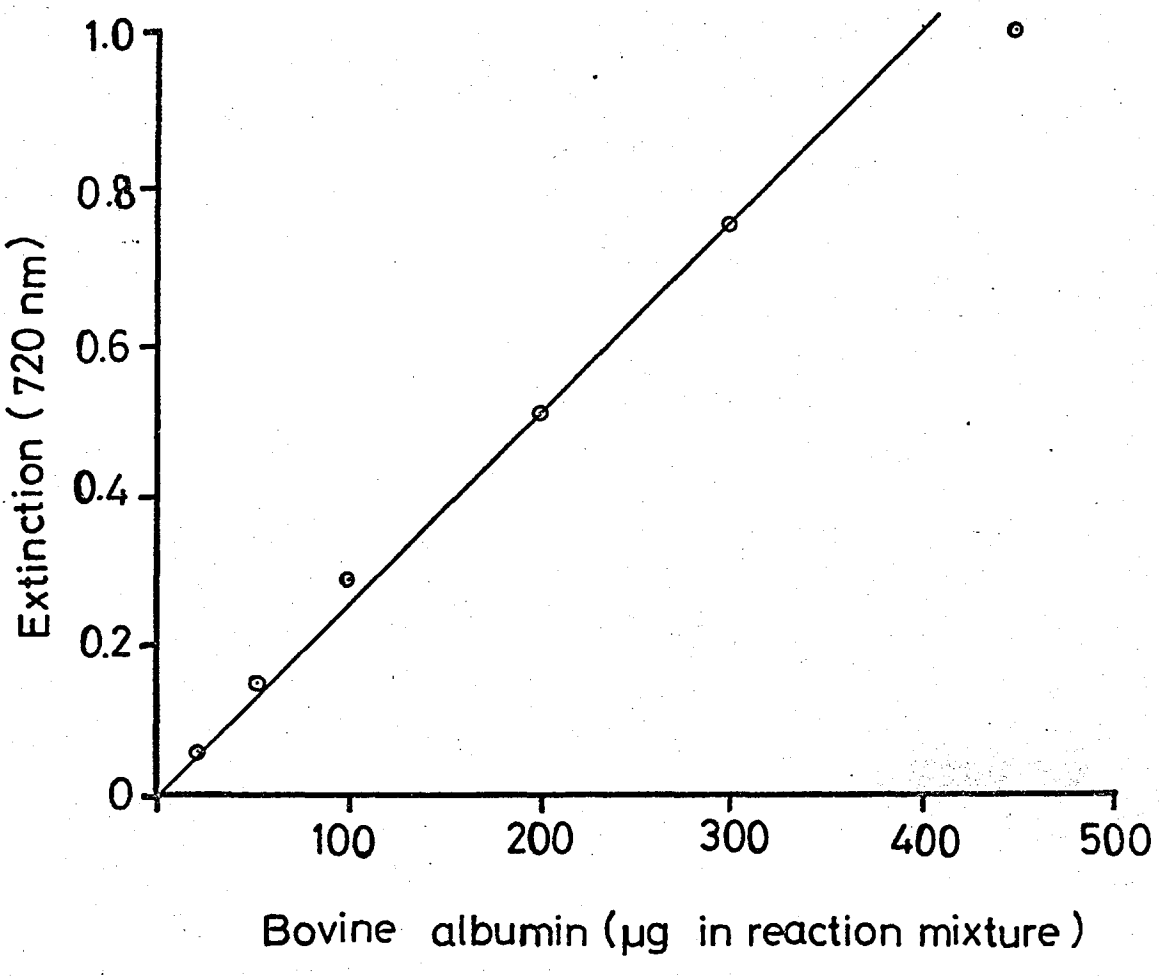


Figure 2.2: Standard curve for protein estimation.

2.4.1 Preparation of Nucleotide 3'5'-phosphodiesterase

Female Wistar albino rats (250-300g) were killed by cervical fracture and the brains quickly removed as follows:- The head was cut off and held dorsal side uppermost, a mid-line cut was made through the interparietal and parietal bone to between the eyes (scissor points kept up), then the cranium levered off to expose the cerebral hemispheres and cerebellum. The brain was lifted out with a spatula, dropped into ice-cold Tris (60 mM, pH 8) containing 2-mercaptoethanol (5 mM). After weighing and adjusting volume to 3 ml per g fresh weight, the tissue was homogenised (Potter-Elvehjem) and centrifuged for 30 minutes at 30,000 g (MSE High Speed 18, 8 x 50 rotor at 4°C, 15,500 rpm). Subsequent steps were carried out in the cold-room (0-4°C). Finely powdered ammonium sulphate was added gradually to the supernatant with stirring over a period of about 15 minutes to achieve 50% saturation (0.38g/ml). The precipitate was collected by centrifugation (10 min at 30,000g), resuspended in Tris-mercaptoethanol (as above, i.e. 3 volumes per g fresh weight) and dialysed overnight with stirring against two changes of this buffer. The preparation was divided into 0.5 ml aliquots, which can be kept at 0°C for 3-4 weeks or at -20°C for 4-5 months without appreciable loss of activity. Protein concentration was determined by the Lowry procedure.
The phosphodiesterase activity was measured by estimation of the inorganic phosphorus released on enzymic breakdown of cyclic AMP in the presence of excess 5'-nucleotidase (Butcher &

Sutherland, 1962), or by use of [^{3}H]-adenosine release from tritiated cyclic AMP.

2.4.2 Determination of the Activity of Rat Brain Phosphodiesterase

(i) In this procedure (Butcher & Sutherland, 1962) the release of inorganic phosphate was measured after a known incubation time.

$$\text{Cyclic AMP} \xrightarrow[\text{(limiting)}]{\text{Phosphodiesterase}} \text{AMP} \xrightarrow[\text{(excess)}]{\text{Snake Venom nucleotidase}} \text{Adenosine} + \text{Pi}$$

The initial reaction mixture (0.9 ml) contained:-

0.36	µmol	cyclic AMP
1.80	µmol	$MgSO_4$
35	µmol	Tris (pH 7.5)
50	µl	of a suitable dilution* of the phosphodiesterase preparation.

(* generally this was achieved by diluting the original 1:10 with 50 mM Tris, pH 7.5, so that the 50 µl addition contained about 12 µg protein).

After incubating for 20 min at 30°C, 0.1 ml of Crotalus atrox venom solution (1 mg/ml in 10 mM Tris buffer, pH 7.5) was added and the incubation continued for a further 10 min. The reaction was terminated after 30 min by the addition of 1.5 ml of ice-cold 6% trichloroacetic acid and the precipitate formed spun-down using a bench centrifuge, (top setting) for about 5 min. The complete supernatant was poured off and to it added:- 1.5 ml of 5% perchloric acid containing 0.6% ammonium molybdate, followed by 0.5 ml of freshly prepared 0.2% ascorbic acid solution. After mixing and standing for

at least 30 minutes the extinctions were measured at 720 nm, together with appropriate controls and standards. Figure 2.3 shows a typical standard curve for phosphorus estimation. Using this procedure a specific activity of about 300 pmol phosphate released/min/mg protein was obtained for the phosphodiesterase preparations.

(ii) Later, activity was measured by the breakdown of [^{3}H]-cyclic AMP to liberate tritiated adenosine. This procedure is described in section 2.5.2 the 'cold' cyclic AMP standard or unknown being replaced by 50 µl of 60 mM Tris-buffer, pH 8.0, containing 5 mM 2-mercaptoethanol.

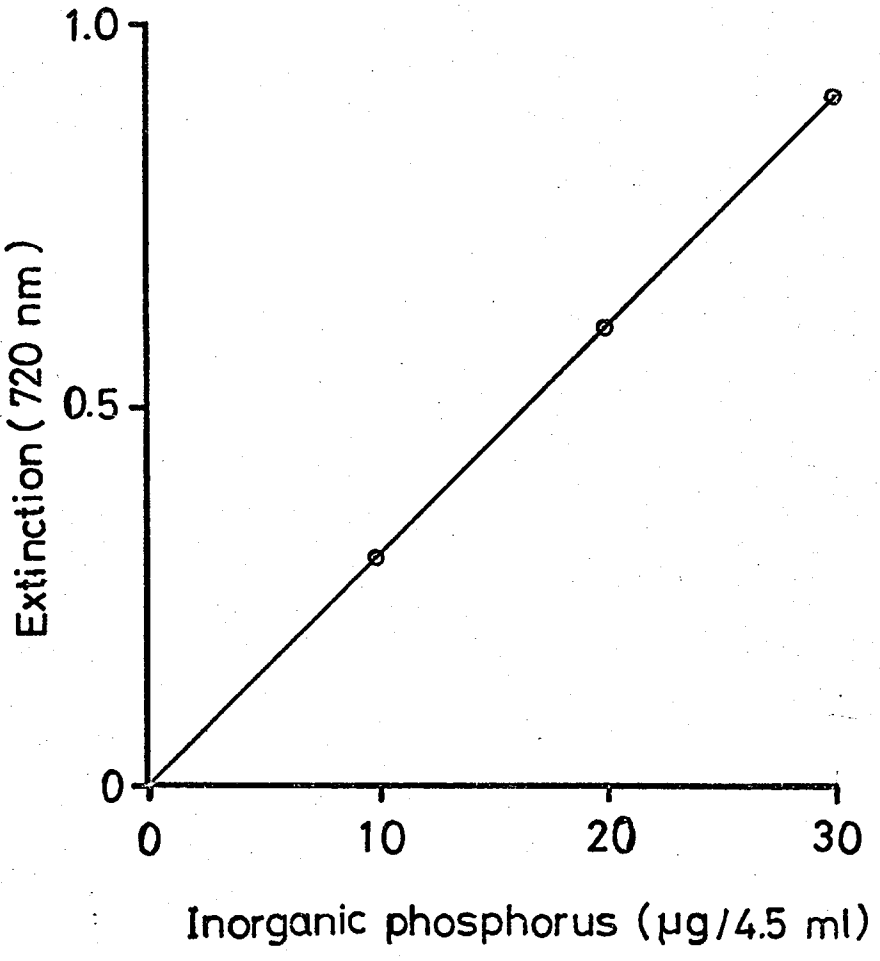


Figure 23: Standard curve for phosphorus.

2.5: ENZYMIC RADIOISOTOPIC DISPLACEMENT TECHNIQUE

2.5.1. Sample preparation

Samples were thawed out immediately before preparation for assay. To one ml urine, 0.2 ml, 0.3 M, zinc sulphate and 0.2 ml, 0.3 M, barium hydroxide* were added, well mixed ('Whirlimixer') and centrifuged for 10 minutes (bench centrifuge, top setting). This precipitated proteins, phosphates, pyrophosphates and all nucleotides except cyclic AMP (Krishna et al., 1968). Due to the amount of protein present in plasma its purification was modified:- To 2 ml of plasma were added equal volumes of zinc sulphate and barium hydroxide* and the mixture centrifuged as above.

The supernatants were loaded drop by drop on to one column for urine and two for plasma. Each column consists of 3 x 0.6 cm of 'Dowex 50W' resin, prewashed with at least 5 volumes of distilled water, and with a filter paper disc on top to avoid resin disturbance. The loaded columns were eluted with glass distilled water. Cyclic AMP, freed of cations, appeared in the second to tenth ml of effluent as monitored by the appearance of tritium label or UV absorbance (Figure 2.4). The third to the seventh ml (inclusive) of effluent were pooled and freeze-dried. The dried material was taken up in 50 µl of 60 mM Tris-buffer, pH 8.0, containing 5 mM 2-mercaptoethanol for plasma and 50-500 µl for urine.

To estimate the loss of cyclic AMP during this purification, tracer amounts of tritiated cyclic AMP (about 1000 dpm) were added to 1 ml of sample which was then passed through the

(* freshly prepared)

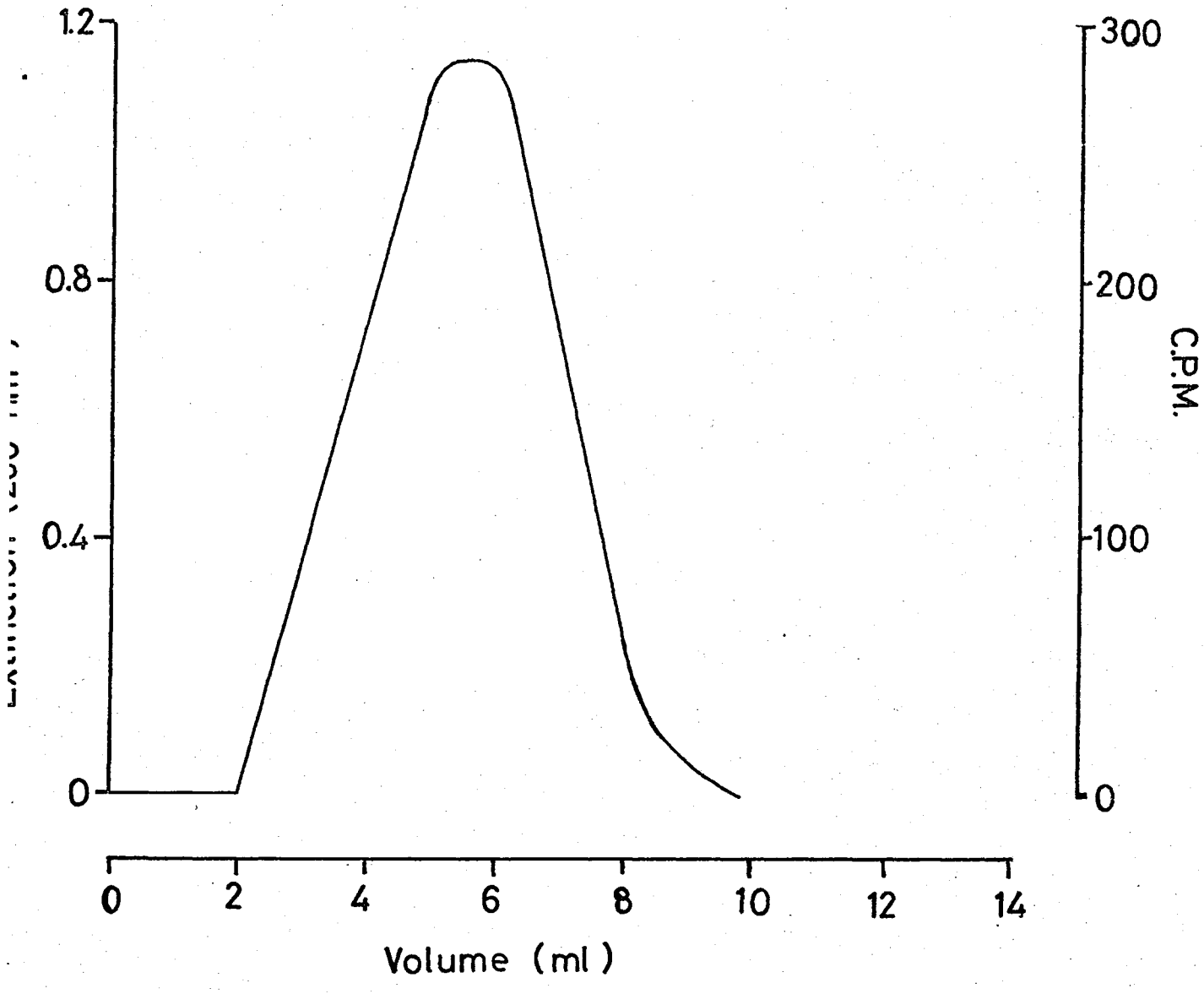


Figure 2.4: Elution curve for the purification of cyclic AMP on a column (0.6 X 3.0 cm) of 'Dowex 50W' resin.

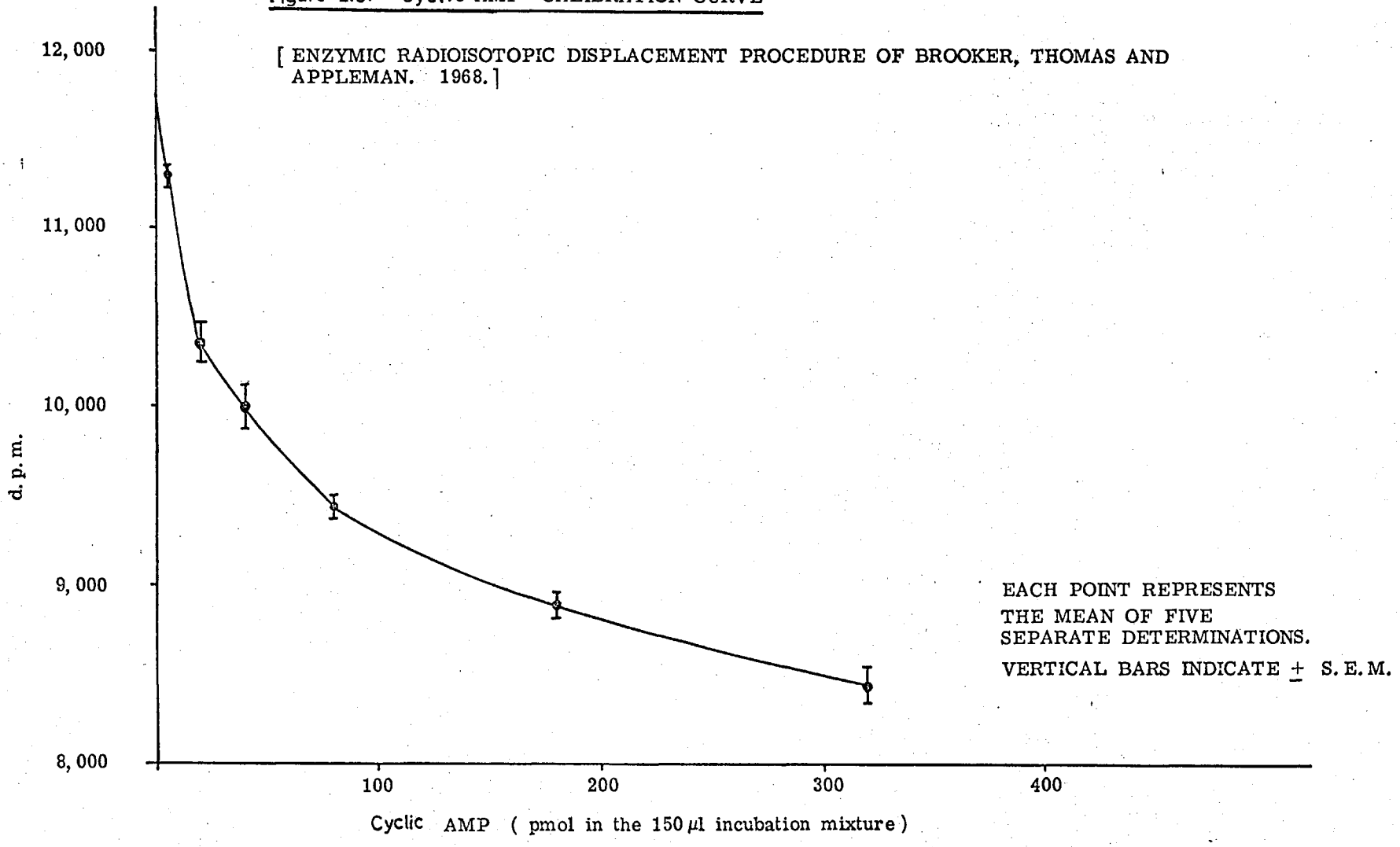
procedure described above and the resultant 50 µl solution in buffer counted in 15 ml of scintillant. 20% out of each batch of estimations were checked for recovery. Values in the range 44 to 51% of the added [^{3}H] cyclic AMP were obtained. Part of the loss was due to collection of the central region of the elution peak rather than the whole peak.

2.5.2. Cyclic AMP Assay

A purified sample (50 µl, containing 5-320 pmol of cyclic AMP) was pipetted into the bottom of a 20 ml conical glass-stoppered centrifuge tube; to this was added an equal volume of substrate containing 6 µmol Tris-HCl, pH 8.0, 6 µmol $MgCl_2$, 6 nmol 5' AMP and 8 pmol tritiated cyclic AMP (about 50 nCi). The reaction was started by the addition of 50 µl of an enzyme mixture which contains the brain phosphodiesterase (about 48 µg of protein/ml), Crotalus atrox venom (1mg/ml), 60 mM Tris-HCl, pH 8.0, and 5 mM 2-mercaptoethanol. The amount of phosphodiesterase (about 2.4 µg/incubation tube) was previously determined so that it was just sufficient to hydrolyse 30-40% of the tritiated nucleotide without the addition of non-radioactive cyclic AMP. The reaction was stopped, after 10 minutes at 30°C, by the addition of 0.8 ml of 50% (w/v) slurry* of 'Dowex 1' resin. The mixture was left to equilibrate for 10 minutes, then 15 ml of scintillant added and thoroughly mixed ('Whirlimixer') about 1 minute. After standing for a further 10 minutes the mixture was spun (shielded bench centrifuge) at top-setting for 10 minutes and the supernatant transferred as fully as possible to scintillation vials for counting. The

(* constantly mixed by magnetic stirrer)

Figure 2.5: Cyclic AMP CALIBRATION CURVE



values of unknown samples were obtained by reference to a standard curve (Figure 2.5). This curve is prepared by measurement of known concentrations of cyclic AMP through the described procedure.

2.5.3. Specificity Characteristics of Brain Phosphodiesterase

The rat brain phosphodiesterase preparation was investigated by replacing the cyclic AMP standards with concentration ranges of each of the following:- cyclic GMP, cyclic UMP, cyclic IMP, cyclic CMP, adenosine, 5' AMP, ADP and ATP. The cyclic AMP assay was carried out as described in 2.5.2. and the results plotted (Figure 2.6).

2.5.4. Discussion of the Brooker et al. Assay for Cyclic AMP

The assay is based on the competition between unlabelled cyclic nucleotide with labelled cyclic AMP for conversion to 5'-nucleoside by a limiting amount of phosphodiesterase. The amount of labelled adenosine formed is inversely proportional to the quantity of unlabelled cyclic AMP present (Brooker & Appleman, 1968).

Cyclic AMP concentrations in body fluids are obtained from the standard curve in conjunction with the calculated recovery factor for the 'clean-up' procedure (2.5.1).

The enzymic radioisotopic displacement assay for the measurement of cyclic AMP in body fluids is easily set up and maintained. The assay is sensitive enough for the levels of the nucleotide in urine. However, determination of the much lower concentrations of cyclic AMP in plasma requires at least 2 ml to achieve a borderline sensitivity.

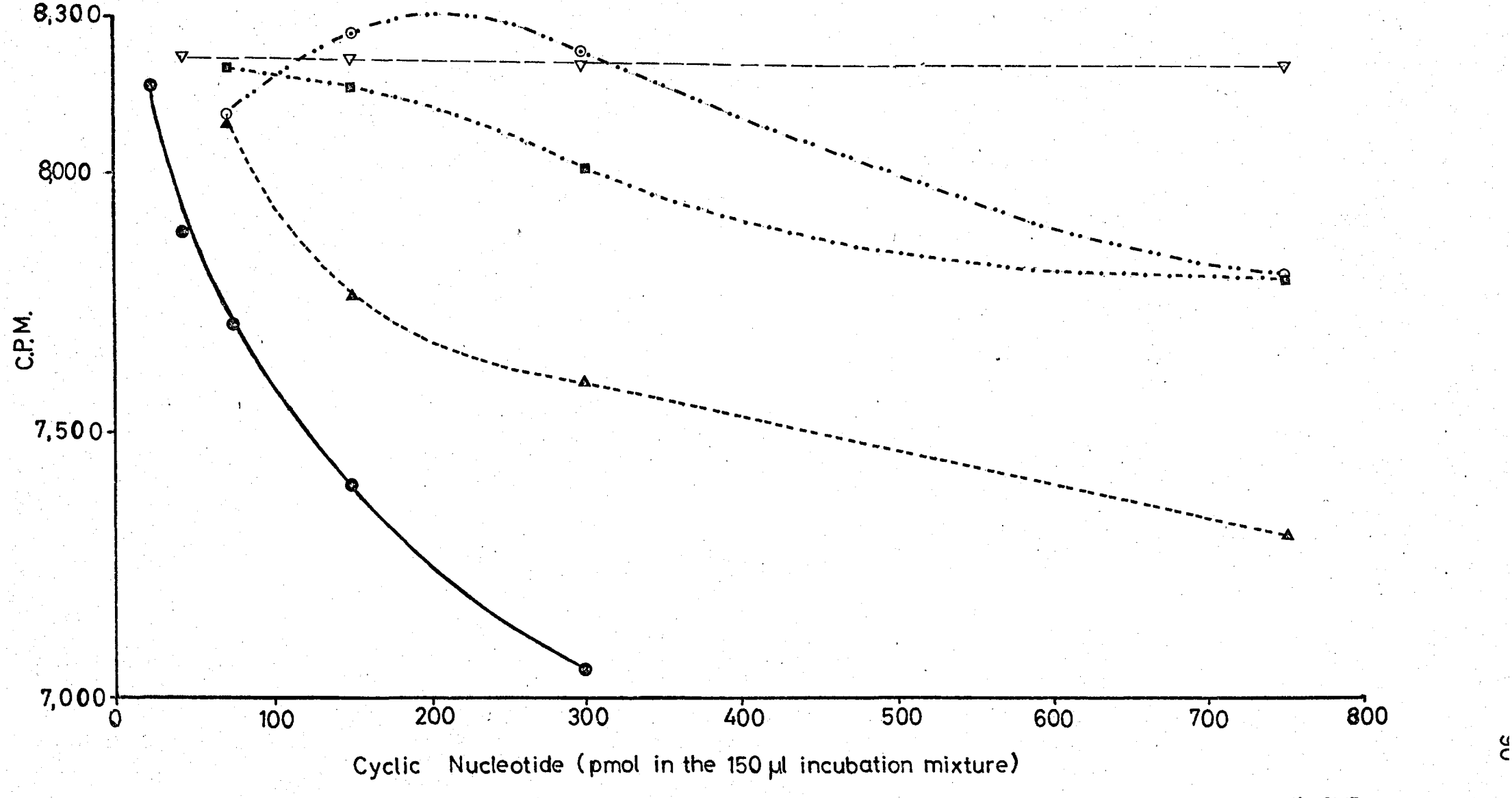


Figure 2.6: Specificity study for the radioisotopic displacement technique. ⊙—⊙ Cyclic AMP; ▲---▲ cyclic IMP; ■---■ cyclic GMP; ⊙-·-·-⊙ cyclic CMP; ▽—▽ cyclic UMP. ATP, ADP up to 100 nmol, adenosine up to 25 nmol and 5'AMP up to 75 nmol per incubation mixture showed no cross reaction.

It has been suggested by Rodbell (1971a) that as much as 5 - 25% endogenous nucleotide could be lost by treatment of the body fluid with zinc sulphate and barium hydroxide. However, Krishna (1968) argued that less than 1% of the nucleotide is lost via this route. Radioactivity measurements on several samples in this study indicate a loss of 1 - 5%.

The venom used was found not to show any phosphodiesterase activity towards the cyclic AMP under the conditions of the assay. 5' - AMP is included in the assay mixture to act, after hydrolysis by the venom, as a carrier for the minute quantities of labelled adenosine, the reaction product. Although the amount of the 5' - AMP used inhibits the phosphodiesterase to some extent (Brooker et al., 1968), we found that as much as 1.5 mM 5' - AMP still gave a functional assay system.

The reaction is stopped by the addition of anion exchange resin, which binds unhydrolysed cyclic AMP, and thus differentiates substrate from product. The removal of resin improves the sensitivity for urine measurement, the trapped nucleotide giving a distinct count rate in contradiction to the findings of Brooker et al. (1968). Resin removal by centrifugation is vital for attempts at plasma measurements.

Table 2.2 shows various experiments conducted to confirm the validity of the assay. Prior destruction of cyclic AMP with phosphodiesterase reduced the assay value to zero; boiling and then readdition of cyclic AMP agreed well with standards.

Interference with the assay of cyclic AMP by other nucleotides is shown in Figure 2.6. Of the nucleotides tested

Table 2.2: Validation for the Brooker, Thomas and Appleman Procedure

		pmol of cyclic AMP	
		*Measured	Expected
1.	50 µl of purified urine	36	-
2.	20 µl of purified urine	16	14
3.	50 µl of purified urine + 80 pmol of cyclic AMP	110	116
4.	20 µl of purified urine + 80 pmol of cyclic AMP	90	94
5.	100 µl of freeze-dried plasma (2 ml original plasma)	12	-
6.	50 µl of freeze-dried plasma (1 ml original plasma)	6.5	6.0
7.	100 µl of freeze-dried plasma + 80 pmol of cyclic AMP	87	92
8.	50 µl of freeze-dried plasma + 80 pmol of cyclic AMP	80	86
∓ 9.	50 µl of purified urine pretreated with excess phosphodiesterase	0	0
∓ 10.	50 µl of freeze-dried plasma pretreated with phosphodiesterase	0	0
∓ 11.	50 µl of purified urine + 80 pmol of cyclic AMP pretreated with phosphodiesterase	0	0
∓ 12.	50 µl of freeze-dried plasma + 80 pmol of cyclic AMP pretreated with phosphodiesterase	0	0
13.	As in 11 except 80 pmol of cyclic AMP added after boiling	75	80
14.	As in 12 except 80 pmol of cyclic AMP added after boiling	74	80

* The mean of 5 separate estimations.

∓ Brain phosphodiesterase preparation (48 µg) was incubated for 10 minutes with the sample and then destroyed by boiling (100°C) for 5 minutes.

cyclic CMP and cyclic UMP showed negligible cross reaction at concentrations as high as 300 pmol/incubation. Cyclic IMP was found to have a third of the effect of cyclic AMP, and cyclic GMP had about a tenth of the effect. Cyclic GMP has been detected in urine (Broadus *et al.*, 1971) but to a lesser extent than cyclic AMP (0.4 - 3.0 μmol/24h). Other cyclic nucleotides, including cyclic IMP, are barely detectable in urine (Hardman, 1971). Treatment of the body fluids with zinc sulphate and barium hydroxide removes a range of other possible interfering substances. There is now evidence for two distinct brain phosphodiesterases - one for cyclic AMP and one for cyclic GMP (Thompson & Appleman, 1971), both activities would be expected in the rat brain preparation described (Section 2.4.1.).

One of the requirements of this type of assay is that the enzyme used must have a Michaelis constant, which is not appreciably higher than the concentrations which are to be measured. The K_m for the rat brain phosphodiesterase as used in this study was 9.7×10^{-7}M (Figure 2.7). This is in general agreement with the value of 8.3×10^{-7}M obtained by Somerville *et al.* (1970), and 8×10^{-7}M by Skidmore and Hindley (1970). Thompson and Appleman (1971) have reported values of 5×10^{-6}M for the K_m for cyclic AMP phosphodiesterase, and 1×10^{-4}M for cyclic GMP phosphodiesterase.

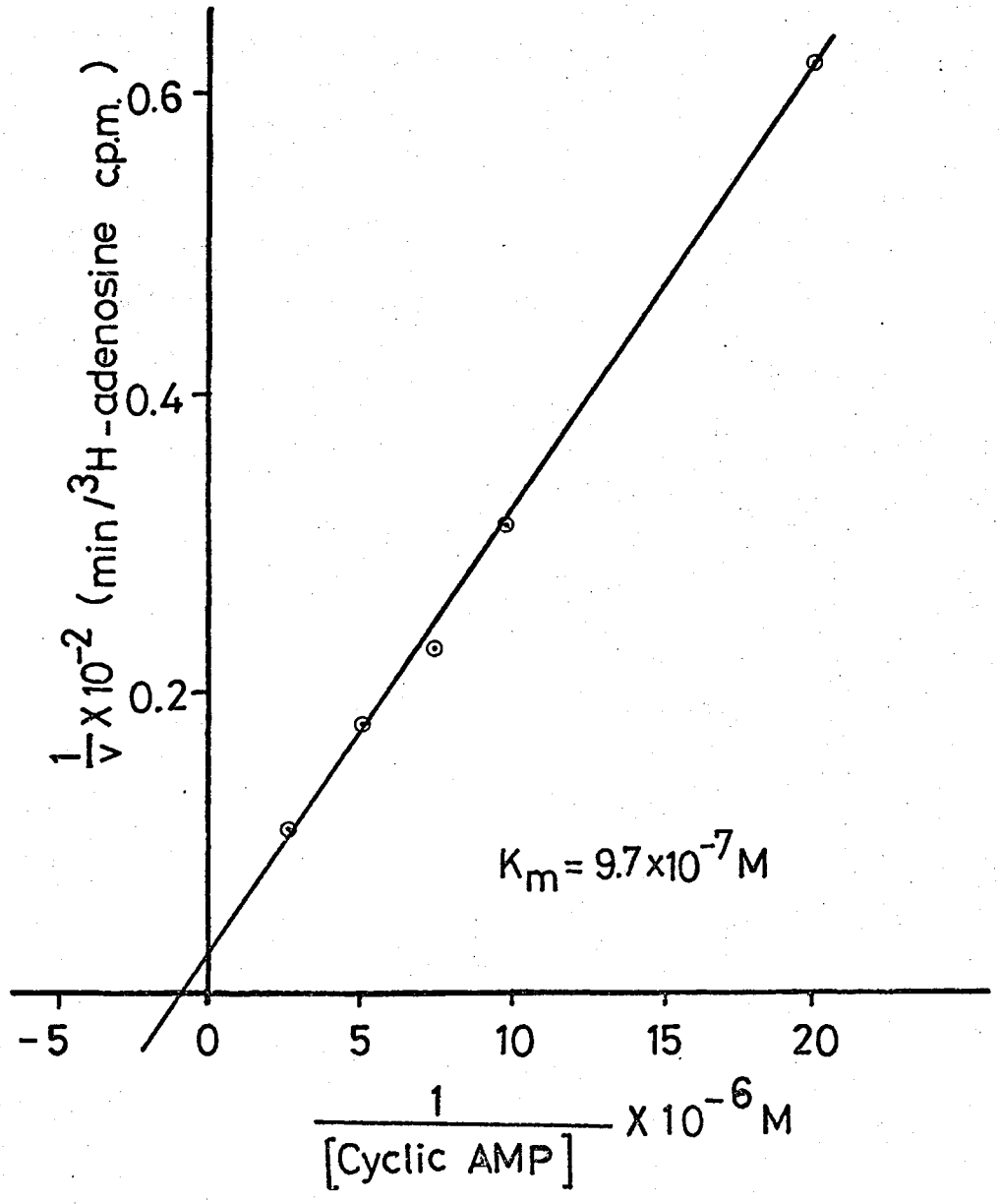


Figure 2.7: Lineweaver - Burk plot for rat brain 3',5'-cyclic AMP phosphodiesterase.

2.6: SATURATION ASSAY

2.6.1. Preparation of the Binding Protein

The procedure was modified from that given by Brown et al. (1971):- Bovine adrenals collected as soon as possible after slaughter, were transported packed in ice. Preparation of binding protein was carried out at 4°C (cold room).

The adrenals were freed from the surrounding fat and the cortices were separated from the medulla and the capsule as follows: the adrenals were cut into halves which were pinned on to a dissection board. The medulla was stripped away from the cortex with a pair of curved forceps, then the cortex was scraped off the capsule with a scalpel. The pooled cortices were disrupted in an MSE homogenizer, with 1.5 volumes of ice-cold Littlefields medium (0.25 M sucrose, 0.5 M Tris-HCl buffer, pH 7.4, 0.05 M magnesium chloride, 0.025 M potassium chloride). The homogenate was centrifuged for 10 minutes at 10,000 *g* at 4°C in an MSE High Speed 18 centrifuge (10,500 rpm, 8 x 50 rotor). The resulting supernatant was stored in 0.5 - 1.0 ml portions at -20°C. Immediately before use one such portion was thawed and an aliquot diluted; activity is lost on refreezing. Using this one freeze-thaw procedure negligible loss of binding activity was found after 3 months. The diluent used is 50 mM Tris, pH 7.4, containing 8 mM theophylline and 6 mM 2-mercaptoethanol ("Buffer").

2.6.2. Binding Protein Dilution Curve (Fig. 2.8)

This was set up for each preparation of binding protein to determine the dilution of binding protein to be used in subsequent assays. Serial dilutions of binding protein were

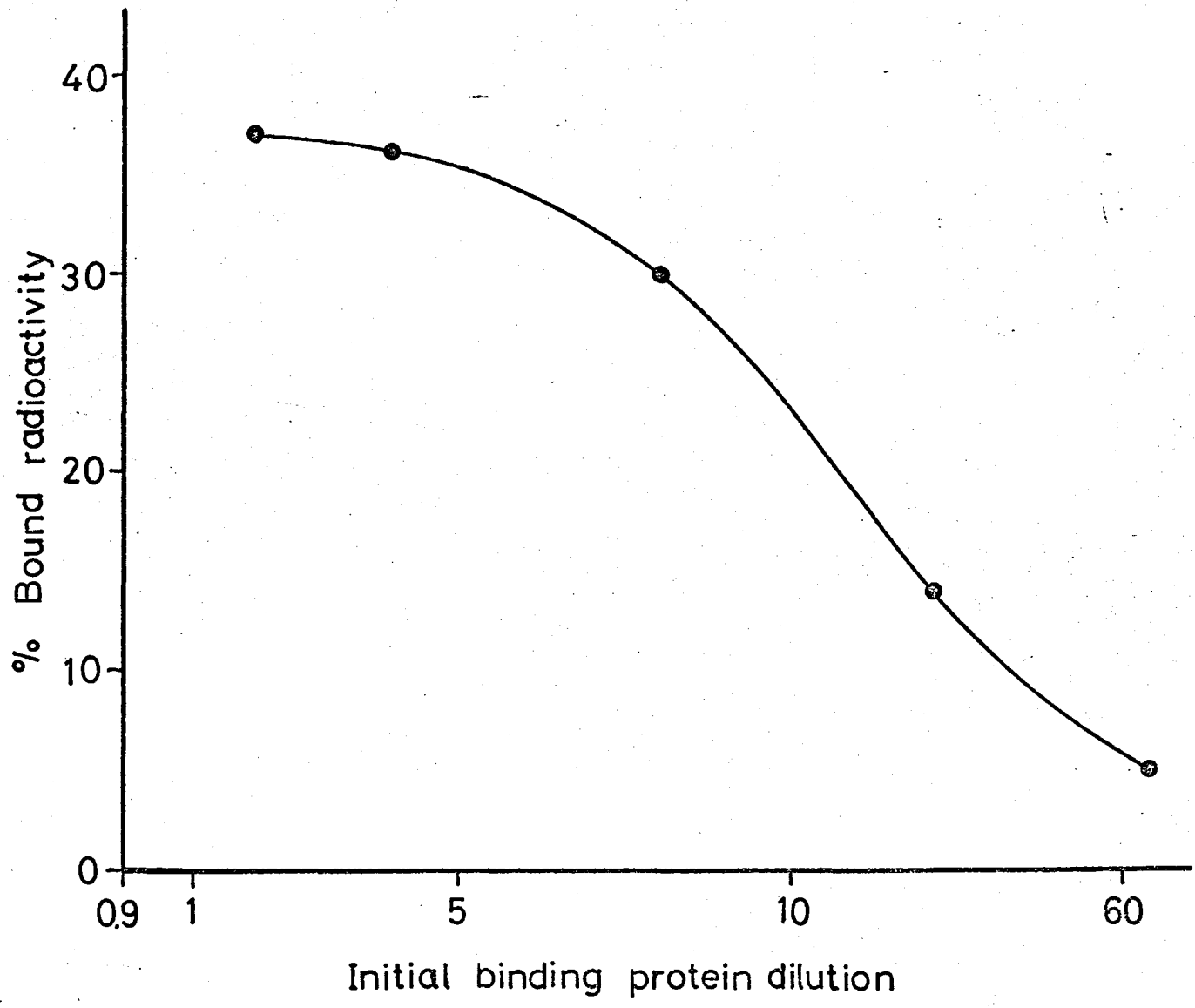


Figure 2.8: Binding protein dilution curve (semi-log plot).

prepared (dilutions used were: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64) and the incubation tubes (50 x 9.75 mm round bottom) were set up as follows:-

100 µl "Buffer"

100 µl binding protein (each dilution)

50 µl [^{3}H] - cyclic AMP (0.36 pmol i.e. 10nCi)

The tubes content was mixed ('Whirlimixer') and incubated (without shaking) in an ice-bath for 90 minutes.

2.6.3. Separation of Free and Bound Fractions

After this incubation 100 µl of a suspension of 1g/10 ml of charcoal in buffer containing 2% (W/V) of bovine albumin was added to each tube. The suspension (initially at 0°C) was agitated (magnetic stirrer) for about 5 minutes and then, with continuous stirring, 100 µl portions were rapidly transferred using an 'Oxford' pipette. Immediately after receiving the charcoal each tube was agitated for a few seconds on a 'Whirlimixer' then placed back in the ice-bath. The complete batch (in this study 24 tubes) took 3-4 minutes to complete; it was then centrifuged for 10 minutes at 4,000 rpm (MSE 'Multex' 24 x 15 angle head rotor) at room temperature. The batch was replaced in an ice-bath and 100 µl of the supernatants of each tube transferred to 6 ml scintillant and counted.

It is important to work to a precise time sequence as it is found that if tubes were not rapidly centrifuged but left to stand at room temperature reversal of the binding reaction occurs (Table 2.3). However, the rapid reversal of the binding reaction is slowed down by keeping the tubes in an ice-bath.

Table 2.3: Reversal of Binding Reaction on Standing at Room Temperature 22°C), before Centrifugation

Time (min)	% [^{3}H] - cyclic AMP bound (mean of 2 estimations)	% Reversal of Binding
0 (10 sec)	36.0	0.0
1	34.0	5.6
2	34.0	5.6
5	27.0	25.0
10	23.5	34.7
20	16.8	53.5

The binding protein dilution was plotted (Figure 2.8) as % bound (ordinate, linear scale) against dilution of the binding protein (abscissa, log scale). The 100% binding value was obtained by setting up tubes containing everything but the charcoal (i.e. 100 µl of buffer added, instead of the Norit suspension, to the 250 µl incubation mixture). An aliquot (100 µl) was counted as above. Sample tubes were expressed as a percentage of this standard.

The dilution of the binding protein that gave 20 - 30% binding of the tracer was read off Figure 2.8. This was the dilution to be used in the assay (Ekins and Newman, 1970).

2.6.4. Preparation of Sample

a. Urine

Samples were thawed out immediately before cyclic AMP determination and assayed for creatinine. Dilutions (1:10 to 1:50) in 'Buffer' were prepared and 100 µl of the diluted samples were taken for estimation of cyclic AMP.

b. Plasma

Freshly thawed heparinised plasma (0.2 ml) was deproteinised by the addition of 0.2 ml, 0.3 M zinc sulphate and 0.2 ml, 0.3 M barium hydroxide (Krishna et al., 1968; see section 2.5.1). The supernatant was freeze-dried and the residue taken up in 100 µl 'Buffer'. Recovery values for this plasma procedure were checked by the addition of a known amount of [^{3}H] - cyclic AMP to about 20% of the plasma samples in each batch (see section 2.5.1). After freeze-drying and redissolving in 'Buffer' the sample was counted. Normally about 90% of the labelled nucleotide was recovered.

Both plasma and urine cyclic AMP in the appropriately prepared 100 µl samples was determined by the saturation method of Brown et al. (1971).

2.6.5. Protein Binding (Saturation) Assay for Cyclic AMP

Each assay tube contained 300 µl for incubation:-

50 µl of cyclic AMP standard (0-15 pmol) OR

50 - 150 µl of unknown.

50 µl of [^{3}H] - cyclic AMP (10nCi; 0.36 pmol i.e. 120 pg)

100 µl of an appropriate dilution of binding protein (normally diluted 1:16).

'Buffer' to 300 µl.

The tubes content was mixed ('Whirlimixer') and incubated at 0°C for 90 minutes (without shaking). At the end of the incubation period 100 µl of charcoal suspension was added and the procedure described in section 2.6.3 was followed.

The values of unknown samples were obtained by reference to a standard curve (Figure 2.9). The linear range was

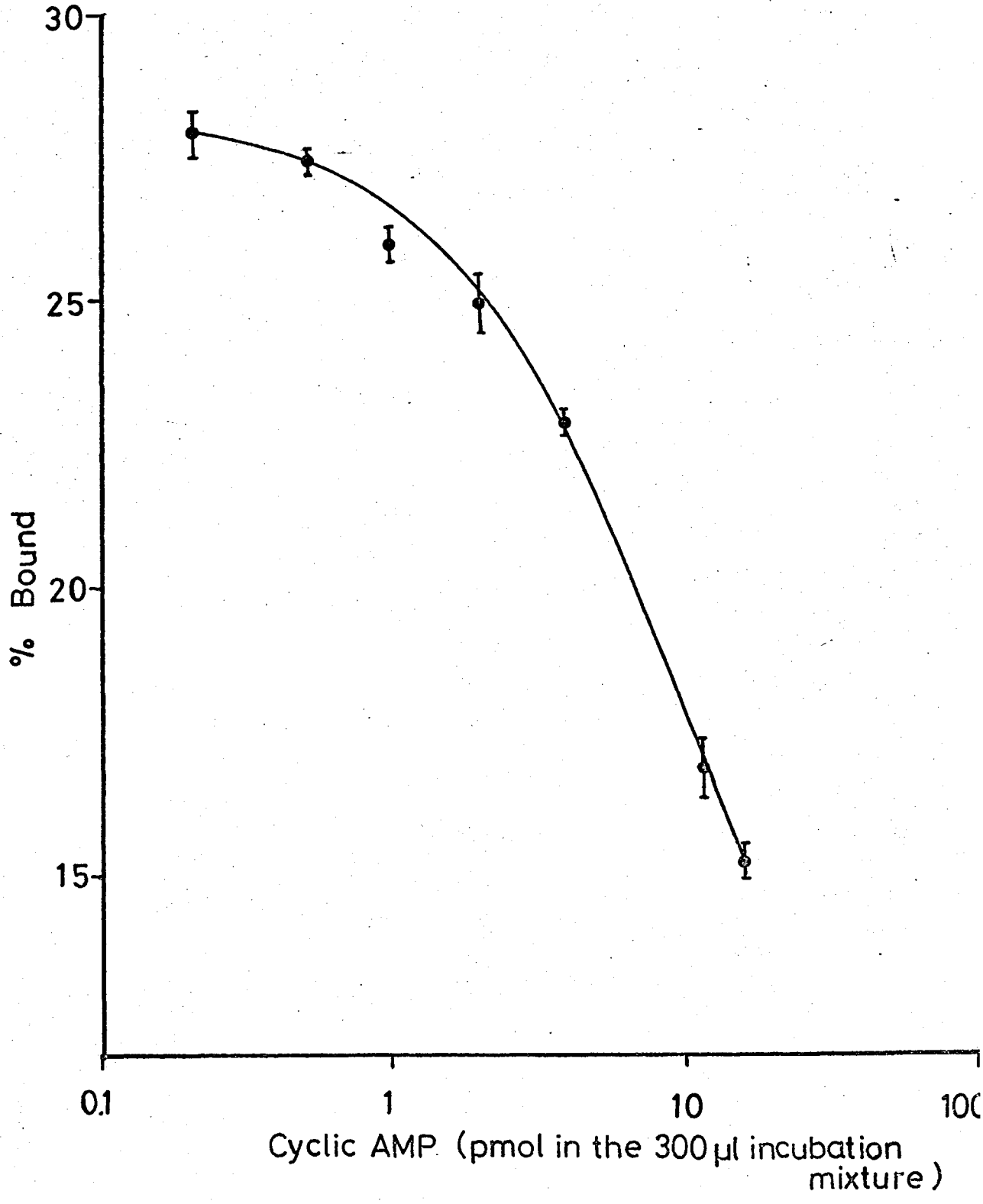


Figure 2.9: Cyclic AMP calibration curve for the protein binding assay. Each point represents the mean of seven separate determinations. The vertical bars indicate ± S.E.M..

1 - 15 pmol.

Control tubes containing 100 µl buffer instead of binding protein were prepared; these were used to monitor the efficiency of the charcoal in absorbing the free nucleotide and also as a check for non-specific effects. It was found that the Norit did not completely remove radioactivity. In general aqueous standards or urine dilutions gave 40 - 60 cpm, plasma samples gave 90 - 100 cpm (scintillant alone gave 20 cpm). These values were subtracted from standards and unknowns as appropriate.

2.6.6. Specificity Characteristics of the Binding Protein

This was investigated by incubating dilutions of cyclic CMP, cyclic GMP, cyclic IMP, cyclic UMP, adenosine, 5' - AMP, ADP and ATP with $[^3H]$ cyclic AMP and binding protein under the specified assay conditions (figure 2.10).

2.6.7. Presentation of Results

Standard plots can be presented in a number of ways:-

a. Percentage of cyclic AMP bound (see section 2.6.3) as ordinate, against the log of concentration of unlabelled cyclic AMP (pmol per assay tube; figure 2.9) as the abscissa (Brown et al., 1971; Latner & Prudhoe, 1973; Zettner, 1973).

b. Percentage of cyclic AMP bound as ordinate against the concentration of unlabelled cyclic AMP added (pmol per assay tube; figure 2.11) as the abscissa (Barling, 1974).

c. $Co/_{Cx}$ as ordinate against concentration of unlabelled cyclic AMP (pmol per assay tube; figure 2.12) as abscissa (Weller et al., 1972), where Co is the amount

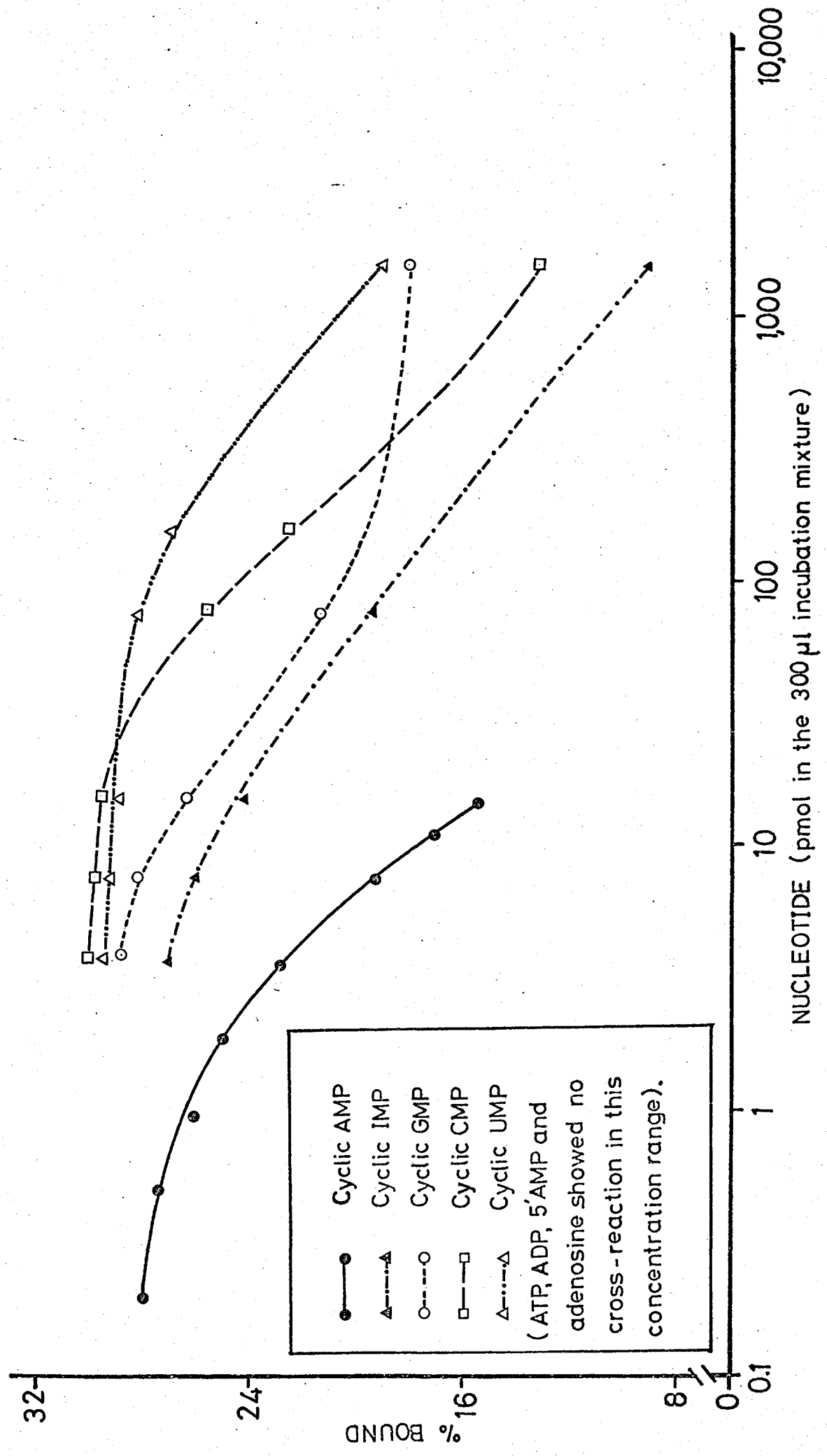


Figure 2.10: Binding protein-specificity study.

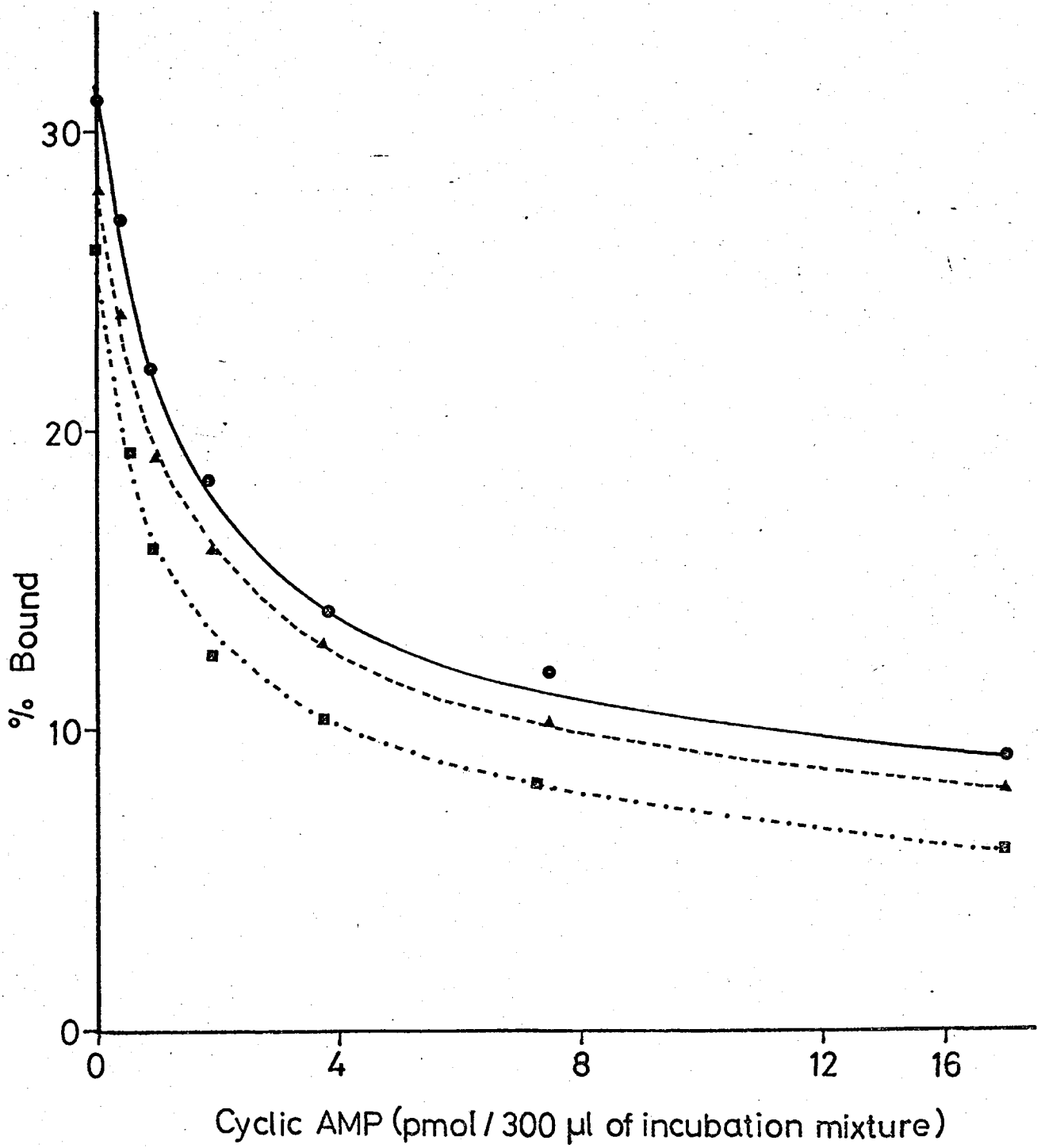


Figure 2.11: Effect of plasma on the cyclic AMP response curve. Three assays were set up with the same reagents: ▪ . . . . ▪ Assay curve for standards in buffer; ▲ - - - ▲ and ● —— ● assays supplemented with blank plasma (50 µl) from two different subjects in place of an equal volume of buffer.

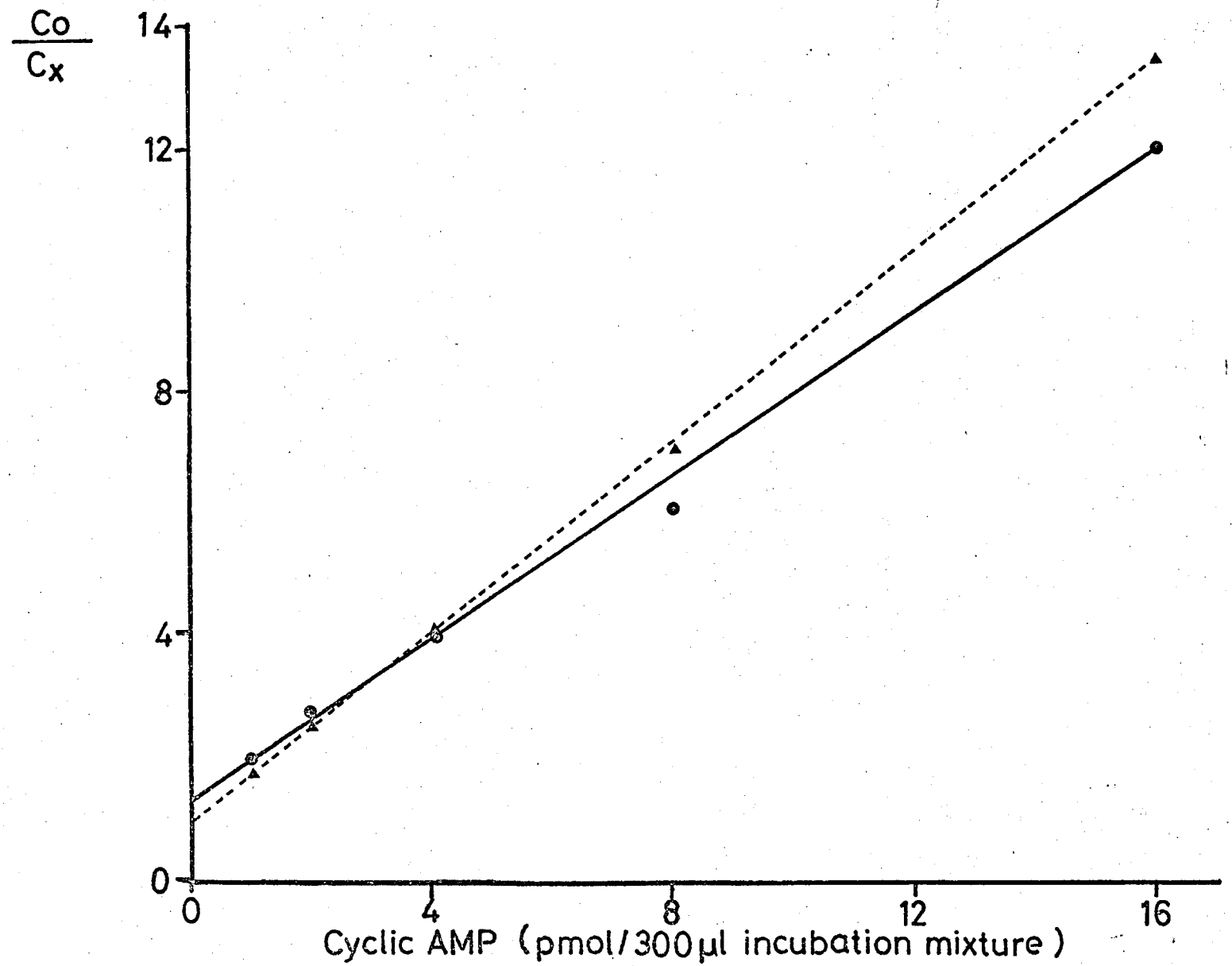


Figure 2.12: Cyclic AMP calibration curve. ▲--▲ Purified binding protein from bovine muscle;

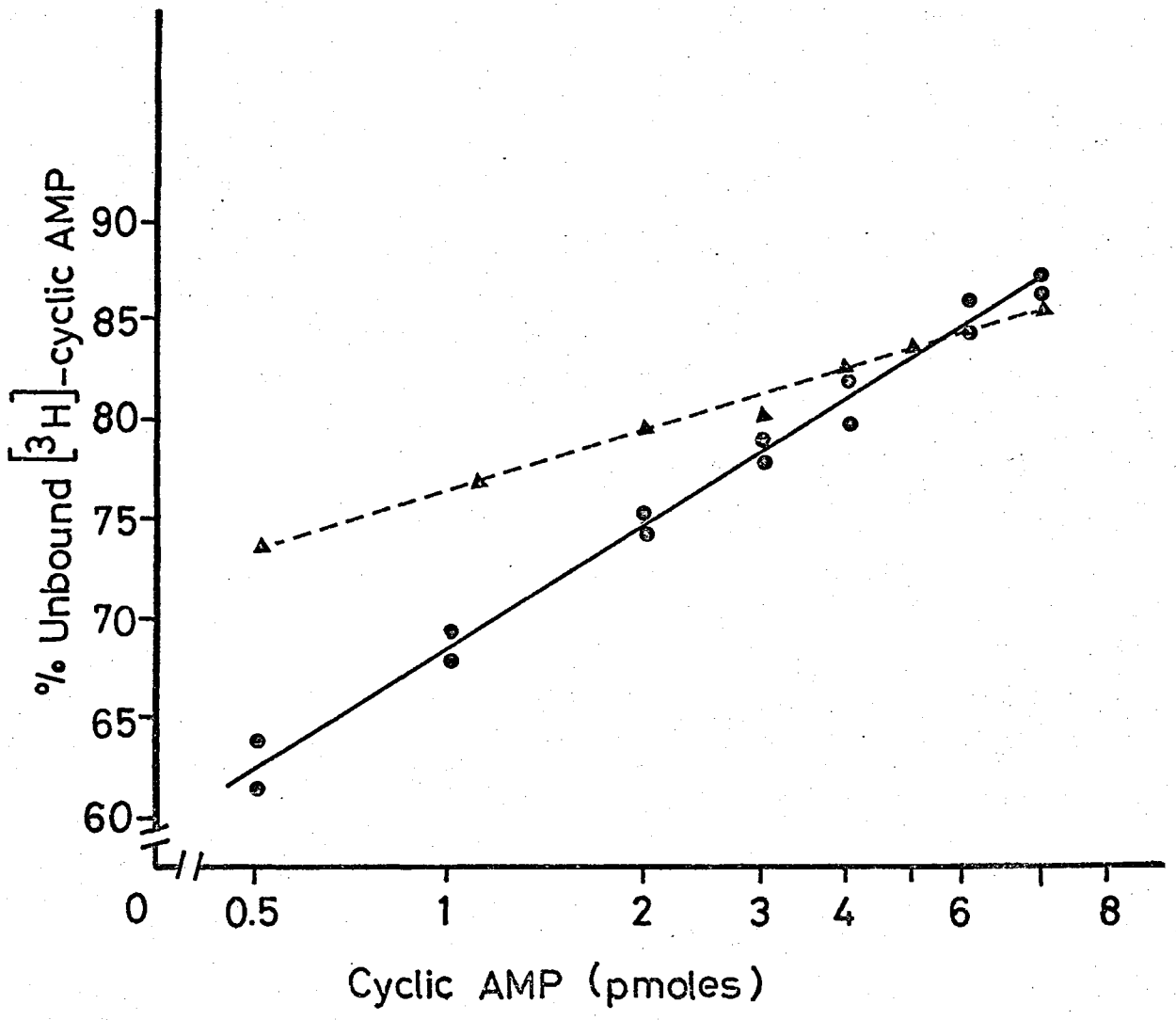


Figure 2.13: Standard curve for cyclic AMP. Solid line, 0.1 ml of eightfold diluted extract of binding protein. Broken line, 12-fold dilution (Rabinowitz and Katz 1973).

of radioactivity bound in the absence of unlabelled cyclic AMP and Cx is the amount of radioactivity bound in the presence of standard or unknown unlabelled cyclic AMP.

d. Percentage of free $[^3H]$ - cyclic AMP as ordinate against unlabelled cyclic AMP (pmol/incubation tube; figure 2.13) as abscissa (Rabinowitz & Katz, 1973).

Of these four methods the former two give curves the latter two are linear. Plot (c) is now widely used giving a straight line of intercept 1.0 on the ordinate.

2.6.8. Standardization Problems

A major problem with the measurement of cyclic AMP in plasma is the presence of phosphodiesterase, which is active even in frozen samples (Brown, personal communication). Thus unless the enzyme is inactivated the plasma concentration of cyclic AMP is found to fall by as much as 100% after storage at - 20°C for a month. Attempts to inactivate the enzyme by collecting blood into lithium-heparin tubes which are either coated with theophylline or contained a small volume of isotonic solution of this inhibitor, proved unsatisfactory in terms of reproducibility. Recently EDTA has been used with success (Oldham*, personal communication). To avoid excessive loss of cyclic AMP heparinised plasma was snap-frozen within 5 - 7 minutes of blood collection, stored at -20°C and deproteinized as described in section 2.6.4 within 24 hours. Recoveries were estimated by the addition of known amounts of $[^3H]$ - cyclic AMP to the freshly collected plasma. In deproteinized plasma cyclic AMP is stable for long periods of storage.

* Tovey et al., 1974

Brown's group (Barling et al., 1974) found that dilution of heparinised plasma with an equal volume of 'Buffer' allowed prolonged storage of samples at - 20°C with minimum cyclic AMP loss. Thus by slight modification of the saturation assay (i.e. up to 150 µl of diluted plasma) direct measurement of cyclic AMP in plasma is made possible. The previously described (section 2.6.4) deproteinization procedure will remove plasma interfering factors, however These are still present but can be corrected by letting the plasma samples stand at room temperature for 24 hours in the absence of theophylline thus losing all measurable cyclic AMP. The effect of cyclic AMP free plasma (blank plasma) on the assay response curves is shown in figure 2.11. These assay curves are not identical showing that constituents in the plasma of different subjects may alter the curve to different extents. The binding curve for plasma diluted with 'blank plasma' for an individual is concomitant with the standard curve of pure cyclic AMP dissolved up in the same blank plasma. Thus the preparation of standards in blank plasma allows elimination of interfering factors. The ideal is individual standard curves but it is, in general, satisfactory to compromise with a standard curve made up with pooled blank plasma (Latner & Prudhoe, 1973). In urine interfering factors are conveniently removed by dilution, thus eliminating the need for the preparation of a blank urine.

Weller et al. (1972) encountered a similar problem in studies of cyclic AMP determination in cerebral tissue. This was overcome by modifying Gilman's assay (1970) by incorporating

unlabelled internal standards in order to determine the extent of interference.

2.6.9. Discussion of the Protein Binding Assay for Cyclic AMP

The assay is based on the competition of variable unlabelled cyclic nucleotide with a fixed amount of labelled cyclic nucleotide for limited protein binding sites. The amount of tritiated cyclic AMP (cpm) still present in the supernatant (protein bound fraction) at the end of the assay is inversely proportional to the quantity of unlabelled cyclic AMP added.

Bovine adrenal binding protein was found to have a high affinity for cyclic AMP (0.83×10^{-8}M; see figure 2.14). This is in good agreement with the value of 0.75×10^{-8}M quoted by Walton & Garren (1970). In the assay procedure as described the lower limit of detection for cyclic AMP was 0.2 pmol. For measurement, the range used was greater than 1 pmol and less than 15 pmol per assay tube.

Of the nucleotides tested cyclic CMP, cyclic GMP, cyclic IMP and cyclic UMP showed slight cross-reaction (figure 2.10). Cyclic IMP seems to be about 10 times less potent than cyclic AMP, and cyclic CMP, cyclic GMP and cyclic UMP are at least 100 times less effective. Urinary levels of cyclic GMP are not sufficiently high to interfere with the assay of cyclic AMP (Goldberg *et al.*, 1969; Ishikawa *et al.*, 1969), while cyclic UMP could not be detected in the urine (Hardman, 1971). There are no published reports of cyclic IMP in urine. Adenosine at concentrations up to 30 nmol/incubation tube, 5' - AMP at concentrations up to 75 nmol/incubation tube and ADP and ATP

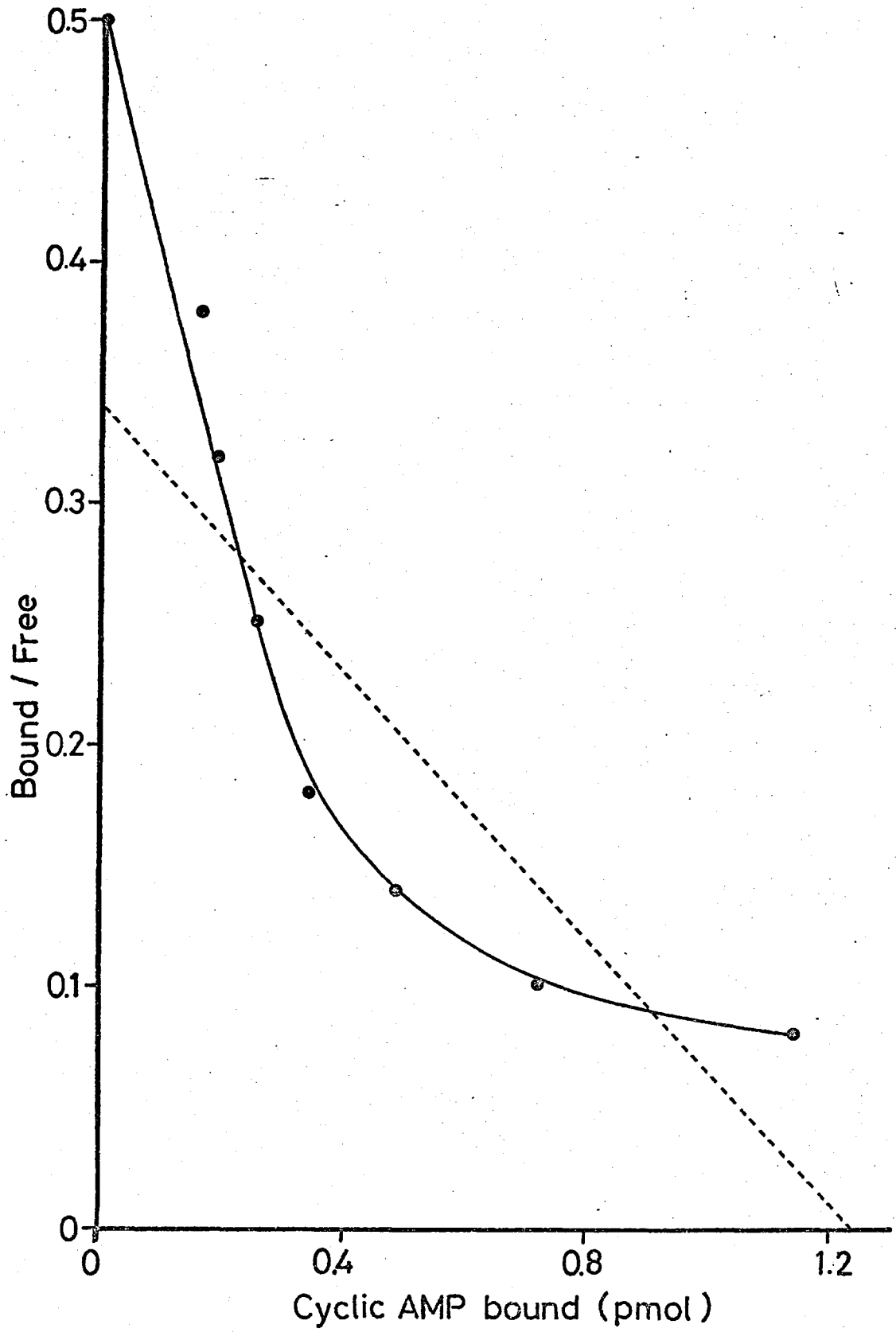


Figure 2.14: Scatchard plot (Scatchard, 1949) for the saturation assay of cyclic AMP. The resulting graph (solid line) is not a straight line because of the heterogeneity of the binding protein preparation (Zettner, 1973). The broken line shows an average slope of -8.3×10^{7} M, indicating the average affinity constant of this binding protein preparation to be 8.3×10^{7} M, and the dissociation constant to be 1.2×10^{-8} M.

at concentrations up to 100 nmol/incubation tube did not appear to cross-react with the system. Thus for urine, after ten to fifty fold dilution, the assay may be taken as specific for cyclic AMP.

The partial competition of cyclic CMP, cyclic GMP, cyclic IMP and cyclic UMP but lack of competition by adenosine, 5' - AMP, ADP and ATP suggests that the specificity resides in the cyclic 3',5' ring. Serial dilutions of binding protein were assayed with the intention of distinguishing non-specific effects (Ekins & Newman, 1970). Though such data does not constitute complete evidence for assay specificity, parallel dilution curves on semilogarithmic plots with respect to a standard cyclic AMP range were observed.

Various experiments were conducted to check the validity of the assay (table 2.4). Treatment of urine with cyclic AMP phosphodiesterase reduced the cyclic AMP content to zero; readdition of a known amount of cyclic AMP to this urine gave a value in accordance with aqueous standards.

Table 2.4: Protein Binding Assay for cyclic AMP in Urine

	Cyclic AMP (pmol/50µl)		
	Measured	Expected	% Recovery
1. 50 µl of urine (1:50 dilution)	2.8 (3)	-	-
2. 50 µl of urine (1:50 dilution) + 7.5 pmol of cyclic AMP	10.1 (3)	10.3	98
*3. 50 µl of urine (1:50 dilution) pretreated with phosphodiesterase	0.0 (3)	0.0	-
*4. As 3 except 15 pmol of cyclic AMP added after boiling	15.9 (3)	15.0	106

*Brain phosphodiesterase preparation (60µg) was incubated for 10 minutes with the sample and then destroyed by boiling (100°C) for 5 minutes

( ) Number of estimations

To check the reproducibility of the urinary cyclic AMP determinations, three separate urine specimens were collected. Each specimen was put through the dilution and assay procedure on 4 separate occasions (table 2.5).

Table 2.5: Check for Assay Reproducibility

Specimen	Cyclic AMP (pmol/50µl urine) ± S.D.	Interassay Coefficient of Variation
1	78.0 ± 4.0 (4)	5.1
2	47.3 ± 5.7 (4)	12.0
3	47.9 ± 8.1 (4)	16.9
Mean interassay coefficient of variation		11.4

CHAPTER III

Factors Affecting the Plasma and Urinary Levels of Cyclic AMP

3.1 Diurnal Variation of Plasma and Urinary Cyclic AMP in Normal Human Subjects.

Cyclic AMP has been implicated in a number of clinical conditions including pseudohypoparathyroidism (Chase et al., 1969; Marcus et al., 1971; Murad and Pak, 1972), mania and depression (Abdulla and Hamadah, 1970; Paul et al., 1970 ; Cramer et al., 1972; Naylor et al., 1974), bronchial asthma (Szentivanyi, 1968; Alston et al., 1974), and diabetes insipidus (Fichman and Brooker, 1972). In these conditions cyclic AMP levels have been studied mainly in urine and plasma. The interpretation of cyclic AMP levels as an aspect of clinical biochemistry must rely on a clear understanding of the "normal range" for this nucleotide. Thus the occurrence of diurnal variation of cyclic AMP in body fluids could produce a misleading interpretation if reliance is placed on data obtained from random specimens.

A number of hormones are known both to initiate their biological actions by altering the intracellular concentrations of cyclic AMP (section 1.4) and to exhibit circadian rhythms (Conroy and Mills, 1970; table 3.1). It would therefore seem possible that cyclic AMP should exhibit a diurnal variation. Cyclic AMP has been reported to exhibit such a pattern in mouse epidermis (Marks and Grimm, 1972), rat gastric mucosa (Domschke et al., 1972) and rat adrenal cortex (Moore and Quavi, 1971). In this latter case the rhythm disappeared after appropriate denervation in the hypothalamic region.

Table 3.1 Hormones Exhibiting Circadian Rhythms
(Conroy & Mills, 1970)

Hormone	Peak	Trough
Adrenaline	11.00 - 20.00	23.00 - 05.00
Noradrenaline	08.00 - 14.00	20.00 - 02.00
ACTH	06.00	18.00
*ADH	Morning	Night
MSH	Early morning	18.00
TSH	20.00 - 08.00	11.00 - 20.00

* Goodwin, et al. (1968) have reported considerable individual variation in the diurnal rhythm of urinary excretion of ADH.

At present there is no detailed information on the diurnal variation of plasma cyclic AMP. The situation with regard to human urinary excretion of cyclic AMP is confused. Chase *et al.* (1969) did not find any diurnal variation in the excretion of cyclic AMP in four normal volunteers. However, at the same time as this investigation, Murad and Pak (1972) reported circadian rhythms for both cyclic AMP and cyclic GMP in an unspecified number of subjects. Recently Sagel *et al.* (1973; 16 normal volunteers) and Somerville (1973; 5 normal volunteers) have found a diurnal variation in urinary excretion of cyclic AMP.

Plasma and urinary levels of cyclic AMP were investigated in 8 male and 10 female volunteers and urinary levels alone were studied in a further 3 male and 7 female volunteers (doctors, nurses and students) aged 18 - 55 years (all but four being under 30 years). Serum levels of PTH and TSH were measured on 8 volunteers. There was no attempt to modify the diet and physical activity of the volunteers. In fact apart from 4-hourly attendance at the collecting centre they were asked to pursue normal activities. Disturbance of sleep was kept to a minimum, the 04.00 and 08.00 sample being collected at the bedside. Samples were obtained at 4-hourly intervals over a 24-hour period commencing at 08.00. As we have evidence of a circadian rhythm associated with the menstrual cycle (section 3.2), repeat collections were made eight weeks later. On the second 24-hour study the volunteers were asked to avoid the ingestion of alcohol. Both plasma and urine

samples were collected (2.3.1) and prepared for the assay as described in section 2.6.4. Cyclic AMP was determined by the saturation method of Brown et al. (1971) section 2.6.5.

Figure 3.1 summarises data for plasma and urine from all subjects studied. The bimodal distribution observed for plasma led us to look for two groups within this population. Consideration of the first 24-hour collection period indicated two distinct urinary patterns: 21 subjects (14 female, 7 male) showed maximum levels of cyclic AMP in the morning (04.00 - 08.00), whereas 7 subjects (3 female, 4 male) showed a peak in the afternoon (16.00 - 20.00). Plasma values also gave two patterns; 16 subjects (9 female, 7 male) showed a cyclic AMP peak at about midnight, whereas 2 subjects (1 female, 1 male) had their peak value at noon. Plasma and urinary cyclic AMP levels were remeasured in 10 of the subjects (6 female, 4 male) eight weeks after the first 24-hour survey and eight of these (5 female, 3 male) showed the same morning peak pattern as before (figure 3.2). The two exceptions had shifted from a morning peak to an afternoon peak pattern. The male had had a markedly higher alcohol intake on the first occasion; and the female was at the beginning of her menstrual cycle for the first collection but at the end of her cycle for the second.

Figure 3.3 summarises data for urine and plasma of the morning peak type, figure 3.4 shows the afternoon peak pattern. Figure 3.5 illustrates the reproducibility of an individual pattern for the urinary cyclic AMP in collections made at the mid-point of three consecutive menstrual cycles. The recovery

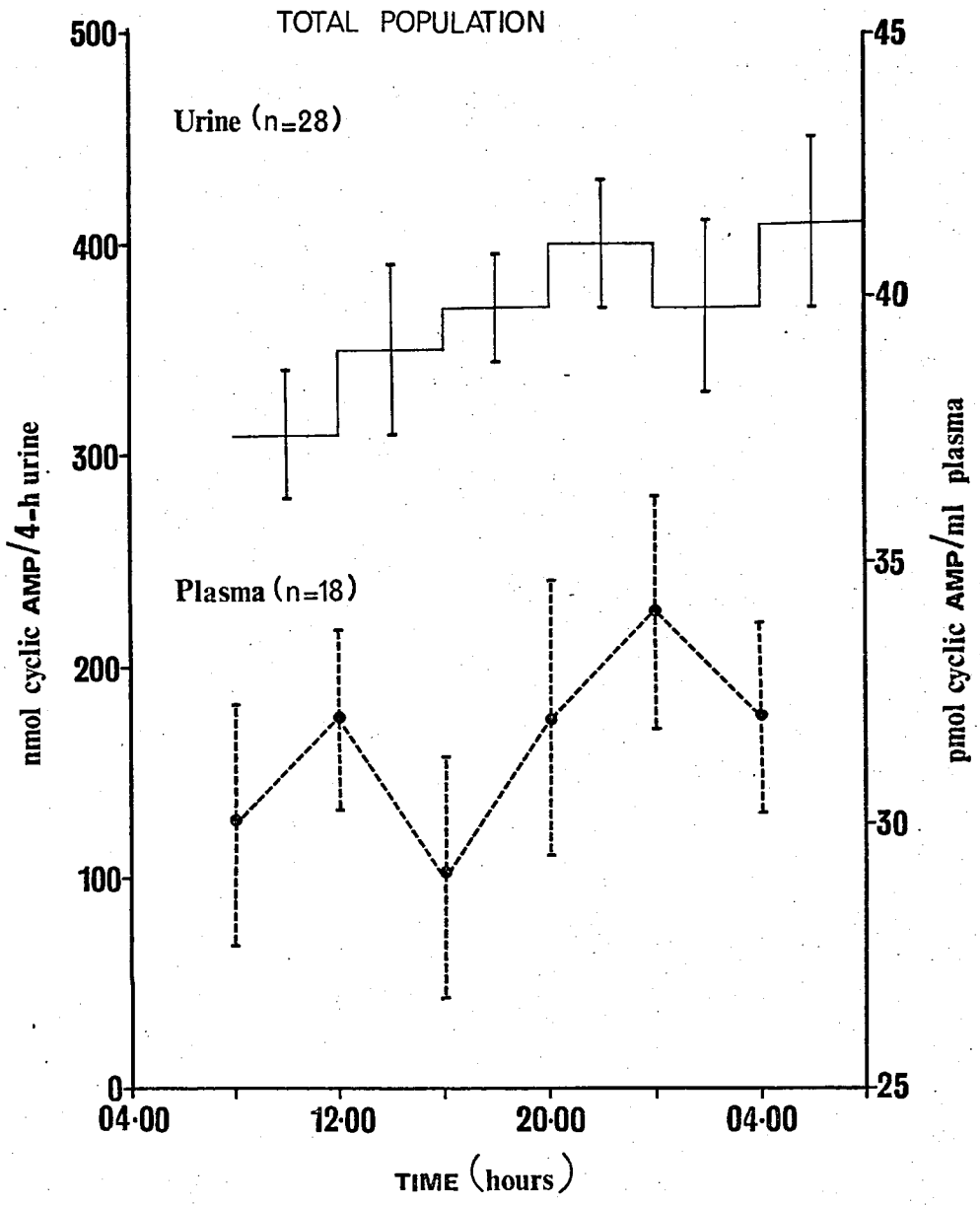


Figure 3.1: Diurnal variation of urinary and plasma cyclic AMP levels in normal human subjects. Solid line shows variation in urinary cyclic AMP, broken line shows variation in plasma cyclic AMP levels; S.E.M. values are indicated by the vertical bars. Comparison of the paired data (Student's t test; Bailey, 1964) for minimum with maximum cyclic AMP in the 4 h urine collection periods gives a difference significant at the level $p < 0.05$. For plasma the minimum value at 16.00 h is lower than the 12.00 h ($p = 0.1$) and the 24.00 h ($p < 0.02$).

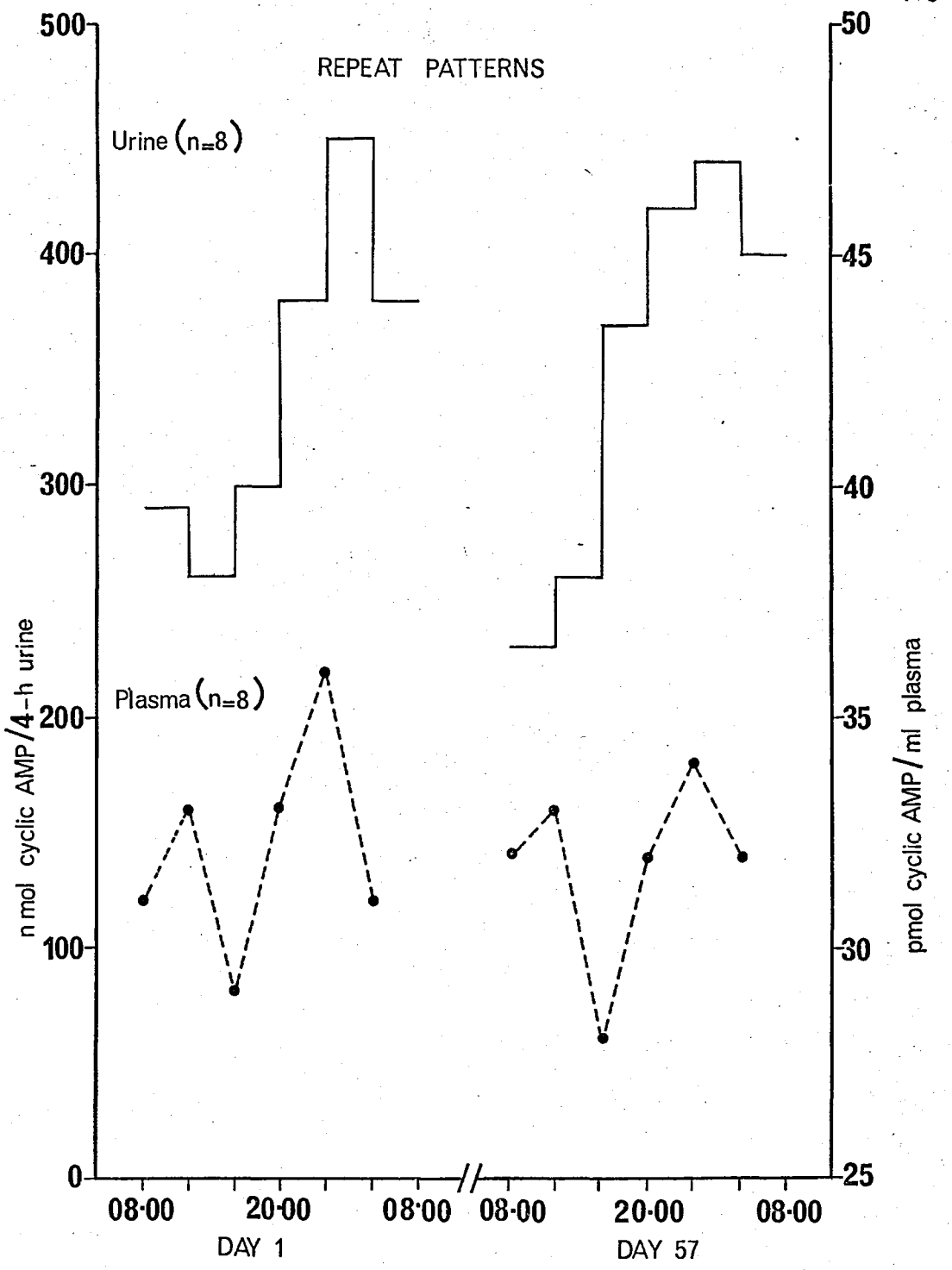


Figure 3.2: Diurnal pattern for plasma and urinary levels of cyclic AMP in normal humans (mean of 8). Specimens were collected on two separate occasions eight weeks apart.

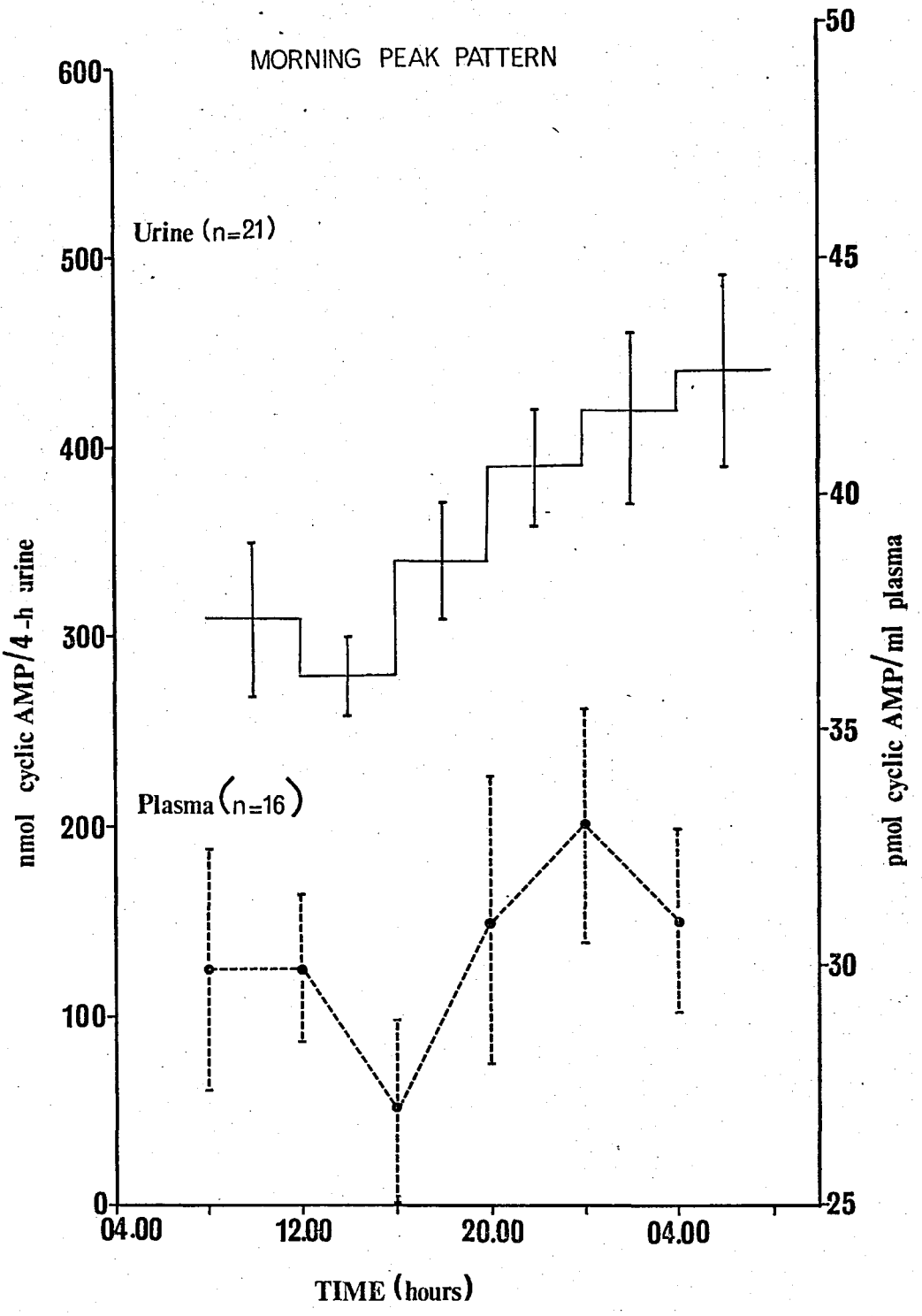


Figure 3.3:Diurnal rhythm-morning peak pattern-for plasma and urinary levels of cyclic AMP in normal humans. For urine (n=21) the 1200-1600h value is lower than the 0400-0800h value ($p<0.02$). For plasma (n=16) the minimum value at 1600h is lower than the 2400h value ($p<0.01$).

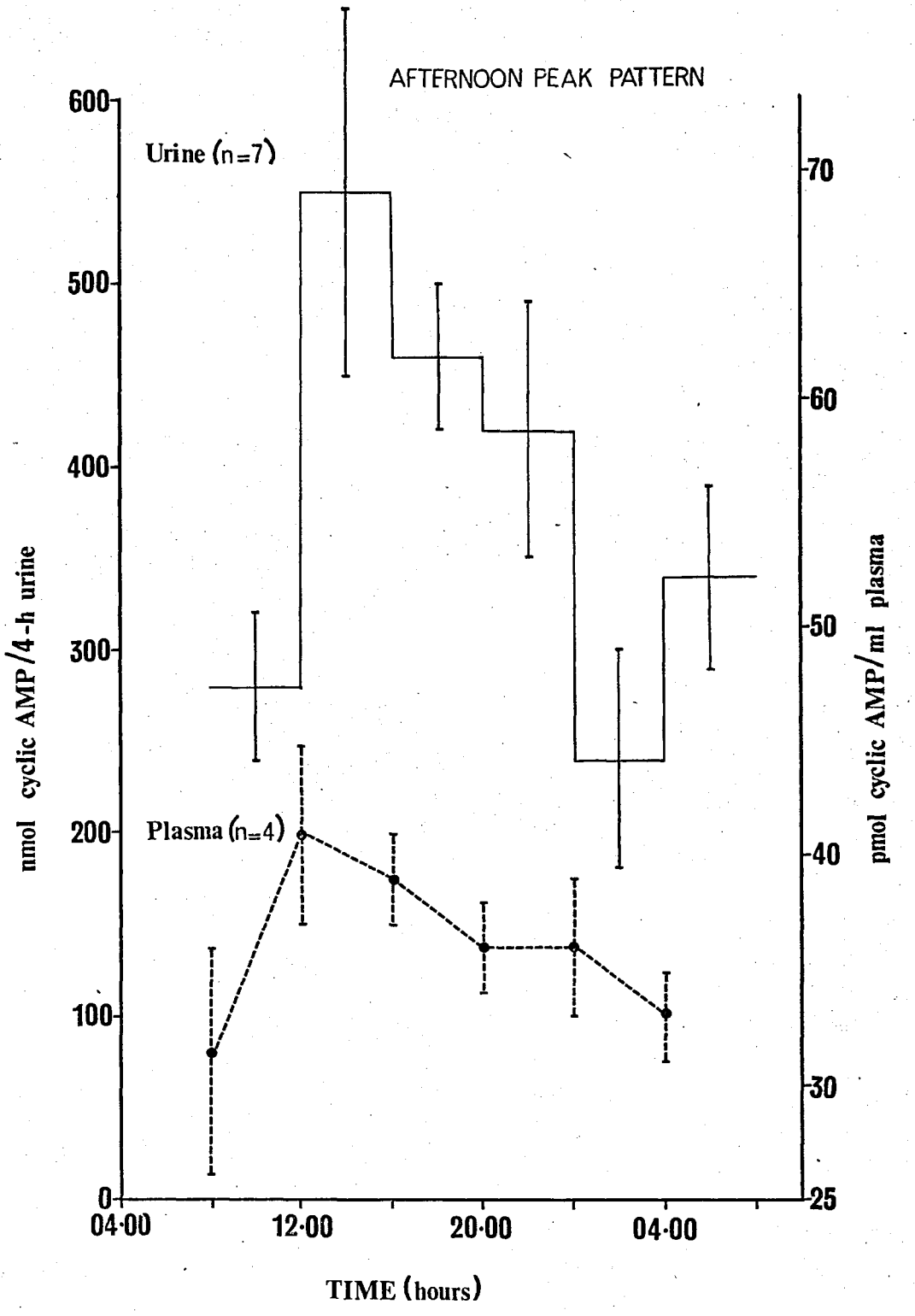


Figure 3.4 : Diurnal rhythm - afternoon peak pattern - for plasma and urinary levels of cyclic AMP in normal humans. For urine (n=7) the 2400-0400h and 0800-1200h values are lower than the 1200-1600h ($p<0.01$). The plasma pattern is for 4 subjects (2 of these showing a pattern shift from one collection to the next), the individual points are not significantly different at the 10% level.

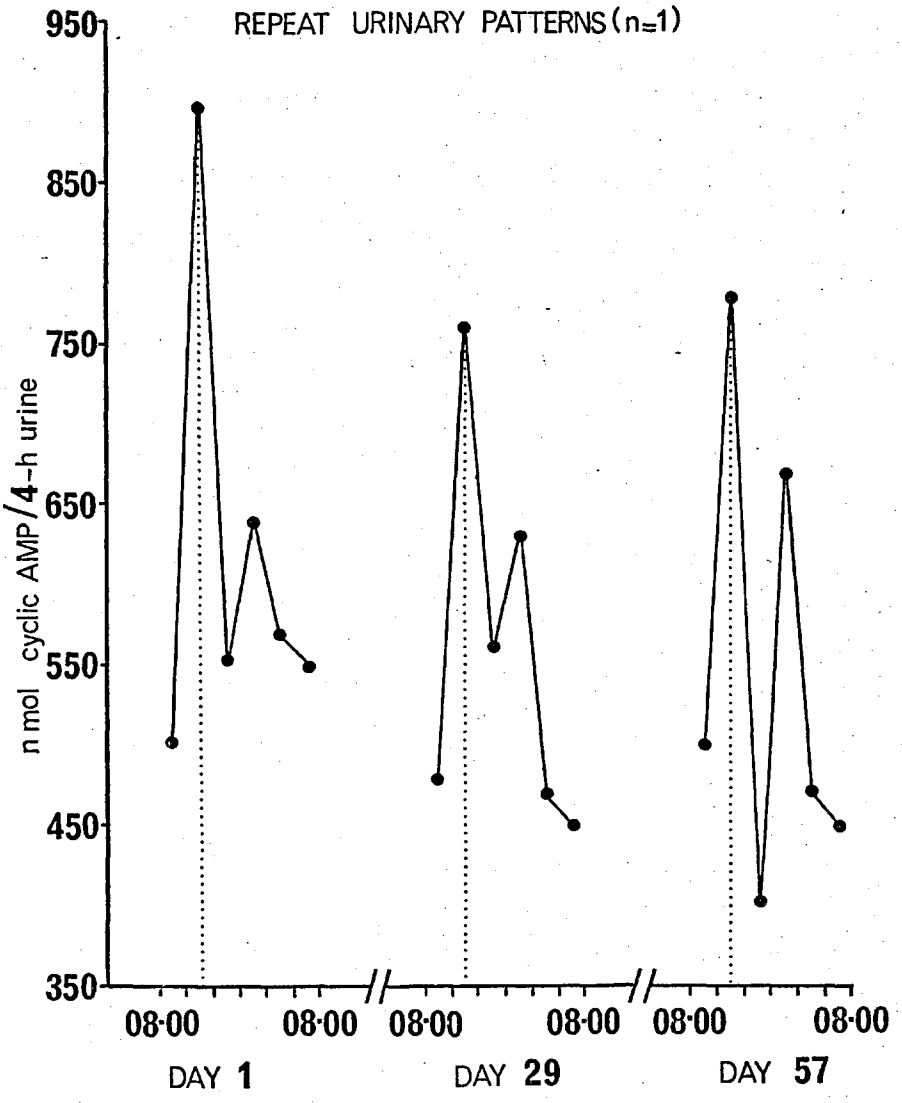


Figure 3.5: Illustration of reproducibility of an individual diurnal pattern for urinary cyclic AMP. Specimens were collected from a normal volunteer at the mid-point of three consecutive menstrual cycles.

values for the plasma procedure ranged between 70-80%. The 24-hour urine values for each individual investigated on the two occasions varied by not more than 10%, with one exception (27%) of the female who was at different points in her menstrual cycle. All 24-hour creatinine values fell within the normal range. Variations in plasma levels of PTH and TSH were measured through the co-operation of Mr. Brian Morris and Mr. Peter Wood. Their findings are depicted in figures 3.6 and 3.7.

The results obtained demonstrate diurnal rhythms for plasma and urinary cyclic AMP. The urinary excretion pattern was, in fact, similar whether the results were expressed as nmol/4h or nmol/mg creatinine. This latter method of expressing data has been used by some workers (e.g. Murad and Pak, 1972), however in agreement with Curtis and Fogel (1970) we found a circadian rhythm for creatinine itself and hence find no purpose in presenting data obtained from the ratio of a variable with a variable.

The patterns obtained indicate two populations with regard to peak times. In our study the major group showed maximum values in the early morning for both plasma and urinary cyclic AMP, the minor group showed their peak values in the afternoon. Some individuals investigated fitted in with both these groups in that they showed a bimodal distribution. The urinary pattern does not seem simply to be a reflection of plasma concentration. Liddle and Hardman in their review (1971) point out a proportion of cyclic AMP in urine is formed in the

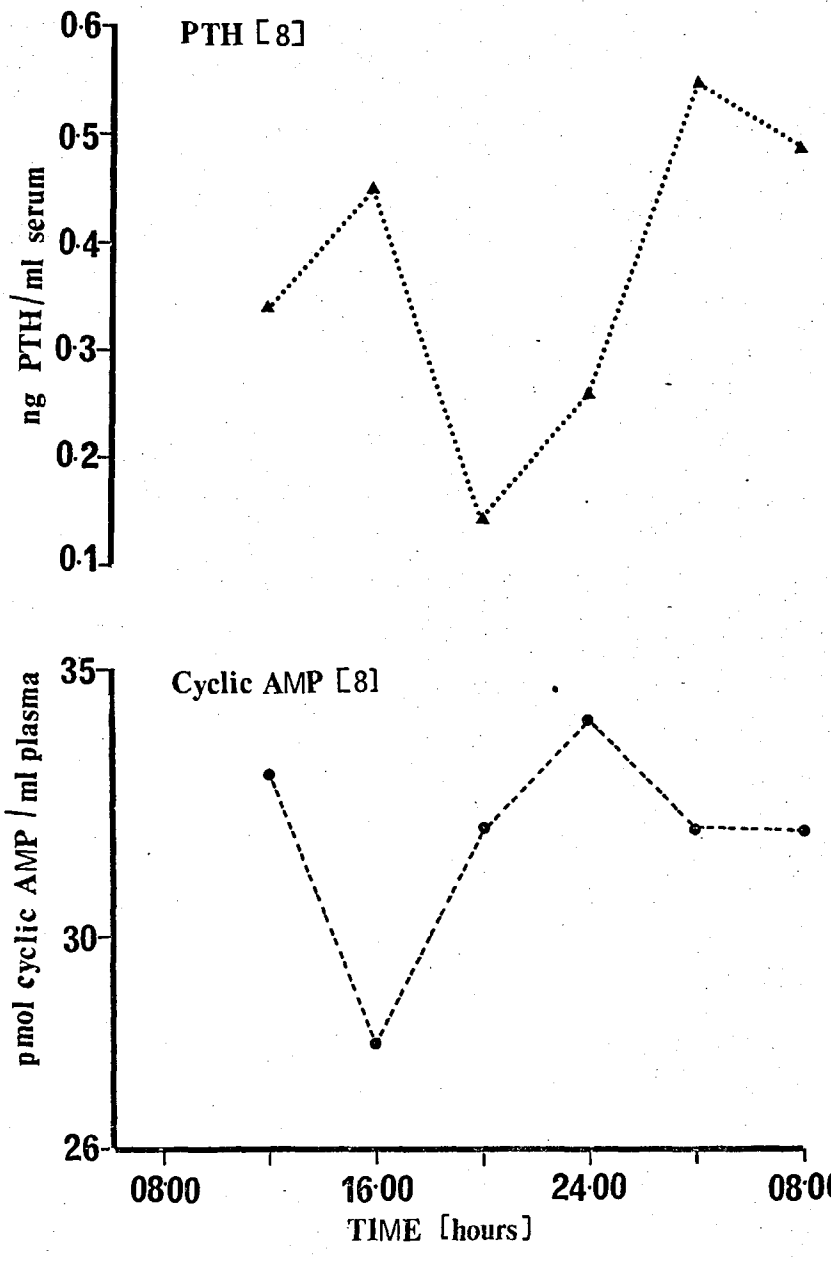


Figure 3.6: Diurnal variations in plasma levels of PTH and cyclic AMP. Each point represents the mean value for eight normal subjects.

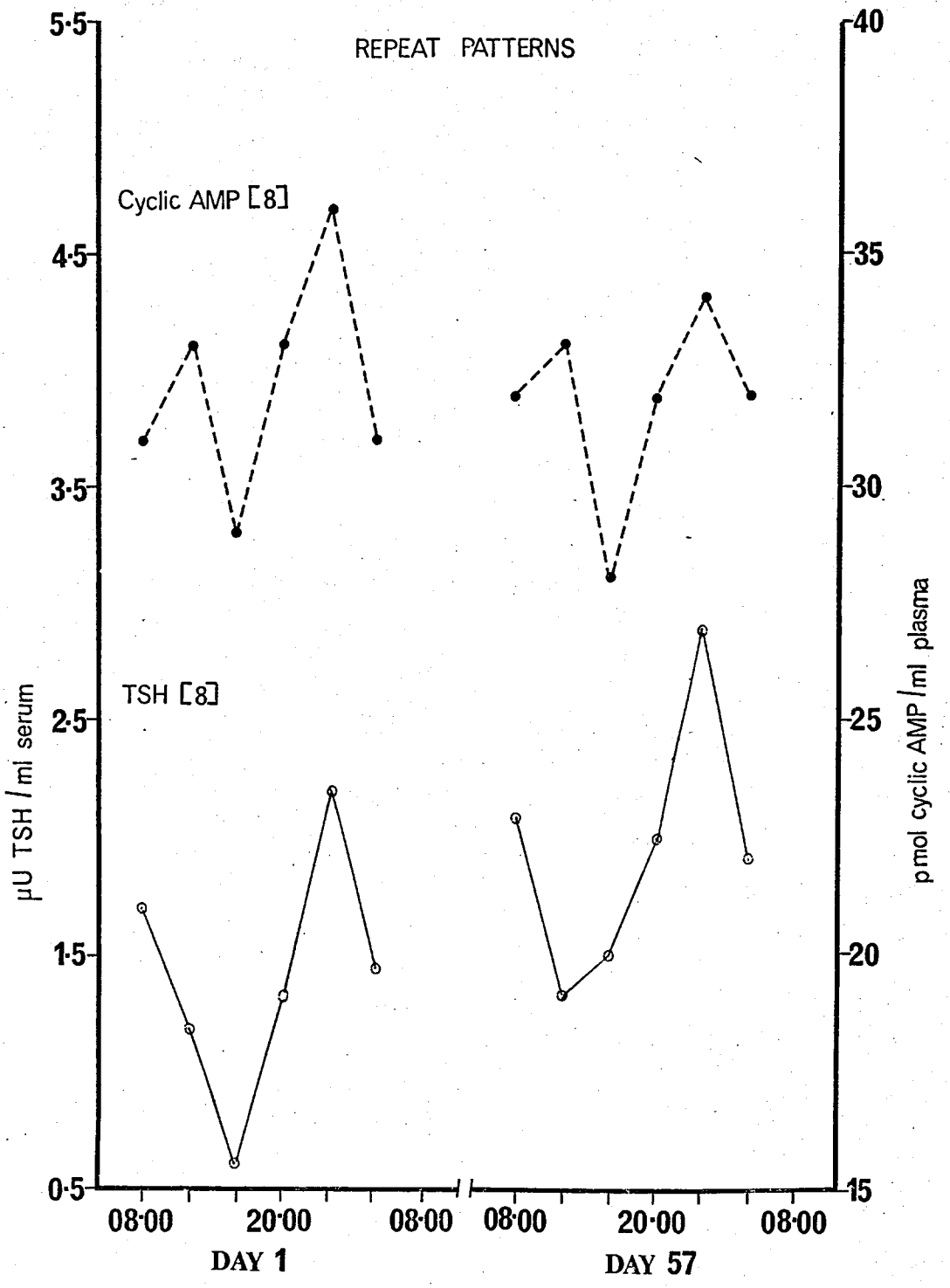


Figure 3.7: Diurnal variations in plasma levels of TSH and cyclic AMP. Each point represents the value for eight normal subjects. Determinations were carried out on two separate occasions eight weeks apart.

kidney ("nephrogenous") and is excreted directly without traversing the general circulation.

The pathways to the appearance of cyclic AMP in body fluids are still far from clear (Hardman *et al.*, 1971) and the mechanisms involved in the control of diurnal variation in cyclic AMP remain to be established. We chose PTH and TSH on the criteria that they affect cyclic AMP levels (Robison *et al.*, 1971) and that assays exist in this Department. There is a need to investigate a variety of hormones including ADH (Jenner *et al.*, 1972) and glucagon (Park & Exton, 1972) before attempting to correlate patterns.

Our findings indicate the need for caution in interpreting cyclic AMP levels in clinical disorders. It would seem that investigation of patterns of variation will be of greater value than the determination of the concentration of a random specimen.

3.2 Variation of Urinary Cyclic AMP during the Human Menstrual Cycle.

The plasma concentrations of luteinising hormone (LH) and follicle-stimulating hormone (FSH), show a marked rise at about mid-cycle (figure 3.8) and this leads to ovulation (Watson, 1973). In the corpus luteum LH activates adenyl cyclase leading to an increased progesterone synthesis (Marsh *et al.*, 1966). Oestradiol shows a peak plasma concentration about one day preceding the gonadotrophin peak (Dodson et al., 1973), and one action of this hormone is to restore uterine cyclic AMP levels in ovariectomised rats (Szego and Davis, 1967). Ryan and Coronel (1969) have reported that the injection of cyclic AMP in female Swiss mice prevented pregnancy and led to a considerable reduction in ovarian mass. The treated mice were still infertile 5 weeks after cessation of the injections. However, the dose level was, to say the least, pharmacological (5 mg per day). A further confusion is that the dibutyryl analogue was ineffective even at this massive dose level as was 5'-AMP. These variations in hormonal levels associated with the menstrual cycle may influence urinary excretion of cyclic AMP and Taylor *et al.* (1970) reported a mid-cycle rise of this nucleotide in three women.

Twelve normally menstruating women, two women with secondary amenorrhoea, one pregnancy (second trimester) and six men were investigated (the overall age range being 19-38 years). Five of those with a normal ovulatory cycle were classified as suffering from premenstrual tension. The classification was based on a questionnaire (see pp.129-130) which was compiled and assessed by

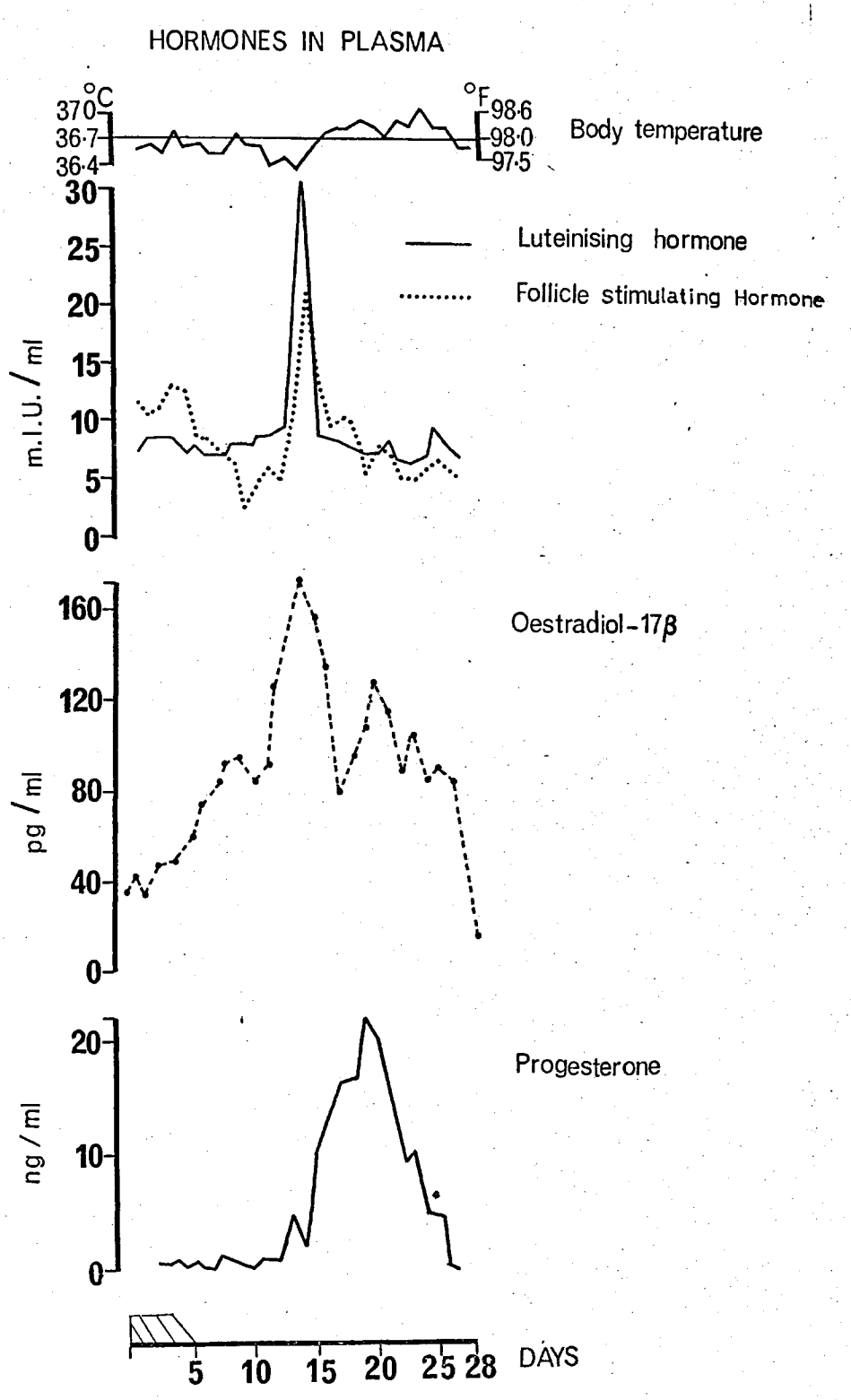


Figure 3.8: Circadian variations in plasma levels of hormones associated with human menstrual cycle. The secretion of progesterone by the corpus luteum leads to a rise in basal body temperature. A temperature rise and the maintenance of a plateu provides some indication that ovulation has taken place. (Reproduced from a chart by Searle Diagnostic Hormone Assay Laboratory, High Wycombe, Bucks., U.K.).

Questionnaire devised for the Assessment of Premenstrual Tension Syndrome

Name ..... Age .....

Marital Status ..... No. of children .....

1. Are your periods regular and normal? Yes .....
   No .....
2. How long does the period last? No. of days .....
3. How many days between the periods? .....
4. Do you experience feeling of tension before or during the period? Yes .....
   No .....
   If yes,
   (a) How many days before the period? .....
   (b) How long does the tension last? .....
   (c) Is it relieved by the onset of the period? .....
   (d) Does the tension last throughout the period? .....
5. Do you feel pain before or during the period? Yes .....
   No .....
   If yes, please specify .....
   .....

If you suffer from tension before or during the period:-

6. Do you feel slight irritation with other people? .....
7. Do you get bad tempered? .....
8. Do you take it out on yourself or others? .....

9. Do you feel depressed and weepy? .....

10. Do you entertain any suicidal .....
thoughts? .....

11. Do you imagine husband or boyfriend .....
does not want you, etc. - "No one .....
cares about me!" .....

12. Do you want to go out? .....

13. Do you feel bloated and ugly? .....

14. Do you put on weight? .....

15. How long have you had this tension? .....

16. When in cycle does it start? .....

17. How does it affect you? .....
.....

18. What relieves the symptoms? .....
.....

Menstrual History

1. At what age did menstruation start? .....

2. What pattern was it in the first .....
two years? .....

3. What pattern was it before you .....
started having tension, if any? .....

4. If you suffer from premenstrual .....
tension, has there been a difference .....
in the symptoms over the years? .....
Please specify .....

Comments

Please feel free to add any information you wish.

.....

.....

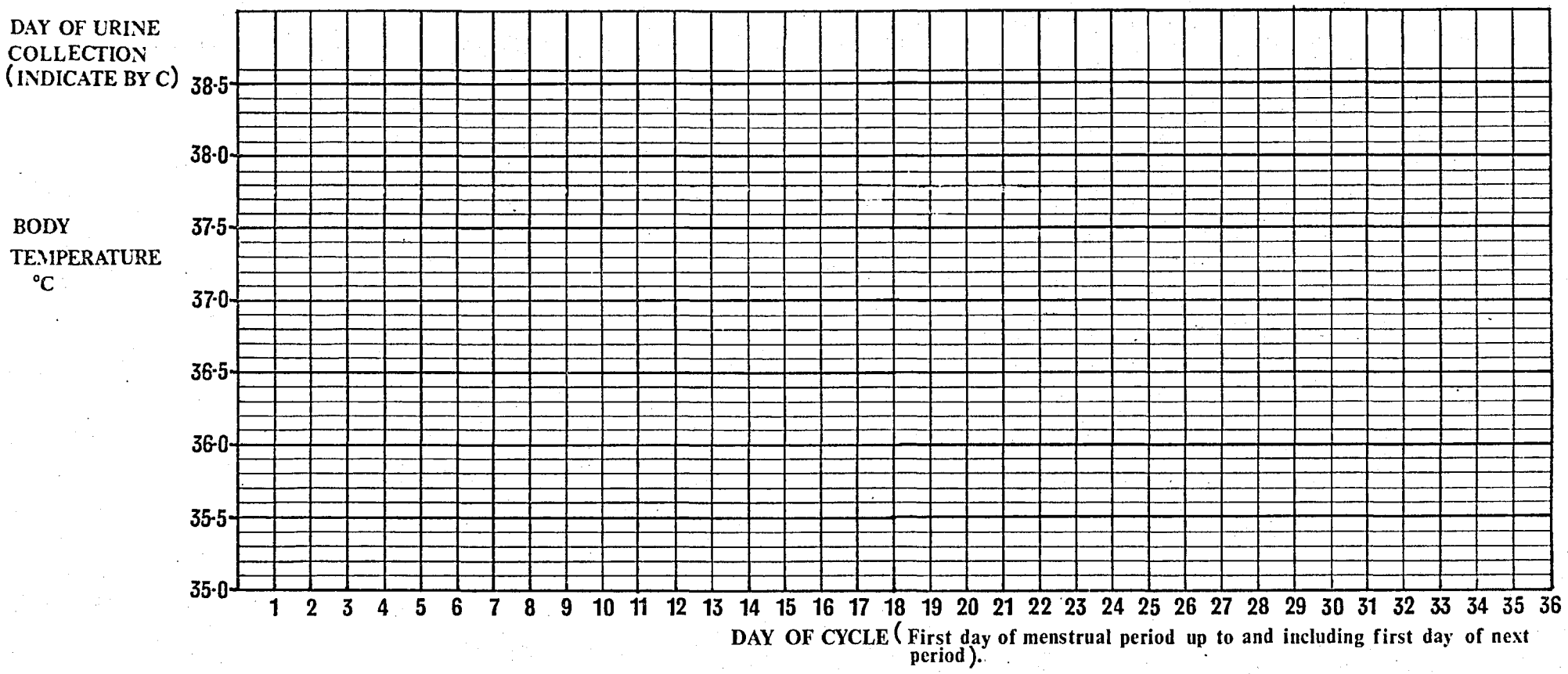
Miss Mary L. Stokes, Consultant Gynaecologist at the South London Hospital for Women. To obtain a rough estimate of the time of ovulation first morning temperature charts (figure 3.9) were completed by some of the volunteers. Urines (24-hours) were collected at weekly intervals for up to two months. Completeness of the collection was checked by creatinine estimation and the samples were stored at -20°C until cyclic AMP determination by the protein binding method of Brown *et al.* (1971).

The results obtained for the twelve normally menstruating women are shown (two cycles) in figures 3.10 and 3.11. A peak (3.5 ± 0.3 μmol cyclic AMP/24 hours) was obtained at day 14 (where day one is defined as the onset of menstruation). This maximum was significantly higher than the value on day 7 (2.5 ± 0.2 μmol cyclic AMP/24 hours; $P<0.02$) and on day 21 (2.9 ± 0.2 μmol cyclic AMP/24 hours; $P = 0.02$). The pregnant female (3.7 ± 0.17 μmol cyclic AMP/24 hours; 6 samples) and the six males (3.1 ± 0.15 μmol cyclic AMP/24 hours; 36 samples) showed no significant variation during six to eight weeks. The two subjects with amenorrhoea excreted cyclic AMP (1.9 ± 0.18 μmol cyclic AMP/24 hours; 9 samples) at a level comparable to the minimum in the menstrual cycle studies (table 3.1) but again there was no significant change in four weeks.

These findings indicate a maximum urinary excretion of cyclic AMP associated with temperature rise and thus, presumably, ovulation. Our results suggest that the premenstrual tension syndrome may be associated with a rhythm of greater amplitude (figure 3.11a) the significance of which is not yet understood.

FIGURE 3.9 :- FIRST MORNING TEMPERATURE CHART

NAME :- _ DATE ON FIRST DAY OF CYCLE :- (i.e. FIRST DAY OF PERIOD) :-



Days when menstruation occurs (indicate by X)

Days of tension or depression. Indicate degree of severity i.e. + = mild, 2+ = marked, 3+ = severe. (If no tension, leave blank).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

DAY OF CYCLE

Collection procedure

Collect a 24 h urine (blood-free) at least four times during the cycle. A possible scheme is days 7, 14, 21 and 28. It is important to collect at about the time of ovulation (temperature change) and during the time (if any) of tension.

Any other comments, e.g. illness, drugs and medicines :-

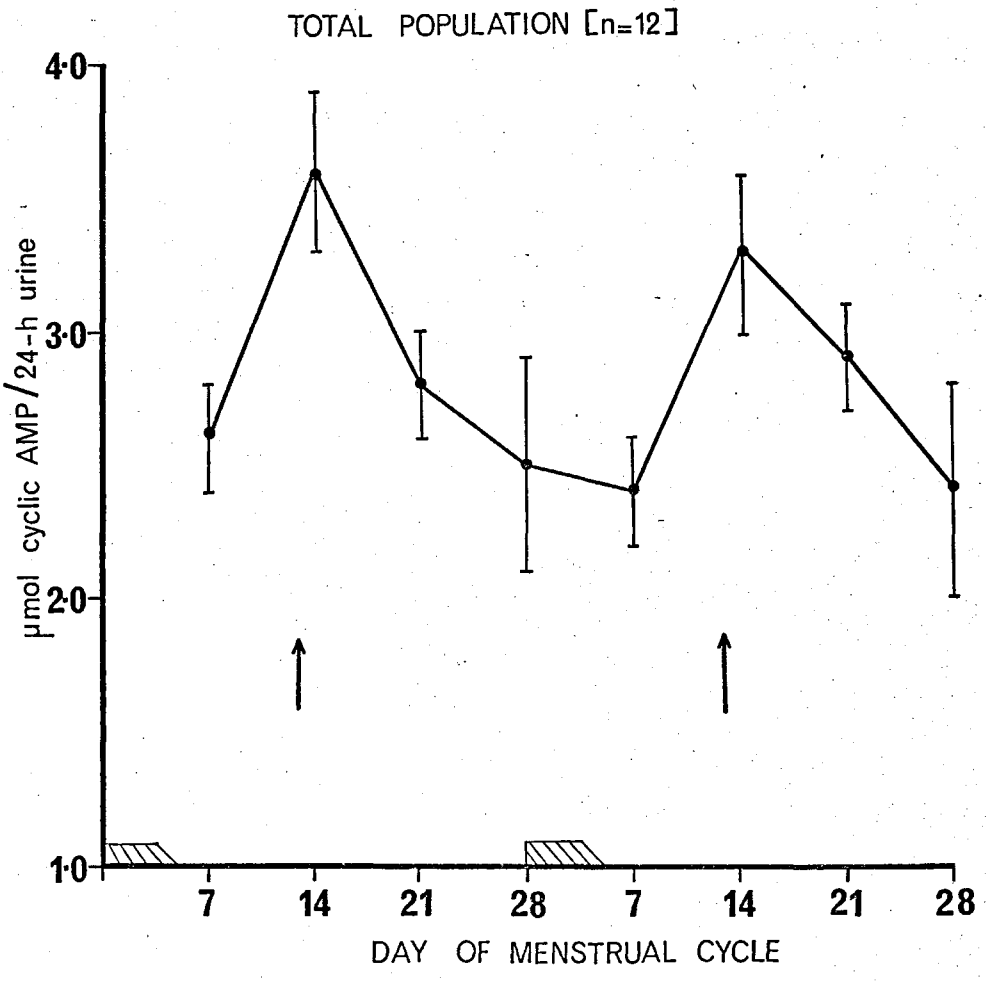


Figure 3.10: Urinary cyclic AMP excretion during two menstrual cycles. Each point represents the mean value for 12 normally menstruating women; the vertical bars indicate S.E.M. values. The shaded areas show periods of menstruation, and the arrows the time of first morning temperature shift (approximate time of ovulation).

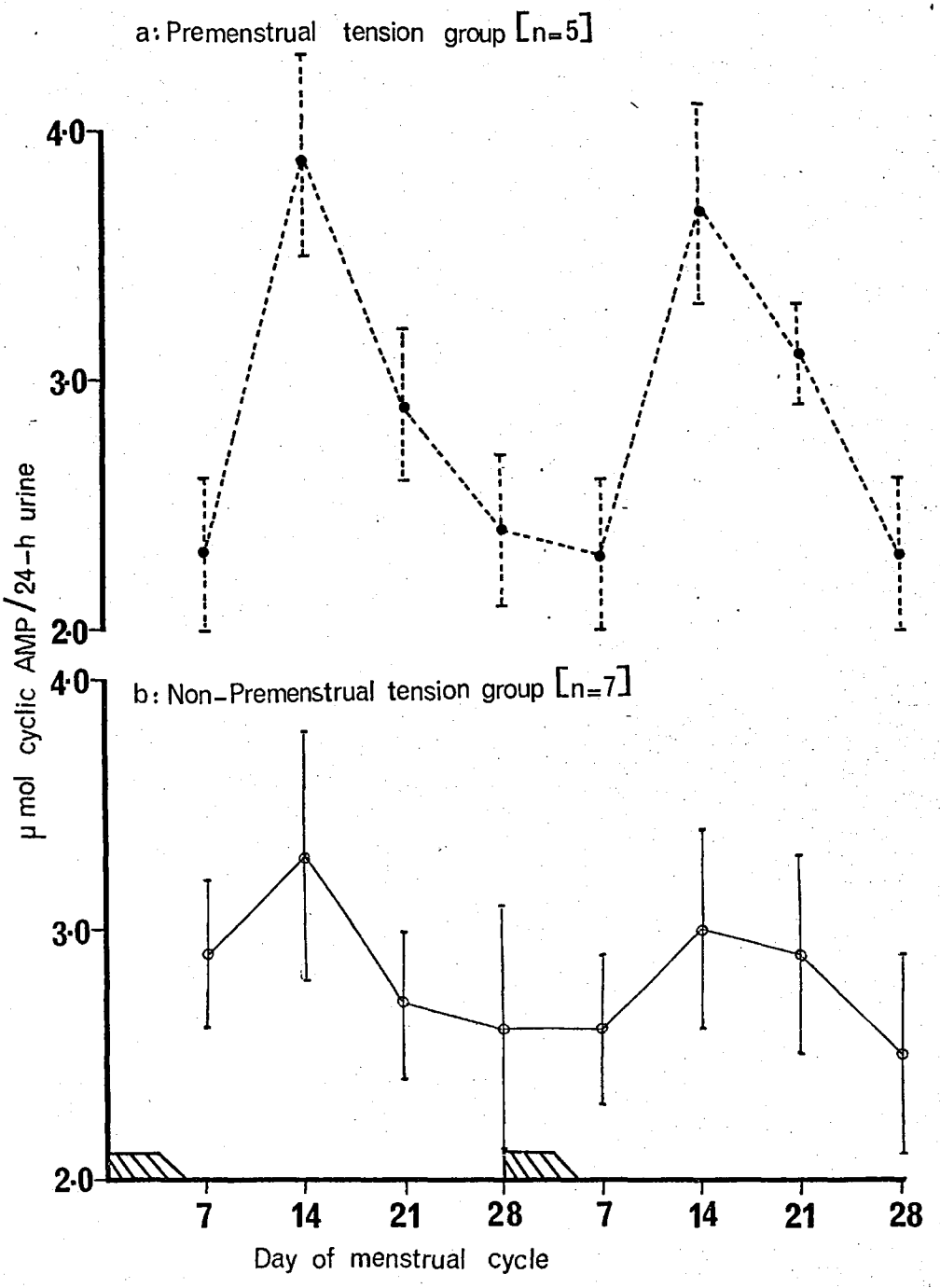


Figure 3.11: Urinary cyclic AMP excretion during two menstrual cycles. The shaded areas represent periods of menstruation. S.E.M. values are indicated by vertical bars.

TABLE 3.1:

URINARY CYCLIC AMP (μmol/24 hours)

	Normal ovulatory cycle					
Day of cycle	Overall group	Non-premenstrual tension	Premenstrual tension	*Secondary Amenorrhoea	*Pregnancy	*Males
7	2.6 ± 0.2	2.9 ± 0.3	2.3 ± 0.3	2.1 ± 0.7	3.8	3.4 ± 0.3
14	3.6 ± 0.3	3.3 ± 0.5	3.9 ± 0.4	2.2 ± 0.4	4.2	3.3 ± 0.4
21	2.8 ± 0.2	2.7 ± 0.3	2.9 ± 0.3	1.9 ± 0,6	3.0	3.3 ± 0.5
28	2.5 ± 0.4	2.6 ± 0.5	2.4 ± 0.3	1.8 ± 0.2	3.7	3.0 ± 0.5
7	2.4 ± 0.2	2.6 ± 0.3	2.3 ± 0.3		3.4	3.0 ± 0.4
14	3.3 ± 0.3	3.0 ± 0.4	3.7 ± 0.4		3.8	3.4 ± 0.5
21	2.9 ± 0.2	2.9 ± 0.4	3.1 ± 0.2			
28	2.4 ± 0.4	2.5 ± 0.4	2.3 ± 0.3			

Values given here are the means ± S.E.M.

*Urines (24 hours) were collected at weekly intervals.

One hypothesis is a loss of fine control, i.e. an overswing. We could find no evidence of a significantly low urinary level of cyclic AMP during premenstrual tension. However, this variation of cyclic AMP excretion associated with the menstrual cycle again indicates the need for care in studying urinary cyclic AMP levels in clinical states.

3.3 The Effect of Oral Contraceptives on Urinary Excretion of Adenosine 3'5'-Cyclic Monophosphate in Normal Humans.

The precise mechanism whereby conception is prevented by the use of oral contraceptives is uncertain. Dufau et al. (1970) have shown that the oestrogen peak and the output of pituitary gonadotrophins, FSH and LH, which are largely responsible for the events of a normal menstrual cycle and pregnancy, are suppressed or diminished during the first cycle of the treatment with an oestrogen-progestagen contraceptive preparation. However, there is evidence that long term treatment with oral progestational agents suppresses ovarian activity and ovulation but does not inhibit pituitary function (Loraine et al., 1963). Oestradiol-17β (Szego and Davis, 1967) and LH (Marsh et al., 1966) have been shown to exert their actions via cyclic AMP. As described in the previous section there is a mid-cycle peak in the urinary excretion of cyclic AMP, in normally menstruating women, while volunteers with suppressed ovulation (secondary amenorrhoea and pregnancy) and males had little or no variation. It is therefore possible that the administration of oral contraceptives could suppress or abolish the nucleotide peak.

Seven normal healthy women (18-27 years) taking oral progestagens were investigated. Five were using 'Norilyn-1', one 'Myrovlar' and one 'Minulyn'; the dose for all drugs used was one tablet per day orally from day 5 to 25 of the cycle. Each tablet of 'Norilyn-1' contains 1 mg norethisterone (17α-ethinyl-19-nortestosterone) plus 0.05 mg mestranol (ethinyl oestradiol-3-methyl ether). 'Myrovlar' contains 1 mg norethisterone

acetate and 0.05 mg ethinyl oestradiol. 'Minulyn' contains 2.5 mg lynesterol plus 0.05 mg ethinyl oestradiol. Six of the women had been taking oral contraceptives 6 to 18 months prior to this investigation. Two of these were followed up 2 months after they had discontinued the ingestion of progestagens. One of the volunteers was investigated for two cycles prior to administration of oral contraceptives and during the first 3 cycles of treatment with 'Norilyn-1'. Urines (24h) were collected at weekly intervals. Completeness of collection was checked by creatinine estimation and the samples were stored at -20°C until estimation of cyclic AMP by the protein binding method.

The results obtained are presented in figure 3.12*; the six women who had taken progestagens for 6 to 18 months showed an excretion of cyclic AMP in the normal range (1.5 - 3.7 umol/24h), with no significant variation between the eight collections during two cycles. The two women (figure 3.13 A & B) showed no variation in cyclic AMP excretion during 2 cycles on progestagens, but two months after withdrawal of the contraceptive agents a marked mid-cycle peak in cyclic AMP excretion was obtained. The single volunteer (figure 3.13 C) investigated for two cycles prior to administration of 'Norilyn-1', showed a mid-cycle peak in cyclic AMP excretion which disappeared during the third month of taking the oral contraceptive. Depressive symptoms caused the cessation of ingestion of the drug at this point.

This study indicates that during the administration of oestrogen-progestagen contraceptive preparations the mid-cycle

(* these should be compared with the pattern shown in Figure 3.10).

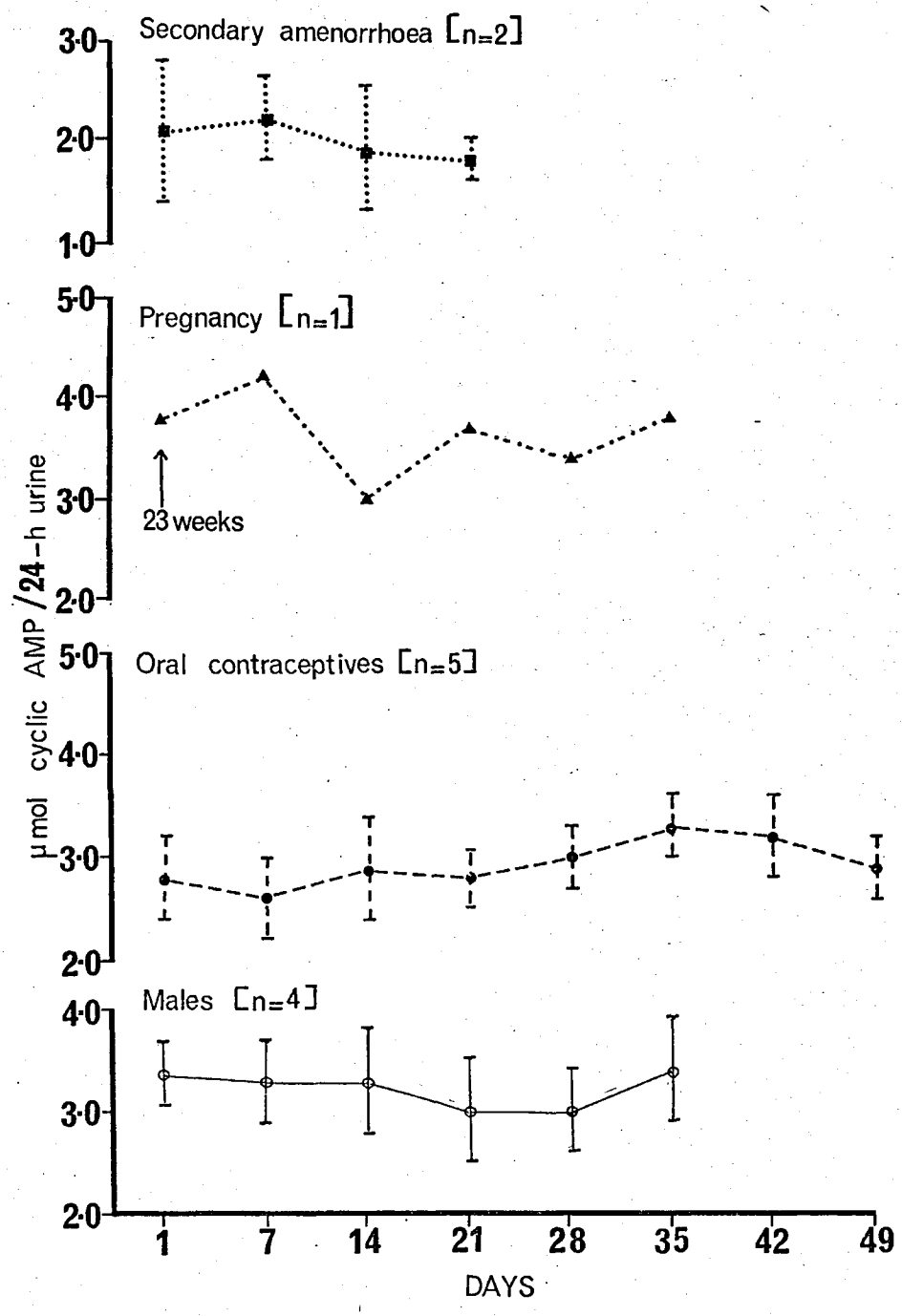


Figure 3.12: Urinary cyclic AMP excretion during periods of up to 8 consecutive weeks. Vertical bars represent S.E.M. values.

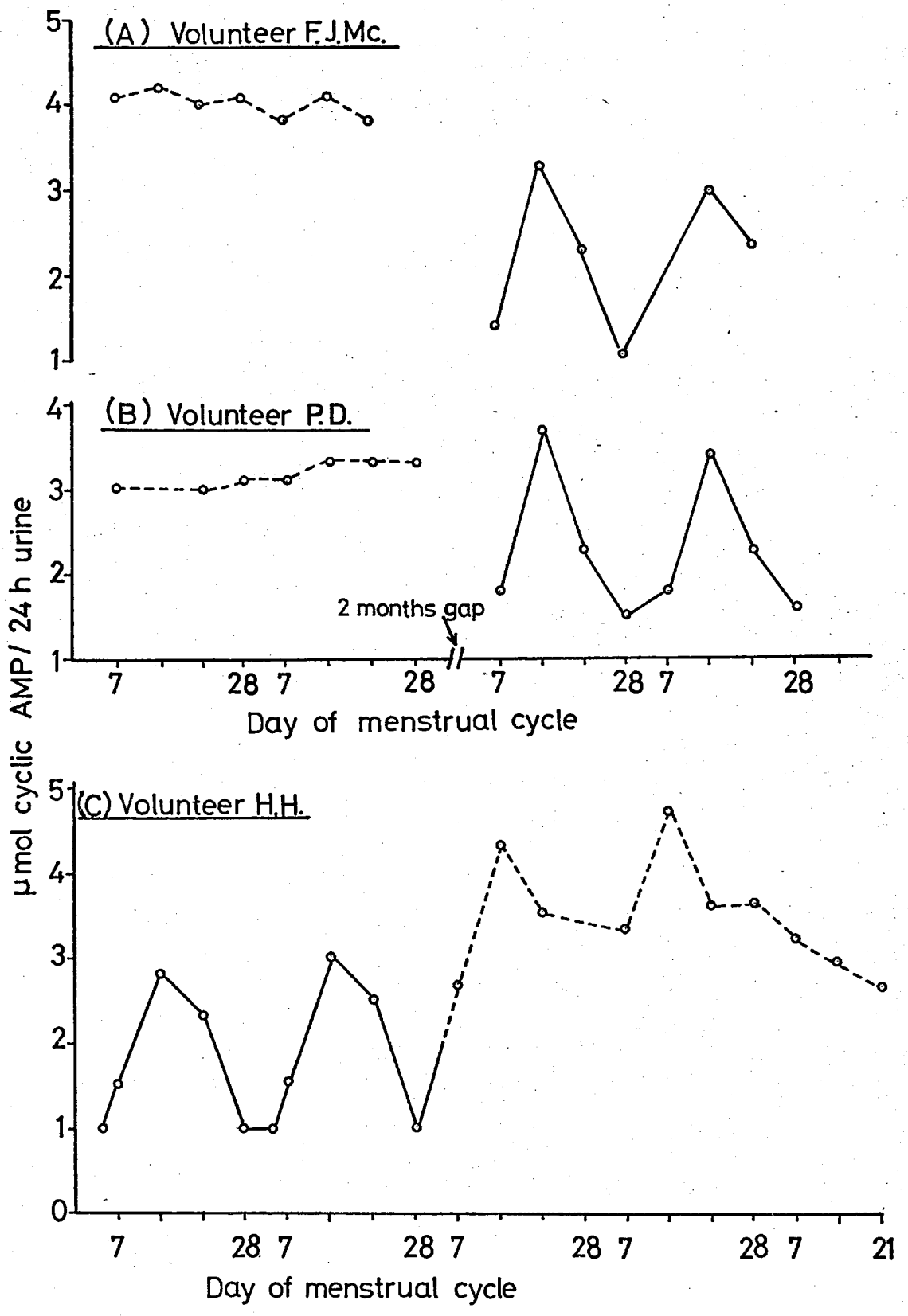


Figure 3.13: Effect of oral contraceptives on urinary cyclic AMP. Solid lines represent cyclic AMP excretion during cycles free from contraceptives agents; broken lines show cyclic AMP excretion during cycles on progestagens. The oral contraceptive agent was 'minulyn' for F.J.Mc.,'myrovlar' for P.D. and 'norilyn-1' for H.H..

urinary cyclic AMP peak is suppressed. It is possible that ovarian activity and ovulation are mediated through an increased production of cyclic AMP in the ovary. It is interesting that depressive symptoms were absent in the volunteers taking oral contraceptives (figure 3.13 A & B) but premenstrual tension recurred after discontinuation of progestagens.

3.4 The Effect of Exercise and Diet on the Plasma Levels of Cyclic AMP.

Cyclic AMP has been implicated in many biological systems (section 1.7) and because of its relation to β-adrenergic receptors it is thought that changes in muscular activity could result in changes in cellular cyclic AMP. Prolonged physical activity - 90 minutes of football or stair climbing - was found to have no significant effect (Paul *et al.*, 1971b; Owen, 1975; Murad and Pak, 1972). Williams *et al.* (1972) reported a marginal rise in cyclic AMP excretion in four volunteers after a 2 or 3 mile run (but no change in cyclic GMP). Paul *et al.* (1971b) could not find any significant difference in cyclic AMP excretion between 10 hyperkinetic children and an age-matched control group. He also reported that physically active and inactive depressed patients show similar (low) levels of cyclic AMP. No significant difference in urinary cyclic AMP levels were found between the normal and psychotic phases of two patients with periodic catatonia; one of whom exhibited episodes of prolonged stupor, whereas the other exhibited violent and destructive episodes. However, Eccleston and his group (1970) in an investigation of 3 volunteers who walked 5 or 10 miles in hilly country found a doubling in cyclic AMP excretion (24h output) in two of these subjects. Heath *et al.* (1973), have reported a rise in plasma cyclic AMP and in the urinary cyclic AMP/creatinine ratio after exercise in an unspecified number of subjects.

Twelve normal volunteers, 7 males and 5 females (age 18 to 24 years) were investigated. No food was consumed by any of

the subjects for at least 12 hours prior to the investigation. All volunteers were confined to bed for 8 - 9 hours before the collection of the first blood specimen (08.00 - 10.00 h). After the first blood collection, four controls, two males and two females, remained in their beds for the rest of the period of this investigation. Six volunteers (3 males, 3 females) underwent the Harvard step procedure (Consalazio et al., 1963) for up to 5 minutes (20 inch step for males, 17 inch for females: 30 steps a minute); they then returned to bed till the end of the investigation. The remaining 2 male volunteers stayed in bed and ingested a meal of 760 calories (Glaxo and Farley baby rice cereal, chocolate flavour, consisting of:

	carbohydrates	80%
	protein	10%
	fat	3%
and milk:	protein	3.3 g
	fat	3.6 g).

Within a minute after completion of exercise or food intake, a second blood specimen was collected. A specimen was taken from the members of the control group at the same time. Forty minutes later a third and final blood specimen was collected from all volunteers.

Plasma was isolated and frozen within seven minutes (see section 2.3.1). Part of each sample was deproteinised and freeze-dried (section 2.5.1) and the remainder was diluted with an equal volume of 50 mM Tris buffer, pH 7.4, containing 8 mM theophylline. Cyclic AMP was measured in 50 and 100 µl

aliquots of the appropriately prepared plasma by the protein binding technique.

The changes in plasma cyclic AMP resulting from exercise are shown in table 3.2. All six volunteers showed a significant rise in plasma cyclic AMP one minute after exercise (mean value 17.7 pmol/ml) compared with a mean of 9.2 pmol/ml plasma before exercise ($P<0.001$). However, this effect was only transient; 40 minutes later plasma cyclic AMP had fallen back to almost pre-exercise level (11.2 pmol/ml plasma). The four controls did not show any significant change throughout the period of this investigation. Only two of the originally arranged four volunteers consumed the 760 calories meal. They showed no significant change in plasma cyclic AMP levels.

The influence of haemolysis was checked by dividing blood samples into two parts. One part was used to obtain plasma under careful conditions, the other was freeze-thawed twice to get massive haemolysis. This latter sample showed considerably higher cyclic AMP content (eg. 9 pmol/ml rising to 35 pmol/ml). Thus haemolysis must be avoided in this estimation.

It is clear from this investigation that a transient rise in plasma cyclic AMP is associated with vigorous exercise. This is in agreement with the findings of Heath *et al.* (1973). However, no significant variation could be detected in pre- and post-exercise urine samples. This finding contradicting that of Eccleston *et al.* (1970) but agreeing with several studies:- Paul *et al.*, 1971b; Moffat and Owen, 1972; Murad and Pak, 1972; Williams *et al.*, 1972. The consumption of a large meal (760 calories) did not influence plasma cyclic AMP levels in the two subjects studied.

TABLE 3.2: Effect of Exercise and Food Intake on Plasma Levels of Cyclic AMP in Humans

Subject	Weight (kg)	Sex	Cyclic AMP pmol/ml plasma					
			*First Specimen (At rest)	*Second Specimen	*Third Specimen	**First Specimen	**Second Specimen	**Third Specimen
CONTROL GROUP								
J.C.H.	80	M	8.0	7.1 (66)	6.8	12.7	11.2	6.6
S.E.S.	77	M	12.5	8.3 (74)	10.9	12.5	8.3	4.1
A.E.J.	49	F	9.9	10.1 (78)	9.8	10.7	10.0	12.8
H.J.M.	52	F	4.2	4.2 (77)		10.1	6.4	
Mean ± S.E.M.			8.7 ± 1.8	7.4 ± 1.2	9.2 ± 1.2	11.5 ± 0.7	9.0 ± 1.0	7.8 ± 2.6
EXERCISE GROUP								
M.S.P.	73	M	8.4	18.7 (126)	8.0	8.1	12.5	7.4
N.C.P.	75	M	8.2	15.2 (128)	15.5	5.0	8.3	10.1
A.G.	86	M	8.8	16.6 (160)	9.2	6.6	26.8	10.7
K.M.J.	44	F	7.3	16.1 (140)	10.6	5.0	14.4	10.6
M.D.B.	50	F	6.7	13.4 (124)	6.8	5.0	13.0	5.5
L.B.	46	F	15.6	26.0 (104)	16.8	13.4	18.7	8.7
Mean ± S.E.M.			9.2 ± 1.3	17.7 ± 1.8	11.2 ± 1.7	7.2 ± 1.3	15.6 ± 2.6	8.8 ± 0.8
DIET GROUP								
P.M.	79	M	11.7	9.2	11.4	16.5	6.3	6.2
R.S.J.	83	M	8.3	9.0	8.2	6.6	7.6	9.3
Mean			10.0	9.1	9.8	11.6	7.0	7.8

* Plasma diluted with an equal volume of "Buffer".

** Plasma deproteinised with equal volumes of $ZnSO_4$ and $Ba(OH)_2$, freeze-dried and then taken up in "Buffer".

( ) Indicate pulse/min.

Comparison of the paired data for post with pre-exercise cyclic AMP levels in plasma gives a difference significant at the level $P<0.001$ (number of subjects = 6).

Clearly a larger study is needed to establish the effect of exercise and diet on cyclic AMP levels in plasma. This limited sample does, however, show that conditions must be carefully controlled both in terms of muscular activity immediately prior to venepuncturing and in the avoidance of haemolysis.

3.5 In vitro Studies on the Effect of Vitamins on Cyclic 3'5'-Nucleotide Phosphodiesterase Activity.

A significant fall in the intracellular concentration of cyclic AMP, including the central nervous system, has been postulated to account for depressive illness (Abdulla and Hamadah, 1970). It has been suggested that some antidepressant drugs may act through the inhibition of phosphodiesterase (Abdulla and Hamadah, 1970; Ramsden, 1970) thus allowing the concentration of cyclic AMP in the cells to build up to a 'normal' level.

Vitamin deficiency is known to precipitate depressive illness (Slater and Roth, 1969) and the administration of the vitamin B complex has been proved effective in the treatment of some psychotic states.

In this study the in vitro effects of a number of vitamins on phosphodiesterase activity were investigated. For each compound tested, six reaction mixtures comprising three tests and three controls were prepared. Phosphodiesterase activity was measured essentially as previously described (section 2.5.2). Each test mixture contained 50 µl [^{3}H]-cyclic AMP, 50 µl of the compound dissolved in Tris-buffer, pH 8.0, and 50 µl of the rat brain preparation. The controls contained all but the test compound. The phosphodiesterase activity was measured by the breakdown of [^{3}H]-cyclic AMP to liberate tritiated AMP and thence [^{3}H]-adenosine.

The results are given in table 3.3. Of the vitamins examined only ascorbic acid and cyanocobalamin were inhibitory at concentrations lower than millimolar. These findings are in

agreement with those of Weinryb *et al.* (1972) on vitamin B_{12} and Moffat *et al.* (1972) on vitamin C.

Table 3.3: Inhibition of Phosphodiesterase from Rat Brain

Drug/Vitamin	Minimum Inhibitory Concentration (Molar)
Ascorbic acid	1×10^{-4}
Cyanocobalamin	4×10^{-4}
Theophylline	1×10^{-3}
Caffeine	1×10^{-2}
Nicotinamide	4×10^{-2}
Pyridoxine hydrochloride	4×10^{-2}
Nicotinic acid	4×10^{-2}
Riboflavin	No activity at 1×10^{-2}
Tocopherol acetate	No activity at 1×10^{-1}
Na-Pantothenate	No activity at 5×10^{-1}

CHAPTER IV

Cyclic AMP Levels in Depressive Illness and the Effect of Electro-Convulsive Therapy.

4.1: Comparison of Plasma and Urinary Levels of Cyclic AMP between Normal Volunteers and Patients suffering from Depressive Illness.

Abdulla and Hamadah (1970) reported from a London study that the cyclic AMP urinary output was significantly lower in a depressed group of 21 female patients (0.52 ± 0.08 μmol/24h) compared with 18 female controls (2.3 ± 0.1 μmol/24h). There was the suggestion that the level was raised in mania. At about the same time Paul et al. (1970) working at NIH, Bethesda gave findings of similar pattern (psychotic depression 3.6 ± 0.2 μmol/24h; controls 5.6 ± 0.7 μmol/24h; manic 9.9 ± 1.9 μmol/24h). In a later study Paul et al. (1971a) reported a marked but transient elevation in urinary cyclic AMP at the time of the switch from depression into mania.

Abdulla and Hamadah (1970) suggested a causal relationship :- "that intracellular cyclic AMP deficiency is related to depressive illness and that a striking increase leads to mania". This was controversial, thus Eccleston and his colleagues (1970) suggested that muscular activity was the key rather than the clinical states of depression and mania (see previously 3.4). Brown et al. (1972) in a longitudinal study on a group notable for its small sample size, diversity and lack of controls, and their more recent single patient study (Hullin et al., 1974), could not show any correlation between cyclic AMP excretion and "mood" (see 1.11). Jenner et al. (1972) similarly reported a failure to correlate cyclic AMP excretion and "mood" in an undefined number of depressed patients. Their paper included a more detailed account of a study on a patient with a regular 48h cycle of mania and depression. In this case there was excellent

correlation both with urinary cyclic AMP and ADH. It was suggested that ADH is the 'driver' and cyclic AMP the 'passenger' - this pattern reflecting pituitary or hypothalamic function.

Hansen (1972) has reported that cyclic AMP in blood is significantly lower in a group of depressed patients compared with controls. He suggests, however, that this is a reflection of changes in ATP concentration. His methodology is idiosyncratic and his reported blood levels for cyclic AMP (2.3 μg/ml) are 3 orders of magnitude higher than other workers. Robison et al. (1970a) found no correlation between clinical condition and cyclic AMP level in cerebrospinal fluid pooled from a manic group, or a depressed group, or a 'neurological' control group. Cramer et al. (1972, 1973) supported this lack of correlation in a study of 'baseline' CSF samples. However, administration of 'probenecid' led them to conclude that the 'turnover' of cyclic AMP in brain is significantly greater in the manic group than in the depressed or the 'neurological' control group. The measured level in a body fluid such as CSF is a resultant of the dynamic interplay between input and removal.

Thus the years pass but the controversy is not resolved. The various investigations have the common feature of small sample size. In this study cyclic AMP levels in urine and plasma have been investigated. Ethical criteria prevented the use of CSF.

The urinary levels of cyclic AMP were measured in 47 normal volunteers (13 males; 34 females) ranging from 18 - 59 years of age; and in 35 female (24 - 67 years) inpatients suffering from depressive illness. Plasma levels of cyclic AMP were measured

in 50 normal subjects (32 males; 18 females) of age range 18 - 59 years; and 11 female (37-64 yrs) inpatients who were diagnosed as suffering from endogenous depression. All patients received antidepressants or tranquillisers or both, the doses of which were fixed throughout the period of the study. All participants avoided certain foods which might affect indole and catecholamine excretion (Paul et al., 1970); no attempt was made to modify physical activity.

Twenty-four hour urine specimens (08.00 - 08.00) and venous blood samples were collected on two separate occasions from the majority of the subjects. In 11 of the patients (7 endogenous depression and 4 reactive depression) the 24-hour urinary excretion of cyclic AMP was measured both during the depressed phase and after recovery. Plasma and urine samples were collected (section 2.3.1) and prepared for assay as previously described (2.5.1; 2.6.4). Cyclic AMP was determined by either the enzymic radioisotopic displacement technique (2.5.2) or, later, by the protein-binding method (2.6.5).

Table 4.1 summarises data for plasma and urine for all subjects studied. There was no significant difference in the levels of either urinary or plasma cyclic AMP between the male and female normal volunteers. The nucleotide levels were consistently lower in the depressed group as compared with controls, irrespective of the measurement technique employed. Moreover, patients diagnosed as suffering from endogenous depression showed significantly lower ($P < 0.001$) urinary excretion for cyclic AMP (0.9 ± 0.1 μmol/24h) than the patients suffering from reactive depression (1.9 ± 0.2 μmol/24h). The latter were

Table 4.1: Influence of Depressive Illness on Plasma and Urinary Levels of Cyclic AMP.

Group	Urinary Cyclic AMP (µmol/24h)	Significance	Plasma Cyclic AMP (pmol/ml)	Significance	Method Used
Normals	7.1 ± 0.3 (12 ♀)	P<0.001	15.8 ± 2.5 (27 ♂, 3 ♀)		Enzymic radioisotopic displacement
Depression	2.2 ± 0.2 (18 ♀)		-		
Normals	2.1 ± 0.2 (13 ♂)	NS	24.1 ± 3.5 (16 ♂)	NS	Protein-Binding assay
	2.3 ± 0.1 (22 ♀)		20.4 ± 2.8 (15 ♀)		
Endogenous Depression	0.9 ± 0.1 (9 ♀)	P<0.001	9.2 ± 0.8 (11 ♀)	P<0.002	
Reactive Depression	1.9 ± 0.2 (8 ♀)	NS	-		

( ) indicate the number of subjects investigated and their sex.

not significantly different from normal subjects (2.2 ± 0.1 μmol/24h). On improvement the 11 depressed patients showed an increase in urinary cyclic AMP excretion. This increase was not significant at the 10% level for the reactive depression group but was highly significant ($P<0.001$) for the endogenous depression group (Table 4.2). The lower levels of plasma and urinary cyclic AMP in patients suffering from endogenous depression could not be attributed to a reduction in muscular activity, as both physically inactive and agitated patients in this group showed similarly low levels of cyclic AMP. In any case, studies on controls (3.4) indicated that vigorous exercise produced a transient increase in plasma cyclic AMP levels which was not detected in the urine.

This present study thus confirms Abdulla and Hamadah's (1970) earlier report; added weight being given by the use of a 'blind' procedure involving the analysis of 'coded' urines, the code being resolved only after the completion of results. Recently two other groups, Naylor *et al.* (1974) and Sinanan *et al.* (1975) provided further evidence in support of these findings.

Table 4.2: Comparison between Cyclic AMP Excretion in Depressed Patients before and after Improvement.

Group	Cyclic AMP µmol/24h Urine: Depressed Phase	Improved Phase	n	Reference	P<
Endogenous Depression	1.0 ± 0.2	2.2 ± 0.3	7	This Study	0.001
Reactive Depression	2.0 ± 0.4	2.9 ± 0.3	4		NS
Depression	0.5 ± 0.008	1.3 ± 0.02	15	Abdulla & Hamadah (1970)	0.001
Depression	*4.5 ± 0.4	*6.0 ± 0.6	12	Naylor et al. (1974)	0.02

* µmol cyclic AMP/g creatinine.

4.2: Diurnal Variation of Urinary Cyclic AMP in Depressive Illness.

An important and significant symptom of depressive illness is the daily fluctuation of mood and of the total state. A worsening of depression in the morning has been associated with endogenous depression while a worsening in the evening has been thought as characteristic of reactive depression (Winokur et al., 1969). In depressive illness alterations in the circadian rhythms of plasma and urinary corticosteroids (Bridges & Jones, 1966), urinary sodium and chloride excretion (Elithorn et al., 1966), salivary electrolyte excretion (Palmai & Blackwell, 1965) have been reported. Murad and Pak (1972) have reported a diurnal rhythm for the urinary excretion of cyclic AMP in normal humans, a finding repeated and extended to include plasma in section 3.1. The purpose of this study is to demonstrate possible alterations in circadian rhythm of cyclic AMP in depressive illness before and after improvement.

Eleven women, of age range 37 - 67 years, who were inpatients at Tooting Bec Hospital and had been diagnosed as suffering from either endogenous depression (7) or reactive depression (4) were investigated. All patients received tricyclic antidepressants and a night sedative, and some were also given tranquillisers. The doses were fixed throughout the period of study. Seven of the patients were also treated with E.C.T. Urine samples were collected at 4-hourly intervals over a 24-hour period commencing at 08.00 h on two occasions. The first collection was obtained from the patients after they were stabilised on drugs (approximately one week after admission), and the second on improvement one to five months later. For patients who received E.C.T. the second collection was carried

out at least two weeks after completion of this treatment (Hamadah et al., 1972). Urine samples were collected (2.3.1) and prepared as described in section 2.6.4. Cyclic AMP was determined by the protein-binding method (2.6.5).

During the depressed phase of endogenous depression the diurnal pattern observed was of the form shown in figure 4.1. The maximum being at 08.00 - 12.00 h for the group. After improvement the group pattern became bimodal with peaks at 04.00 - 08.00 and 16.00 - 20.00 h. As well as this qualitative change in pattern there was a quantitative change in that the 24-hour output increased by 115% (Table 4.3). The reproducibility of the 'depressive pattern' is indicated in figure 4.2 in which the same patient was investigated at various dates during a depressed phase of three months duration.

The reactive depressive group showed no qualitative pattern change, maintaining maxima at 24.00 - 04.00 and 12.00 - 16.00 h both when depressed and after improvement (Figure 4.3). Quantitatively there was no significant increase in the 24-hour urinary output of cyclic AMP (Table 4.3).

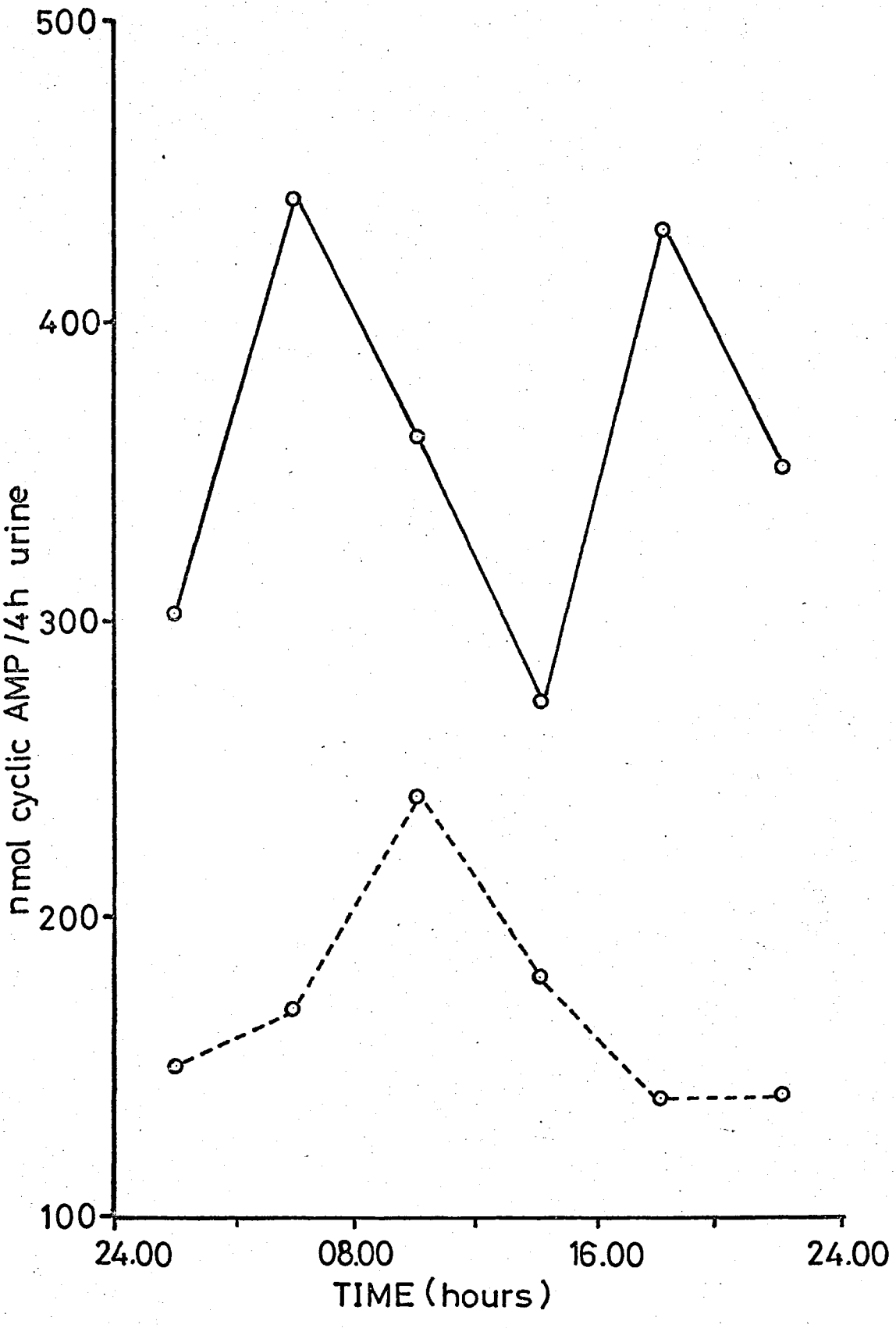


Figure 4.1: Diurnal variation of urinary cyclic AMP levels in patients suffering from endogenous depressio (n=7). The broken line shows variation in cyclic AMP excretion during the depressed phase; the variation ir the urinary levels of the nucleotide after improvement

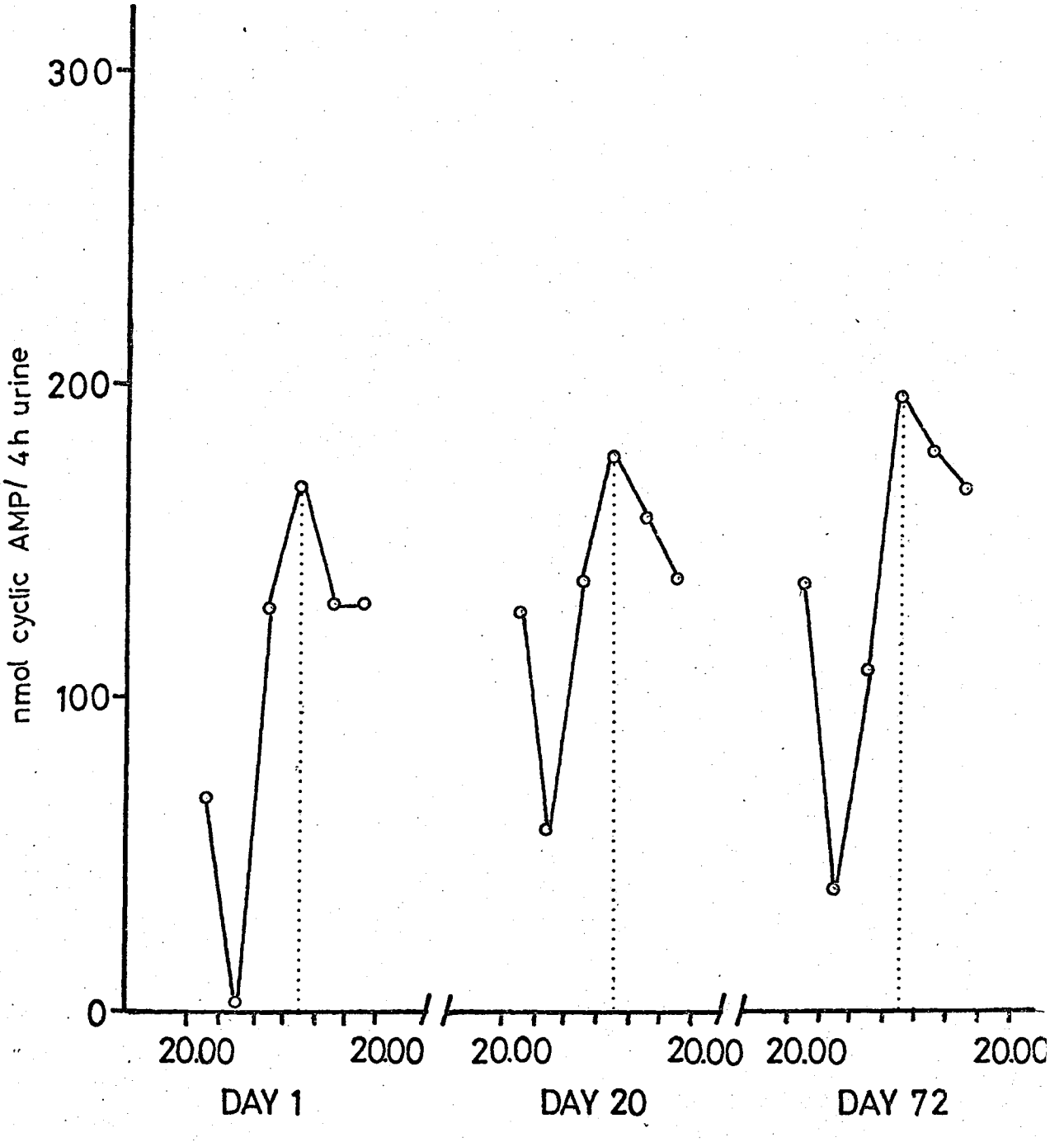


Figure 4.2 : Diurnal variation in the urinary cyclic AMP excretion of a single patient suffering from endogenous depression. Specimens were collected on 3 separate occasions during the depressed phase.

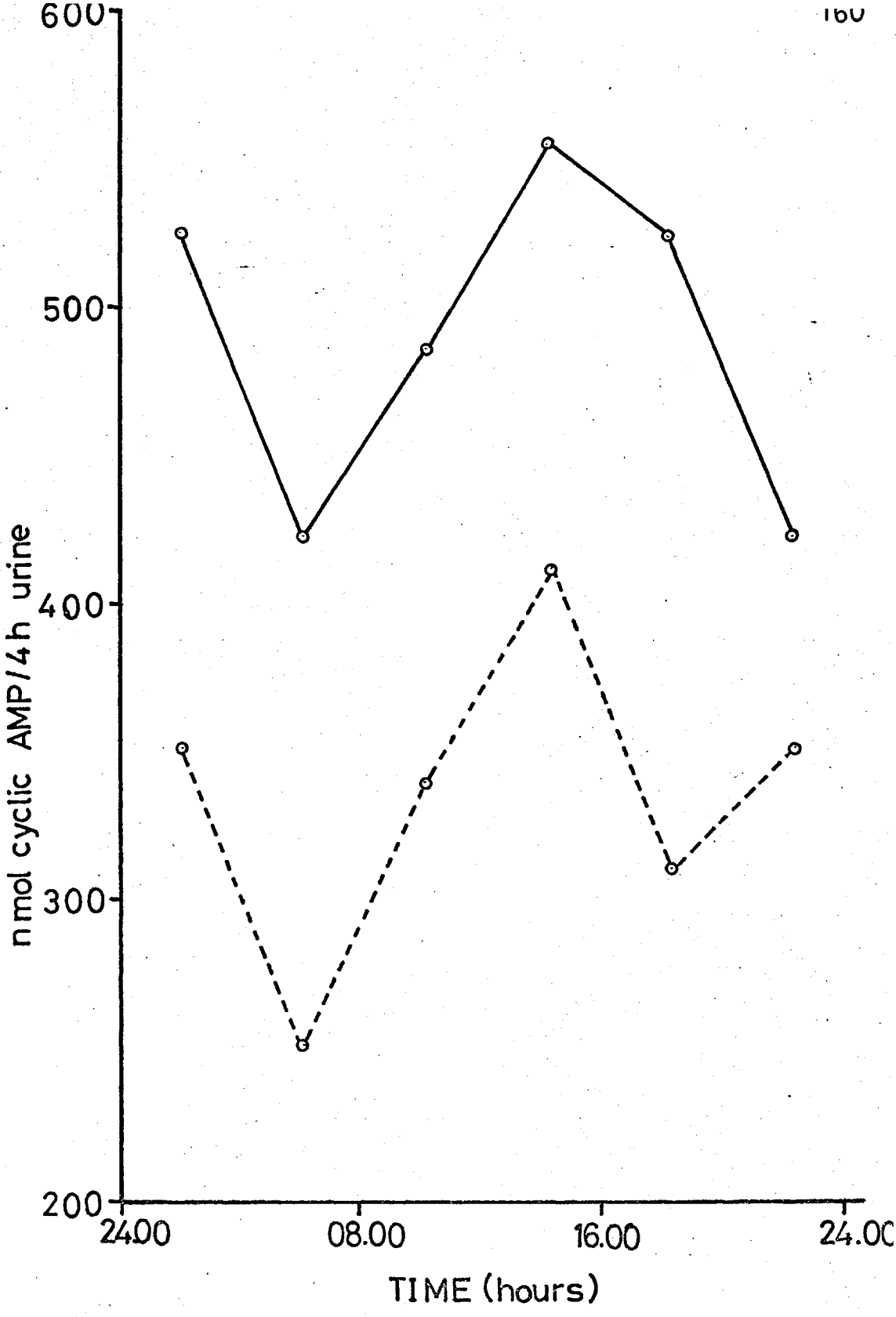


Figure 4.3: Diurnal variation of urinary cyclic AMP levels in patients suffering from reactive depression (n=4). The broken line shows variation in cyclic AMP excretion during the depressed phas the solid line represents variation in the urinary levels of the nucleotide after improvement.

Table 4.3:

Time (hours)	URINARY CYCLIC AMP (n mol)			
	ENDOGENOUS DEPRESSION		REACTIVE DEPRESSION	
	During depression	After improvement	During depression	After improvement
24.00-04.00	150 ± 37	300 ± 38	350 ± 30	520 ± 18
04.00-08.00	170 ± 49	440 ± 97	250 ± 48	420 ± 70
08.00-12.00	240 ± 48	360 ± 46	340 ± 108	480 ± 70
12.00-16.00	180 ± 29	270 ± 42	410 ± 147	550 ± 123
16.00-20.00	140 ± 17	430 ± 68	310 ± 57	520 ± 93
20.00-24.00	140 ± 9	350 ± 50	350 ± 100	420 ± 78
Total for 24 h	1000 ± 145	2150 ± 311	2010 ± 388	2910 ± 317
	% increase of the mean 24 h value = 115 (significant at the level $P < 0.001$)		% increase of the mean 24 h value = 45 (not significant at the level $P = 0.1$)	

The results presented are the means of the determinations (7 for endogenous depression, 4 for reactive depression) ± S.E.M.

(Interassay coefficient of variation for the cyclic AMP estimation in urine = 9.6%).

4.3: Effect of Electro-Convulsive Therapy on Urinary and Plasma Levels of Cyclic AMP.

Significantly decreased urinary and plasma levels of cyclic AMP have been shown in patients with depressive illness (sections 4.1 and 4.2). Abdulla and Hamadah (1970) have suggested that depressive illness is the manifestation of a fall in the intracellular levels of cyclic AMP in all tissues, including those of the central nervous system.

The levels of cyclic AMP in tissues and body fluids will reflect such processes as rates of synthesis, breakdown and secretion. Thus affecting one or more of these processes may change cyclic AMP concentrations. The tricyclic antidepressants (e.g. imipramine, desipramine, amitriptyline) inhibit cyclic AMP phosphodiesterase (Abdulla and Hamadah, 1970; Ramsden, 1970; Beer et al., 1972; Weinryb et al., 1972) and their administration leads to an increase in urinary level of cyclic AMP. Studies by Kodama et al. (1971) also showed that these compounds stimulate adenyl cyclase activity in brain slices.

Cerebral cortex slice studies have also indicated that electrical stimulation increases the concentration of cyclic AMP (Kakiuchi et al., 1969; Shimizu et al., 1970; Reddington et al., 1973). A similar finding has been reported in the superior cervical ganglion of the rabbit (McAfee et al., 1971).

Putative transmitters: noradrenaline, 5-hydroxytryptamine and histamine (Kakiuchi & Rall, 1968 a,b; Kakiuchi et al., 1969; Chasin et al., 1971, 1973), adenine ribose compounds (Sattin & Rall, 1970) and certain depolarising agents such as ouabain, veratridine and batrachotoxin (Shimizu et al., 1970; Huang et al.,

1972) have been found to increase cyclic AMP levels in respiring brain slices. Adrenaline, isoproterenol, noradrenaline and to a lesser extent dopamine, have also been shown to increase the endogenous cyclic AMP in the brain when injected *in vivo* (Burkard, 1972). Electrical stimulation of brain slices results in a release of neurohumoral agents from the tissue including noradrenaline, 5-hydroxytryptamine (Katz & Chase, 1970) and adenosine (McIlwain, 1971) any of which may act as intermediates in the increase of cyclic AMP concentration by electrical stimulation.

Electrotherapy has a long history (possibly back to the Roman Emperors - see Alexander and Selesnick, 1967). Cerletti and Bini first used E.C.T. in 1938 and its simplicity of procedure and favourable results (e.g. in involutional melancholia) led to it rapidly replacing insulin - shock treatment. Extensive clinical experience (Lehmann, 1965) and a number of controlled studies (Greenblatt *et al.*, 1964) agree that approximately 80% of depressed patients experience significant, sustained improvement or disappearance of symptoms in the course of an E.C.T. series given at intervals of one to a few days.

Urinary levels of cyclic AMP were investigated in 14 women (ages 24 - 61) and plasma levels were measured in a further 7 women (ages 37 - 64), all of whom were inpatients at Tooting Bec Hospital diagnosed as suffering from depressive symptoms and requiring E.C.T. Patients with heart or kidney diseases were excluded. The only additional criteria for selection were the willingness and the ability of the patients to co-operate in the

investigation.

Throughout the period of the study the patients were given a diet that lacked foods which might affect indole and catecholamine excretion (Paul et al., 1970). No alcohol was permitted and tea and coffee intake was stabilised. Patients were clinically assessed on the day before E.C.T., on the day of the treatment, and on the following day to detect any sudden or pronounced change in 'mood'. All participants received antidepressants or tranquillisers, or both, the doses of which were fixed throughout the period of the study.

E.C.T. was administered between 09.30 and 11.00 hours on Tuesdays and Fridays. All patients received 0.6 mg of atropine sulphate as premedication half to one hour before treatment. In 18 patients, who were investigated over a total of 28 treatments, the convulsion was modified by the intravenous injection of 60 - 70 mg of methohexitone sodium and 25 - 50 mg of suxamethonium chloride immediately before E.C.T. In two patients (investigated over seven treatments) the convulsion was modified by the intravenous injection of 250 mg of thiopentone sodium and 30 - 50 mg of suxamethonium bromide. The E.C.T. was administered with an 'Ectonus' mark 3, A.C. mains model (Ectron Ltd). The electrodes (Ectonus head-band) were lightly soaked in 'Ectronolyte' solution and applied bitemporofrontally. The rotary control was used to apply the potential in a series of increasing steps, reaching a maximum of 140 V; the current was passed for about one second. The patients were well oxygenated with 100% oxygen.

During this study three patients received all the preliminary

treatment but for various reasons, including instrument failure, no shock was delivered. As they had experienced the anticipatory anxiety and had received atropine sulphate, methohexitone sodium, suxamethonium chloride, but not the electric shock they were regarded as controls on these occasions. A fourth patient, who received the atropine sulphate but panicked before further treatment could be given and refused it, was regarded as a separate control. Thus the controls for urinary cyclic AMP (Table 4.4) were achieved by chance rather than design. It was felt to be unacceptable on ethical grounds to enlarge this control group.

Twenty-four hour specimens of urine (07.00 - 07.00 hours) were collected for up to three days before the first E.C.T. to establish a baseline for each individual patient and to determine the day-to-day variation of cyclic AMP excretion. Twenty-four hour specimens were also collected on the day of E.C.T., for two days after E.C.T. and, where appropriate, on the days between subsequent treatments. Venous blood samples were collected on the day before E.C.T. (between 09.30 - 11.00 hours) and on the day of E.C.T., about one minute before treatment (i.e. after the administration of atropine sulphate but before muscle relaxant and anaesthetic). Blood samples were also collected at 10, 30, 90 and 180 minute intervals after the administration of the electric shock.

Urine and blood specimens were collected as described in section 2.3.1 and prepared for assay according to sections 2.5.1 and 2.6.4 respectively. Urinary levels of cyclic AMP were determined by the enzymic radioisotopic displacement technique

(section 2.5.2) whilst plasma levels were determined by the saturation assay (section 2.6.5).

The changes in urinary and plasma cyclic AMP resulting from the administration of E.C.T. in a typical case are shown in figure 4.4 (a and b). Twelve of the 13 patients who received E.C.T. (Table 4.4) showed a significant rise in urinary cyclic AMP excretion on the day of the treatment. The mean value for the 13 patients (26 treatments) was 14.2 μmol/24h on the day of treatment compared with a mean of 4.2 μmol/24h for the period before E.C.T. ($P<0.001$). In general the excretion of cyclic AMP fell on the following day but not necessarily to the pre- E.C.T. level. The four controls were markedly different in that they showed a fall in the urinary excretion of cyclic AMP on the day of (abortive) treatment, (Table 4.4). The plasma levels of cyclic AMP did not show any significant variation during the 24 h prior to the treatment (Table 4.5). However, five out of seven patients who received E.C.T. showed a marked rise in plasma cyclic AMP. This increase was obtained within 10 minutes of administration of the electric shock, when the mean value was 18.6 pmol/ml plasma, compared with 10.3 pmol/ml plasma at one minute before E.C.T. The plasma level was still raised 30 minutes after treatment but had fallen to pre- E.C.T. level by 3 hours (Table 4.5). It was noted that the three patients who did not respond to E.C.T. in terms of cyclic AMP increase also showed no clinical improvement, whereas those who showed an increase in cyclic AMP were psychiatrically assessed as improved.

Brown et al. (1972) reported that E.C.T. does not affect

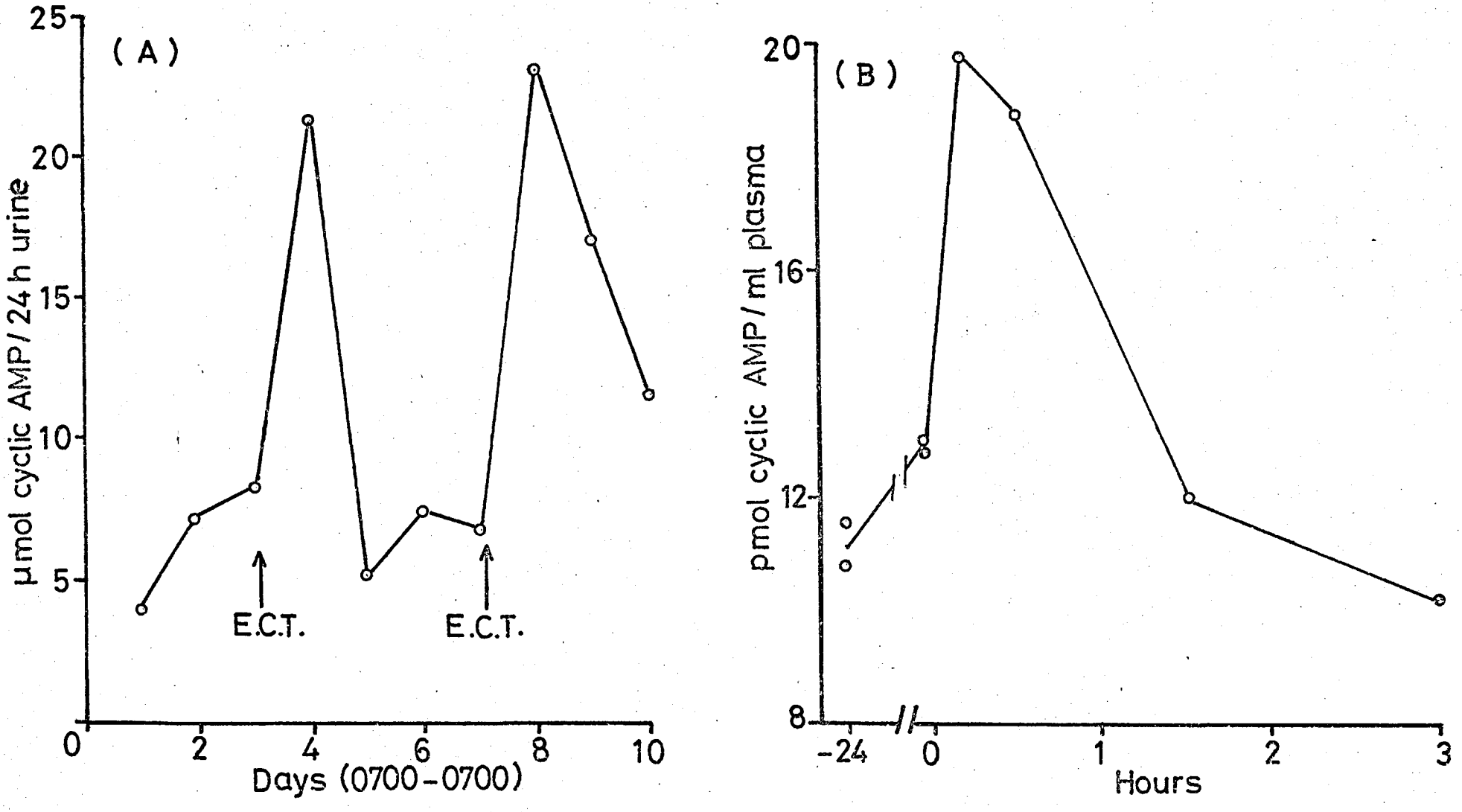


Figure 4.4: (A), Daily urinary cyclic AMP excretion in a patient who received E.C.T. on days 4 and 8. (B), Plasma levels of cyclic AMP in a patient who received E.C.T.; the above graph represents the combined results from two treatments

Table 4.4: Effect of E.C.T. on Urinary 3'5', Cyclic Adenosine Monophosphate

Case No.	Urinary Excretion of cAMP (μmol cAMP/24h)			% Change in cAMP Excretion on Day of E.C.T. compared with Before E.C.T.
	Before E.C.T.*	Day of E.C.T.	After E.C.T.*	
		E.C.T. Group		
1	3.6	7.8	4.3	+120
2	2.6	6.9	2.9	+170
	(1.4	8.3	7.5	+480
3	(3.9	14.5	15.1	+280
	(6.5	21.2	6.6	+230
	(6.6	23.0	14.4	+250
4	9.1	24.8	4.7	+170
5	12.2	28.6	5.7	+130
6	(2.4	4.3	1.5	+80
	(1.5	6.8	3.5	+370
7	(1.9	3.6	2.0	+90
	(2.0	3.7	3.6	+90
8	(3.8	6.9	4.6	+80
	(4.6	27.0	4.2	+490
9	(1.2	3.5	3.1	+180
	(5.2	21.5	19.1	+310
10	6.2	38.1	25.2	+520
11	(2.0	2.3	2.1	+20
	(2.1	2.0	1.9	0
	(1.7	2.8	2.3	+60
‡ 12	(2.3	14.4	4.5	+540
	(4.5	28.3	2.2	+530
	(4.9	20.0	2.0	+310
‡ 13	(2.0	29.7	4.4	+1,360
	(1.9	3.9	13.2	+110
	(1.2	14.1	2.2	+1,070
Mean Values	4.2 (0.4; n = 51)	14.2 (2.1; n = 26)	6.3 (0.7; n = 44)	+310 (50; n = 26)
		Control Group (No E.C.T.)		
4	26.1	24.6	20.3	-6
5	9.3	7.9	5.0	-16
14	5.1	3.7	0.9	-27
‡ 10	3.0	1.0	0.9	-67

*Mean values (for up to three days, where appropriate).

**Mean values with S.E. of mean and number of samples in parentheses.

‡Patient received thiopentone sodium instead of methohexitone sodium.

‡‡Patient received 0.6 mg atropine sulphate only.

Comparison of the paired data from cyclic AMP excretion on the day of E.C.T. with excretion on the day of E.C.T. gives a significant difference P 0.001. For cases 9 - 13 a double-blind procedure was used; only after assaying all specimens was the code disclosed.

Table 4.5: Effect of E.C.T. on Plasma Levels of Cyclic AMP

Patient	Plasma Cyclic AMP (pmol/ml)						% Change in plasma cyclic AMP 10 or 30 minutes after E.C.T. compared with the mean value of samples taken before E.C.T.
	Before E.C.T.		After E.C.T.				
	24 hours	1 min	10 min	30 min	90 min	180 min	
1	6.8	7.8	9.0	-	9.0	-	+ 23
	10.4	9.6	-	9.8	-	10.8	- 2
2	10.8	12.8	19.8	-	12.0	-	+ 68
	11.6	13.0	-	18.8	-	10.2	+ 53
3	9.0	8.2	18.0	-	11.0	-	+109
4	5.8	5.8	14.0	-	10.6	-	+141
5	10.0	12.0	-	18.0	-	12.0	+ 64
6	6.0	6.0	-	6.0	-	5.0	0
7	14.0	16.0	32.0	-	28.0	-	+113
Mean	9.4	10.1	18.6	13.2	14.1	9.5	+ 63
± S.E.M.	± 0.9	± 1.2	± 3.8	± 3.1	± 3.5	± 1.6	± 17

Comparison of plasma cyclic AMP before E.C.T. with plasma cyclic AMP 10 min after E.C.T. gives $P < 0.02$.

cyclic AMP excretion. This was a single patient study and 14% of the group here investigated (Tables 4.4; 4.5) showed no significant increase in the plasma and urinary levels of cyclic AMP after E.C.T. A rise in plasma and urinary cyclic AMP levels after E.C.T. could be due to one or more of the following factors: anticipatory anxiety, premedication, anaesthesia, the administration of muscle relaxant, hypoxia, or electrical stimulation. The failure of the four controls to show any increase in cyclic AMP excretion suggests that E.C.T. was the major factor responsible for the rise in urinary and plasma cyclic AMP concentrations observed in this investigation. This conclusion is supported by the results of other studies. The work of Cox and Potkonjuk (1969) and Robison et al. (1970) suggests that atropine would have little or no effect at the dose level used. Wilson (1969) showed that barbiturates lower cyclic AMP levels in the rat brain, and Havens et al. (1959) showed that thiopentone and suxamethonium reduce the post-convulsive levels of catecholamines in the plasma of patients undergoing E.C.T. and hence probably also lower cyclic AMP levels (Robison et al., 1971).

The procedure used in E.C.T. needs to be carefully controlled to prevent hypoxia, which can result in various biochemical changes. Thus Havens et al. (1959) showed that the post-convulsive plasma level of adrenaline bears an inverse relation to the degree of oxygenation of the patient, while hypoxia leads to a definite increase in the level of cyclic AMP in mouse and rat brain (Goldberg et al., 1970; Ditzion et al., 1970). In this study the patients were well oxygenated, and the lack of increase in cyclic AMP level

in the three suxamethonium treated controls again points to convulsive electric shock as the causative factor.

The mechanism by which electrical stimulation increases the level of cyclic AMP in brain remains to be established. Direct administration of noradrenaline, 5-hydroxytryptamine, histamine (Chou et al., 1971; Chasin et al., 1971; Reddington et al., 1973) and adenosine-ribose compounds (Sattin and Rall, 1970) to respiring guinea pig cerebral cortex slices causes activation of adenyl cyclase. Excitation of slices with electrical stimulation results in a release of neurohumoral agents from the tissue including noradrenaline, 5-hydroxytryptamine (Katz and Chase, 1970) and adenosine (McIlwain, 1971; Zanella and Rall, 1973) any of which may act as intermediates in the effect of electric shock on cyclic AMP content. Since the response produced by histamine and 5-hydroxytryptamine is additive with that of electrical stimulation (Reddington et al., 1973; Williams and Rodnight, 1974) these agents are made less plausible as intermediates in the effect of E.C.T. on cyclic AMP level. Currently the most likely suggestion is that depolarisation results in increased release of adenine-ribose compounds and noradrenaline which then stimulate cyclic AMP formation (Zanella and Rall, 1973; Reddington et al., 1973).

This study has shown that E.C.T. produces an increase in both plasma and urinary levels of cyclic AMP. The direct stimulation of slices of guinea pig cerebral cortex rapidly produces a greatly increased level of cortical cyclic AMP (Reddington et al., 1973; Zanella et al., 1973). Furthermore,

evidence has been obtained to support the suggestion that cyclic AMP mediates the physiological actions of neuro-transmitter substances in the central nervous system (Florendo et al., 1971; Greengard and Kuo, 1970). Possibly, therefore, the antidepressant action of E.C.T. is mediated through an increased production of cyclic AMP in the brain, which is reflected in the increase observed in the plasma and urine of E.C.T. -treated patients.

CHAPTER V

Discussion

5.1. The Use of Plasma and Urine for Studying Changes in Cyclic AMP associated with Depressive Illness

Affective disorders are a group of illnesses associated with biochemical changes in the central nervous system. The first biological fluid which comes to mind in studying these changes is cerebrospinal fluid, especially as there is good evidence of ready exchange between brain extracellular fluid and the cerebrospinal fluid. Blood and especially urine are more remote from metabolic changes in brain tissue, the signal to noise ratio will be decreased by the action of all the other tissues. However in studies on patients, whereas blood and urine may be readily available, the procedure of lumbar puncture in the interest of establishing a biochemical hypothesis may be considered unethical. Blood and urine were used in this study as a matter of necessity. If, as suggested by Abdulla and Hamadah (1970), depressive illness is a systemic disorder remoteness from the central nervous system becomes unimportant.

The major problem in interpretation is that the sources of normal plasma cyclic AMP are far from clear. There is evidence to indicate that under appropriate hormonal stimulation both liver and kidney are capable of adding cyclic AMP to plasma (Broadus *et al.*, 1971). Albano and Brown (1974) showed that *in vitro* stimulation of rat adrenals by ACTH causes an increase in the intracellular cyclic AMP which is then released into the perfusing fluid. Similar findings were obtained *in vivo* by Peytremann *et al.*, (1973). Studies

in vitro, with rat testes have shown that after stimulation with human chorionic gonadotrophin, the intracellular cyclic AMP concentration reaches a maximum and then declines as cyclic AMP is extruded into the surrounding medium (Dufau *et al.*, 1973). Cramer and Lindl (1974), in an *in vitro* study, have shown that cyclic AMP is released from the peripheral sympathetic ganglion of the rat following stimulation of its intracellular formation by biogenic amines such as adrenaline and histamine. They suggested that under physiological conditions the release or overflow of cyclic AMP after stimulation by neurohormones could have some distinct peripheral function, or could serve to remove and thus effectively inactivate any excess in nucleotide that is not bound to protein kinases in the nervous system. Cramer *et al.* (1972b) have evidence of the *in vivo* release of cyclic AMP from the central nervous system.

This investigation (Chapter 4) has shown that the plasma and urinary cyclic AMP levels correlate with mood changes in patients suffering from depressive illness. Cramer *et al.* (1972a) suggested that the 'turnover' of cyclic AMP in brain as measured in cerebrospinal fluid with and without probenecid correlates with mental condition, i.e. higher in mania than in depression. Other workers (Abdulla and Hamadah, 1970; Paul *et al.*, 1971b) have provided evidence that the urinary levels of cyclic AMP in manic depressive patients correlate with clinical change and improvement.

This is not without controversy but recently support has come from Naylor et al. (1974) in Dundee and from an extensive survey by Sinanan et al. (1975) in Dublin.

Plasma cyclic AMP is derived from a number of tissues including the central nervous system; it is not yet feasible to draw up a balance sheet; however, Broadus et al. (1970) have presented evidence that approximately 50% of the urinary cyclic AMP is derived by direct glomerular filtration of plasma while the other 50% is nephrogenous in humans under basal conditions.

5.2. Factors affecting the Plasma and Urinary Levels of Cyclic AMP in Normality and Depressive Illness

In this study human plasma and urinary levels of cyclic AMP were found to be affected by the following factors: diurnal rhythms, menstrual cycle (in turn affected by oral contraceptives), exercise and depressive illness (in turn affected by E.C.T.).

As discussed in Chapter 3.1, there seem to be two populations with regard to diurnal patterns of cyclic AMP in both plasma and urine from controls. The two patterns do not seem to be mutually exclusive. It would be worthwhile investigating a normal group in more depth with regard to this patterns. A somewhat superficial evaluation suggested that introverts tend to the afternoon peak whereas extraverts have a morning peak of cyclic AMP. It is possible that an evaluation of cyclic AMP patterns in normal subjects in

conjunction with other evidence (e.g. history of affective disorders in close relatives) may be of prognostic value.

Patients suffering from reactive depression had a bimodal diurnal pattern for urinary cyclic AMP with similar timing to that of the controls. This group of patients did not show any qualitative change in the urinary cyclic AMP rhythm on clinical improvement. However, the patients suffering from endogenous depression had a diurnal pattern for urinary cyclic AMP with a single peak (08.00 - 12.00); on clinical improvement the group pattern tends towards the normal bimodal rhythm.

The mechanisms involved in the control of diurnal variation of cyclic AMP are not yet established. However, a number of hormones are known both to initiate their biological actions by altering the intracellular concentrations of cyclic AMP and to exhibit circadian rhythms (table 3.1). Thus it is possible that diurnal patterns in plasma and urinary cyclic AMP could reflect the sum of the hormonal patterns. This only shifts the problem back to what controls the hormonal patterns and we are led back to hypothalamic function. There may also be non-hormonal effects to consider. In this study diurnal variations in plasma TSH and PTH were measured (figures 3.6 and 3.7). The TSH pattern showed quite a good correlation with the plasma cyclic AMP pattern, whereas the pattern for PTH, a hormone acting predominantly on the kidney, showed a correlation with the diurnal rhythm of urinary cyclic AMP. This latter finding has been mentioned

by Sagel et al. (1973). There is need to investigate a larger number of hormones before a causal relationship can be established. Thus at present there is lack of information on ADH which could well affect urinary patterns (Jenner et al., 1972). Dietary factors can influence hormonal levels e.g. glucagon and insulin. In the rat adrenal cortex cyclic AMP circadian rhythm has been shown to be under hypothalamic control, as the rhythm disappears after appropriate denervation of the hypothalamic region (Moore and Quavi, 1971). In gastric mucosa the periodicity of cyclic AMP content seems to be connected to changes in gastric secretion (Domschke et al., 1972). In mouse epidermis the periodicity in cyclic AMP may be related to the control of mitotic activity. It has been suggested that cyclic AMP depresses mitotic activity whereas cyclic GMP is an enhancer (Hadden et al., 1972).

Hormonal involvement in the control of circadian rhythm of urinary cyclic AMP is further strengthened by studies of changes of this nucleotide during the human menstrual cycle. As shown in section 3.2 when women with normal ovulatory cycles were investigated a mid-cycle peak in cyclic AMP was obtained.

Muscular activity can have no more than a minor influence on the diurnal patterns as strenous physical exercise was found to cause only a transient increase in plasma cyclic AMP and this rise was not reflected in the urine. In any case the diurnal peak position tends to coincide with minimal

physical activity.

In this investigation plasma and urinary levels of cyclic AMP were found to be significantly lower in patients suffering from endogenous depression compared to controls and patients suffering from reactive depression. Abdulla and Hamadah (1970) presented a cause and effect relationship in that they suggested that depressive illness is the result of a lowering of cyclic AMP in all tissues including those of central nervous system. During a depressive phase for an endogenous group the cyclic AMP levels are low whereas on clinical improvement the nucleotide level rises towards normality; this does not show which is passenger and which is driver. Jenner et al. (1972) proposed that ADH leads and cyclic AMP follows in a dramatic example of the regular switch from mania to depression. Thus pituitary or hypothalamus may be the driver. Hansen (1972) suggested that the lowering of blood cyclic AMP in depression is a reflection of a decrease in ATP concentration. If this is the case we are back to a central feature of metabolic control, let alone the possibility of compartmentalization. Lack of exercise has also been proposed as the cause of lower excretion of cyclic AMP in patients, i.e. a change in life style which comes from the change in mood (Eccleston et al., 1970). However, physical activity as a main contributor to changes in plasma and urinary cyclic AMP in depressive illness has now been eliminated. Thus physically active and inactive depressed patients show similar (low) levels of

cyclic AMP (this study; Paul et al., 1971b), and Perry et al. (1973) could not find a significant difference in urinary cyclic AMP levels between the normal and psychotic phases of two patients with periodic catatonia.

The quest for a single precipitating factor in a group of related illnesses such as cancer or depression may be as fruitless as the search for the 'Philosopher's Stone' ! The finding of this study supported by several other investigations is that plasma and urinary cyclic AMP is decreased in endogenous depression. It would seem fruitful to try to arrive at reasons for this decrease:

(a) Lack of stimulus, i.e. hormone; this would tie in with the catecholamine hypothesis of mental illness and the fact that some patients do respond to monoamine oxidase inhibitors.

(b) Faulty hormone receptors, and/or adenyl cyclase.

(c) An overactive phosphodiesterase - such cases should respond to tricyclic antidepressants if a major action of these drugs is the inhibition of this enzyme.

(d) A faulty cyclic AMP-dependent protein kinase receptor, and/or active centre, and thus a lack of physiological response in the presence of normal cyclic AMP concentrations.

Further investigations on these various factors are required before more conclusive evidence can be presented to support the role of cyclic AMP in depressive illness.

E.C.T. has been shown to cause significant increase in

both plasma and urinary levels of cyclic AMP (section 4.3). As discussed in greater detail in 4.3 electric shock seems to be the cause of the rise in plasma and urinary cyclic AMP. The mechanisms by which electrical stimulation increases the level of cyclic AMP in brain remains to be established. Direct administration of noradrenaline, 5-hydroxytryptamine, histamine (Chou et al., 1971; Chasin et al., 1971; Reddington et al., 1973) and adenosine-ribose compounds (Sattin and Rall, 1970) to respiring guinea pig cerebral cortex slices causes activation of adenyl cyclase. Excitation of slices with electrical stimulation results in a release of neurohumoral agents from the tissue including noradrenaline, 5-hydroxytryptamine (Katz and Chase, 1970; Essman, 1973) and adenosine (McIlwain, 1971; Zanella and Rall, 1973) any of which may act as intermediates in the effect of electric shock on cyclic AMP content. However, the response produced by histamine and 5-hydroxytryptamine is additive with that of electrical stimulation (Reddington et al., 1973; Williams and Rodnight, 1974) suggesting separate modes of action. Currently the most likely suggestion is that E.C.T. causes depolarisation of synaptic structures thus resulting in an increased release of noradrenaline and adenine-ribose compounds (Rodnight, 1975; McIlwain, 1972; Shimizu and Daly, 1972 ; Kuroda and McIlwain, 1973). These in turn increase intracellular concentrations of cyclic AMP by

stimulation of adenyl cyclase through receptors located on the post-synaptic membrane (Schultz and Daly, 1973a); further adenosine can act as a precursor for ATP (section 1.8). Electrical stimulation has also been shown to increase cerebrovascular permeability (Essman, 1973), and thus raised levels in blood and urine may reflect increased leakage as well as production.

Zanella and Rall (1973) have presented evidence that the major part of the extra cyclic AMP formed on depolarisation with electrical pulses is due to adenosine release; however other workers question the importance of this nucleotide in the mechanism. The diverse actions of cyclic AMP are mediated by protein kinases (see figure 1.6), and this raises the problem that adenosine is an effective kinase inhibitor (Weller and Rodnight, 1973; Ueda et al., 1973) blocking the protein phosphorylation response to histamine in slices (Reddington et al., 1973). Exogenous adenosine does not prevent protein phosphorylation following electrical stimulation or the administration of serotonin and noradrenaline (Williams and Rodnight, 1974), and currently the weight of evidence is against its involvement in protein phosphorylation mediated by E.C.T. (Rodnight, 1975). Noradrenaline and possibly dopamine (Thierry et al., 1973; Hungen and Roberts, 1973; Williams, 1974) remain the most likely mediators of the physiological response to E.C.T.

Certainly E.C.T. can have a marked effect on the clinical course of depression (section 4.3) as well as on cyclic AMP levels in plasma and urine. Where does E.C.T. act? : the brain is insulted (the exorcist might suggest the devils are driven away) but the precise sites of action are still a mystery.

5.3. Possible Physiological Role of Cyclic AMP in the Nervous System

In the rabbit superior cervical ganglion system, it has been shown (Greengard et al., 1972) that alterations in cyclic AMP content of the postganglionic nerve cell results in hyperpolarisation leading to a modulation of cholinergic transmission. It was suggested that the link between the increase in cyclic AMP and the membrane hyperpolarisation might be a cyclic AMP-stimulated protein kinase.

Williams (1974) has shown that a noradrenergic mechanism involving an increase in cyclic AMP, results in raised protein phosphorylation in the synaptic region of guinea pig neocortical neurones. He suggested that this phosphorylation is involved in the modulation of synaptic transmission. Such a supposition was supported by the relatively long time of turnover of the protein phosphorylated on application of electrical pulses ($t_{\frac{1}{2}}$ = 5 min), which is comparable in some respects to the dopamine-dependent modulation of cholinergic transmission (Libet and Tosaka,

1970) and the suggested time course of noradrenaline effects on central neurones (Salmoriaghi, 1966).

Changes in the phosphorylation state of the post-synaptic nerve cell membrane mediated by cyclic AMP-dependent protein kinases were suggested by Greengard and Kuo (1970) and Routtenberg et al. (1975) to be part of the molecular basis of learning and memory.

It is generally accepted that the associative processes occurring in the brain during learning result in the repetitive activation of new neuronal pathways, with the progressive development of persistent changes in the properties of their component synapses (Kety, 1972). These changes presumably involve molecular variations affecting the efficiency of synaptic transmission (Glassman et al., 1972).

Drugs that affect the release of noradrenaline or enhance its effects, have been shown to affect memory consolidation processes (Kety, 1972). Bilateral lesions of the locus coeruleus in rats, which cause a reduction in the neocortical noradrenaline concentration, stops the learning process (Anzelark et al., 1973). Similarly, lesions in both the dorsal and median midbrain raphe nuclei in rats, which cause a reduction in the forebrain serotonin and possibly noradrenaline (Hole and Lorens, 1975) result in a deficit in learning. The coerulocortical pathway is one of two noradrenergic pathways which are considered to

function as 'reinforcement' systems in the consolidation of memory (Gilbert, 1975). The firing of Purkinje cells is inhibited by noradrenaline via cyclic AMP (Hoffer et al., 1972), and Gilbert (1975) has recently presented evidence that this inhibition is involved in the system, for learning and storage of information with regard to movement. Thus cyclic AMP is implicated in the modulation of processes involved in learning and memory consolidation. Indeed the behavioural changes occurring on the destruction of the noradrenergic pathway have been compared (Stein et al., 1972) to those associated with schizophrenia. Endogenous 6-hydroxydopamine can cause such destruction and Altsman et al. (1972) have suggested that this is the primary lesion in the onset of this disorder.

It is well established (Kety, 1972) that drugs like reserpine which deplete nerve endings of their catecholamine content, lead to a depression in behaviour (ptosis) when administered to rat or mice in vivo and this effect is reversed by cyclic AMP and its dibutyryl derivative (Abdulla and Hamadah, 1970). On the other hand drugs like D-amphetamine which both block re-uptake (Glowinski and Axelrod, 1965) and increase the release of noradrenaline (Zianie et al., 1972) have the opposite effect to the reserpine-like drugs when given in vivo, counteracting drowsiness and leading to hyperactivity and aggressiveness. From such studies Schildkrout (1965) has proposed that the

biochemical lesion of depressive illness is a reduction in the availability of endogenous amines. A more recent hypothesis for the aetiology of affective illness formulated by Ashcroft et al. (1972) states that the activities of amine mediated synapses in brain are modified in affective illness either as a result of altered input into neuronal systems from other centres or as a result of altered sensitivity of postsynaptic receptors. Evidence for such a modification include a report of increased adenyl cyclase sensitivity to noradrenaline in rat brain, following a lesion in the noradrenergic pathway (Palmer, 1972; Weiss and Strada, 1972). Janowsky et al. (1972) postulated a cholinergic-adrenergic hypothesis of depression which states that the affective state may represent a balance between central cholinergic and adrenergic neurotransmitter activity in those areas of the brain which regulate affect, with depression being a disease of cholinergic dominance and mania being an illness of adrenergic dominance. Cyclic AMP has been shown to affect behaviour, its lipid soluble dibutyryl analogue producing catatonia and convulsions when injected into the lateral ventricles of rat brain (Gessa et al., 1970).

This present investigation shows cyclic AMP to be related to behavioural changes. This relationship is based on the following findings:-

(i) Both plasma and urinary levels of cyclic AMP were significantly lower in patients suffering from

endogenous depression compared to normals.

(ii) A correlation between cyclic AMP excretion and mood was obtained, thus patients suffering from endogenous depression excreted least cyclic AMP in the morning when depression was most severe.

(iii) The low levels of urinary cyclic AMP observed during the depressed phase increased quite significantly on clinical improvement.

(iv) Approximately 80% of depressed patients experienced significant and sustained improvement, or disappearance of symptoms, in the course of an E.C.T. series (Kety et al., 1967). In this investigation 86% of the patients who received E.C.T. showed an increase in plasma and urinary level of cyclic AMP accompanied by improvement in clinical condition. The 14% of the cases that did not show any change in cyclic AMP levels did not show clinical improvement.

(v) Women suffering from premenstrual tension, a syndrome with symptoms characteristic of depressive illness, showed fluctuations in urinary cyclic AMP. This fluctuation was smoothed by the administration of oral contraceptives with concomitant relief of the tension.

A number of recent publications provide strong support for the biological dualism of cyclic AMP and cyclic GMP in the mediation of a number of biological processes. Watson et al. (1973) suggested that cyclic AMP and cyclic GMP mediate

the intracellular signals which determine whether antigen-sensitive cells follow an inductive or non-inductive (paralytic) pathway. Cyclic AMP and cyclic GMP have also been implicated in cell division (Hadden et al., 1972; Moens et al., 1975), mobility (Estensen et al., 1973) and differentiation (Silverman and Epstein, 1975). An increase in the cellular cyclic GMP is thought to represent an active signal for cell proliferation whereas an elevation of cyclic AMP concentration may limit or inhibit cell division. The contractile force of the heart is regulated by these two nucleotides, cyclic AMP mediating the positive inotropic response (Robison et al., 1965) and cyclic GMP mediating the negative response (George et al., 1973). Costa and his colleague (1975) provided evidence for an increase of cyclic AMP/cyclic GMP ratio as the earliest detectable biochemical change in the adrenal medulla during the trans-synaptic induction of tyrosine-3-monooxygenase. In the central nervous system cyclic AMP has been shown to mediate noradrenergic and dopaminergic responses whereas cyclic GMP mediates muscarinic responses to acetylcholine (Taylor et al., 1975). Greengard and his group (1974) provided evidence for the involvement of cyclic GMP in synaptic transmission in sympathetic ganglia.

In view of this evidence and Janowsky's cholinergic-adrenergic hypothesis of depression it is possible that the low levels of cyclic AMP seen in the endogenous depression

could be accompanied by raised cellular cyclic GMP levels: thus the lowering of the cyclic AMP/cyclic GMP ratio could act as a trigger mechanism for endogenous depression.

It is, to say the least, unfortunate that five years after the initial publications there is still controversy over what is apparently easily verifiable - the levels of cyclic AMP in body fluids in mania and depression. Certainly we can expect the discovery of other second messengers, with stress at present on cyclic GMP and its relationships to cyclic AMP. Future work on patients should include:- an investigation of cyclic AMP/cyclic GMP ratios; cyclase receptor sensitivity in, for example, platelets; phosphodiesterase activity (including isoenzyme patterns). It would be of value to examine brain protein kinases but this is not feasible in human studies. As was indicated in a recent brief review in the Lancet (1975) there are many clues to follow.

CHAPTER VI

References

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The Effect of an Ovulatory Dose of Human Chorionic Gonadotrophin on Preovulatory Adenosine 3′:5′-Cyclic Monophosphate Concentrations in Rabbit Ovarian Tissue*

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In bovine corpus-luteum tissue and rabbit ovarian tissue *in vitro* evidence suggests that cyclic AMP is an intermediate in the action of luteinizing hormone on steroidogenesis (Marsh & Savard, 1966; Dorrington & Kilpatrick, 1967).

Injection of luteinizing hormone or human chorionic gonadotrophin also promotes ovulation in oestrus rabbits, and the effect of the latter on concentrations of cyclic AMP *in vivo* in oestrus rabbits has now been investigated. The animals were anaesthetized with Nembutal (2ml) and 37% urethane (ethyl carbamate). The ovaries were freeze-clamped *in situ* and transferred to liquid N_2. Cyclic AMP was extracted and assayed by the method of Brown *et al.* (1971). Basal concentrations (± S.E.M.) of 1.75±0.25nmol/g of tissue were found in oestrus rabbits. Cyclic AMP concentrations rose to a peak of 7.3± 0.93nmol/g of tissue 30min after the injection of human chorionic gonadotrophin and they remained high for about 4h. Concentrations of cyclic AMP then fell and had reached values similar to those found in controls by 8h after the injection of human chorionic gonadotrophin and remained low throughout the ovulatory period, 10–12h after administration of the gonadotrophin.

ATP concentrations were also measured, as described by Lowry *et al.* (1964); these tended to fall from 1.2±0.09μmol/g of tissue (control) to 0.52±0.03μmol/g of tissue at 8h after the injection of human chorionic gonadotrophin, but a significant increase to 1.03±0.02μmol/g of tissue was observed at the onset of ovulation (10h).

These results show that human chorionic gonadotrophin can increase cyclic AMP production and that changes of cyclic AMP concentrations occur in rabbit ovaries *in vivo* with a dose of the hormone that produces the ovulatory response. They also support the view that cyclic AMP may mediate the steroidogenic action of luteinizing hormone, since the direction and duration of these changes was similar to changes observed in steroid production after mating (Hilliard *et al.*, 1964).

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Diurnal Variation of Plasma and Urinary Adenosine 3′: 5′-Cyclic Monophosphate Contents in Normal Human Subjects

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A number of hormones are known to initiate their biological actions by altering the intracellular concentrations of cyclic AMP (Robison *et al.*, 1971), and to exhibit circadian rhythms (Conroy & Mills, 1970). It would therefore seem likely that periodic changes of

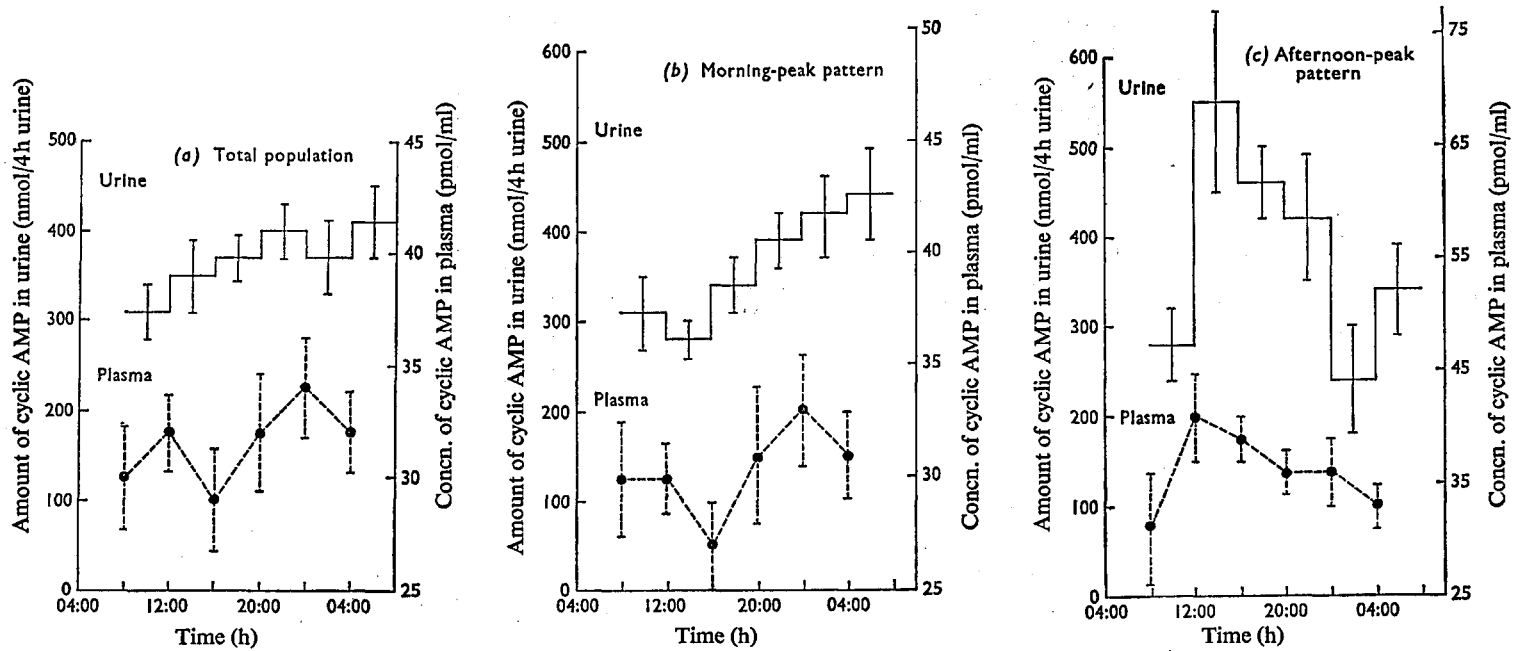

Fig. 1. *Diurnal variation of urinary and plasma cyclic AMP contents in normal human subjects*

——, Variation in urinary cyclic AMP content; – – – –, variation in plasma cyclic AMP concentration; the vertical bars indicate S.E.M. values. (*a*) Total population. Comparison of the paired data (Student's *t* test) for minimum with maximum cyclic AMP in the 4h urine collection periods gives a difference significant at the level $P<0.05$ (number of subjects = $n = 28$). For plasma ($n = 18$) the minimum value at 16:00h is lower than the 12:00h ($P = 0.1$) and the 24:00h value ($P<0.02$). (*b*) Morning-peak pattern. For urine ($n = 21$) the 12:00–16:00h value is lower than the 04:00–08:00h value ($P<0.02$). For plasma ($n = 16$) the minimum value at 16:00h is lower than the 24:00h value ($P<0.01$). (*c*) Afternoon-peak pattern. For urine ($n = 7$) the 24:00–04:00h and 08:00–12:00h values are lower than the 12:00–16:00h value ($P<0.01$). The plasma pattern is for four subjects (two of these showing a pattern shift from one collection to the next); the individual points are not significantly different at the 10% level.

cyclic AMP could occur in plasma and urine. Cyclic AMP has been reported to exhibit diurnal variation in mouse epidermis (Marks & Grimm, 1972), rat gastric mucosa (Domschke *et al.*, 1972) and rat adrenal cortex (Moore & Quavi, 1971). In this last case the rhythm disappeared after appropriate denervation in the hypothalamic region.

At present there is no available information on the diurnal variation of plasma cyclic AMP. The situation with regard to human urinary excretion of cyclic AMP is confused. Chase *et al.* (1969) did not find any diurnal variation in the excretion of cyclic AMP in four normal volunteers. However, Murad & Pak (1972) reported circadian rhythms for both cyclic AMP and cyclic GMP in an unspecified number of subjects.

Plasma and urinary concentrations of cyclic AMP were investigated in eight men and ten women, and urinary concentrations alone were studied in a further three men and seven women aged 18–55 years. There was no attempt to modify the diet and physical activity of these volunteers, and disturbance of sleep was kept to a minimum. Samples were obtained at 4h intervals over a 24h period commencing at 08:00h.

Blood was obtained by venepuncture (antecubital fossa), and heparinized plasma was isolated and snap-frozen (solid CO_2) within 5min. These plasma samples and portions from each 4h urine specimen were stored at −20°C until determination of cyclic AMP. Samples were thawed immediately before assay. Then 0.2ml portions of plasma were deproteinized by the addition of 0.2ml of 0.3M-$ZnSO_4$ and 0.2ml of 0.3M-$Ba(OH)_2$ (Krishna *et al.*, 1968). The deproteinized supernatants were freeze-dried and the residues were taken up in 0.1ml of buffer [50mM-Tris–HCl buffer, pH7.4, containing theophylline (8mM) and 2-mercaptoethanol (6mM)]. The urine samples were assayed for creatinine and a range of dilutions was prepared (1:10 to 1:50) in the buffer specified above. For both plasma and urine, cyclic AMP in the appropriately prepared 0.1ml samples was determined by the saturation method of Brown *et al.* (1971). Recovery values for the plasma procedure were checked by the addition of a known amount of cyclic [8-H^3]-AMP and were found to be in the range 70–80%.

Fig. 1(*a*) summarizes data for plasma and urine from all the subjects studied. The bimodal distribution observed for plasma led us to look for two groups within this population. Consideration of the first 24h collection period indicated two distinct urinary patterns: the majority (14 female, seven male) showed maximum concentrations of cyclic AMP in the morning (04:00–0:800h); the minority (three female, four male) showed a peak in the afternoon (16:00–20:00h), Plasma values also gave two patterns: 16 subjects (nine female, seven male) showed a cyclic AMP peak at about midnight (24:00h), whereas two subjects (one female, one male) had their peak value at noon (12:00h). Plasma and urinary cyclic AMP concentrations were remeasured in ten of the subjects 8 weeks after the first 24h survey, and eight of these showed the same morning-peak pattern as before. Fig. 1(*b*) summarizes data for urine and plasma of the morning-peak type, and Fig. 1(*c*) shows the afternoon-peak pattern. The 24h urine values for each individual investigated on the two occasions varied by not more than 10%, with one exception (27%) of the female who was at different points in her menstrual cycle. All 24h creatinine values fell within the normal range.

The patterns obtained indicate two groups with regard to peak times, and some individuals fitted in with both in that they showed a bimodal distribution. The urinary pattern does not seem simply to be a reflection of plasma concentration, but this is not surprising if a proportion of urinary cyclic AMP is formed in the kidney and is excreted directly (Liddle & Hardman, 1971). Our findings indicate the need for caution in interpreting cyclic AMP concentrations in clinical disorders. It would seem that investigation of patterns of variation will be of greater value than the determination of the concentration of a random specimen.

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Diurnal Variation of Urinary Adenosine 3′:5′-Cyclic Monophosphate Content in Depressive Illness

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An important and significant symptom of depressive illness is the daily fluctuation of mood and of the total state. A worsening of depression in the morning has been associated with endogenous depression, whereas a worsening in the evening has been thought as characteristic of reactive depression (Winokur *et al.*, 1969). In depressive illness alterations in the circadian rhythms of plasma and urinary contents of corticosteroids (Bridges & Jones, 1966), urinary Na^+ and Cl^- excretion (Elithorn *et al.*, 1966), salivary flow and salivary electrolyte excretion (Palmai & Blackwell, 1965) have been reported. Abdulla & Hamadah (1970) observed abnormally low excretion of cyclic AMP in patients suffering from depression. Murad & Pak (1972) have reported a diurnal rhythm for the urinary excretion of cyclic AMP in normal humans, a finding repeated and extended to include plasma in a study reported by us in the preceding paper (Holmes *et al.*, 1974). The purpose of the present study is to demonstrate possible alterations in the circadian rhythm of cyclic AMP in depressive illness before and after improvement.

We have investigated 11 women, of age range 37–67 years, who were inpatients at Tooting Bec Hospital and had been diagnosed either as endogenous depression (seven patients) or reactive depression (four patients). All patients received tricyclic antidepressants and a night sedative, and some were also given tranquillizers. The doses were fixed throughout the period of study. Seven of the patients were also treated with electroconvulsive therapy. Urine samples were collected at 4h intervals over a 24h period commencing at 08:00h on two occasions. The first collection was obtained from the patients after they were stabilized on drugs (about 1 week after admission), and the second on improvement 1–5 months later. For patients who received electroconvulsive therapy the second collection was carried out at least 2 weeks after completion of this treatment (Hamadah *et al.*, 1972). Urine samples were stored at −20°C until cyclic AMP determination by the protein binding method of Brown *et al.* (1971).

All patients in the endogenous-depression group changed from a maximum value for urinary cyclic AMP at 08:00–12:00h when depressed to maxima at 04:00–08:00h and 16:00–20:00h when improved (Table 1). On the other hand all members of the reactive-depression group maintained maxima at 24:00–04:00h and 12:00–16:00h both when depressed and after improvement (Table 1). A comparison of the 24h urinary cyclic AMP values for the two occasions showed, on improvement, a rise of 115% in the endogenous-depression group and 45% in the reactive-depression group. Comparison of paired data (Student's *t* test) showed the former to be significant ($P<0.001$).

These results indicate a pattern change in the urinary excretion of cyclic AMP during clinical improvement in patients suffering from endogenous depression but not in those

Table 1. *Diurnal variation of the urinary cyclic AMP content in patients with depressive illness*

The results presented are the means ± S.E.M. of the determinations (seven for endogenous depression, four for reactive depression). Inter-assay coefficient of variation for the cyclic AMP determination in urine = 9.6%.

	Amount of cyclic AMP in urine (nmol/4h urine)			
	Endogenous depression		Reactive depression	
Time (h)	During depression	After improvement	During depression	After improvement
24:00–04:00	150 ± 37	300 ± 38	350 ± 30	520 ± 18
04:00–08:00	170 ± 49	440 ± 97	250 ± 48	420 ± 70
08:00–12:00	240 ± 48	360 ± 46	340 ± 108	480 ± 70
12:00–16:00	180 ± 29	270 ± 42	410 ± 147	550 ± 123
16:00–20:00	140 ± 17	430 ± 68	310 ± 57	520 ± 93
20:00–24:00	140 ± 9	350 ± 50	350 ± 100	420 ± 78
Total for 24h	1000 ± 145	2150 ± 311	2010 ± 388	2910 ± 317
	% increase of the mean 24h value = 115 (significant at the level $P < 0.001$)		% increase of the mean 24h value = 45 (not significant at the level $P = 0.1$)	

with reactive depression. During the depressed phase urinary cyclic AMP excretion is significantly lower in the endogenously depressed patients than in the normal group described by Holmes *et al.* (1974) ($P < 0.001$ for either the whole normal group or for the female members of the group). These findings may prove to be of diagnostic and prognostic value.

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Variation in Urinary Adenosine 3′:5′-Cyclic Monophosphate Content during the Human Menstrual Cycle

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The plasma concentrations of luteinizing hormone and follicle-stimulating hormone show a marked rise at about mid-menstrual cycle and this surge leads to ovulation (Watson, 1973). In the corpus luteum luteinizing hormone activates adenylate cyclase, leading to an increased concentration of cyclic AMP, which in turn leads to increased progesterone synthesis (Marsh *et al.*, 1966). Oestradiol shows a peak plasma concentration about 1 day before the gonadotrophin peak (Dodson *et al.*, 1973), and one action of this hormone is to restore uterine cyclic AMP concentrations in ovariectomized rats (Szego & Davis, 1967). There is evidence that the administration of oestrogen–progestagen contraceptive preparations initially suppresses both the oestrogen peak and the output of follicle-stimulating hormone and lutenizing hormone (Dufau *et al.*, 1970), but the gonadotrophins may return to their pre-administration concentration on long-term treatment (Loraine *et al.*, 1963). These variations in hormonal concentrations associated with the menstrual cycle may influence urinary excretion of cyclic AMP, and Taylor *et al.* (1970) reported a mid-menstrual cycle rise in the excretion of this nucleotide in three women.

Twelve normally menstruating women, two women with secondary amenorrhoea, one pregnancy (second trimester), five women receiving high-progestagen oral contraceptives and four men were investigated (the overall age range being 19–38 years). Five

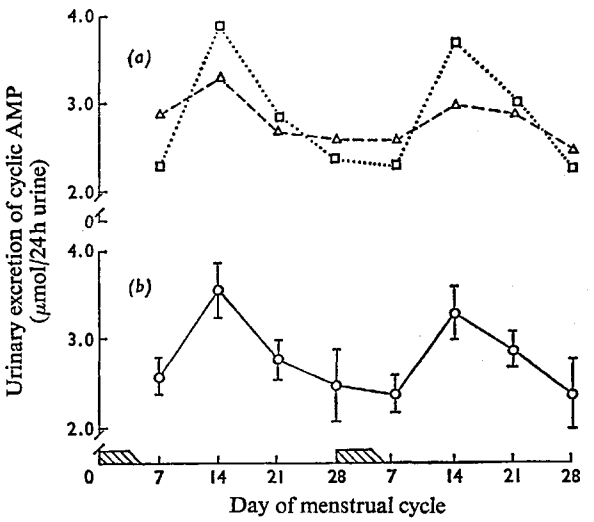

Fig. 1. *Urinary cyclic AMP excretion during two menstrual cycles*

○, Overall pattern (12); vertical bars indicate S.E.M. values; △, non-pre-menstrual-tension subgroup (7); □, pre-menstrual-tension subgroup (5). The shaded areas indicate periods of menstruation.

of those with a normal ovulatory cycle were classified as suffering from pre-menstrual tension. Urines (24h) were collected at weekly intervals for a period of up to 2 months. Completeness of collection was checked by creatinine determination and the samples were stored at −20°C until cyclic AMP determination by the protein binding method of Brown *et al.* (1971).

The results obtained from the 12 normally menstruating women are shown (two cycles) in Fig. 1. A peak ($3.5\pm0.3\,\mu$mol of cyclic AMP/24h urine) was obtained at day 14 (where day 1 is defined as the onset of menstruation). This maximum was significantly higher than the value on day 7 ($2.5\pm0.2\,\mu$mol/24h urine; $P<0.02$) and on day 21 ($2.9\pm0.2\,\mu$mol/24h urine; $P=0.02$). The five women receiving oral contraceptives ($2.9\pm0.12\,\mu$mol of cyclic AMP/24h urine; 32 samples), the pregnant female ($3.7\pm0.17\,\mu$mol/24h urine; six samples) and the four males ($3.1\pm0.15\,\mu$mol/24h urine; 27 samples) showed no significant variation during 6–8 weeks. The two subjects with amenorrhoea excreted cyclic AMP ($1.9\pm0.18\,\mu$mol/24h urine; nine samples) at a rate comparable with the minimum in the menstrual-cycle studies, but again there was no significant change in 4 weeks.

These findings indicate a maximum urinary excretion of cyclic AMP associated with ovulation. Our results suggest that the pre-menstrual tension syndrome may be associated with a rhythm of greater amplitude (Fig. 1), the significance of which is not yet understood. This variation of cyclic AMP excretion associated with the menstrual cycle again indicates the need for care in studying urinary cyclic AMP contents in clinical states.

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The Effect of Electro-convulsive Therapy on the Urinary Excretion of Adenosine 3′:5′-Cyclic Monophosphate

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Abdulla & Hamadah (1970) have suggested that depressive illness is due to a severe fall in the intracellular concentration of cyclic AMP (adenosine 3′:5′-cyclic monophosphate) in all tissues. They also report that urinary excretion of cyclic AMP is lowered in depressive illness, but an increase in the urinary excretion occurred after administration of tricyclic antidepressants.

Electrical stimulation has been found to increase the concentration of cyclic AMP in cerebral-cortex slices (Pull & McIlwain, 1972), and electro-convulsive therapy has long been accepted as an effective form of treatment in some types of depression. We have therefore measured the urinary excretion of cyclic AMP in 14 female patients (aged 24–61 years) undergoing electro-convulsive therapy for depressive illness. The administration of antidepressant or tranquillizing drugs and of potential dietary inhibitors of phosphodiesterase (e.g. methylxanthines in tea and coffee) was stabilized (Paul *et al.*, 1971). Patients were well oxygenated through the period of electro-convulsive therapy, as it has been noted that hypoxia in guinea-pig brain slices results in a marked increase in the output of adenosine derivatives (Pull & McIlwain, 1972).

Urines (07:00–07:00h) were collected and stored at −20°C, and the cyclic AMP content was determined by an enzymic radioisotope-displacement technique (Brooker *et al.*, 1968). It has been suggested that this method may lack the specificity inherent in the protein binding assay (Brown *et al.*, 1971), but this has not been our experience with determinations in urine.

Twelve of the patients showed a significant rise in the excretion of cyclic AMP on the day of electro-convulsive therapy (14.2 compared with 4.2μmol/24h before the therapy). Four controls (failed electro-convulsive therapy) showed no significant increase. This finding is not in agreement with the single-patient study made by Brown *et al.* (1972), but we have found that about 10% of our patients showed no significant increase in the excretion of cyclic AMP on the day of electro-convulsive therapy.

The role of cyclic AMP as a transmitter in the central nervous system is under investigation (Greengard, 1972). It is possible that the antidepressant action of electro-convulsive therapy is mediated through an increased production of cyclic AMP in brain, and that this is reflected in an increased urinary excretion.

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Effect of Electric Convulsion Therapy on Urinary Excretion of 3′, 5′ Cyclic Adenosine Monophosphate

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Summary

Electric convulsion therapy (E.C.T.) was used in the treatment of 13 women inpatients suffering from depressive symptoms. Twelve of the patients showed a significant increase in urinary excretion of 3′, 5′ cyclic adenosine monophosphate (cAMP) on the day of treatment, whereas four controls who received all or part of the preliminary treatment but no electric shock showed a reduction. The results of this study are consistent with the hypothesis that the antidepressant action of E.C.T. is mediated through an increased production of cAMP in brain tissue.

Introduction

Abnormally low excretion of 3′, 5′ cyclic adenosine monophosphate (cAMP) has been reported in patients with depressive illness (Abdulla and Hamadah, 1970; Paul *et al.*, 1970). Abdulla and Hamadah suggested that depressive illness is due to a severe fall in the intracellular level of cAMP in all tissues, including those of the central nervous system. Several factors have been found to influence cAMP levels in body fluids and tissues. An increase in the urinary level has been noted after the administration of tricyclic antidepressants, and these have been shown to be inhibitors of cAMP phosphodiesterase (Abdulla and Hamadah, 1970; Ramsden, 1970). Recent studies by Kodama *et al.* (1971) on brain slices suggest that imipramine, desipramine, and amitriptyline stimulate the formation of cAMP. Electrical stimulation has also been found to increase the level of cAMP in cerebral cortical slices (Kakiuchi *et al.*, 1969; Shimizu *et al.*, 1970), and this response was synergistic with that of histamine or noradrenaline but was prevented by previous treatment with a methylxanthine such as theophylline (Kakiuchi *et al.*, 1969; Sattin and Rall, 1970).

Electric convulsion therapy (E.C.T.) has long been accepted as an effective form of treatment in some types of depression, and we therefore decided to examine the effect of E.C.T. on the urinary excretion of cAMP.

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Patients and Methods

The investigation was carried out on 14 women inpatients at Tooting Bec Hospital suffering from depressive symptoms who were judged clinically to require treatment with E.C.T. The only additional criteria for selection were the willingness and the ability of the patients to co-operate in the investigation. Patients with heart or kidney diseases were excluded. The age range was from 24 to 61 years. Throughout the period of the study the patients were given a diet that lacked foods which might affect indole and catecholamine excretion (Paul *et al.*, 1970). No alcohol was permitted and tea and coffee intake was stabilized. Patients were clinically assessed on the day before E.C.T., on the day of treatment, and on the following day to detect any sudden or pronounced change in mood. All participants received antidepressants or tranquillizers, or both, the doses of which were fixed throughout the period of the study.

E.C.T. was administered regularly between 09.30 and 11.00 hours on Tuesdays and Fridays. All patients received 0·6 mg of atropine sulphate as premedication half to one hour before treatment. In 11 patients, who received a total of 19 treatments, the convulsion was modified by the intravenous injection of 60-70 mg of methohexitone sodium and 25-50 mg of suxamethonium chloride immediately before E.C.T. In the last two patients in the E.C.T. group, who received a total of seven treatments, the convulsion was modified by the intravenous injection of 250 mg of thiopentone sodium and 30-50 mg of suxamethonium bromide. The E.C.T. was administered with an Ectonus mark 3 A.C. mains model (Ectron Ltd.). The electrodes (Ectonus head-band) were lightly soaked in Ectronolyte solution and applied bitemporofrontally. The rotary control was used to apply the potential in a series of increasing steps, reaching a maximum of 140 V; the current was passed for about one second. The patients were well oxygenated with 100% oxygen.

On three occasions patients received all the preliminary treatment but for various reasons, including instrument failure, no shock was delivered. As they had experienced the anticipatory anxiety and had received atropine sulphate, methohexitone sodium, suxamethonium chloride, but not the electric shock they were regarded as controls on these occasions. A fourth patient, who received the atropine sulphate but panicked before further treatment could be given and refused it, was regarded as a separate control. Thus the controls (see Table) were achieved by chance rather than design. It was felt to be unacceptable on ethical grounds to enlarge this control group.

Twenty-four-hour specimens of urine (07.00-07.00 hours) were collected for up to three days before the first E.C.T. to establish a baseline for each individual patient and to determine the day-to-day variation of cAMP excretion. Twenty-four-hour specimens were also collected on the day of E.C.T., for two days after E.C.T., and, where appropriate, on the days between subsequent treatments. The total urinary output over each

Effect of E.C.T., on Urinary 3', 5' Cyclic Adenosine Monophosphate

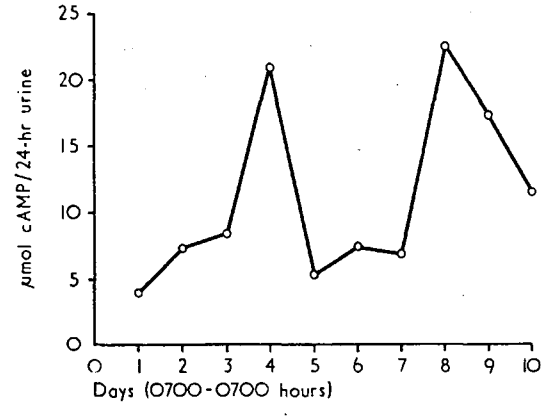
Case No.	Urinary Excretion of cAMP (μmol cAMP/24 hr)			% Change in cAMP Excretion on Day of E.C.T. compared with Before E.C.T.
	Before E.C.T.*	Day of E.C.T.	After E.C.T.*	
		E.C.T. Group		
1	3·6	7·8	4·3	+120
2	2·6	6·9	2·9	+170
3	1·4	8·3	7·5	+480
	3·9	14·5	15·1	+280
	6·5	21·2	6·6	+230
	6·6	23·0	14·4	+250
4	9·1	24·8	4·7	+170
5	12·2	28·6	5·7	+130
6	2·4	4·3	1·5	+80
	1·5	6·8	3·5	+370
7	1·9	3·6	2·0	+90
	2·0	3·7	3·6	+90
8	3·8	6·9	4·6	+80
	4·6	27·0	4·2	+490
9	1·2	3·5	3·1	+180
	5·2	21·5	19·1	+310
10	6·2	38·1	25·2	+520
11	2·0	2·3	2·1	+20
	2·1	2·0	1·9	0
12†	1·7	2·8	2·3	+60
	2·3	14·4	4·5	+540
	4·5	28·3	2·2	+530
13†	4·9	20·0	2·0	+310
	2·0	29·7	4·4	+1,360
	1·9	3·9	13·2	+110
	1·2	14·1	2·2	+1,070
Mean values‡	4·2 (0·4; n = 51)	14·2 (2·1; n = 26)	6·3 (0·7; n = 44)	+310 (50; n = 26)
		Control Group (No E.C.T.)		
4	26·1	24·6	20·3	−6
5	9·3	7·9	5·0	−16
14	5·1	3·7	0·9	−27
10§	3·0	1·0	0·9	−67

*Mean value (where appropriate).
†Patient received thiopentone sodium instead of methohexitone sodium.
‡Mean values with S.E. of mean and number of samples in parentheses.
§Patient received 0·6 mg atropine sulphate only.
Comparison of the paired data for cAMP excretion before E.C.T. with excretion on the day of E.C.T. gives P <0·001. For Cases 9-13 a double-blind procedure was used. Only after assaying all specimens was the code disclosed.

24-hour period was pooled over 1 ml of chloroform, thoroughly mixed, and a 10-ml sample stored at −20°C until analysis. The completeness of the collection was checked by determination of the creatinine content as described by Varley (1969). cAMP was determined by the enzymic-radioisotopic displacement method essentially as described by Brooker *et al.* (1968).

Results

The changes in cAMP excretion resulting from the administration of E.C.T. in a typical case are shown in the Chart. Twelve of the 13 patients who received E.C.T. (see Table) showed a significant rise in urinary cAMP excretion on the day of treatment, when the mean value for the 13 patients (26 treatments) was 14·2 μmol/24 hr compared with a mean of 4·2 μmol/24 hr for the period before E.C.T. (P <0·001). In general the excretion of cAMP fell on the following day but not necessarily to the pre-E.C.T. level. The four controls showed a fall in the urinary excretion of cAMP on the day of (abortive) treatment (see Table).



Daily urinary cAMP excretion in a patient who received E.C.T. on days 4 and 8.

Discussion

A rise in urinary cAMP excretion after E.C.T. could be due to one or more of the following factors: anticipatory anxiety, premedication, anaesthesia, the administration of muscle relaxant, hypoxia, or electrical stimulation. The failure of the four controls to show any increase in cAMP excretion suggests that E.C.T. was the major factor responsible for the rise in cAMP excretion observed in this investigation. This conclusion is supported by the results of other studies. The work of Cox and Potkonjuk (1969), and of Robison *et al.* (1971) suggests that atropine would have little or no effect at the dose level used. Wilson (1969) showed that barbiturates lower cAMP levels in the rat brain, and Havens *et al.* (1959) showed that thiopentone and suxamethonium reduce the postconvulsive levels of catecholamines in the plasma of patients undergoing E.C.T. and hence probably also lower cAMP levels (Robison *et al.*, 1971).

The procedure used in E.C.T. needs to be carefully controlled to prevent hypoxia, which can result in various biochemical changes. Thus Havens *et al.* (1959) showed that the postconvulsive plasma level of adrenaline bears an inverse relation to the degree of oxygenation of the patient, while anoxia leads to a definite increase in the level of cAMP in mouse and rat brain (Goldberg *et al.*, 1970; Ditzion *et al.*, 1970). In the present study the patients were well oxygenated, and the lack of increase in cAMP level in the three suxamethonium-treated controls again points to convulsive electric shock as the causative factor.

The mechanism by which electrical stimulation increases the

level of cAMP in the brain remains to be established. Although the direct administration of the monoamines noradrenaline and histamine into rat cerebral cortex causes prolonged activation of adenyl cyclase (Chou *et al.*, 1971) the E.C.T.-induced increase in brain cAMP content cannot be completely explained by the action of these amines, since electrical stimulation of guinea-pig cerebral cortex produces an accumulation of cAMP over and above that produced by either noradrenaline or histamine (Rall and Sattin, 1970). Currently the most plausible suggestion is that depolarization results in increased release of adenine-ribose compounds, which then stimulate cAMP formation (Rall and Sattin, 1970).

This study has shown that E.C.T. produced an increase in the urinary excretion of cAMP, which may be due to increased tissue levels of cAMP or to an increased rate of elimination. The direct electrical stimulation of slices of guinea-pig cerebral cortex, however, rapidly produces a greatly increased level of cortical cAMP (Kakiuchi *et al.*, 1969; Shimizu *et al.*, 1970), and, furthermore, evidence has been obtained to support the suggestion that cAMP mediates the physiological actions of neural transmitter substances in the central nervous system (Florendo *et al.*, 1971; Greengard and Kuo, 1970). Possibly, therefore, the antidepressant action of E.C.T. is mediated through an increased production of cAMP in the brain, which is reflected in the increase observed in the urine of E.C.T.-treated patients.

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