THE EFFECTS OF CYCLOPHOSPHAMIDE AND ITS DERIVATIVES ON CYCLIC NUCLEOTIDE METABOLISM

by

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A thesis submitted to the University of Warwick in fulfilment of the requirements for the degree of Doctor of Philosophy

The research described in this thesis was carried out in the Department of Molecular Sciences, University of Warwick, between November 1978 and October 1981

ACKNOWLEDGEMENTS

I would like to thank Professor K.R. Jennings for providing laboratory facilities and Dr B.E.P. Swoboda for his guidance and advice throughout the course of this project. I would also like to thank the Science Research Council for their financial support.

The production of 4-hydroperoxycyclophosphamide and 4-ketocyclophosphamide by ozonisation and the subsequent purification of these compounds was achieved in collaboration with Miss L.A. Fitton (Warwick University) whose help is very gratefully acknowledged. I am also indebted to Miss L.Y. Lian (Warwick) for running carbon-13 N.M.R. spectra and to Mr I. Katal (Warwick) who produced the mass spectra presented in this thesis.

Thanks to all my friends at Warwick, especially those of the 2nd floor for numerous coffee breaks and for making my time spent at Warwick University most enjoyable. Thanks are also due to all my family for their support and encouragement throughout my academic career.

SUMMARY

(1) Three methods for the production of an "activated" (4-hydroxylated or 4-hydroperoxylated) derivative of cyclophosphamide were investigated:

(i) Photooxidation of cyclophosphamide by polymer-bound Rose Bengal was unsuccessful. No products could be detected by high performance liquid chromatography and no change in alkylating activity during incubation was observed.

(ii) Incubation of cyclophosphamide with rat liver microsomes followed by deproteinisation with ethanol to produce 4-ethoxycyclophosphamide was partially successful. The product was purified by flash chromatography but was too unstable for further concentration and characterisation.

(iii) Ozonisation of cyclophosphamide produced both 4-hydroperoxycyclophosphamide and 4-ketocyclophosphamide. The latter was easily purified by fractional crystallisation and the former by flash chromatography. Both of these derivatives were used in the enzyme studies in this project.

(2) The compounds used in these enzyme studies were : cyclophosphamide, 4-hydroperoxycyclophosphamide, 4-ketocyclophosphamide, phosphoramide mustard and nitrogen mustard. All cyclophosphamide derivatives were fully characterised and checked for purity by melting point determination, nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C), infra red and mass spectroscopy and high performance liquid chromatography. Their behaviour in thin layer chromatographic systems and in a chemical assay for alkylating activity was also measured.

(3) The spontaneous production of the primary cyclophosphamide metabolite, 4-hydroxycyclophosphamide from 4-hydroperoxycyclophosphamide in aqueous solution was demonstrated by changes in alkylating activity and thiol-binding properties of the product.

(4) Normal rat hepatic plasma membranes were prepared and the adenylate cyclase activity characterised. The effect of cyclophosphamide derivatives on basal (Mg^{2+} ions present) and glucagon and fluoride-stimulated activity was investigated. Only 4-hydroperoxycyclophosphamide was found to produce any significant difference in enzyme activity and inhibited basal activity by 50% at a concentration of 5 mM. Glucagon stimulation was totally abolished and fluoride stimulation only partially inhibited at this concentration.

(5) Guanylate cyclase of normal rat hepatic cytosol was characterised and the effects of cyclophosphamide derivatives on its activity was investigated. Phosphoramide mustard was found to stimulate (40% at 30 mM concentration) and 4-hydroperoxycyclophosphamide to inhibit (50% at 30 mM concentration) the basal (Mn^{2+} ions present) activity of the enzyme. All derivatives were found to inhibit the carcinogen (N-methyl-N'-nitro-N-nitrosoguanidine)-stimulated guanylate cyclase activity with varying effectiveness. Using a carcinogen concentration of 40 μM, 50% inhibition of enzyme activity was observed with 4-hydroperoxycyclophosphamide (1.97 mM), phosphoramide mustard (16.72 mM), 4-ketocyclophosphamide (17.70 mM), cyclophosphamide (18.20 mM) and nitrogen mustard (>30 mM).

(6) The possible mechanisms of action of cyclophosphamide derivatives on the cyclase enzymes and possible relevance of these results to cyclophosphamide chemotherapy and its mode of action are discussed.

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ABBREVIATIONS AND NOMENCLATURE

ADP	adenosine diphosphate
AMP	adenosine 5'-monophosphate
AP	aldophosphamide
A-PDE	cyclic AMP phosphodiesterase
A-PK	cyclic AMP dependent protein kinase
ATP	adenosine triphosphate
BCNU	N,N-Bis(2-chlorethyl)-N-nitroso-urea
BSA	bovine serum albumin
C	catalytic component or subunit
CarbP	carboxyphosphamide
CaM-AC	calmodulin dependent adenylate cyclase
CaM-PDE	calmodulin dependent phosphodiesterase
CB1954	5-aziridinyl-2,4-dinitrobenzamide
CD50	curative dose -50%
Chlorambucil	4-(p-[bis(2-chlorethyl)amino]phenyl) butyric acid
CP	cyclophosphamide (2-bis(2-chloroethyl)amino tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide
cyclic AMP	adenosine 3',5'-cyclic monophosphate
cyclic CMP	cytosine 3',5'-cyclic monophosphate
cyclic GMP	guanosine 3',5'-cyclic monophosphate
cyclic IMP	inosine 3',5'-cyclic monophosphate
DEAE	diethylaminoethyl
dibutryl cyclic AMP	N^6-2'-O-dibutyryl cyclic AMP
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DTNB	5-5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol

ED_{50}	effective dose -50%
EDTA	ethylenediaminetetracetic acid
EGTA	ethyleneglycol-bis(2-aminoethylether),N,N'-tetra acetic acid
GDP	guanosine diphosphate
GMP	guanosine 5'-monophosphate
G-PDE	cyclic GMP phosphodiesterase
G-PK	cyclic GMP dependent protein kinase
Gpp(NH)p	guanosine diphosphate iminophosphate
GTP	guanosine triphosphate
HN2	nitrogen mustard (methylbis(β-chloroethyl)amine)
HP	4-hydroperoxycyclophosphamide
ITP	inosine triphosphate
Ka	association constant
Km	Michaelis-Menten constant
KP	4-ketocyclophosphamide
LD_{50}	lethal dose -50%
MFO	mixed function oxidase
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
Mr	relative mass (molecular weight)
MS	mass spectroscopy
N	nucleotide binding (regulatory) component or subunit
NAD(H)	nicotinamide adenine dinucleotide (reduced form)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced form)
NBP	γ-(P-nitrobenzyl)pyridine
NMR	nuclear magnetic resonance
nor-HN2	nor-nitrogen mustard (bis(β-chloroethyl)amine)

NNPG	N'-nitro-N-nitroso-N-propylguanidine
OHCP	4-hydroxycyclophosphamide
OZ	3-(2-chloroethyl)-1,3-oxazolidine
Pi	inorganic phosphate
PDE	phosphodiesterase
PM	phosphoramide mustard (N,N-bis(2-chloroethyl) phosphorodiamidic acid)
POPOP	1,4-bis-2(4-methyl-5-phenyloxazole)benzene
PPi	inorganic pyrophosphate
PPO	2,5-diphenyloxazole
PZ	N-N'-bis(2-chloroethyl)piperazine
R	receptor or regulatory component or subunit
RNA	ribonucleic acid
Rose Bengal	4,5,6,7-Tetrachloro-2',4',5',7'-tetraiodofluorescein potassium (or sodium) derivative potassium (or sodium) salt
SDS	sodium dodecyl sulphate (sodium lauryl sulphate)
SKF-525A	2-diethylaminoethyl-2,2-diphenylvalerate
SQ20009	1-ethyl-4(isopropylidenehydrazine)-1H-pyrazole-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester hydrochloride
Theophylline	1,3-dimethylxanthine
TI	therapeutic index
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
Triton-X-100	polyethylene glycol-p-isooctylphenylether
Vmax	maximum velocity (of an enzymatic reaction)

ENZYMES		E.C.number
Adenylate cyclase	ATP pyrophosphate-lyase (cyclizing)	4.6.1.1.
Creatine phosphokinase	ATP:creatine N-phosphotransferase	2.7.3.2.
Glucose-6-phosphate dehydrogenase	D-Glucose-6-phosphate:$NADP^+$ 1-oxidoreductase	1.1.1.49.
Guanylate cyclase	GTP pyrophosphate-lyase (cyclizing)	4.6.1.2.
Phosphodiesterase	3',5'-cyclic nucleotide 5'-nucleotido-hydrolase	3.1.4.17.
Protein kinase	ATP:protein phosphotransferase (3',5'-cyclic nucleotide dependent)	2.7.1.37.

INTRODUCTION

CHAPTER 1

INTRODUCTION

PART 1 CYCLIC NUCLEOTIDE METABOLISM

1.1 Cyclic Nucleotides

Naturally occurring 3',5'-cyclic nucleotides are nucleotide monophosphates whose phosphate group is cyclised by esterification to the ribose ring at the 3' and 5' positions. They have the structure shown in Fig. 1.1. Cyclic AMP and cyclic GMP are purine nucleotides that are generally thought to influence or regulate numerous cell functions and biological events. Other cyclic nucleotides, such as cyclic CMP and cyclic IMP, have been found in extremely low concentrations but nothing is known of their function or importance in biological processes.

The intracellular level of the cyclic nucleotides is governed by the enzymes catalysing their synthesis and degradation. A cyclase enzyme, acting on the corresponding nucleotide 5'-triphosphate, produces the cyclic nucleotide and pyrophosphate. A phosphodiesterase, acting on the cyclic nucleotide, produces the corresponding nucleotide 5'-monophosphate (reaction 1).

$$\text{5'-NTP} \xrightarrow{\text{CYCLASE}} \text{cyclic NMP} + \text{PPi} \xrightarrow{\text{PHOSPHODIESTERASE}} \text{5'-NMP} \qquad (1)$$

Fig. 1.1 The Structures of 3',5'-cyclic Nucleotides

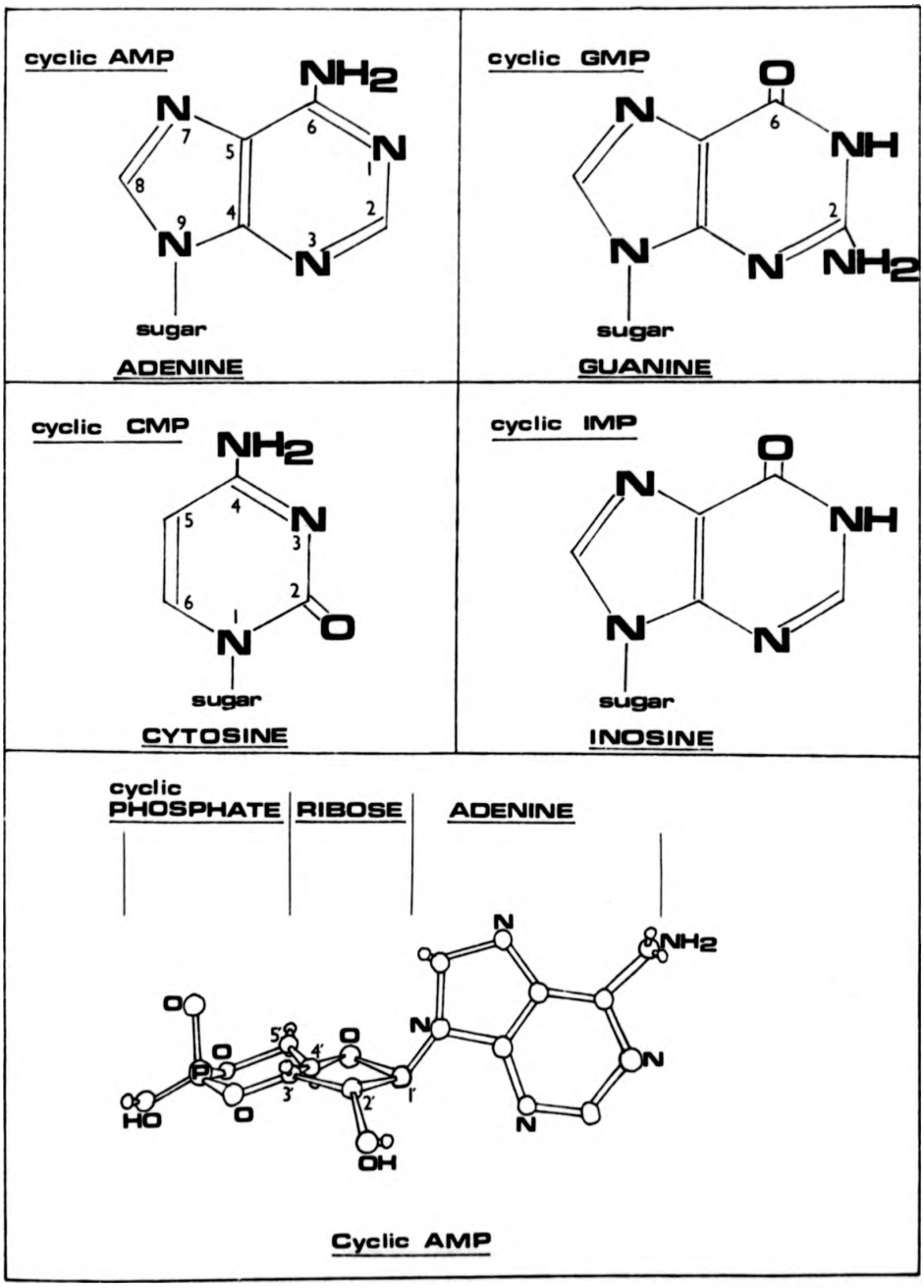


Fig. 1.1 Continued

The main figure shows the structure of cyclic AMP; other 3',5'-cyclic nucleotides differ only in their base composition (insets).

The ribose ring, of 3',5'-cyclic nucleotides is twisted slightly out of a planar orientation by the cyclic phosphate ring which is in a "chair" conformation. The plane of the base ring structure lies at right angles to the approximate plane of the ribose ring.

It follows, therefore, that the concentration of a specific cyclic nucleotide may be controlled by alteration in the activity of a specific cyclase or phosphodiesterase.

In the following account I have restricted the discussion, so far as possible, to mammalian systems. The effects of calcium ions on the various processes discussed has been described separately in Section 1.10. The relevance of cyclic nucleotide metabolism to cancer has been discussed in Section 1.11.

1.2 3',5'-cyclic AMP

Cyclic AMP is the most abundant cyclic nucleotide, intracellular concentrations being in the range of 0.1 to 1.0 μmoles/kg in animal tissues (1). It was originally discovered as a component of the mammalian hormonal system whereby adrenaline could stimulate liver glycogenolysis. The role of cyclic AMP in such systems is that of the well documented "second messenger", mediating the response of the cell to a hormonal "signal" (1). Cyclic AMP has many diverse effects upon different organisms and cell types, some of these being summarised in Table 1.1. The mechanism of cyclic AMP action is quite well understood and involves the interaction of cyclic AMP with a protein kinase.

1.3 Cyclic AMP-dependent Protein Kinase

Cellular processes regulated by cyclic nucleotides originate with binding of the nucleotide to a specific receptor protein. These sites of binding in eukaryotic organisms are protein kinases. Cyclic AMP-dependent protein kinase (A-PK) was first discovered by Walsh *et al.* (2) in skeletal muscle. Studies from several laboratories indicate that A-PK consists of two types of subunit , a regulatory (R) subunit which binds cyclic AMP, and

TABLE 1.1 Effects of 3',5'-cyclic AMP

PROCESS	SITE and ACTIVITY
Membrane permeability	(i) Ions (synapse, neuromuscular junction, adrenal medulla, retina) (ii) Water (kidney, toad bladder, skin, antidiuretic hormone)
Steroidogenesis	Corpus luteum, adrenal cortex, Leydig cells
Secretory responses	Hypothalamic releasing factors, salivary gland, exocrine pancreas, thyroid, insulin, gastric HCl
Triglyceride, cholesterol ester hydrolysis	Adipose tissue, liver, steroid-producing cells
Inhibition of lipogenesis	Liver, adipose tissue
Movement of intracellular structures	Melanophore dispersion, sperm mobility, cell process maintenance, (fibroblasts)
Glycogenolysis, stimulation and inhibition	Liver, adipose tissue, muscle
Gluconeogenesis	Liver, kidney
Gene transcription	Microorganisms (lac operon), enzyme induction in foetal liver
Protein synthesis and translation	General inhibition of protein synthesis, selective protein synthesis (adrenal cortex), stimulation in microorganisms (tryptophan synthesis)
Motility and aggregation	Slime mould aggregation

Reproduced from ref. (9)

a catalytic (C) subunit which catalyses phosphorylation of substrate proteins by ATP (3). The holoenzyme (RC) is a tetramer containing two R and two C subunits. The mechanism of action of cyclic AMP is that it binds to the R subunit and causes dissociation of the inactive holoenzyme to yield fully active C subunit (reaction 2).

	R_2C_2	+	2 cyclic AMP $\rightleftharpoons$	R_2.cyclic AMP_2	+	2C	(2)
	INACTIVE					ACTIVE	
Mr =	186,000			110,000		38,000	

The association constant, Ka, for cyclic AMP ranges from 0.01 to 0.08 μM, and binding of cyclic AMP and subsequent activation of A-PK are directly related to the concentration of cyclic AMP (4).

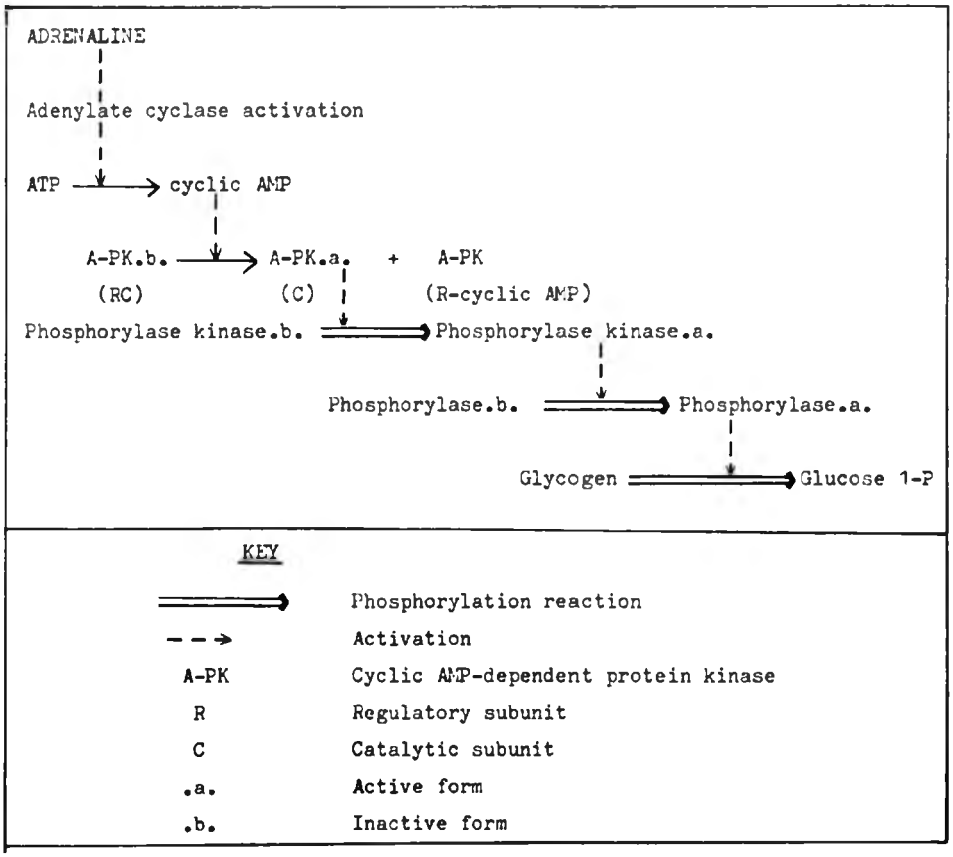
There are two isoenzymes of A-PK, type I and type II, which differ only in the R subunit. Type I is characterised by its high affinity binding site and its inability to self-phosphorylate the R subunit. Mg.ATP decreases the affinity of the type I A-PK for cyclic AMP by 10-fold and increases the affinity of the type II A-PK for cyclic AMP by 6-fold. The biological significance of the two isoenzymes is not clear but it may be that the type I enzyme is involved in cell proliferation and tissue growth and the type II is involved in cell differentiation (5) with a possible role in cancer (Section 1.11).

A-PK catalyses the transfer of the γ-phosphate group of ATP to serine and threonine residue hydroxy groups of several enzymes and structural proteins, thereby affecting their biological functions in a variety of ways but it is not known which of these are true physiological substrates (4).

The initial phosphorylation of a protein molecule may only be the first step in a series of reactions ultimately leading to an expression of activity. For example, the action of cyclic AMP in glycogenolysis in liver occurs by a "phosphorylation cascade" involving three phosphorylations (Fig. 1.2). The phosphorylated active form of phosphorylase catalyses a reaction which results in the expression of the glycogenolytic action of adrenaline since it is this enzyme which catalyses the conversion of glycogen to glucose 1-phosphate (Fig. 1.2) (6).

The phosphorylation of phosphorylase kinase is, however, rather more complicated than the scheme in Fig. 1.2 suggests, and involves multisite phosphorylation (7). Phosphorylase kinase is composed of four subunits and has the structure $(\alpha\beta\gamma\delta)_4$; the γ subunit contains the active site of the enzyme and the δ subunit contains the calcium binding site. The β subunit is phosphorylated quickly by A-PK (which is necessary for activation) and its dephosphorylation (and hence its deactivation) is enhanced by the slower phosphorylation of the α subunit, which therefore serves a regulatory role. Dephosphorylation of the α subunit also occurs and serves to inhibit the rate of deactivation, thus in this system, A-PK serves the dual role of (i) activating the enzyme by phosphorylating the β subunit and (ii) determining the time that deactivation may start by the slower phosphorylation of the α subunit. This mechanism ensures that for a period of time, determined by the different kinetics of α and β subunit phosphorylation, there is no competition between A-PK and β subunit dephosphorylation, prolonging the activation of phosphorylase kinase and facilitating the conversion of inactive phosphorylase to active phosphorylase. Such enzymatic regulation by multisite phosphorylation occurs *in vivo* and may be the rule rather than the exception (8).

Fig. 1.2 Cascade Activation of Glycogenolysis



Not all reactions of A-PK are of an "activating" nature, and it should be noted that the action of A-PK on glycogen synthetase serves to de-activate this particular enzyme. This has been noted in view of the previous discussion of A-PK and glycogenolysis. It can be seen that A-PK, in this case, serves two functions, (i) it activates glycogenolytic enzymes and (ii) it deactivates the glycogen synthesising enzyme.

This system may at first sight seem to be extremely unselective. A rise in intracellular cyclic AMP concentration leads to an activation of A-PK which then acts by phosphorylating several types of proteins with various effects. However, selectivity may be exhibited by the presence or absence of such proteins. This would be governed by the control of expression of the genes for these proteins. Hence, the cellular response to a rise in cyclic AMP levels would depend upon the particular cell, its stage in the cell cycle and other effects influencing gene expression such as induction or repression.

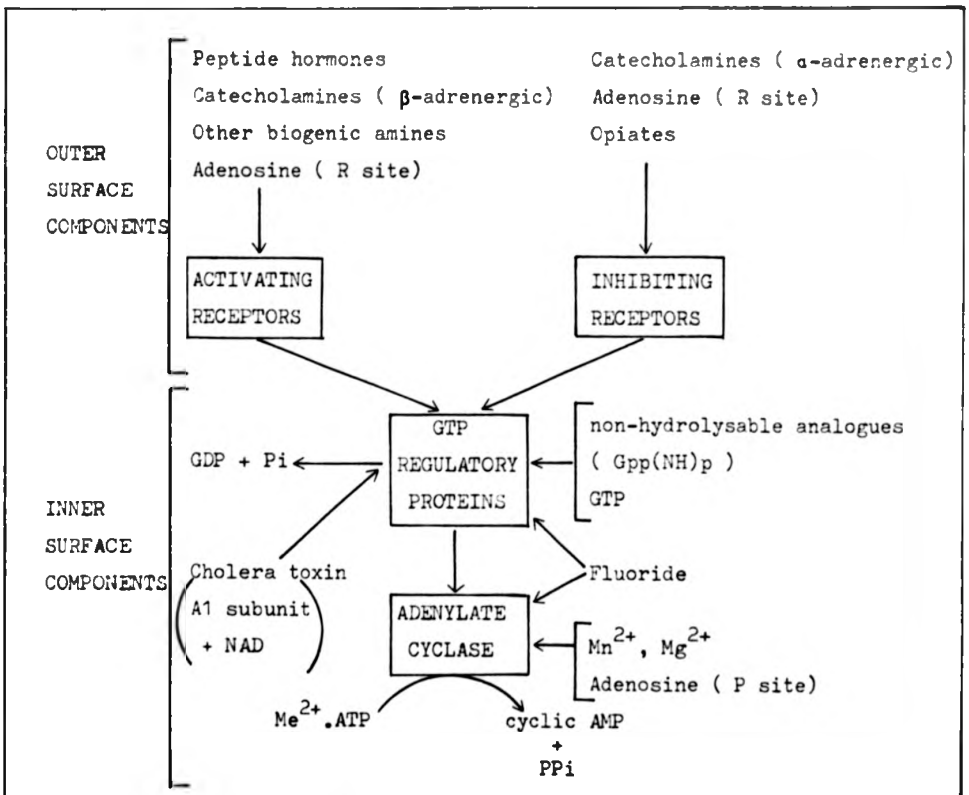
Another degree of selectivity is achieved at the hormonal level by the receptor component of the adenylate cyclase system.

1.4 Adenylate Cyclase

(i) Structure

Adenylate cyclase catalyses the conversion of ATP to cyclic AMP and pyrophosphate (1). It is a membrane-bound enzyme whose activity is controlled by a number of regulatory sites (Fig. 1.3) (10). The protein constituents, structured within the lipid framework of the cell membrane, are of at least three types. At the outer membrane is the receptor component (R) which contains a specific binding site. At the inner membrane surface are the catalytic (C) and the nucleotide regulatory (N)

Fig. 1.3 The Structure of Adenylate Cyclase



Redrawn from ref. (10).

components (11). The latter contains a GTP binding site and is responsible for mediating the effects of hormones and GTP on the activity of C. Much of the work on adenylate cyclase has involved investigations of target analysis (12) where high energy radiation is used to produce ionisation of the target molecule. The energy imparted is so high (1500 kcal/mol) that a single "hit" anywhere in a protein molecule destroys its function. The analysis of the radiation inactivation curves (exposure vs. log. activity/original activity) leads to a value for the size of the "functional unit" under investigation (13). The usefulness of such a method is that the measured "functional unit" may be the catalytic unit (C) if this activity alone is measured, or the entire cyclase complex if cyclase activity is measured in the presence of hormone and GTP. Thus values for the size of various subunits may be calculated. For such studies the enzyme is preincubated with the appropriate ligand(s). The "ground state" target sizes are measured by irradiating the enzyme sizes obtained are presumed to represent precursor forms antecedent to those obtained after preincubation of the enzyme system (10).

Using this method, Schlegel et al. (12) have investigated the adenylate cyclase system of hepatic plasma membranes. One of their major conclusions was that the adenylate cyclase system increases in molecular size with increasing complexity of regulation, Table 1.2. This was taken to indicate the binding of regulatory proteins (R and N) to the enzyme (C). Furthermore, the large target sizes discovered from ground state and hormone-binding studies (Table 1.2) indicated that multimeric complexes probably exist of the R and N components and that these break down to smaller units in the process of combining with the C subunit. Rodbell (11) has postulated the possible structure of such multimeric components, Fig. 1.4. It is possible that such structuring of the cyclase system is

TABLE 1.2 Target size analysis of adenylate cyclase in liver membranes

ASSAY	LIGANDS	TARGET SIZE ($x10^5$ daltons)	HYPOTHETICAL TARGET COMPONENTS	TARGET IF ONLY C_2 ACTIVE§
Adenylate cyclase	Mn.ATP	1.5	C	C_2
(Ground state)	Mg.ATP	2.4	NC	$(NC)_2$
	Gpp(NH)p + glucagon	13.0	$(RNC)_n$	$[(RNC)_2]_4$
Preactivated	Gpp(NH)p, GTP or NaF	2.3	NC	$(NC)_2$
	GTP + glucagon	3.5	RNC	$(RNC)_2$
Binding	Glucagon GTP displaceable	6.7	$(RN)_n$	$[(RN)_2]_3$

Target size : C = 1.5, N = 0.8, R = 1.2 ($x10^5$ daltons)

If N = 42,000 (from ref. (19)) then C = 75,000, N = 42,000, R = 60,000

Data is reproduced in modified form from ref. (12).

§ Target analysis based on N = 42,000, from ref. (10).

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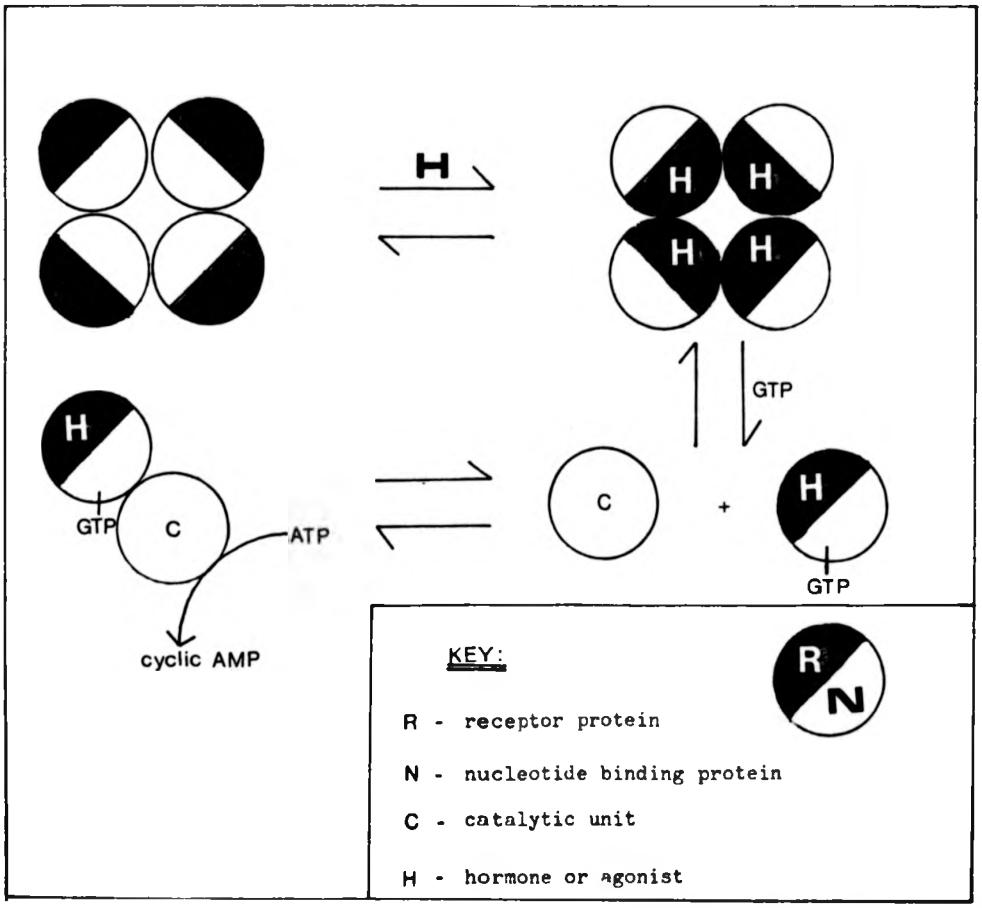
Fig. 1.4 A model for the coupling of the receptor-nucleotide regulatory units (RN) to the catalytic unit (C) of adenylate cyclase and the role of hormone and GTP in this process. Redrawn from ref. (11).



necessary for the activity of the catalytic units when Mn.ATP is used as a substrate. That such structures are needed for further coupling to the C component may explain the effects of other effectors such as membrane lipids (14). Such oligomers of the RN components can be used to explain the kinetics observed with adenylate cyclase systems. In Fig. 1.4 the GTP-binding site is drawn to indicate that binding of GTP cannot occur until binding of the hormone has occurred. Differences in the number of active RN "monomers" compared with the catalytic until could explain the effects observed for glucagon binding and the degree of activation by hormone (15).

The proposed functional units, R, N and C have since been isolated by Citri and Schramm and reconstituted in their work on the action of a hormone receptor (16).

(ii) Hormonal Interactions - The R Component

A large number of hormones and neurotransmitters activate adenylate cyclase in the course of eliciting their physiological response (Table 1.3). For each of these effectors, there exists a specific receptor (R component) containing a binding site for the hormone in question (1) (10). Cells may contain one or many such receptors on their surface.

Using the information of Kaslow *et al.* (19) that the N peptide has a molecular weight of 42,000, the results of Schlegel *et al.* (12) Table 1.2 suggest that the hepatic glucagon receptor has the corresponding molecular weight of 60,000. Their radiation inactivation data (19) suggest the Mr = 120,000 and they therefore conclude that this is due to the activity of a larger "functional unit"($(RNC)_2$) which is measured and corresponds to two R components (Table 1.2). These results are incompatible with the previously reported data of Houslay *et al.* (31).

TABLE 1.3 Selected examples of hormone-cyclase interactions

STIMULATORY HORMONES	INHIBITORY HORMONES
Adrenaline	α-Adrenergic agonists (i)
Nor-adrenaline	Cholinergic agents (i)
Glucagon (liver)	Opiates (i)
Adrenocorticotrophic hormone	Adenosine (adipocytes)
Leutenising hormone	ADP (platelets)
Thyroid stimulating hormone	Prostaglandins PGE_2 PGE_2 (adipocytes)
Parathormone	Nicotinic acid (adipocytes)
Vasopressin	Dopamine (adenoma)
Calcitonin (kidney)	Angiotensin II (liver)

(i) Demonstrated with neuroblastoma x glioma hybrid cells

Various physical factors in the sample preparation, however, were different. Dried samples were used instead of frozen and a rather low activity [^{32}P] ATP used as substrate. Although they reported that the target size of the glucagon receptor plus that of the cyclase measured in the presence of sodium fluoride added up to the total measured glucagon-stimulated unit, no analysis of the contribution of the N unit could have been included. No mention was made of "ground state target sizes" considered by Schlegel et al. (12) to be critically important. From their data a value of 41,000 may be calculated for the molecular weight of the receptor component.

Other systems have also been studied in a similar way, notably, the turkey erythrocyte adenylate cyclase which is different from the liver enzyme basically due to the N component. In this system, the hormone receptor has been calculated to have a molecular weight of approximately 90,000.

(iii) The Regulatory N Component

The regulatory component of the adenylate cyclase system (termed the N, G/F or G component) mediates the effects of hormone receptors, GTP, or its various analogues, F^- ions and cholera toxin on the catalytic component.

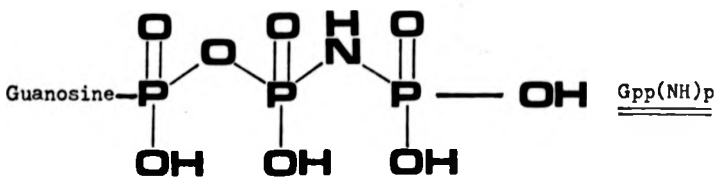
To explain the stimulatory and inhibitory effects of hormones, Rodbell has postulated the existence of not only different R components but also different N units. Ns units which couple with Rs components of stimulatory hormones and Ni units which interact with Ri components of inhibitory hormones (11), both acting through the same catalytic component.

Work on elucidating the mechanisms involved in cyclase stimulation and inhibition has been aided by the ability to isolate and manipulate such components by detergent extraction from cell membranes (16) (17)

and the isolation of various murine lymphoma S49 variants. The S49 variant clone, cyc$^-$, is deficient in adenylate cyclase activity (18). Cyc$^-$ cells, which can be selected because cyclic AMP produced within wild-type S49 cells in response to catecholamine or prostaglandin PGE_1 is cytocidal, lack the N component completely (19). Other S49 mutants have been utilised; unc cells have N units but these fail to interact with R units, and these N units have been shown to have different physical characteristics (20). βd Cells have normal N and C units but only 10 to 20% of the R units relative to wild-type cells (21).

Reconstruction experiments by Kaslow et al. (19) using the cyc$^-$ mutant and several other cell types, revealed that a complete cyclase system (R, N and C) took on the characteristics of the cyclase system from which the N component originated. Citri and Schramm (16) using similar methods and turkey erythrocyte and cyc$^-$ membrane extracts, found that in the presence of hormone all the R components were in the RN complex form. This is in agreement with the mechanism shown in Fig. 1.4, which postulates that the hormone-RN interaction reveals the GTP binding site of the N component.

The observation that GTP was required for hormonal activation of adenylate cyclase and that the non-hydrolysable analogue Gpp(NH)p gave



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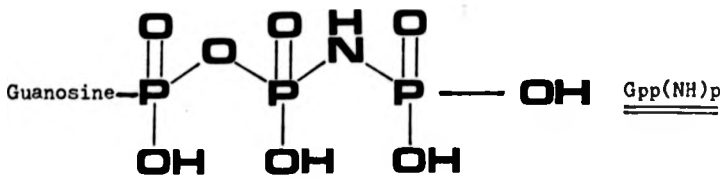
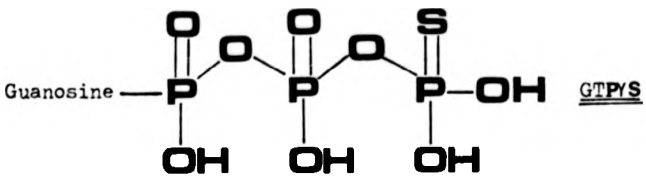


consistent activation (22) led to the idea that a GTPase activity was to be found in the adenylate cyclase system. Furthermore, using a system designed to measure the hydrolysis of GTP, under conditions which minimised the "background" hydrolytic activity found to be present in the turkey erythrocyte membranes used, it was found that catecholamines interacting with the β-adrenergic receptor increased this activity (23). It was also discovered that the hormone, upon binding, causes release of bound GDP (or $Gpp(NH)_p$) (24).

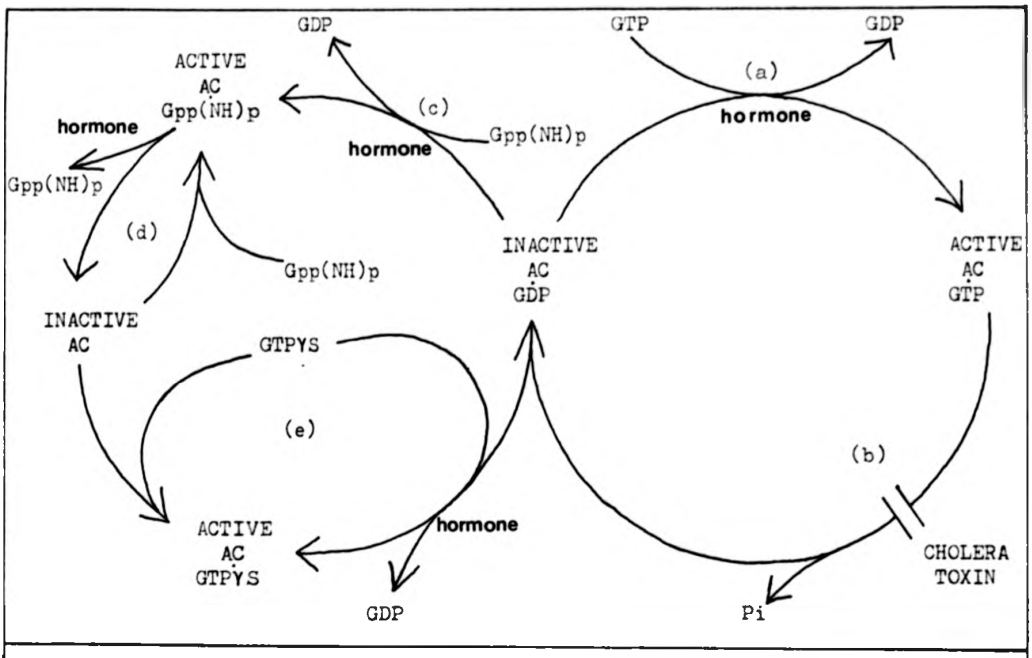


GTPγS was found to be an analogue which would stimulate adenylate cyclase activity consistently, would not be released by hormone binding to its receptor and, therefore, inhibited the GTPase activity of the enzyme system. From these results the regulatory role of both hormone and GTP were understood (Fig. 1.5). The hormone stimulates the enzyme by facilitating the release of GDP. Binding of GTP then activates the cyclase and the GTPase reaction results in the formation of inactive enzyme with tightly bound GDP.

The discovery that the stimulatory action of cholera toxin on adenylate cyclase was by inhibition of the GTPase reaction (25) led to the selective labeling of the N component by using cholera toxin and $[^{32}P]$NAD (26).

Cells deficient in N by functional criteria were also found to lack the toxin labelled protein. In avian erythrocytes N is a heat stable

Fig. 1.5 The Regulatory GTPase Cycle



ACTIVATION - DEACTIVATION BY GTP AND HORMONE

(a) Hormone binding facilitates the release of GDP allowing GTP binding to activate the enzyme.

(b) GTPase reaction inactivates the enzyme. GDP is tightly bound. Cholera toxin stimulates adenylate cyclase activity by its inhibition at this stage.

EFFECT OF GTP ANALOGUES

(c) Gpp(NH)p replaces GTP in stimulating the enzyme. It is not hydrolysable so stimulation is consistent, however

(d) hormone can facilitate its release (24).

(e) GTPγS activates the cyclase system. It is not hydrolysable and cannot be released by hormone action.

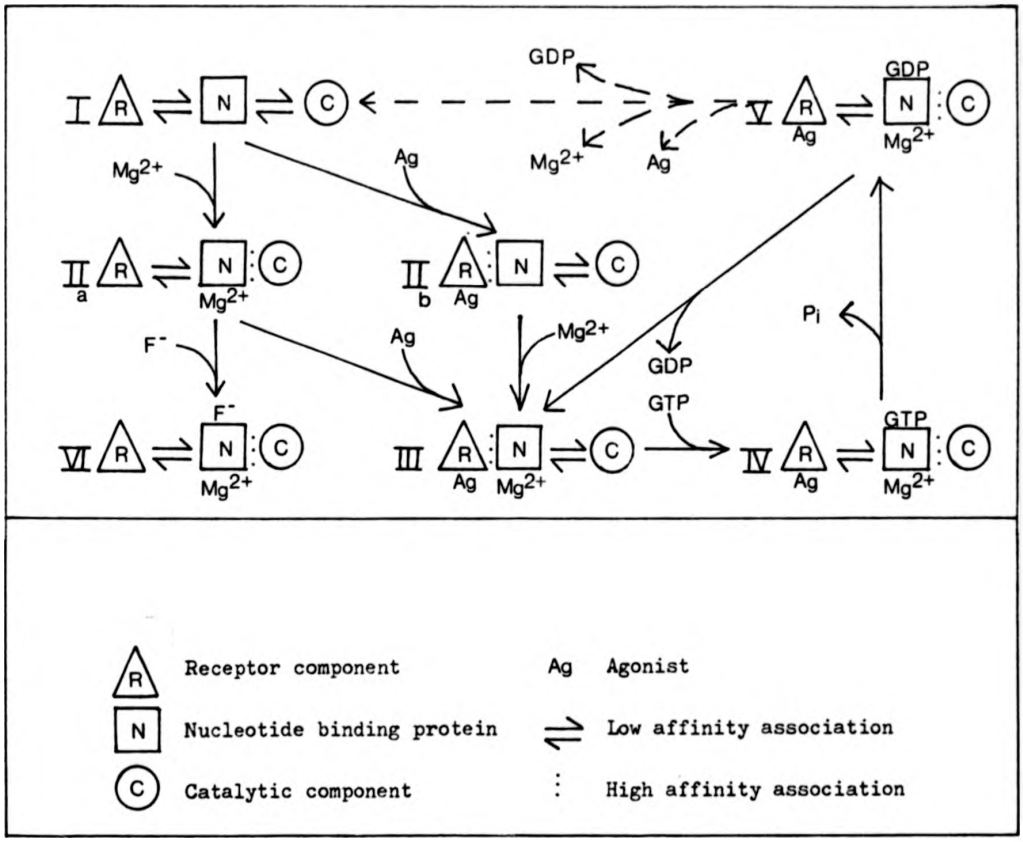
protein of Mr = 42,000 (19). Addition of detergent extracts containing N to membranes from cells genetically deficient in N (cyc$^-$) reconstitutes the ability of hormones, guanine nucleotides, F^- ions and cholera toxin to activate the cyclase (11).

Although the N peptide of Mr = 42,000 has been identified in avian erythrocytes, it also exists as an oligomer of Mr = 126,000 (26). Cholera toxin has been found to ADP-ribosylate other peptides in membranes of other cells including S49 mouse lymphoma (19) and rabbit liver (27). Three major proteins have been observed with molecular weights of 45,000, 35,000 and 52,000, in purified extracts of the N protein but the protein of 35,000 Mr has not been observed to be labelled by cholera toxin and [^{32}P]NAD (27). Neither the 35,000 nor the 52,000 proteins appear to be necessary for measured activities of N but may be related to each other in that N may exist as complexes of different subunit composition with different functional properties (27). It has been noted, however, that in the A9 mouse and 380-6 hamster transformed lung fibroblasts, cholera toxin and [^{32}P]NAD labels an Mr = 52,000 peptide but not an Mr = 42,000 one (28). The 52,000 peptide appears to be the functionally active form of the N component in such cells.

(iv) Adenylate Cyclase Reaction Sequence

Fig. 1.6 shows the reaction sequence of adenylate cyclase, and summarises much that has already been discussed. Structure I represents the cyclase components as they might appear when isolated after EDTA pretreatment or as they actually exist in isolated S49 membranes structure. It shows the effect of Mg^{2+} ions, which would presumably be the "natural state", with N tightly bound to C. Under such conditions the catalytic unit is active. Such activity represents the "basal activity" of the enzyme, although the C component can act alone, on the unnatural sub-

Fig. 1.6 Reaction Sequences for Adenylate Cyclase



Modified from ref. (21)

strate, Mn.ATP. Structures III to V represent the GTPase cycle which occurs in the presence of GTP and agonist. Structure IV is the active unit. In structure V, GDP inhibits the cyclase but is released by agonist binding to R, returning to structure III. Structure VI shows the complex formed in the presence of F^- ions. Similar complexes exist with guanine nucleotides and analogues.

1.5 Cyclic AMP - Phosphodiesterase

Cyclic nucleotide phosphodiesterases (PDE) catalyse the irreversible hydrolysis of the 3'-bond of cyclic nucleotides to produce the non-cyclic nucleotide monophosphate. The action of PDE is the only catabolic process known for the cyclic nucleotides. PDE is not only responsible for "quenching" a cyclic nucleotide "signal" but also constitutes a potential mechanism for modulating the intracellular levels of cyclic nucleotides. Some confusion may arise when discussing PDE's because of the highly heterogeneous nature of these enzymes. The individual enzymes differ with respect to tissue distribution, subcellular location, physico-chemical properties, substrate specificities, kinetic characteristics and susceptibility to activators and inhibitors. However, on DEAE-cellulose ion exchange chromatography, three PDE's are resolved. Fraction I is predominantly active with cyclic GMP and fraction II has a relatively low affinity for both cyclic AMP and cyclic GMP. Fraction III has a predominant action on cyclic AMP and may therefore be termed cyclic AMP-phosphodiesterase (A-PDE).

Most tissues seem to have two forms of A-PDE with different affinities for cyclic AMP. However, since this conclusion is based on the non-linearity of Lineweaver-Burk plots, it could also demonstrate one enzyme exhibiting negative cooperativity (32).

In human lung, A-PDE exhibits a Km for cyclic AMP of 0.5 μM and demonstrates negative cooperativity effects. It is predominantly membrane bound and probably corresponds to the Mr = 200,000 protein isolated by agarose gel filtration (32). Multiple forms of A-PDE have been shown to exist (33) and four active forms have been found in Walker carcinoma cell lines of apparent molecular weights of 10^6, 430,000, 350,000 and 225,000 (35). These forms are apparently interconvertible and are composed of subunits of Mr = 15,000. A-PDE isolated from monocytes and lymphocytes is thought to be prototypical (35). The lymphocyte enzyme demonstrates an extremely high substrate affinity (Km = 0.2 μM) but can be resolved into two species, one with a molecular weight of 45,000 daltons (3.6 S) and the other of 98,000 daltons (6.0 S) which may be a dimer of the first. The monocyte enzyme is monomeric and is identical to the lymphocyte lower molecular weight enzyme, of lower affinity (Km = 0.5 μM).

Interestingly, physical manipulation of lymphocytes and "ageing" by storage of the homogenate causes the lymphocyte enzyme to convert to the lower affinity, lower molecular weight enzyme form identical to the monocyte enzyme (32).

It has been shown that many hormones can effect A-PDE activity and thus alter the level of cyclic AMP (32). The effects of insulin have been studied most but the biological significance of its actions on A-PDE is not known. It has been shown to increase the Vmax of A-PDE without affecting the high affinity particulate form of the enzyme (36). Since cycloheximide has no effect on this stimulation, it must be concluded that protein synthesis is not required (37). Cyclic AMP itself and dibutyryl-cyclic AMP were found to mimic the effect of glucagon, activating A-PDE. Thus, it seemed, cyclic AMP was acting as a mediator for this hormone.

Over a longer period of time, it was found that noradrenaline and dibutyryl-cyclic AMP induced low Km and high Km A-PDE of C-6 glioma cells (38). For this to occur, both protein synthesis and RNA production were shown to be necessary.

PDE activity has also been shown to be dependent upon the redox state of the cell. Since it is inhibited with consequent reduction in Vmax, by NADH (39) and is therefore dependent upon the nutritional state and hormonal control of the cell.

1.6 3',5'-Cyclic GMP

The intracellular levels of cyclic GMP are in general ten times lower than the level of cyclic AMP, and range from 0.01 to 1.0 μmoles/kg in animal tissue (40). The biological role of this cyclic nucleotide is still poorly understood, most information on the subject being restricted to the knowledge that some physiological compounds can effect its concentration. It has become increasingly clear, however, that a similar system to that regulating the levels and effects of cyclic AMP also exists for cyclic GMP.

1.7 Cyclic GMP-dependent Protein Kinase

A class of protein kinases has been found in mammalian tissues which are preferentially activated by cyclic GMP (41). These cyclic GMP-dependent protein kinases (G-PK) have been found in lung (42), cerebellum (43), and femoral artery (44), and these tissues have also been shown to contain high cyclic GMP levels (4). In contrast to A-PK, G-PK is a predominantly soluble enzyme. The Ka for cyclic GMP is 0.01 to 0.12 μM which is similar to that of A-PK for cyclic AMP, and ATP is the phosphoryl

donor. Unlike A-PK, dissociation of the enzyme complex does not seem to be necessary for activation (reaction 3).

$$R_2C_2 \quad + \quad 2 \text{ cyclic GMP} \rightleftharpoons R_2C_2\text{.cyclic GMP}_2 \qquad (3)$$

	INACTIVE	ACTIVE
Mr =	165,000	165,000

Evidence to show that subunit structure exists comes from SDS-polyacrylamide gel electrophoresis, where, in the presence of a reducing agent the Mr = 165,000 holoenzyme can be resolved into a polypeptide of Mr = 74,000 to 81,000 (45). This suggests that the holoenzyme is a dimer of identical subunits, each of which can bind a molecule of cyclic GMP. Like A-PK, G-PK is self-phosphorylating and such activity appears to inhibit the enzyme, A-PK is also capable of inhibiting G-PK by this mechanism. Arginine residues are also necessary for inhibiting G-PK activity : activation of G-PK can be achieved by the action of either 2,3-butanedione (an arginine blocking agent), or trypsin (hydrolysing at the arginine site), in the presence of mercaptoethanol which yields active monomers (46).

A-PK and G-PK appear to be very similar enzymes with respect to physical characteristics, cyclic nucleotide binding, ATP binding, amino-acid composition (70 to 90% homology), self-phosphorylation and substrate protein specificity. Two major differences are their relative activities and the existence of different modulator proteins for each.

Although G-PK is capable of phosphorylating most of the proteins regarded as substrates for A-PK (Section 1.3) its activity is much lower. The only proteins to be phosphorylated to any comparable degree are

histones (47). A-PK phosphorylates serine residues 32 and 36 of histone protein H2B, whereas G-PK preferentially phosphorylates serine residue 32 (47).

In mammalian tissues there are two separate modulator proteins of PK activity; inhibitory modulator of A-PK and stimulatory modulator of G-PK (4). The activities of these modulators is different in various tissues and represents a way of controlling the specificity of A-PK and G-PK. The stimulatory modulator of G-PK acts by reacting with substrate histone molecules, rather than on G-PK directly, rendering them a better substrate. This modulator is also required for G-PK maximal phosphorylation of ribosomal proteins and appears to operate through polyanionic interactions rendering the substrate a better phosphate acceptor. Other polyanions such as DNA, polydeoxyribonucleotides, polyglutamate and heparin are also capable of stimulating G-PK phosphorylation of histones (47).

1.8 Guanylate Cyclase

(i) Guanylate cyclase catalyses the conversion of GTP to cyclic GMP and pyrophosphate in the presence of a divalent metal ion (48). It is widely distributed throughout mammalian tissues and can be distinguished from adenylate cyclase by its subcellular distribution, substrate specificity, cation requirements and its response to activating agents and detergents.

(ii) Subcellular Distribution

Guanylate cyclase is found in both the particulate and soluble fractions of cells. The relative activities of these forms of the enzyme varies with the tissue and species used. In rat lung, spleen and liver 80 to 90% of the total guanylate cyclase activity is found in the soluble fraction, whereas adenylate cyclase is predominantly particulate (48).

Treatment with non-ionic detergents increases the enzyme activity in both particulate (3 to 10-fold) and soluble (1.5 to 2-fold) fractions (49). In mouse intestinal mucosa, the activity is predominantly particulate, approximately 90% of the total after treatment with detergents (40). While in liver, particulate activity is only 20% of the total (49). Several subcellular membrane fractions have been associated with guanylate cyclase activity including Golgi, endoplasmic reticulum and other organelles (50) (51). The soluble and particulate forms seem to represent different structures, this view being supported by the difference in properties between the two (52), Table 1.4. These results could be explained by the existence of multiple forms of the enzyme (ie. different proteins or components) or a different distribution of factors affecting these properties (52). Garbers has shown that antibody prepared against particulate guanylate cyclase from sea urchin sperm inhibited mammalian particulate guanylate cyclase but had no effect on the soluble form (53). This apparent demonstration of antigenically distinct forms has been refuted. Zwiller et al. (54) have demonstrated an antigenic similarity between particulate and soluble forms of rat brain guanylate cyclase. Furthermore, immunological studies suggest that the soluble guanylate cyclase is derived from the membrane-bound enzyme (55). Goldberg and Haddox (56) have suggested the possibility of the soluble form of the enzyme being the result of an artifactual release of guanylate cyclase from the particulate fraction into the cytosol during sample preparation.

(iii) Guanylate Cyclase Activity

Guanylate cyclase is most active when Mn.GTP is used as substrate, whereas Mg.GTP and Ca.GTP result in less than 10% of the observed Mn^{2+}-stimulated activity (48). Mg.GTP, however, must be regarded as the

TABLE 1.4 Some properties of soluble and particulate guanylate cyclase from rat heart, lung and liver

PROPERTY	SOLUBLE	PARTICULATE
Apparent molecular weight	ca. 150,000	ca. 300,000
Km for GTP	12 to 65 μM	50 to 100 μM (sigmoidal kinetics)
Metal-nucleotide sites	1	2 or more
Ka for free Mn^{2+}	ca. 0.2 mM	ca. 0.2 mM
Activity with Mg^{2+}	ca. 10% of Mn^{2+}	ca. 10% of Mn^{2+}
Effect of Ca^{2+}	generally stimulates	generally inhibits
Effect of detergents	stimulates 30 to 100%	stimulates 300%
50% inhibition by ATP	ca. 0.4 mM	1 mM
Cyclic AMP formation	not detectable	not detectable
50% inhibition by p-chloromercuriphenyl	10 to 100 nM	1000 to 10000 nM

Reproduced from ref. (52)

natural substrate due to the usually low intracellular concentration of Mn^{2+} ions (56). Copper has been found to inhibit plasma membrane guanylate cyclase activity in rat liver (57), while other divalent cations such as cadmium, zinc and mercury also inhibit the particulate enzyme (48), their effects being reversed by either of the thiol compounds glutathione or dithiothreitol (DTT).

There are a number of cellular metabolites which have been found to be inhibitory to guanylate cyclase. These include phosphoenolpyruvate, oxaloacetate, ATP, ADP, ITP, dGTP (48), AMP (58), and pyrophosphate (56). The intracellular concentration of these compounds is probably too low to have any physiological significance with regard to guanylate cyclase activity, with the possible exception of ATP. As well as inhibition, however, it has been noted that in rat renal cortex, ATP increased the activity of particulate guanylate cyclase but had no effect on the soluble form. It is possible that metal ions may act by chelating such inhibitors as well as by acting as a substrate/cofactor and binding to a separate activating site on the enzyme (50).

The importance of -SH groups for the activity of guanylate cyclase has been demonstrated. Inhibition of the rat liver enzyme by p-chloromercuriphenyl suphate is reversed by DTT (61). Furthermore, the inhibition of guanylate cyclase by arsenite which can be potentiated by equimolar concentrations of 2,3-dimercaptopropanol but completely reversed by its excess has been considered evidence for the existence of juxtaposed -SH groups on the enzyme (70).

Table 1.4 lists some properties of guanylate cyclase. In general, the soluble form has been shown to be of lower molecular weight (150,000 to

450,000) compared to the particulate cyclase (300,000 to 600,000) and to express a higher affinity for substrate.

(iv) Hormonal Effects

Hardman and Sutherland showed that glucagon, insulin and adrenaline had no effect on guanylate cyclase activity (48) although there have been some reports of stimulation by insulin which have not, however, been corroborated (52). The action of acetylcholine and the α-adrenergic effects of noradrenaline increase cyclic GMP levels and may stimulate guanylate cyclase, but only in the presence of calcium ions (52), and may not, therefore, be a direct stimulation of the enzyme. Liang and Sacktor found that several catecholamines in low concentration activated guanylate cyclase and that neither α nor β blockers prevented this effect (63). Although some neurotransmitters (noradrenaline, acetylcholine, histamine and glutamic acid) are capable of causing the release of cyclic GMP at nerve terminals, the distribution of guanylate cyclase does not parallel the distribution of nerve terminals using these transmitters (62). There have been reports of other hormone activators of guanylate cyclase, such as secretin and pancreozymin and inhibitors such as the catecholamines adrenaline and noradrenaline (61) (64) but these early reports are largely uncorroborated and have been considered to have probably resulted from (i) nonenzymic formation of cyclic GMP (65) (ii) contaminants in preparations (66) and/or (iii) alterations in the redox state of incubations and free radical formation (52).

(v) Non-physiological Activators

Although guanylate cyclase does not seem to be activated by any hormones, there is a wide variety of nonhormonal (and non-physiological) compounds capable of stimulating the enzyme. These include certain lipids hydrogen peroxide, various oxidising agents and nucleophilic nitrogen compounds. Recent evidence suggests that although the precise mechanism of this stimulation is unknown, cellular processes involving alterations in the redox state of the cell and the formation of free radicals may be the general mechanism by which such stimulation can be modulated.

Soluble guanylate cyclase undergoes spontaneous, time-dependent "auto-activation", apparently involving an oxidation, in that oxygen is a requirement and thiol compounds completely block this process (67) led to the idea that hydroxyl radicals may be involved in this process, an idea which will be returned to later.

(vi) Activation By Nucleophilic Nitrogen Compounds

Sodium azide has been shown to activate guanylate cyclase in some tissues but not in others (52) (68). Mixing of responsive and non-responsive tissues revealed the need for a protein "activating factor" in unresponsive tissues (which was present in responsive tissues) and also that certain inhibitors should be absent for sodium azide to be active (52) (68). This explained the previously reported time lag and protein dependency of activation (68). Purification of the activator protein from rat liver by DEAE-cellulose chromatography revealed a heat-labile, protease and α-chymotrypsin-inactivated protein which co-chromatographed with, and could be replaced by, the enzyme catalase (69). Peroxidase, cytochrome b_2 and cytochrome c reductase can also substitute for the activating factor. The inhibitory compounds present in some

tissue preparations were purified from rat heart and lung and identified as haemoglobin and myoglobin (52).

The effect of catalase and other activator proteins on sodium azide results in the production of nitric oxide which is a potent activator of guanylate cyclase (71). It is apparent that the effects of other nitrogen containing activators are mediated through the production of nitric oxide. Activation by azide, hydroxylamine and possibly some others is enzymatic, as described, and requires the presence of hydrogen peroxide or oxygen.

The formation of nitric oxide from several other activators is spontaneous under the appropriate conditions and does not, therefore, require the activator protein. Nitrite, nitroglycerin, nitroprusside, nitrosamines and nitrosoureas are all capable of activating guanylate cyclase (52), an activation which is inhibited by haemoglobin and myoglobin. Some nitroso compounds such as dimethyl-nitrosamine, N-nitrosopiperidine have been found to give rise to nitric oxide and N-methyl-N-nitrosourea, only on photoirradiation a process which accelerates its spontaneous release from other nitroso compounds, including MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) (72) a potent guanylate cyclase activator (73).

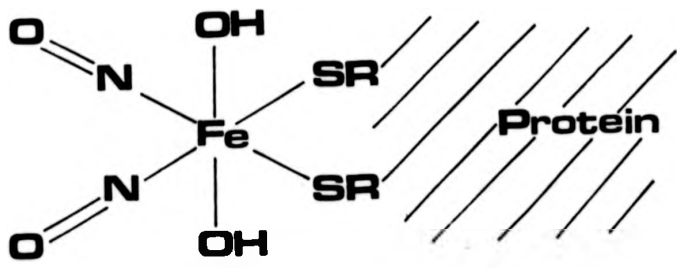
DeRubertis and Craven (73) showed that MNNG is the most potent of the nitroso compound activators, increasing basal Mn^{2+} levels by 36-fold at 1 mM concentration.

Using electron spin resonance techniques, Nagata _et al_. (72) demonstrated the production of free radicals by MNNG and found that the radicals produced in liver cytosol were different from those produced in solution unless sulphydryl compounds and ferrous (iron (II)) chloride were included in the solution. Under the latter conditions, Nagata _et al_. concluded that an iron-nitric oxide-thiol complex was formed with an unpaired electron mainly localised on the iron moiety, giving rise to the

free radical electron spin resonance signal observed. In liver cytosol incubated with MNNG, similar radicals were observed, presumably similar in structure, and iron-nitric oxide-thiol protein complexes were suggested. Considering the importance of thiol groups to guanylate cyclase activity it is perhaps not difficult to envisage an iron-nitric oxide-cyclase complex being involved in cyclase activation. This idea is supported by the observation that ferrous chloride enhances the activity of guanylate cyclase activated by sodium azide (69).

Although heat-treated liver supernatant and MNNG gave rise to free radical formation, the enzymes xanthine oxidase and diaphorase were found to give rise to free radicals without the need of ferrous chloride (72). Other thiol-containing enzymes including urease, aldolase and alcohol dehydrogenase gave no radical formation.

The iron-nitric oxide-thiol protein complexes formed may be similar, or at least analogous, to the iron-nitric oxide complexes observed with amino acids, imidazole (74) hydroxy groups, phosphates and cysteine (75). A proposed structure is:



(vii) Activation By Hydroxyl Radical

An alternative pathway for guanylate cyclase activation is suggested by the observation that superoxide dismutase activates the enzyme in the absence of sodium azide (76). Catalase, inhibitors of superoxide dismutase, such as cyanide ions or thiols, and hydroxyl radical scavengers such as catecholamines, hydroquinone or butylated hydroxyanisole prevent this activation.

Nitrate reductase, which catalyses the formation of superoxide anion (O_2^-) from molecular oxygen, enhances the activation produced by superoxide dismutase. This activation therefore requires both superoxide anion and hydrogen peroxide probably leading to the formation of hydroxyl radicals (reaction 4), which are necessary for the activation of the

$$O_2^- + H_2O_2 \longrightarrow .H + OH^- + O_2 \qquad (4)$$

superoxide anion	hydrogen peroxide	hydroxyl radical	hydroxyl anion	molecular oxygen

guanylate cyclase enzyme.

Under the appropriate conditions, catecholamines and Mn^{2+} ions may give rise to hydroxyl radical production (52) which probably explains the stimulatory effect of these agents in some preparations. Such a mechanism would be highly unlikely to have any physiological significance.

(viii) Fatty Acid Activation

Highly specific structural requirements must be fulfilled if fatty acids are to serve as activators for guanylate cyclase, presumably because only those fatty acids which are substrates for fatty acid cyclooxygenases seem to be capable of activating the enzyme (77). Fatty acids are

metabolised to highly reactive hydroperoxide and endoperoxide intermediates by this enzyme.

The prostaglandin endoperoxides PGG_2 and PGH_2 have been shown to be potent activators but their effects are dependent upon the presence of molecular oxygen. The fatty acid hydroperoxides, 15-OOH-20:4, 12-OOH-20:4, and 13-OOH-18:2 also stimulate guanylate cyclase in a concentration dependent manner.

15-OOH-20:4 :

$$CH_3(CH_2)_4\overset{OOH}{C}{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$$

12-OOH-20:4 :

$$CH_3(CH_2)_4CH{=}CHCH_2\overset{OOH}{C}{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$$

13-OOH-18:2 :

$$CH_3(CH_2)_4\overset{OOH}{C}{=}CHCH_2CH{=}CH(CH_2)_7COOH$$

(ix) Physiological Relevance Of Guanylate Cyclase Activation

Fig. 1.7 summarises the pathways for guanylate cyclase activation already discussed and Table 1.5 shows the changes which occur in some of the properties of the enzyme upon its activation.

The most important alteration observed is the ability of the cyclase to use Mg^{2+} ions equally as effectively as Mn^{2+} ions which may not be present in high enough concentration under physiological conditions to have any effect. It may be considered, therefore, that the extremely low activity of guanylate cyclase in the presence of only Mg^{2+} ions results in a negligible physiological role until or unless it is activated.

Another important change in the activated enzyme is its ability to utilise ATP as a substrate for the production of cyclic AMP. Activated guanylate cyclase may be an important route for the production of both cyclic AMP and cyclic GMP and this may have important consequences in the regulation of intracellular levels of both nucleotides (78).

Fig. 1.7 Free Radical Formation and Redox Regulation of Guanylate Cyclase

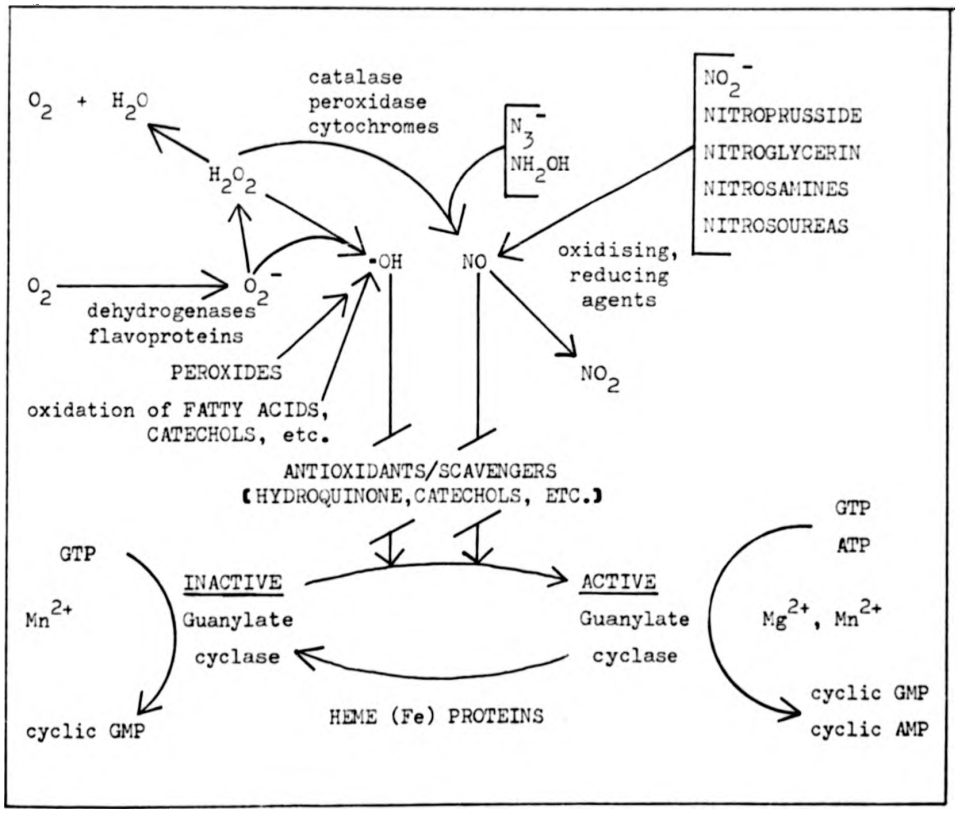


TABLE 1.5 Alteration in properties of guanylate cyclase with activation

PROPERTY	EFFECT OF ACTIVATION
Km for GTP	unaltered or increased
Requirement for Mn^{2+} ions	decreased
Effect of Mg^{2+} ions as sole cation	as effective as Mn^{2+} ions
Stability	more labile
Effect of Ca^{2+} ions	decreased
Cyclic AMP formation	ca. 5 to 15% of cyclic GMP formation

Reproduced from ref. (52)

Oxides of nitrogen are common environmental pollutants and the formation of nitroso compounds from dietary precursors may explain the high guanylate cyclase activities observed in lung and intestinal mucosa respectively (52). Since the concentration of nitric oxide required for activation is approximately 1 μM or less, concentrations of nitrite, nitrate, amines and other potential precursors are sufficiently high in tissues that humoral and/or physiological alterations in redox states could influence nitric oxide formation and guanylate cyclase activity (52).

Cyclic GMP formation and the production of hydroxyl radicals has been reported in leukocyte phagocytosis, fatty acid oxidation, prostaglandin synthesis and platelet aggregation. The altered formation of hydroxyl radicals could explain some of the observed effects of hormones and other agents on cyclic GMP production.

1.9 Cyclic GMP Phosphodiesterase

Like A-PDE activity, cyclic GMP phosphodiesterase (G-PDE) activity commonly occurs as a function of two distinct proteins separable by DEAE-cellulose chromatography (Section 1.5). One of these proteins can also hydrolyse cyclic AMP and has a low affinity for cyclic GMP (Km cyclic GMP = 20 μM) and is therefore inhibited by cyclic AMP. The other protein, separated as fraction I, has a high affinity for cyclic GMP (Km = 0.3 μM) with little or no activity with cyclic AMP (79).

Hormonal effects on G-PDE activity have been reported (32). For example, insulin can increase the activity of the enzyme and raise the intracellular cyclic GMP levels in cultured cells. Thyroxine and glucocorticoids decrease G-PDE activity in the soluble fraction of the thyroid and in homogenates of liver and muscle respectively. The role of G-PDE and that of hormonal regulation is not known.

1.10 Calcium and Cyclic Nucleotide Regulation

(i) Intracellular concentrations of Ca^{2+} ions can be altered by the action of hormones and neurotransmitters and this provides another mechanism by which cyclic nucleotide metabolism may be controlled.

Ca^{2+} ions have direct effects upon the activity of adenylate and guanylate cyclase. The effects of calcium upon adenylate cyclase are somewhat obscure (21) and it may act at a distinct site on the cyclase complex or it may interact with Mg^{2+} ions, either at the binding site or in the form of the non-productive substrate Ca.ATP. Even in the presence of excess Mg^{2+} ions, Ca^{2+} ions inhibit adenylate cyclase in many tissues, including liver membranes (80). Maximum inhibition is produced with 1 mM Ca^{2+} in the presence of millimolar Mg^{2+}. This effect results in a decreased Vmax for the enzyme with no change in the Km for ATP. Inhibition can be reversed to some extent with Mn^{2+} ions presumably because they act at a separate binding site, and this inhibition is completely reversed by EGTA. Levitski et al. (81) proposed the existence of allosteric binding sites for free Ca^{2+} ions in turkey erythrocyte membrane adenylate cyclase which are distinct from the Mg^{2+}-binding site. This conclusion has been questioned by Cech et al. (21) and criticized as a misinterpretation of their results. The data of Rodan et al. (82) suggests a competitive binding of Ca^{2+} ions to the Mg^{2+}-binding site of the enzyme.

In contrast to their effect on adenylate cyclase, Ca^{2+} ions have been demonstrated to increase the activity of guanylate cyclase in millimolar concentrations (83) but are less effective than Mn^{2+} ions. In the presence of Mn^{2+} ions, guanylate cyclase can be inhibited or stimulated by Ca^{2+} ions depending upon their concentration (84), but higher concentrations of Ca^{2+} ions are required than would be expected to

exist intracellularly under physiological conditions.

As well as these "direct" effects of calcium ions, other actions of Ca^{2+} ions can be mediated by a regulatory protein, calmodulin.

(ii) Calmodulin

Calmodulin is an endogenous, calcium-binding protein first discovered as a PDE-activating protein (85). It has since been characterised as an acidic protein with a molecular weight of 16,723 and has the ability to bind four calcium ions per molecule with a dissociation constant of 4 to 18 μM (86). It has been calculated that under physiological conditions, calmodulin may be found binding 3 Ca^{2+} ions and 1 Mg^{2+} ion (87). The binding of calcium converts the protein to a more helical configuration which is apparently necessary for its binding to calmodulin-sensitive enzymes. The activation of enzymes by calmodulin therefore requires two steps (Scheme 5).

Ca^{2+}	+	Calmodulin	$\rightleftharpoons$	Ca^{2+}-Calmodulin*	(i)
		INACTIVE		ACTIVE	
Ca^{2+}-Calmodulin*	+	E	$\rightleftharpoons$	Ca^{2+}-Calmodulin*-E	(ii)
		INACTIVE		ACTIVE	

Scheme (5)

Calmodulin is found in all subcellular fractions although it seems to be mainly cytosolic and in most tissues, organs and species so far examined (87). It has been found to activate a number of enzymes including ATPase, phospholipase A2, PDE, adenylate and guanylate cyclases and protein kinases and may have a role in the control of intestinal secretion, disassembly of microtubules and smooth muscle contraction. In

some tissues therefore, calmodulin may modify the biosynthesis and biodegradation as well as the actions of cyclic AMP and cyclic GMP.

(iii) Calmodulin-dependent Phosphodiesterase (CaM-PDE)

There is evidence that at least one of the multiple forms of PDE (Section 1.5) is activated or stimulated by Calmodulin. The hydrolysing activity of CaM-PDE is mixed, but seems to have a higher affinity for cyclic GMP than cyclic AMP. CaM-PDE often parallels the concentration of calmodulin in many tissues (e.g. brain, liver, kidneys, adrenals) although some tissues which contain much calmodulin possess little or no CaM-PDE activity (e.g. testes, lung, adrenal medulla, lymphoblast) (87). At saturating concentrations of Ca^{2+}-Calmodulin* (Scheme 5) the Km for cyclic nucleotides was reduced 20-fold for both cyclic AMP (Km = 180 μM) and cyclic GMP (Km = 8 μM), with no change in Vmax (79). Cheung, however, has reported both increased Vmax and decreased Km upon activation of bovine brain cyclic AMP-PDE activity by Calmodulin (88). Under saturating concentrations of Calmodulin, half maximal velocity (Vmax/2) was found at 4 μM Ca^{2+} for CaM-PDE activity and no time lag has been reported for Ca^{2+} ion-activation in the presence of Calmodulin.

Endogenous protein stimulators of CaM-PDE have been found in adipocytes, retina, testes and brain (79) with molecular weights ranging from 1000 to 95,000. These seem to act by binding to the activated Ca^{2+}-Calmodulin* complex (Scheme 5) preventing its binding to Calmodulin-sensitive enzymes. One other inhibitory binding protein (Mr = 540,000) has been found, however, which predominantly inhibits the Ca^{2+}-independent PDE activity of bovine retina.

(iv) Calmodulin-dependent Cyclases

Calmodulin-dependent guanylate cyclase activity has been demonstrated in *Tetrahymena* (89) but little is known of this enzyme in mammalian tissues.

There appear to be two forms of adenylate cyclase present in animals, one is inhibited by Ca^{2+} ions, the other is stimulated by Ca^{2+} and seems to be restricted to neuronal tissues. Ca^{2+}-dependent adenylate cyclase, has however been reported in adipocytes and adrenal cortex (90). Ca^{2+}-stimulated adenylate cyclase from brain tissue is calmodulin-dependent (CaM-AC) and shows differential distribution within this tissue; medulla, thalamus and hypothalamus having high CaM-AC activity, cerebrum, and cerebellum intermediate and pons having low CaM-AC activity (87).

Catecholamine stimulation of brain adenylate cyclase is Ca^{2+}-dependent. Although the release of Calmodulin from membranes has been shown to decrease the responsiveness of this adenylate cyclase to dopamine in the corpus striatum, dopamine increases adenylate cyclase activity in the presence of EGTA (87).

Calmodulin has been reported to increase the Vmax of CaM-AC without affecting the Km for ATP (91). In contrast, however, Brostrom *et al.* (92) have reported that Calmodulin does not change the Vmax but decreases the Km for ATP of rat brain fluoride or GTP-stimulated cyclase activity. The kinetic basis of Calmodulin activation is therefore not at all clear.

The degree of activation of adenylate cyclase by Calmodulin is not large and its mode of action is not known. The presence of Calmodulin in the cell does not necessarily mean that the adenylate cyclase is sensitive to its effects and both sensitive and Calmodulin-insensitive adenylate cyclase may be present in the same cell (21). Studies of CaM-AC

have been complicated by the nature of the adenylate cyclase complex (Section 1.4) but future radiation inactivation studies of CaM-AC should reveal much of its site of action within the complex and its interaction with the various modulatory ligands of adenylate cyclase activity.

(v) Calmodulin-dependent Kinases

Several kinases have been found to be stimulated by Calmodulin which are involved in stimulating glycogenolysis in skeletal muscle.

Ca^{2+}-dependent phosphorylation of various membrane proteins have been reported in lung, spleen, skeletal muscle, vas deferens, heart and adrenals and shown to be Calmodulin-dependent. Furthermore, Calmodulin may be involved in the Ca^{2+}-stimulated release of neurotransmitters (87).

1.11 Cyclic Nucleotide Metabolism and Cancer

(i) It has been observed that cyclic nucleotides play an important part in cellular proliferation and differentiation. The intracellular concentration of cyclic AMP changes during the cell cycle and is lowest at mitosis (93) is inversely related to growth rate (94) and is higher in contact-inhibited cells than in less dense populations (95). The action of cyclic GMP appears to be antagonistic to those of cyclic AMP in many cases and its concentration has been shown to increase in proliferating cells (96). Cyclic GMP induces nucleic acid synthesis under the appropriate conditions (97) and counter acts the growth-inhibitory effects of cyclic AMP (98). Such observations led Goldberg *et al*. (99) to propose the "yin-yang" hypothesis of biological control. This general theory of cyclic nucleotide action applied to systems where the action of cyclic AMP could be either inhibitory or stimulatory, and cyclic GMP antagonistic to that of cyclic AMP, states that it is the ratio of cyclic AMP to cyclic GMP

levels which is important. Furthermore, Beridge (100) has pointed out the importance of Ca^{2+} ions and proposed that these gave the primary "signal" for cell proliferation and division, cyclic GMP levels rising as a result of Ca^{2+} ion interactions with guanylate cyclase. If cyclic AMP levels were high enough, however, differentiation of the cell would be favoured. Cyclic AMP promotes the expression of differentiated properties of many cells and processes, such as those discussed in Section 1.2, and addition of cyclic AMP to cells accentuates the properties that are typical of that cell type.

It has been a useful generalisation that cyclic AMP inhibits and cyclic GMP promotes cell growth and that cancer cells have low cyclic AMP and high cyclic GMP levels (101). This holds true for some but not all cancerous tissues, nor indeed for many cell systems studied derived from normal tissue (102). No single alteration in cyclic nucleotide metabolism has so far been identified to distinguish neoplastic from normal tissue. Although cyclic GMP has been shown to stimulate nucleic acid synthesis in lymphocytes (103) and seems to be the signal for proliferation in such systems, cyclic AMP has also been shown to induce DNA synthesis in cultured thymocytes (104). Various tumours have also been found to have elevated cyclic AMP levels (105) and changes toward the increased ability to accumulate cyclic AMP during carcinogenesis have been observed (106).

With such conflicting evidence available it would be difficult to conclude that cyclic nucleotides play no part in the regulation of cellular proliferation and/or carcinogenesis. It is most probable, however, that different cell types respond to cyclic nucleotides in fundamentally different ways (102) and thus explain the difficulty encountered in formulating a generalisation of cyclic nucleotide action. It has also been suggested that there may be greater similarities than seems evident

and that apparent discrepancies in observations may be accounted for, at least in part, by differences in experimental design (102).

(ii) Guanylate Cyclase

Guanylate cyclase activity has been shown to differ in foetal, neonatal and adult rat heart (107). Increased activity was observed (approximately 2.5-fold) in foetal and neonatal tissue, suggesting a possible role for cyclic GMP in these highly active, proliferative tissues. At the same time corresponding to the decline of DNA synthesis in these cells, the cyclase activity was also found to "return" to "normal" adult levels of activity (107). This would correlate well with a DNA synthesis-inducing effect for cyclic GMP.

Kimura and Murad have found that particulate guanylate cyclase activity was increased and soluble decreased in both foetal and regenerating rat liver and that this phenomenon was also expressed by the hepatoma 3924A (108). Soluble activity in the cancer tissue was found to be decreased by 2-fold and particulate increased by 9-fold. Earlier studies on Morris hepatomas showed a marked decrease in cyclase activity, its presence being undetectable in some cell lines (109). A similar decrease has been reported in renal cortical tumour tissue (110). In contrast to these reports, guanylate cyclase activity has been observed to be increased in ethionine-induced hepatomas (111), in both acute (childhood), and chronic (adult), lymphocytic leukaemia lymphocytes (112) and in 10-methyl-choranthrene-induced rat prostate sarcoma (113). A consistent finding, however, is that the enzyme is located to a greater extent in the particulate fraction and has a reduced responsiveness to activators, particularly azide and hydroxylamine. It is not certain whether this reflects a property of the cyclase or the protein activator required for

such stimulation (Section 1.8(vi)). It is interesting to note that many carcinogens have been shown to generate free radicals (114) and are involved in binding of the carcinogen to nucleic acid bases. The effects of certain free radicals and the redox state of the cell on guanylate cyclase activity is well known (Section 1.8). Many carcinogens have been reported to activate soluble guanylate cyclase activity in a variety of tissues including phorbol myristate acetate (a lymphocyte mutagen) in lymphocytes (115) hydrazine (a carcinogen in tobacco smoke) in a variety of tissues including lung (116) butadiene diepoxide (117) streptozotocin (an antibiotic and nitrosourea derivative) (118) and a wide range of others including the nitroso compounds in many different tissue types (73) (119) (120).

Further interest has arisen from the report of Vesely and Levey (121) that the guanylate cyclase stimulated activity induced by N'-nitro-N-nitroso-N-propylguanidine can be inhibited by a variety of anticancer agents. These include antimetabolites (methotrexate and 6-mercaptopurine), antitumour antibiotics (adriamycin and actinomycin D) and alkylating agents (cyclophosphamide, uracil mustard, isophosphamide, chlornaphazine and 1-propanol-3,3'-iminodimethane sulphonate). All act in an "irregular" dose-response manner (see Fig. 8.2F). It is possible that chemical carcinogenesis in some instances is mediated by activation of guanylate cyclase. The resultant rise in cyclic GMP levels, stimulating DNA synthesis and cellular proliferation. It is also conceivable that some anticancer agents may act by preventing or reversing this. Some anticancer drugs are known carcinogens themselves and Vesely and Levey found that five of the forementioned drugs (6-mercaptopurine, chlornaphazine, 1-propanol-3,3'-iminomethane sulphonate, isophosphamide and uracil mustard)

could stimulate basal guanylate cyclase activity, but only by 1.6 to 3.0-fold and at very high concentrations (50 to 100 mM) (121).

(iii) Adenylate Cyclase

Adenylate cyclase studied in various tumour systems has been found to exhibit properties different to the enzyme found in normal tissues. Some reports suggest an elevated basal activity in hepatomas (109) while others have reported lower levels (122). Decreased adenylate cyclase activity has also been reported for chick embryo fibroblasts transformed by Rous sarcoma virus with a corresponding change in Km for ATP from 0.25 mM to 1.0 mM (123). This change in Km was not observed when a different strain of virus was used, implying a different mechanism for altering cyclase activity (123). Adenylate cyclase with decreased activity has also been found in lymphocytes of patients with either acute or chronic lymphocytic leukaemia (112) and in rat prostate sarcoma (113) whereas adenylate cyclase activity in thyroid carcinomas is no different to normal tissue (124) and cyclase activity above normal has been found in renal cortical tumours (125). A finding similar to that for guanylate cyclase in transformed cell lines, is that adenylate cyclase in such tissues is less responsive to activating ligands, and in particular to hormones. Lowered glucagon stimulation was reported in hepatomas, but increased responsiveness to adrenaline in these tissues was also observed (102). Adenylate cyclase shows decreased responsiveness to parathormone in renal cortical tumour (125), and to isoproterenol, as well as prostaglandin PGE and PGF_{2a} in leukaemic lymphocytes (112). Klein *et al.* (126) have shown that the histamine stimulation of parathyroid adenoma adenylate cyclase can be abolished by cimetidine.

Adenylate cyclase activity of rat heart is increased in foetal life, an opposite effect to that found for guanylate cyclase, and there is evidence for the delayed development of the glucagon receptor (127). It seems a general phenomenon that adenylate cyclase hormone receptors are less effective in rapidly proliferating tissue. This could occur by (i) alteration in the binding sites or receptors for the hormones or (ii) alteration in the N peptide (Section 1.4 (iii)). Variants of the S49 murine lymphoma have been found with both alterations of receptor binding sites (21) and in N peptides (19) (20) (Section 1.4).

It is interesting to note that cholera toxin, which stimulates adenylate cyclase, when given in only one injection, has been demonstrated to produce almost complete inhibition of proliferation for up to 4 days in intra-peritoneal YAC lymphoma cells in mice without any noticeable noxious effects on the animals (128).

Manipulation of adenylate cyclase may therefore be another method or "target" for chemotherapy. Tisdale and Phillips (129) have shown a reduced response to fluoride ions by adenylate cyclase from Walker carcinoma cells (approximately 1.2 to 1.5-fold increase). Neither of the alkylating agents chlorambucil (bifunctional) nor 5-aziridinyl-2,4-dinitro-benzamide (CB1954, monofunctional) had any effect on either basal or fluroide-stimulated enzyme activity although the carcinoma is responsive to the cytostatic actions of these compounds. A CB1954-protective agent had no effect alone but in combination with CB1954 stimulated basal activity to the level expressed by fluoride ions. Fluoride ions had no effect on this stimulated activity (129).

(iv) Protein Kinase

An increase in the total activity of cyclic AMP-dependent protein kinase (A-PK) has been reported in thyroid adenoma (124). Rapidly growing hepatomas with high cyclic GMP levels were found to have increased cyclic GMP binding activity (109). In liver neoplasia there appears to be a shift towards a less responsive protein kinase system (130) and the lack of steroidal response in adrenal cortical carcinoma was thought to be due to a "defect" in the protein kinase system in this tissue (131). In various hepatoma cell lines, a decreased capacity to bind cyclic AMP was found, and in HTC hepatoma cell line, this was found to be due to the presence of C component of A-PK in the cytoplasm (109) (Section 1.3). Although A-PK in the prostate sarcoma was reported to have a slightly higher affinity for cyclic AMP (Km cyclic AMP; prostate = 0.11 μM, tumour = 0.08 μM) the same responsiveness to cyclic AMP was found (113). Cyclic GMP-dependent protein kinase (G-PK) however, was much more sensitive to cyclic GMP in tumour tissue than in normal prostate (Km cyclic GMP; prostate = 4.85 μM, tumour = 0.88 μM) (113).

Studies like these and the observation by Fossberg et al. (132), that there was a raised type I/type II ratio of A-PK isoenzymes in renal cortical tumours, led Russel to propose that type I A-PK was responsible for stimulatory effects on cell growth (5). ChoChung (133) has more recently proposed protein kinase to be involved as a key step for signaling tumour regression. He suggests the existence, from various lines of evidence, of a cyclic AMP ternary complex of A-PK type II isoenzyme, phosphorylated at a serine residue. This complex, he proposes, is capable of translocating to the nucleus of the cell where the R subunit would bind to an acceptor site, releasing active catalytic components. These would be

capable of interacting with chromatin proteins and trigger tumour cell regression. It is reasoned that if it is this ternary complex and its translocation which is the property of differentiated cells which stop proliferating, then hormone-dependent tumours that retain the properties of differentiated cells should undergo regression with the proper cyclic AMP stimulation (133).

Tisdale and Phillips have demonstrated that the bifunctional alkylating agent, chlorambucil, activates A-PK in Walker carcinoma cells sensitive to the effects of this compound (WS cells) (134). CB1954 and merophan also activated A-PK in WS cells but N-ethyl-chlorambucil had no effect. The degree of activation by 5 μg/ml, chlorambucil was equivalent to 100 μg/ml, dibutyryl cyclic AMP. Chlorambucil was also found to increase A-PK activity in HeLa and chinese hamster cells (135) and this correlated well with the rise of cyclic AMP levels in these cells.

Cyclic AMP binding in Walker cells was observed to decrease as resistance to CB1954 or chlorambucil was increased (in special resistance-developed cell lines) and this was found for both low and high Km binding sites (136).

Of the cyclophosphamide metabolites tested, only 4-hydroperoxy-cyclophosphamide (HP) inhibited cyclic AMP binding in Walker cells to any extent (137) although similar effects were observed for the 4-hydroxy-4-methyl-cyclophosphamide derivative. Since 4-ketocyclophosphamide (KP) was ineffective it was apparent that the presence of an oxygen atom alone in the C4 position was not sufficient to elicit a response. Cyclophosphamide (CP) and cytoxyl alcohol (Section 1.14) were also ineffective in inhibiting binding activity. HP produced competitive binding (Ki = 0.19 mM) corresponding to an interaction with the low affinity (high Km) site.

Furthermore it was found that HP activated the protein kinase in Walker cells with a linear relationship for binding and activation, suggesting that HP bound to the kinase cyclic AMP binding site, causing dissociation of the holoenzyme (137). CP, KP, chlorambucil and phosphoramide mustard had no effect on kinase activity.

(v) Phosphodiesterase

Cyclic AMP phosphodiesterase (A-PDE) and cyclic GMP phosphodiesterase (G-PDE) have been found with a 5 to 10-fold greater activity in leukaemic lymphocytes compared with lymphocytes, spleen, thymus or lymph node PDE from normal mice (139). This increased activity was not due to the action of a calcium-dependent activator protein, although present, and was accompanied by an increase in the Vmax of the enzyme. The PDE's isolated from leukaemic lymphocytes were similar to those isolated from normal cells with respect to pH optima, stability to freezing and thawing and their sensitivity to the inhibitors chlorpronazine, papaverine and isobutylxanthine but were found to be more heat-resistant (139).

The specific activities of adenylate cyclase and A-PDE have been found to increase as the density of cultured cells (chick embryo fibroblasts) is increased (32). In contact-inhibited cells intracellular cyclic AMP levels are increased corresponding to an increase in the ratio of adenylate cyclase : A-PDE activities at higher cell densities. The growth-promoting effect of trypsin may be due to its protyolytic action on membrane-bound A-PDE (32).

The low Km form of A-PDE has been observed to have a high activity in leukaemic (106) hepatoma (109) and human breast cancer (138) cells. The presence of at least two forms of A-PDE, with high and low affinities for cyclic AMP, has been demonstrated in several experimental animal

tumours including Walker 256 rat mammary carcinoma, ADJ/PC6 plasma cytoma, sarcoma 180, NK and TLX5 lymphomas (140). The apparent Vmax of the low Km form A-PDE in Walker 256 and ADJ/PC6 cells, naturally sensitive to alkylating agents, contributed a higher percentage to the total measured Vmax than in any of the other, naturally resistant, cell lines.

The alkylating agents chlorambucil and CB1954 produce an increase in cyclic AMP levels in sensitive Walker 256 cells (141). This effect has been found to be produced by an inhibition of A-PDE activity which can also be produced by the alkylating agent merophan (142). A comparable increase in cyclic AMP levels was found with the PDE inhibitor aminophylline at a concentration which produced the same degree of growth inhibition (141). The N-ethyl derivative of chlorambucil had no effect. In chlorambucil-resistant (WR) cells of the Walker 256 carcinoma, increasing resistance was found to be accompanied by a decreasing activity of low Km form A-PDE (140) not due to the presence of an endogenous inhibitor. The apparent Vmax of this form of the enzyme has been shown to decrease in increasingly resistant WR cells. Furthermore, a change from high molecular weight to low molecular weight forms was demonstrated as Walker cells achieved resistance to chlorambucil, with no change in the low affinity form of the enzyme (34).

PART 2 CYCLOPHOSPHAMIDE

1.12 Origins

Cyclophosphamide (2-[bis(2-chlorethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide, **CP**) is one of the most effective and widely used drugs in clinical cancer chemotherapy and immunosuppression (143). It is effective against more different types of human neoplastic disease than any other chemotherapeutic compound (144). It is an alkylating agent and kills dividing and non-dividing cells by covalently binding to essential cellular components.

The development of "mustard gases" for use as chemical warfare agents led to the production of nitrogen mustard compounds during World War II. The observation that lymphoid tissues and rapidly dividing cells were particularly susceptible to the toxic effects of such compounds suggested that lymphoid tissues would be a good "target" for chemotherapy using nitrogen mustards. The first synthetic compound found to possess anti-tumour activity in humans was methylbis (β-chloroethyl)amine, (nitrogen mustard, HN2) which was used to treat Hodgkin's disease and caused limited regressions in certain lymphosarcomas (Fig. 1.8).

This success led to the production of many congeners of bis-β-chloroethylamines but none proved to be more effective than HN2. The observation by Gomori *et al.* in 1948 that the activity of the enzyme phosphamidase was localised in certain tumours led Friedman and Seligman to develop phosphoramide mustards (Fig. 1.8) as latent derivatives of nitrogen mustards. It was postulated that phosphamidase would cleave the P-N bond of these phosphoramide mustards thus releasing the cytotoxic nitrogen mustard moiety within the cell.

CP was synthesised by Arnold and Bourseaux as a "transport form" of nor-HN2 (Fig. 1.8) using the same rationale. CP does indeed require metabolic activation but this is not brought about by a phosphamidase-mediated release of nor-HN2.

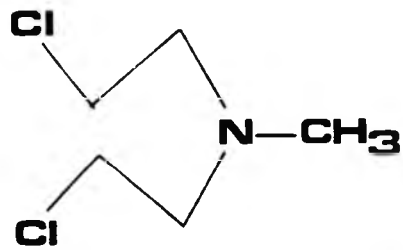
1.13 Metabolism

The metabolic pathways for CP activation, deactivation and degradation are shown in Fig. 1.9. The pathway of activation involves hydroxylation of CP to produce 4-hydroxy-CP (OHCP) which tautomerises to aldophosphamide (AP) with which it is in equilibrium. Spontaneous elimination of acrolein from AP produces the active species, phosphoramide mustard (PM). The major deactivating pathway *in vivo* is the production of 4-keto-CP (KP) and carboxyphosphamide (CarbP) both of which appear as urinary metabolites.

1.14 Microsomal Hydroxylation

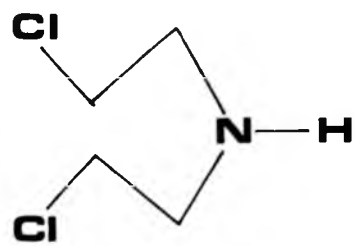
(i) Brock and Hohorst (145) demonstrated that the liver was the primary site of activation of CP, although the production of alkylating metabolites was detected when lung and kidney tissue was used instead of liver. Activation by the liver was found to require NADPH and oxygen and Cohen and Jao (146) confirmed the idea that CP activation was mediated by the mixed function oxidase system. The mixed function oxidase system is well known for its involvement in the metabolism of steroids, prostaglandins and drugs including carcinogens. One of the major purposes of the system appears to be the detoxification of xenobiotics (147) by converting the compound to a more polar derivative, usually by hydroxylation, rendering it more water-soluble and thereby more susceptible to

Fig. 1.8 The Structure of Nitrogen Mustard Compounds



NITROGEN MUSTARD

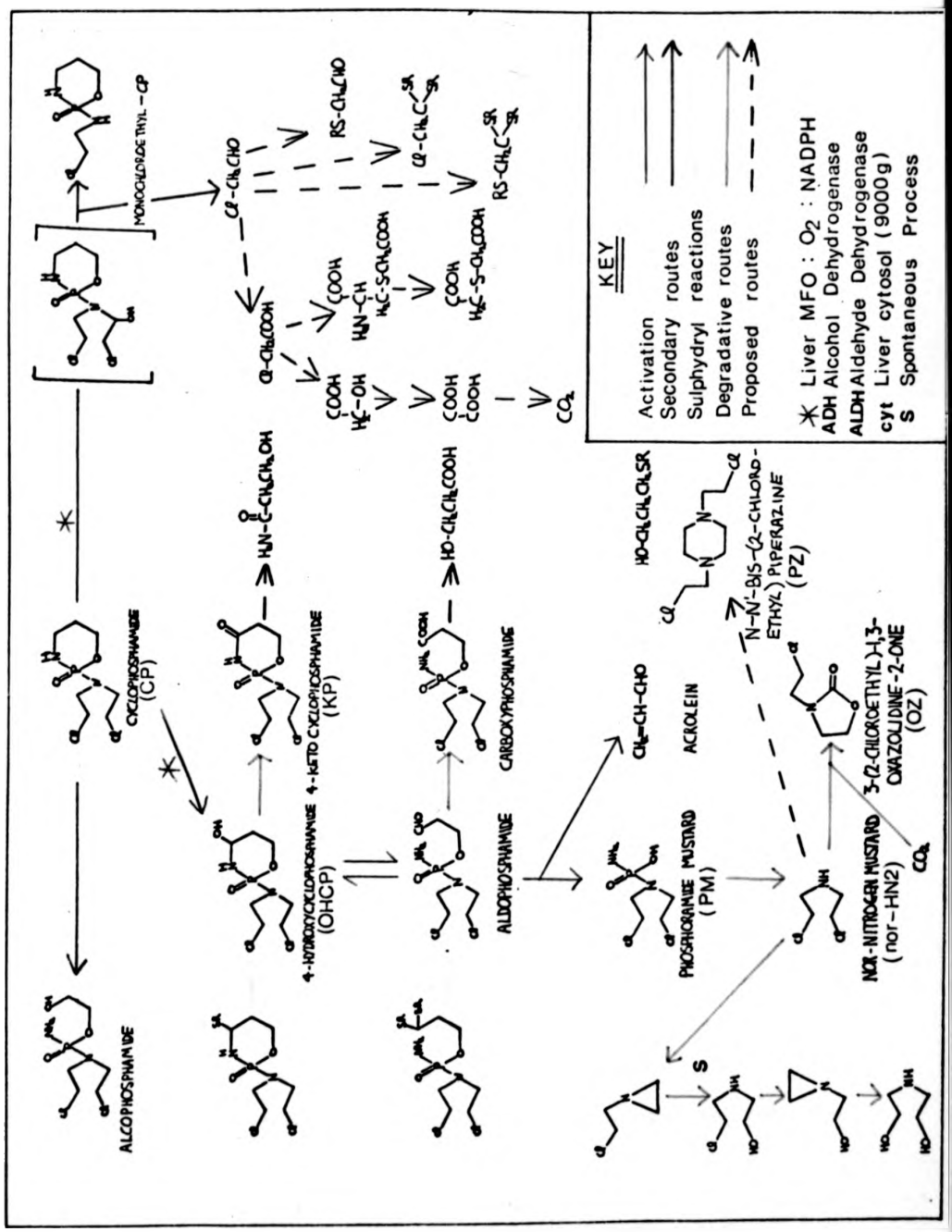
(HN2)



NOR-NITROGEN MUSTARD

(nor-HN2)

Fig. 1.9 The Metabolism of Cyclophosphamide



excretion via the renal tubules. In some cases, however, these oxidative processes may have the opposite effect and produce a reactive (or pharmacologically active) species from an inactive compound. This is true for many drugs including parathion, tremorine, prontosil and CP which express their action only through the production of pharmacologically active metabolites.

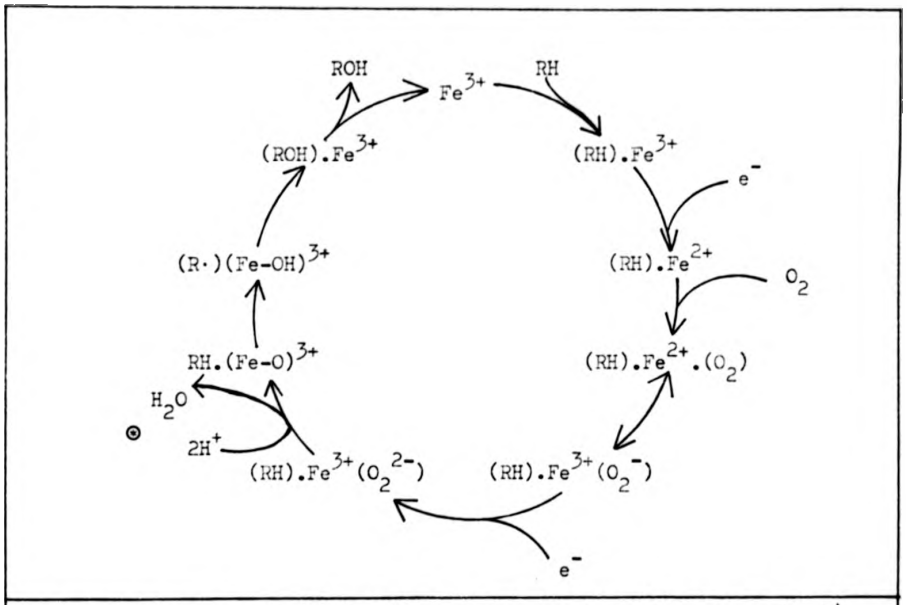
Cytochrome P-450, which is tightly bound to microsomal membranes, plays an important part in the oxidative metabolism of a wide variety of xenobiotics by acting as the oxygen-activating enzyme as well as the site of binding. Its involvement in CP metabolism has been demonstrated by the production of a type I spectrum for cytochrome P-450 in the presence of CP providing evidence for CP binding to the cytochrome (148). The mixed function oxidase reactions obey the equation shown in reaction 6, where

$$RH + O_2 + NAD(P)H + H^+ \longrightarrow ROH + H_2O + NAD(P)^+ \quad (6)$$

RH is the substrate for the hydroxylation.

The mechanism for the action of cytochrome P-450 is probably similar to that described by White and Coon (149) Fig. 1.10. In hepatic microsomes a flavoprotein (NADPH-Cyt.P-450 reductase) containing both FMN and FAD catalyses electron transfer from the electron-donating, reduced pyridine nucleotide (usually NADPH) to the cytochrome. This process involves binding of the substrate to cytochrome P-450 in the resting ferric state, followed by uptake of an electron from the associated flavoprotein and oxygen binding forming an oxyferro complex. Uptake of a second electron leads to the elimination of water leaving the oxylide radical bound to iron. The

Fig. 1.10 Proposed Mechanism of Action For Cyt. P-450 in Substrate Hydroxylation



Reproduced from ref. (149). The precise point in the scheme for H^+ uptake, ⊙, is not known.

dissociation of the hydroxylated product and the restoration of ferric P-450 is the final step and perhaps the least understood part of the mechanism in the reaction sequence (149).

It is not, therefore, surprising that CP activation is carried out predominantly in the microsomal fraction of rat (146) and mouse (150) liver and requires NADPH for full expression of activity, although NADH can be utilised (146), but not to any great extent. The extent of activation by other tissues corresponds to their microsomal content (143), and the optimum pH has been reported as 7.3 (148). Kinetic data for the metabolism of CP by rat and mouse liver varies widely. For example, for CP the Km varies from 0.48 to 7.0 mM and Vmax varies from 0.7 to 65.7 µmoles/g liver/Hr (144).

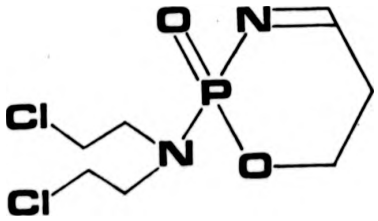
Activation is inhibited by carbon monoxide which binds to cytochrome P-450 and by other compounds which interrupt microsomal electron transport such as p-hydroxymercuribenzoate and cytochrome c (146) (148) (150). Many other compounds have been reported to affect metabolic activation of CP (144) of these SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate) is the most potent competitive inhibitor (Ki = 4.1 µM). H xobarbital (146), ethyl morphine (148) (competitively), and nicotinamide (148) (noncom petitively), inhibit CP transformation as measured by a retardation in the appearance of alkylating activity during CP incubations with microsomal fractions. Steroids (146), atropine and adrenaline (150), also inhibit while chloramphenicol has been shown to retard CP transformation in humans (151). Reduced <u>in vitro</u> activity has also been observed in microsomal preparations which had previously been stored in a frozen state (148).

Activation can be stimulated by pretreatment of the experimental animals with phenobarbital which induces the production of microsomal

enzymes. Furthermore, a sex difference has been reported, male rats metabolised CP at a much faster rate than did female rats (146). This probably correlates with the difference in microsomal content of the liver between the sexes.

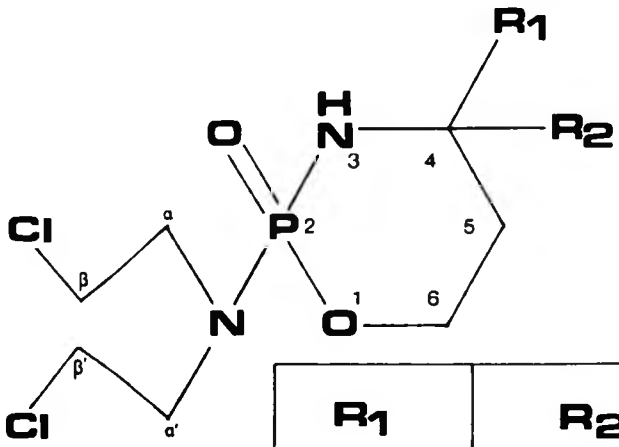
(ii) Activation

The importance of aldophosphamide (AP) as a key intermediate of CP metabolism was first suggested by Connors *et al*. (151). Sladek (152) had already implicated this compound as the primary metabolite of CP, having demonstrated that the activation of CP generated material which gave a positive test for aldehydes. Isolation of AP (Fig. 1.11) from an *in vitro* incubation mixture of CP with mouse liver microsomes and from the plasma of CP-treated patients (154) supports the metabolic scheme (Fig. 1.9) in which the first product of metabolism is 4-hydroxy-CP (OHCP) (Fig. 1.11). AP is formed spontaneously from this hydroxylated intermediate with which it is in equilibrium. Qualitative and quantitative analysis by thin layer chromatography has demonstrated the existence of OHCP and AP in both synthetic and biological sources (153). The presence of 4-ethoxy-CP in ethanol-deproteinised samples was also shown. Isolation and mass spectral identification of 4-ethoxy-CP from microsomal incubations of CP treated with ethanol supported the existence of OHCP and an imino intermediate, iminophosphamide (151) (143) which may

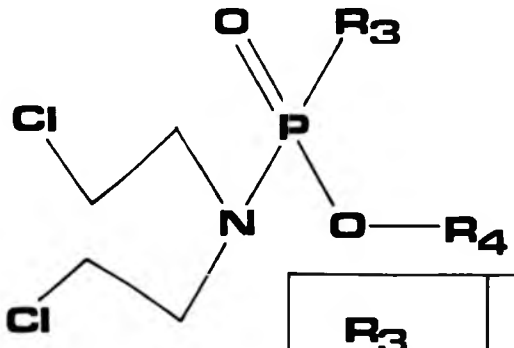


IMINOPHOSPHAMIDE

Fig. 1.11 Structures of Cyclophosphamide Derivatives



		R_1	R_2
CP	CYCLOPHOSPHAMIDE	H	H
KP	4-KETOCYCLOPHOSPHAMIDE	=O	
OHCP	4-HYDROXY-CP	OH	H
HP	4-HYDROPEROXY-CP	OOH	H



		R_3	R_4
PM	PHOSPHORAMIDE MUSTARD	NH_2	H
AP	ALDOPHOSPHAMIDE	NH_2	$(CH_2)_2CHO$
CarbP	CARBOXYPHOSPHAMIDE	NH_2	$(CH_2)_2COOH$
	CYTOXYLAMINE	OH	$(CH_2)_3NH_2$
	ALCOPHOSPHAMIDE	NH_2	$(CH_2)_3OH$
	CYTOXYL ALCOHOL	$NH(CH_2)_3OH$	H

be formed by the elimination of water from OHCP, and is thought to be in equilibrium with OHCP (143). The N=C double bond would be very polar and addition of an alcohol or thiol across the bond may, therefore, be expected to occur readily. Evidence for the existence of iminophosphamide has been obtained by treatment of synthetic OHCP with sodium borodeuteride to produce CP which is monodeuterated at C4 (143).

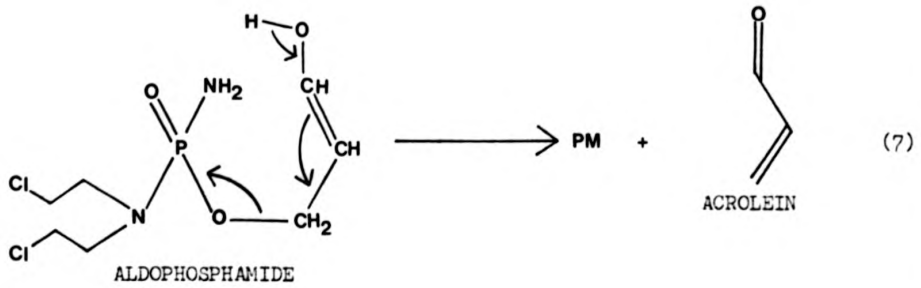
Sladek (152) identified AP by reaction with semicarbazide to produce the corresponding semicarbazone. Struck et al. (158) treated synthetic OHCP with semicarbazide in aqueous solution and subsequently isolated and identified the semicarbazone of AP, identical to the product of Sladek implying the conversion of OHCP to AP. Further evidence for this is that acrolein which is eliminated from AP, can be found as a spontaneous decomposition product of synthetic OHCP (154). AP has also been identified in the plasma of patients receiving CP chemotherapy and in microsomal incubations of CP after reaction with cyanide to yield the cyanohydrin derivative (155) which can also be produced by addition of cyanide to solutions of OHCP (155). Conversion of AP to OHCP is also possible, evidence of which has been provided by the extensive oxidation of alcophosphamide (Fig. 1.11) the product of which is 4-keto-CP (KP). Initial oxidation produces AP which tautomerises to OHCP which is then further oxidised to KP (156). Voelcker et al. (157) have reported the equilibrium of the OHCP $\rightleftharpoons$ AP interconversion reaction to be in favour of OHCP (OHCP : AP = 1.69).

By use of a 4-deuterated analogue (CP-4-d_2) Connors et al. (159) were able to differentiate between initial hydroxylation at either the C4 or C6 position. As well as confirming initial C4 hydroxylation, Connors et al. showed that there was a virtual absence of any isotope effect between the

rate of hydroxylation of CP and CP-4-d_2. This indicated that cleavage of the C-4-D bond is not the rate-determining step in CP 4-hydroxylation.

(iii) β-elimination

Colvin et al. (160) were the first to identify phosphoramide mustard (PM) as a biologically active metabolite of CP by chromatography and mass spectrometry of incubation mixtures of CP with mouse liver microsomes. Connors et al. (151) suggested its mode of formation by spontaneous β-elimination of acrolein from AP (reaction 7).



Struck et al. (161) isolated and identified PM as a blood metabolite in mice treated with CP and PM has also been detected in plasma and urine of patients receiving CP chemotherapy (162).

The production of acrolein during the metabolism of CP has been demonstrated by Alarcon and Meienhofer and its biological effects have been much studied (143). Furthermore, the liberation of acrolein from "activated" derivatives of CP and isophosphamide (an isomer of CP), has recently been utilised in a fluorimetric assay for such compounds by condensation of acrolein with m-aminophenol (163).

(iv) Side Chain Hydroxylation

The action of microsomal mixed function osidase on CP also produces CP hydroxylated at the α-carbon of the 2-chloroethyl side chain (Fig. 1.9) (161). This compound spontaneously decomposes to yield N-dechloroethyl-CP (monochloroethyl-CP) and chloroacetaldehyde, both of which have been detected as blood metabolites of CP (161). Monochloroethyl-CP has also been isolated from microsomal incubation mixtures of CP (151). Although this product is apparently inactive as a cytostatic agent, chloroacetaldehyde may play an important part in the immunosuppresive actions of CP (143) (see Section 1.15(ii)).

1.15 Deactivation Reactions

(i) Urinary Metabolites

Carboxyphosphamide (CarbP) and KP (Fig. 1.11) are detoxification products derived from oxidation of AP and OHCP respectively (152) (Fig. 1.9). These two compounds are the major urinary metabolites of CP KP accounting for approximately 15% and CarbP about 50% of administered CP (143) (157).

CarbP was found to be produced from either OHCP or AP by incubation with the cytosolic fraction of mouse liver cells, or with purified aldehyde oxidase (150). Conversion of OHCP to CarbP is presumably achieved via AP since this is the most likely precursor and substrate for the enzyme.

KP was isolated and identified from the urine of CP-treated dogs by Hill *et al*. (164) and the identification confirmed by chemical synthesis of KP. It has since been reported in the urine of CP-treated rabbits, sheep and man (144). Since KP can be produced by the dehydrogenation of OHCP, the cytosolic enzyme alcohol dehydrogenase has been implicated for this conversion (152).

The spontaneous production of nor-HN2 (Fig. 1.8, 1.9) from PM has been reported after administration of CP (145) to rats. It has been detected both in plasma and as a urinary metabolite (174) in humans.

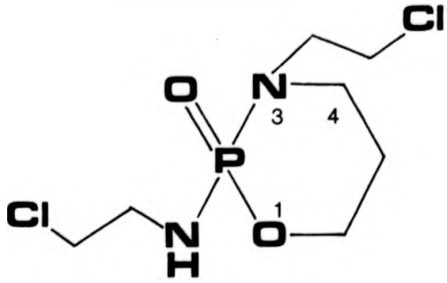
Nor-HN2 is capable of cyclisation to N-2-chloroethyl aziridine under refluxing for 48 hours (165). Even so, the presence of the latter compound *in vivo* has been reported after CP administration (145). Other detoxification products have been isolated which arise from this pathway. For example hydroxylation of N-2-chloroethylaziridine followed by cyclisation of the remaining 2-chloroethyl group to the aziridine structure again, and further hydroxylation yields the products shown in Fig. 1.9 culminating in production of bis(2-hydroxyethyl)amine (166).

Nor-HN2 is also capable of dimerisation to give N-N'-bis(2-chloroethyl) piperazine (PZ) or reaction with CO_2 to yield 3-(2-chloroethyl)-1, 3-oxazolidine (OZ) both of which have been found as urinary metabolites (167) (168) (Fig. 1.9). Although the dimerisation of nor-HN2 to form PZ is not certain *in vivo* (167), OZ may be produced from nor-HN2 and carbon dioxide in blood. In aqueous solution in the presence of bicarbonate ion at neutral pH, OZ is spontaneously produced from nor-HN2 via a carbonate ion intermediate (168). Both PZ and OZ are undoubtedly inactive products (168).

(ii) Enzymic Oxidations

Alcophoshpamide (Fig. 1.11) has been found as a product of CP metabolism, Connors *et al.* (151) observing its formation from CP by a fraction of liver cells, possibly by the action of phosphorodiamide esterase. It has also been observed as a blood metabolite in mice which had been treated with CP (161). It has also been proposed that alcophosphamide may arise from AP by reductase activity (161).

As has already been stated, chloroacetaldehyde is produced by side-chain oxidation of CP. Isophosphamide (IP), an isomer of CP, undergoes side-chain hydroxylations and much work has been done investigating the products of this reaction.



ISOPHOSPHAMIDE

In the case of IP, side chain hydroxylation is not such a minor metabolic pathway as found in CP metabolism (166). Slower ring hydroxylation is probably due to stereochemical hindrance of the C4 position by the chloroethyl side chain of the N3 atom. Chloroacetaldehyde may be converted rapidly *in vivo* to chloroacetic acid and metabolites of this compound have been isolated from the urine of patients treated with IP, namely S-carboxy-methylcysteine and thiodiacetic acid (Fig. 1.9) (166). There is evidence which suggests that these compounds are also present in CP biotransformation. Other metabolites of chloroacetic acid may enter the general metabolic scheme of the cell, leading to production of carbon dioxide (Fig. 1.9).

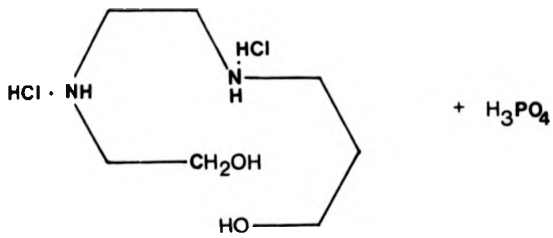
(iii) Sulphydryl Reactions

The reaction of OHCP with thiols (Section 1.18(iii)) may be considered a deactivation reaction although it is reversible and may therefore serve only to "delay" activation. Various other thiol reactions are shown in Fig. 1.9. AP and acrolein have been shown to be capable of binding to thiol groups but the involvement of these reactions in the actions of CP is unknown.

(iv) Hydrolytic Products

In isotonic saline at room temperature, CP is quite stable (165). In unbuffered solution, CP slowly hydrolyses to nor-HN2 and phosphoric acid ester of N-propanolamine and inorganic phosphate. Under conditions which lead to acid-catalysed hydrolysis, CP is converted to cytoxylamine (Fig. 1.11). This compound has been found in the serum of rats and the urine of humans administered CP, and apparently has anti-tumour activity *in vitro*. Decomposition of CP by cleavage of the exocyclic P-N bond has also been shown, over a long period of time (2 weeks).

If CP is refluxed for several hours, internal alkylation of the endocyclic N atom can occur. This leads to the formation of various intermediates (143) (165) of which the most abundant is a dioldiamine:-



This pathway is also believed to occur to some extent on acid hydrolysis and is thought to account for some loss of activity in the NBP test (Chapter 3). The dioldiamine product has also been observed in high yields when cytoxyl alcohol (Fig. 1.11) was hydrolysed in water (143).

Cytoxyl alcohol has been synthesised and could theoretically be a metabolite of CP after endocyclic P-O bond cleavage. It has not, however, been observed, possibly due to its rapid hydrolysis in biological fluids. Unlike CP, cytoxyl alcohol is active against L1210 cells in mice.

1.16 Chemistry

The biological activity of CP and its metabolites is due in part to their chemical reactivity. Alkylating agents are electrophilic reactants which combine with electron rich or nucleophilic centres. They have the ability, therefore, to substitute alkyl groups for the hydrogen atoms of certain organic compounds. The overall, unimolecular reaction which these agents exhibit (reaction 8) can be resolved into two processes. The

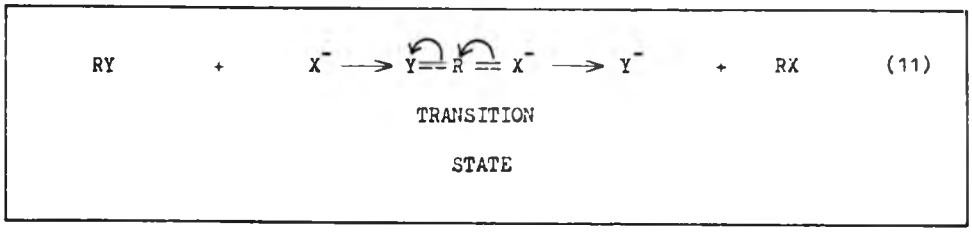
$$\underset{\text{NUCLEOPHILE}}{X^-} + \underset{\text{ALKYLATING AGENT}}{RY} \longrightarrow RX + Y^- \qquad (8)$$

first, rate-determining step is a slow, reversible, heterolytic bond fission or ionisation of the alkylating agent (RY) to yield a carbonium ion (R^+) (reaction 9). This is followed by a much faster bond formation between carbonium anion and nucleophile, completing the first-order

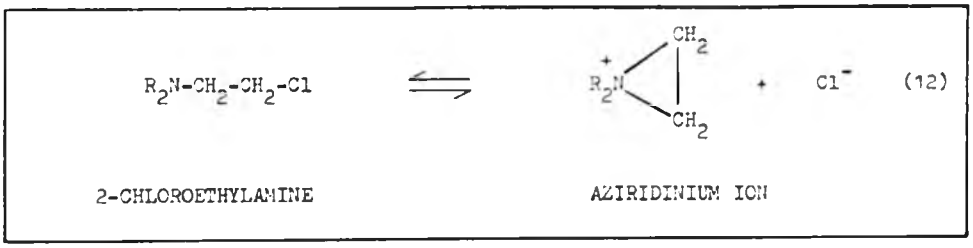
$$RY \xrightarrow[\text{SLOW}]{} R^+ + Y^- \qquad (9)$$

$$R^+ + X^- \xrightarrow[\text{FAST}]{} RX \qquad (10)$$

nucleophilic substitution (S_N1) reaction (reaction 10). Some alkylating agents such as primary and secondary alkyl halides (e.g. methyl iodide, isopropyliodide) undergo second-order nucleophilic substitution (S_N2) involving the formation of a transition state (reaction 11).



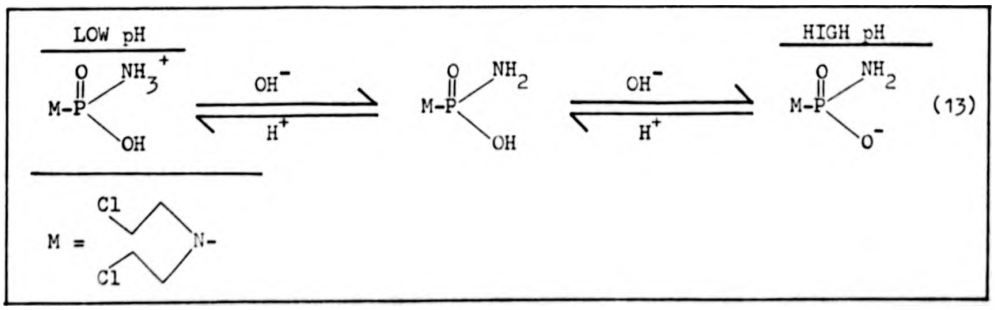
2-chloroethylamines such as the nitrogen mustard derivatives tend to undergo an S_N1 type of reaction producing a cyclic aziridinium (ethyleneimmonium) ion in the step equivalent to reaction 9 (reaction 12).



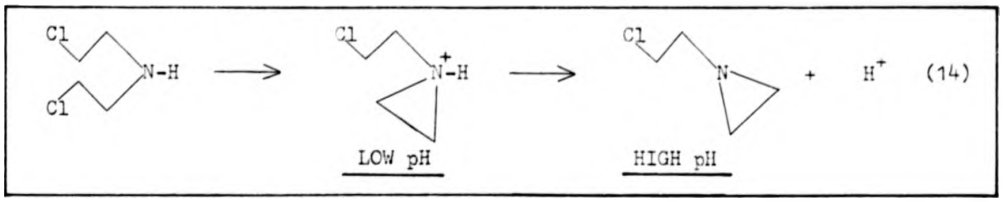
Phosphoramide mustards such as CP have been shown to undergo such reactions by selective deuterium labelling of the side chain (170). Using PM labelled only at the β-carbons (Fig. 1.11) Colvin *et al.* found that upon reaction with ethanethiol, the product contained deuterium atoms distributed uniformly in the α and β positions. This result is explained best in terms of aziridinium ion formation, where α and β carbon atoms of the side chain are equivalent. Reaction of this symmetrical ion with ethanethiol produced scrambling of the label due to the equivalence of the α and β carbons. Direct displacement of the chlorine atoms in the 2-chloroethyl side chain by S_N2 reaction (reaction 11) would not have produced such scrambling.

Cyclisation of the reactive aziridinium ion (reaction 12) is affected by the basicity of the nitrogen atom, and is therefore influenced by the substituents of the nitrogen mustard derivative. Strongly electronegative substituents have the effect of reducing the rate of cyclisation, and this is generally true of fully substituted phosphoramide mustards (esterdiamidates, triamidates, diesteramidates) the phosphoryl group being inherently electronegative. In phosphoramidic acid mustards such as PM (Fig. 1.11) the hydroxyl group is ionised at physiological pH and its electron-repelling effect actually enhances the basicity of the nitrogen atom. This increases both the rate of cyclisation to the reactive intermediate and (therefore) the alkylating activity (143). CP, however, shows no alkylating activity in the NBP test, whereas other esterdiamidates show at least some activity.

Since ionisation is important for reactivity, it is not surprising that the pH of the medium may influence alkylation. Alkylation would be much reduced at a pH below the isoelectric point since ionisation would be repressed, decreasing the negative charge on the molecule. Alkylation would be enhanced at a pH above the isoelectric point since ionisation would be favoured (reaction 13).



PM, HN2 and nor-HN2 are strong alkylating agents at low pH but only PM and HN2 retain some reactivity at physiological pH (7.4). This is due to the fact that nor-HN2 is active when protonated at low pH but is converted to ethylenimine at higher pH (reaction 14).



The ethylene imines derived from PM and HN2 cannot be neutralised by the loss of a protein and so retain their charge at physiological pH. OHCP has very little alkylating activity at pH6.0 and no measureable activity at physiological pH (Table 1.6) as compared to PM. OHCP, like HN2, is a more potent alkylating agent under conditions of low pH.

Another method of assessing the chemical reactivity of CP and derivatives is by estimation of the rate of chloride ion liberation. Such liberation occurs during alkylation (reaction 8), but the interpretation of such measurements is very difficult. The NBP assay is a direct measure of alkylation of the NBP molecule and requires no further considerations. Chloride ion liberation, however, occurs during intra-molecular alkylation (reaction 12) which may theoretically lead to inter-molecular alkylation. It is also possible that intra-molecular alkylation may not lead to further alkylation but result in the formation of an ethylenimine as is the case with nor-HN2 (reaction 14). Intramolecular cyclisations such as that occurring during CP hydrolysis (section 1.15(iv)) will also lead to chloride ion release without alkylation of "target" substrates. Even so, both NBP and chloride ion liberation assays have been used to quantitate chemical reactivity with biological activity for both CP and its metabolites.

TABLE 1.6 Alkylating activity of CP metabolites using the NBP test (161)

pH	nor-HN2	PM	OHCP
4.82	0.663	0.791	-
6.0	0.351	0.270	0.014
6.88	0.052	0.243	-
7.4	0.016	0.148	0.000

1.17 Biological Activity

As has been already discussed, CP is an inactive transport form which gives rise to active derivatives *in vivo*, a process known as "drug latentiation" (144). This process has the effect of reducing the general toxicity of the drug while preserving its chemotherapeutic potency and thus introducing a margin of safety. Since CP undergoes a complex variety of biotransformations, it is important to establish which of the major metabolites is responsible for the biological effects of CP and which may be the transport forms. The active form(s) must spontaneously react with the biological substrate and would therefore be expected to have a high chemical activity as well as a high *in vitro* and *in vivo* cytotoxicity. The transport form would be expected to demonstrate low chemical reactivity and *in vitro* cytotoxicity but a high *in vivo* cytotoxicity. Oncostatic specificity, obviously a prerequisite for an anti-tumour agent, could be exhibited at any stage previously mentioned. CP has a relatively high oncostatic specificity. This has been evaluated quantitatively by determining the therapeutic index (TI) of the drug (171) which is the ratio of the LD_{50} (the concentration of drug required to kill 50% of the animals in the test; Lethal $Dose_{50}$) to the CD_{50} (the concentration required to produce tumour regressions in 50% of the animals treated; Curative $Dose_{50}$). For the potent cytotoxic agent nor-nitrogen mustard, the TI in Yoshida ascitic sarcoma of the rat is 2.5 whereas for CP it is 175 (Table 1.7). It can also be seen from Table 1.7 that CP has a low chemical reactivity compared to that of nor-HN2, in terms of both alkylating activity and chloride ion liberation. These compounds therefore represent the extremes of activity of CP metabolites which show cytotoxic activity. With the exception of KP which is completely inert in terms of chemical

TABLE 1.7 Chemical reactivity and biological activity of CP and its metabolites and derivatives (172)

COMPOUND	in vitro			in vivo		
	ALKYLATING ACTIVITY	CHLORIDE ION LIBERATION	CYTOTOXIC ACTIVITY	CURATIVE DOSE CD_{50}	LETHAL DOSE LD_{50}	THERAPEUTIC INDEX TI
	(a) (%)	(b) (1 val/mol. in :)	(c) (CU/µmol)	(d) (mg/kg)	(d) (mg/kg)	(LD_{50}/CD_{50})
CP	1.3	>7d	<0.03	1.25	220	175
KP	1.2	>7d	<0.07	> 800	> 800	-
OHCP	65	480m	63	1.25	150	120
HP	40	240m	21	1.25	97.5	78
CarbP	85	>7d	0.1	200	≃ 800	≃ 4
PM	90	60m	2.5	20	61	3.5
Acrolein	1.9	-	0.4	2.15	7.3	-
nor-HN2	100	60m	1.35	40	100	2.5
HN2	100	15m	138	0.25	1.1	4.4

TABLE 1.7 Continued

(a) Alkylating activity by NBP test, pH4.6, 70°C.

(b) Chloride ion liberation in bicarbonate buffered solution, pH7.5, 0.026M, 37.5°C. (d = days, m = minutes).

(c) Cytotoxic activity : Cytostatic Unit (CU) defined as that amount of active compound which produces survival in 50% of animals after their inoculation with tumour cells which have been previously treated with the drug and washed.

(d) CD_{50}, LD_{50} determined in rats bearing the Yoshida ascites tumour.

reactivity and biological activity and acrolein which lacks alkylating activity, CP metabolites show gradation of activity between these two limits. OHCP demonstrates some alkylating activity at low pH (Table 1.7) although it exhibits none at physiological pH (Table 1.6). It has a demonstrable cytotoxic activity *in vitro*, slowly liberates chloride ions and exhibits a high TI of 120. All other metabolites tested (172) had TI's below 4. Correspondingly, PM, CarbP and nor-HN2 have relatively high alkylating activity (Table 1.7). This is an interesting observation since OHCP is the primary hydroxylated metabolite and all compounds tested with low therapeutic indices are produced after this in the metabolic scheme (Fig. 1.9) and has been considered evidence that OHCP is responsible for the oncostatic specificity of CP (171).

The use of deuterated analogues of CP has shown that isotope effects of 2.2 and 1.8 obtained for the formation of KP (e.g. the 4-d_2 analogue) and CarbP (4,6-d_4 analogue) respectively caused no change in anti-tumour activity (173). This parameter was measured against advanced ADJ/PC6 tumour in female BALB/C mice. However, in the same study, the 5-d_2 analogue in which β-elimination of acrolein (section 1.4(iii)) was decreased by a factor of 5.3, showed a marked drop in anti-tumour potency, a 7 to 13-fold increase in CD_{50}. Furthermore, when the release of acrolein (or its analogous unsaturated aldehyde) was completely blocked by use of the 5,5-dimethyl-CP derivative, it produced a complete loss of biological activity (172). These data suggest that PM is the active, alkylating metabolite both *in vitro* and *in vivo*.

1.18 Specificity

(i) It is still uncertain how CP attains its anti-tumour specificity and various proposals have been suggested on the basis of several lines of evidence. It is, however, generally accepted now that PM is the CP metabolite responsible for alkylating target macromolecules within the cell (151) (160) (143) and some evidence for this has already been presented. Struck et al. (161) have reported that high levels of PM relative to those of OHCP could be isolated from the blood of mice after administration of [^{14}C] CP. This has also been observed in patients treated with CP (174). It has therefore been suggested that PM may be released from OHCP after its formation in the liver (161). A certain degree of selectivity may be afforded by the different reactivity of PM at different pH values. It is well known that some tumour cells have a reduced intra-cellular pH value which may be as low as 6.8 (175). From Table 1.6 it can be seen that PM demonstrates a 1.6-fold increase in activity at this pH value than at pH7.4, the pH to be found in normal cells. Such a small difference in alkylating activity is probably not responsible for the high oncostatic specificity of CP but could, nevertheless, be a significant factor. It can be seen from Table 1.7 that the therapeutic index for PM is very low (3.5) and this argues against the idea that it is the circulating level of PM in the blood which is responsible for selectivity. Furthermore, PM is less immunosuppressive than CP (176) and does not cause depilation (143), suggesting that these actions of CP are caused by some other metabolite(s).

Brock (172) has reported that the concentration of OHCP required to be cytotoxic to Yoshida sarcoma cells is 25-fold lower than the equivalent cytotoxic dose of PM (Table 1.7). This has also been reported for CP

activated *in vitro* by microsomal incubation and tested for cytotoxic activity against murine L1210 leukaemia cells; the concentration needed was 20-fold less than that of PM required for cytotoxicity (143). The concentration of 'activated' CP required is very similar to the cytotoxic dose of OHCP (154).

4-methyl-CP has been found to be more toxic but of a lower therapeutic index than CP (176). Since this compound is metabolised in a similar way to CP (177) and produces PM, its effect cannot be due simply to the release of this metabolite.

It has been suggested, therefore, that OHCP, AP or iminophosphamide acts as the transport form of PM, being capable of entering the cell where release of the active metabolite occurs.

(ii) Permeation

Permeation of metabolites into cells is an obviously important property since the active metabolite must be within the cell in order to exert its effects. Differences in the permeation rates of CP metabolites into normal or tumour cells could influence the specificity of CP action. It is well known that some molecules may enter cells via a carrier-dependent transport mechanism, HN2 transport has been shown to be mediated by a choline carrier (178). More recently, Byfield and Calabro-Jones (179) have demonstrated that many water-soluble alkylating agents exhibit carrier-dependent uptake (i.e. HN2) and that lipid-soluble alkylating agents are carrier-dependent. Unfortunately, these workers were unable to assess the mechanism of transport of PM due to its negligible effects upon the T-lymphocytes used in their assay. Draeger and Hohorst (180) have studied the permeation of CP metabolites into tumour cells, and their results show that nor-HN2 had the greatest uptake of the derivatives tested and reached

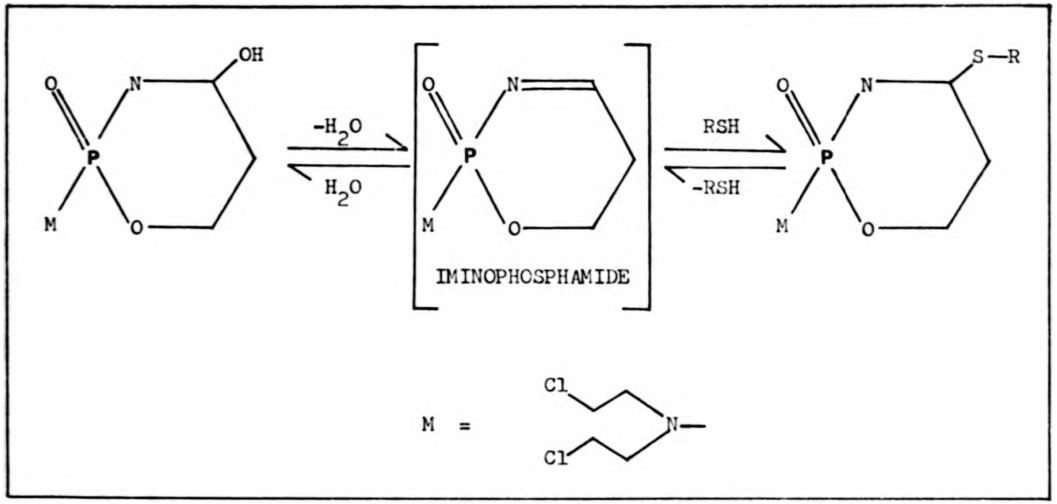
its saturation after 20 minutes incubation. In contrast, CarbP showed very rapid kinetics, reaching saturation within 20 seconds. However, very little uptake of CarbP was observed and these results were interpreted as adsorption of CarbP to the cell surface rather than uptake into the cell. CP and HP showed very nearly the same saturation kinetics and saturation value (≈ 5 nmol/mg protein) which was about half that observed for nor-HN2.

These workers have also shown (180) that while CP can be excluded rapidly from tumour cells, HP shows a certain degree of retention within the cells during sequential washings. They found that retention was greater at 37°C than at 0°C, for HP and that temperature had no effect on the efflux of CP from the cells. The stronger retention of HP was suggested to result from binding of this derivative to cellular components, although no loss in its alkylating activity was detected. Thus a reaction other than alkylation was responsible. It was suggested that retention of HP in the cells may have been due to a reaction between protein sulphydryl groups and OHCP, which is released spontaneously from HP (157).

(iii) Sulphydryl Binding

The reaction between OHCP and thiols is thought to occur via an iminophosphamide intermediate (143) (151). Various sulphydryl compounds have been found to react reversibly with OHCP (169). Protein binding studies have shown that OHCP can bind to the thiol groups of bovine serum albumin yielding a quite stable product (153) capable of being isolated by Sephadex column chromatography. The reaction is shown in Fig. 1.12, and results in an equilibrium between bound OHCP and free OHCP which is capable of spontaneous decomposition to yield PM, via AP, through tautomeric equilibrium. Thus, the release of active alkylating material (PM) is dependent upon the two equilibria of thiol binding of OHCP and its tautomerisation to AP. The concentration of AP is established by the OHCP/

Fig. 1.12 The Reaction of 4-hydroxy-CP (OHCP) With Thiol (RSH) Compounds



thiol equilibrium and therefore the reaction rate of release of PM is inversely proportional to the concentration of free thiol compound (169). This alteration in the rate of production of the alkylating metabolite by thiol compounds may enhance the stability of the metabolites in the plasma (171). It has also been suggested that such reactions could facilitate the entry of metabolites into cells, direct their movement to specific sites inside the cells and delay the release of alkylating phosphoramide mustard. The oncostatic specificity of CP would therefore be due to the particular reactions of OHCP (171). This is supported by the estimated cytotoxic specificity for OHCP *in vitro* (Table 1.8) and also by the very high TI exhibited by OHCP *in vivo* (Table 1.7).

(iv) Selective Detoxification

The detoxification of OHCP and AP by enzymic oxidation to KP and CarbP respectively, (section 1.15(i)), has been suggested by Sladek (181) and Connors *et al.* (151) to be responsible for the selectivity of CP. Cox *et al.* (182) have shown that the ability of tissue soluble-enzyme fractions to "detoxify" biochemically prepared AP, AP analogues and PM varied with the tissue used. The range of deactivating ability obtained was; liver $\gg$ kidney $\gg$ intestinal mucosa $>$ tumour $>$ spleen = bovine serum albumin solution. This finding supports the idea that normal tissues contain more of the enzymes necessary for deactivation than does tumour tissue. Only spleen contained less deactivating activity than the Walker ascites tumour cells used in the study. They have also correlated the deactivating ability of these tissues with NAD^+-dependent aldehyde dehydrogenase activity. Cox *et al.* (177) have also pointed out that although 4-methyl-CP is metabolised in a similar way to CP and produces PM, it cannot form metabolites analogous to KP and CarbP. 4-methyl-CP has a lower therapeutic

TABLE 1.8 Cytotoxic specificities for selected CP metabolites

COMPOUND	CYTOTOXIC SPECIFICITY (a) (in vitro)	THERAPEUTIC INDEX (b) (TI)
OHCP	160	120
PM	1.3	3.5
nor-HN2	4.1	2.5
HN2	12	4.4

(a) (cytotoxic activity/alkylating activity) x 10.
Alkylating activity in nor-HN2 equivalents/mol./min.

(b) See Table 1.7

index than CP, indicating that its metabolites are less selective in their toxicities toward normal and tumour tissues. This further lends weight to the hypothesis that active CP metabolites are not released into normal cells to the same degree found in tumour cells due to the high activity of deactivating enzymes in the former. The primary CP metabolites OHCP and AP are metabolised to non-alkylating derivatives more rapidly in normal tissue than in tumour tissue. As a result, tumour cells are subject to the full cytotoxic effect of PM, released from activated CP, whereas normal cells are only minimally affected.

Hohorst *et al*. (171) however, do not agree with this hypothesis. The enzymatic deactivation of primary CP metabolites, they argue, appears to contribute little to the specificity of CP *in vivo*. It is also unable to explain the specificity of OHCP *in vitro* (estimated by relating the *in vitro* cytotoxicity, Table 1.7, to alkylating activity) (Table 1.8). It is suggested that if *in vitro* cytotoxicity of these compounds is only related to their alkylating activity, then all values for cytotoxic specificity would be approximately equal, and they clearly are not. Furthermore Gurtoo *et al*. (183) have evaluated the role of aldehyde dehydrogenase (ADH) in two strains of mice with differing phenobarbital-inducibilities for ADH and CP-activating enzymes. They conclude that there is no correlation between CP activation and ADH activity and that ADH is not a significant determinant in the metabolism of CP, in the binding of CP to macromolecules or in the chemotherapeutic activity of CP.

1.19 Stereochemical Effects

Because CP contains a chiral phosphorus atom and therefore exists as a mixture of optical isomers (Fig. 1.13) it would not be unreasonable to suppose that there may be differences in the metabolism of the different isomers. 4-hydroxylation introduces another chiral centre to the molecule and differences in the activities of the four resulting isomers of OHCP may also be expected.

Struck *et al.* (176) found that for the 4-methyl derivative of CP, there was no difference in metabolism or activity between its *cis* and *trans* isomers. Cox *et al.* (184) have reported that (-) - CP derivatives were obtained from the urine of patients treated with racemic, (±) - CP. They found up to 83 to 91% of the (-) - isomer in some cases, which suggested stereo-selective metabolism of the R - (+) - isomer. Furthermore, when tested against plasma cytoma cells in mice, the S - (-) - isomer was found to be more effective. The reported TI's were; (-) - CP = 128.1, (+) - CP = 68.9, (±) - CP = 93.

The products of (+) or (-) - CP microsomal incubations were found to have equal toxicities against Walker tumour cells *in vitro*. Mass spectrometry of deuterated or non-deuterated enantiomers of CP metabolites after administration as pseudoracemates showed very little stereoselectivity of hydroxylation since the CP recovered in urine samples resembled that administered (185). The production of KP, however, was found to predominate from the (+) - isomer, whereas production of CarbP predominated from the (-) - isomer, but to a lesser extent.

An apparent species difference has also been reported (186). Stereo-selective metabolism was found in the rabbit but not in the mouse or rat, the S - (-) - isomer showing a 4-fold greater rate of metabolism in

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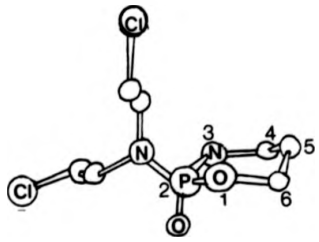
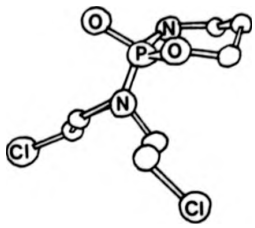
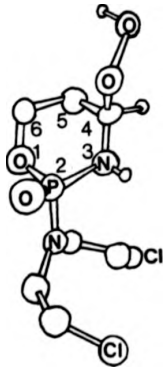
Fig. 1.13 Structure of The Optical Isomers of Cyclophosphamide and Hydroperoxy-CP



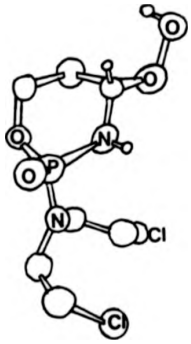
(a) R-(+)-CP



S-(-)-CP



(b) R-Hydroperoxy-R-CP



S-Hydroperoxy-R-CP

In all structures the ring is in the chair conformation.

In R-(+)-CP the phosphoryl oxygen is axial to the ring and trans to the ring nitrogen's hydrogen atom, although the geometries about the exo and endo nitrogen atoms is almost planar.

The R-Hydroperoxy group is axial to the ring and the chloroethyl side chains assume varied conformations. R and S forms of 4-hydroperoxy-CP also exist in the S-(-)-CP conformation, (not drawn).

(a) drawn using data from ref. (189).

(b) drawn using data from ref. (190).

liver microsomal incubations. *In vivo* differences were also reported for R/S isomer ratios of KP recovered in the urine. These were; R/S = 3.0 (mouse), S/R = 3.8 to 11.1 (rabbit), no selectivity (rat).

Tsui *et al.* (187) have demonstrated selective activation of CP isomers by microsomes from phenobarbital-induced mouse livers. Table 1.9 summarises these results. It can be seen that these results indicate a slight difference in kinetic parameters for the two isomers of CP. These workers have also reported that no difference in TI's could be found for the isomers in screening tests against mouse L1210 lymphoid leukaemia.

Jarman *et al.* (188) report that there is no difference between the isomers of CP in the plasma half life of CP or in the urinary output of CarbP from CP treated patients. They have found that the production of urinary KP is greatly increased by administration of (+) - CP compared to the racemate, again suggesting stereoselective metabolism of the (+) - enantiomer regarding KP production. They also report that the enantiomers were found to be equally bound to plasma protein.

There is little evidence for any stereoselectivity between the isomers of CP other than urinary metabolite production. The increased production of KP during (+) - CP metabolism (and presumably an increased deactivation) appears to have little effect upon the therapeutic efficacy of CP (185).

1.20 Biological Effects

(i) Target Molecules

The biological effects of alkylating agents such as CP are considered to be brought about by the alkylation of target molecules within the cell. Since active CP metabolites react with nucleophilic centres (section 1.16) such targets will include organic and inorganic anions, primary,

TABLE 1.9 Stereoselective microsomal activation of CP isomers

	R-(+)-CP	S-(-)-CP	(±)-CP
Vmax (a)	13.8 ± 1.0	20.0 ± 1.5	16.3 ± 1.1
Km (mM)	0.37 ± 0.02	0.56 ± 0.04	0.45 ± 0.02

Vmax, (-)-CP / Vmax, (+)-CP = 1.34 ± 0.17	Km, (-)-CP / Km, (+)-CP = 1.35 ± 0.14

(a) μmol. nor-HN2 equivalents/g. liver/Hr.

secondary and tertiary amines, heterocyclic-ring N-atoms and sulphides (191). Covalent bonding to compounds of low molecular weight is probably not important for the agent's cytotoxic effects since the products of such reactions are probably rapidly removed from the cell and excreted. However, such compounds afford some protection to the cell and may be important if in high (or low) enough concentration in only particular tissues. This effect has been put to good use in a tissue-protective role by the simultaneous adminstration of sulphydryl agents with CP (section vi) (169).

More critical to the functioning of the cell is the alkylation of macromolecules, where such alkylation interferes with their biochemical activity. Many structural proteins and enzymes react with alkylating agents, but at physiological dose levels the alkylation has been shown to be small compared with the number of enzyme molecules present, so that a serious enzyme inhibition does not necessarily result (192).

The prime target for alkylation is thought to be nucleic acid at present the most satisfactory hypothesis is that DNA is most sensitive to alkylation and that inhibition of some aspect of DNA function is the primary cause of cytotoxicity. The most extensively alkylated site in nucleic acids is the N7 position of guanine, less extensively alkylated is the N1 position of adenine, N3 of adenine, N3 of cytosine and O6 of guanine (see Fig. 1.1) (192). Differences in the extent of alkylation of these sites between RNA and DNA are probably due to the availability of the site. In DNA, the N1 of adenine and N3 of cytosine are both involved in hydrogen bonding of base pairs. Whereas these are sites of extensive alkylation in RNA, they are much less extensively alkylated in DNA.

Alkylation of these and other sites in nucleic acids may lead to the loss of the base from the polynucleotide chain. In DNA further breakdown of the chain may occur. Bifunctional alkylating agents such as PM may cross-link the two strands of the DNA helix. Loss of cross-linked residues would be more serious and may result in DNA scission. The cross-linking of adjacent guanine residues in the N7 position was thought to be the predominantly toxic reaction but the cross-linking of a number of nucleophilic centres also occurs. Interstrand cross-linking of DNA would result in an inhibition of mitotic division by inhibiting DNA synthesis and thus constitute an effective block to cell division. Cross-linking can also occur between DNA and protein. HN2-treated L1210 cells have been shown to acquire cross-linked DNA to alkylated residues of large non-histone proteins of the nucleus (195).

(ii) Mutagenicity and Carcinogenicity

As well as being cytotoxic, CP has been shown to be mutagenic and carcinogenic. CP, after metabolic activation, produces mutations in a variety of test systems (144). Gatehouse, however, has also reported mutagenic activity from the breakdown products of CP, formed during its storage in phosphate buffer (193). This may indicate that some breakdown products are similar to CP metabolites found *in vivo*. The most potent mutagenic derivative which seems to fit these criteria is nor-HN2.

CP and HN2 are carcinogenic in mice (194) and produce pulmonary tumours in mice in a dose-dependent manner. HN2 was found to be effective at concentrations 300 times lower than CP, reflecting the fact that only an active CP derivative is responsible for this action. No relationship has been observed between carcinogenicity and chemotherapeutic activity (194).

These effects are certainly produced by the action of CP metabolites on DNA, although the precise mechanism(s) is unknown.

(iii) Teratogenicity

Binding studies of radiolabelled CP to nucleic acids and proteins of embryonic tissues in pregnant mice have suggested that the teratogenic effect of CP is exerted via the alkylation of DNA (196). The production of an active species from CP is dependent upon maternal metabolism since the embryo does not possess the microsomal enzymes necessary for CP activation. Previous *in vitro* studies, however, have indicated that unmetabolised CP (197) and the urinary metabolic KP (198) have teratogenic activity. Recent studies indicate that PM is the metabolite responsible for teratogenic action, having a 10,000-fold greater activity than CP alone (199). HP was also shown to have similar activity to PM, presumably due to the formation of PM from this compound.

(iv) Immunosuppression

CP is known to suppress both the humoral and cellular immune responses in many animals including man (143) and is possibly the most potent immunosuppressive agent yet discovered. Contrary to most other effects of CP, alkylation does not seem to be important for this activity. PM has been found to have very little effect even at doses which induce haemopoietic depression. Of the CP metabolites tested for immunosuppressant activity, chloroacetaldehyde, a product of side chain hydroxylation, has been suggested as the mediator of this effect. The immunosuppressive action of CP has led to clinical usage in the treatment of non-malignant diseases, in two particular fields; (i) suppression of the immune response prior to organ transplantation, especially of bone marrow which has been highly successful in the treatment of acute leukaemia, (ii) treatment of auto-immune diseases including rheumatoid arthritis. The use of CP in the treatment of non-malignant diseases is limited because

of various unfavourable side-effects. In particular, the carcinogenic action of CP has led to the occurrence of acute myelogenous leukaemia in some patients treated with CP for rheumatoid arthritis.

(v) Anti-tumour Activity

CP is effective against Yoshida sarcoma, Walker 256 carcinoma, L1210 murine leukaemia and many other animal tumours including the virally-induced Friend and Rauscher leukaemias (143). CP has been shown to be somewhat less effective against experimental epithelial tumours. PM has been shown to be effective against many of these but its activity relative to that of CP varies, depending on the tumour studied.

The wide spectrum sensitivity to CP seen in animal tumours is reflected in the large number of human tumours responsive to CP therapy. Lymphoid tissues are most sensitive to the effects of CP. In many instances consistent and satisfactory responses to CP therapy have been seen in which a significant prolongation of life and even cure have been obtained (143).

CP is used mostly in combination chemotherapy regimens but has shown a dramatic response when used in Burkitt's lymphoma as a single agent. In early disease CP produces a >90% complete remission rate, with over 80% of children remaining long-term survivors. Treatment of advanced disease results in a 50% long-term survival rate.

One of the most widely used combination regimens is MOPP (Mechlorethamine/HN2, Oncovin/vincristine, Procarbazine and Prednisone) which is used against Hodgkin's disease. Substitution of CP for HN2 has no effect on the therapeutic response of this particular disease.

CP chemotherapy has shown good responses in the treatment of childhood acute lymphocytic leukaemia (40% response rate in previously untreated; 10% in previously treated patients) ovarian cancer, and breast carcinoma (35% overall response rate with CP alone, >50% in combination).

PM has been used clinically (143) and the main differences between it and CP were increased nausea and vomitting, lack of alopecia and few objective anti-tumour responses.

(vi) Side-effects

CP treatment has various side-effects due to the toxicity of CP metabolites. Cardiac toxicity is the dose-limiting effect of treatment with CP but heart failure only occurs at very high dose levels (>100 mg/kg in 48 Hr). Gonadal damage (aspermia, amenorrhea) has been reported. Nausea is apparently caused by PM, since in clinical studies involving treatment with PM, this effect was greatly increased. Water retention (increased urine osmolality) alopecia (hair loss) a side-effect employed for non-mechanical sheep shearing (200) and teratogenic and carcinogenic effects have also been observed.

The latter have been discussed, however, the teratogenicity of CP has been shown to be decreased by administration of thiol compounds (201) or pyrithinoldehydrochloride (202) and the use of thiols to prevent teratogenic effects in pregnant patients treated with CP has been suggested (201).

Haemorrhagic cystitis is caused by the effects of CP metabolites in the urine on the mucosa of the urinary bladder. Cystitis occurs in the rat treated with CP, isophosphamide and, to a lesser extent, PM. Cox (203) has shown that diethyl-CP (H atoms replace the Cl atoms) also causes severe cystitis. Since acrolein is the only reactive metabolite of diethyl CP, it is most likely this compound which causes cystitis. Acrolein has been shown to be capable of binding to proteins and free thiol groups (204). Due to its high reactivity it is probably only present for a short duration and is largely excreted into urine as 3-hydroxypropylmercapturic acid (205). Binding to thiols has been used to prevent damage of the

urinary bladder by local instillation of N-acetyl-L-cysteine (203). Wrabetz et al. (206) have shown that acrolein does not contribute to the cytotoxic action of CP.

1.21 Modification of CP Action By Modification of Its Metabolism

The modification (by stimulation or inhibition) of CP metabolism has been discussed (section 1.14(i)). Changes in the rate of biotransformation of compounds where, unlike CP, the parent compound is the active species, would be expected to produce quantitative changes in therapeutic response. Sladek (207) however, has pointed out that where a metabolite rather than the parent compound is effective, as is the case for CP, no effective change may be expected by alteration of the rate of biotransformation.

Although the rate of activation of CP can be stimulated or depressed by pretreatment with various drugs which effect its metabolism in the liver, the response to activated metabolites which produce an all-or-nothing effect (e.g. tumour cell kill) depends on concentration of metabolite and its time of contact with the cell. In vivo, the increased activation rate of CP due to pretreatment with liver enzyme-inducers (e.g. phenobarbital) is accompanied by an apparent increased disappearance rate of alkylating activity from blood and urine, and vice versa. Altered blood levels are accounted for by alterations in the activation rate of the anti-tumour compound. An increase in the concentration of activated metabolite is observed early with lowered concentration observed later, when metabolism has been stimulated. The reverse is true when metabolism has been depressed.

Sladek states that this inability to alter the therapeutic efficacy of CP by modification of its metabolism, demonstrates the futility of trying to improve the efficacy of CP in patients by pretreatment with drugs which alter CP metabolism (207).

METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

(i) Chemicals

The following compounds were purchased from Sigma (London) Chemical Co. Ltd. :

ATP, Bovine serum albumin (crystallised and lyophilised), cyclic AMP, cyclic GMP, dithiothreitol, EGTA, GTP, glucagon, glucose-6-phosphate, HN2 (hydrochloride), NBP (γ-(p-nitrobenzyl)pyridine), Triton X-100 and MNNG.

Sodium fluoride and sodium lauryl sulphate (SDS) were purchased from Hopkin and Williams and BDH Chemicals Ltd. were the suppliers of Folin reagent E, imidazole and theophylline.

Fisons Chemicals Ltd. supplied PPO and POPOP for scintillation counting and NADP and DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) were obtained from Boehringer Mannheim, GmbH.

Cyclophosphamide was a gift from Boehringer, 4-hydroperoxycyclophosphamide was a gift from Dr M. Colvin, Johns Hopkins Oncology Centre, Baltimore, Maryland and phosphoramide mustard was a gift from the National Institutes of Health, Bethesda, Maryland.

Sensitox II (Chemical Dynamics Corp. NJ.) was a gift from Dr M. Jarman, Chester Beatty Research Institute, London.

(ii) Enzymes

Glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49) type XI, Cobra (Ophiophagus hannah) snake venom and creatine phosphokinase from rabbit muscle, type I, were obtained from the Sigma (London) Chemical Co. Ltd.

(iii) Radiochemicals

Adenosine 5'-[α-^{32}P]triphosphate, [adenine-U-^{14}C]adenosine 3',5'-cyclic phosphate, guanosine 5'-[α-^{32}P]triphosphate and [8-^{14}C]guanosine 3',5'-cyclic phosphate were purchased from the Radiochemical Centre, Amersham, Bucks, UK.

[α-^{32}P]triphosphates were stored in dry ice and utilised within one week, [^{14}C]cyclic nucleotide phosphates were stored at -25°C.

(iv) Chromatography Resins

All Dowex resins were purchased from Sigma (London) Chemical Co. Ltd. Aluminium oxide (neutral grade, activity grade I) was purchased from BDH Chemicals Ltd. Merck supplied kieselgel 60 (0.040 - 0.063mm, 230 - 400 mesh) for use in 'flash' chromatography and kieselgel 60, f254 for thin layer chromatography.

(v) Apparatus

(A) Chromatography

"Econocolumns" were purchased from Bio-Rad Laboratories, polypropylene (4 x 0.7 cm) for use in adenylate cyclase assays and glass (15 x 0.7 cm) for use in guanylate cyclase assays.

A Waters Associates model M-6000A HPLC was used with a C-18 μBondapak reverse-phase column and an R401 differential refractometer for compound detection.

(B) Spectroscopy

[^{13}C]NMR spectra were obtained from a Bruker WH-90 spectrometer by Miss. L.Y. Lian. Mass spectra were obtained from a Carlo Erba/Kratos mass spectrometer by Mr. I.Katai. All other spectra were obtained by myself. [^{1}H]NMR spectra were recorded on a 220 MH_2 Perkin Elmer R34 NMR spectrometer, IR spectra on a Perkin Elmer 257 grating IR spectrophotometer and UV/visible spectra were run on a Unicam SP1800 ultraviolet spectrophotometer. Routine absorbance measurements were made using a Unicam SP600 spectrophotometer.

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(C) Centrifugation

The following centrifuges and rotors were used in the course of this work : BTL bench centrifuge, Sorvall superspeed RC2-B (GSA and SS-34 rotors), Beckman model L-2 ultracentrifuge (swing out SW25.2 rotor) and the Spinco L-50 (40 and 30 rotors).

(D) Miscellaneous

A Gallenkamp melting point determination apparatus was used and a British Oxygen Company B.O.C. Mk. II ozoniser was employed to produce ozone. A Packard 2425 Tricarb liquid scintillation counter was used to measure radioactivity.

(vi) Animals

All rats used were male Wistar, weighing approximately 250 to 300g.

2.2 The Assay of Adenylate Cyclase Activity

(1) Introduction

Adenylate cyclase catalyses the conversion of ATP to cyclic AMP and pyrophosphate in the presence of magnesium ions. This reaction is stimulated by one or more cell-specific hormones in the presence of GTP. In these studies, using plasma membranes prepared from rat liver as the enzyme source, the stimulatory hormone was the peptide, glucagon.

Adenylate cyclase activity was measured using radiolabelled substrate. The formation of cyclic AMP was estimated, after thorough purification on two separate ion exchange columns, by liquid scintillation counting.

The specific activity of adenylate cyclase in eukaryotic tissues is very low and only a small percentage, usually less than 0.05% of the substrate ATP undergoes cyclisation to form cyclic AMP in an *in vitro* assay system. Assay sensitivity and reliability is therefore largely

dependent upon and proportional to the efficiency with which cyclic AMP is separated from ATP and its breakdown products. Such products can accumulate at a high rate due to the activity of other enzymes present in almost every membrane preparation, possibly as contaminants. Due to the presence of such enzymes, cyclic AMP as well as ATP may give rise to the accumulation of adenosine in the reaction mixture.

Adenosine has been shown to stimulate (208) or to inhibit (209) adenylate cyclase activity of various sources. Cyclic AMP phosphodiesterase is capable of hydrolysing any cyclic AMP as it is produced by adenylate cyclase *in vitro*. Methyl xanthines and other inhibitors can be used to reduce product hydrolysis but these do not completely eliminate the problem. If radiolabelled ATP is used as substrate, then a 'cold trap' of unlabelled cyclic AMP may be utilised to advantage. In the presence of 10 mM theophylline and 0.05 mM cyclic AMP, adenylate cyclase may be assayed in rat liver plasma membrane preparations with 95 to 98% recovery of cyclic AMP.

Many techniques for the purification of the radiolabelled product of adenylate cyclase assays have been previously reported. These have been assessed primarily with respect to convenience (i.e. the time involved and complexity of procedure) but also with respect to sensitivity. Sensitivity approaches a maximum as assay blank values approach zero.

A single step chromatographic procedure using neutral aluminium oxide has been described (210) (211) and although rapid, simple and convenient sensitivity is diminished due to high blank values. Q values (see section (10)) using this procedure have been reported as high as 100 to 300 (212). Another system is that of Krishna *et al*. (213) utilising an ion exchange column of Dowex 50 which separates the bulk of radiolabelled

cyclic AMP from the substrate. This is followed by precipitation of nucleotides, other than cyclic AMP, by the nascent production of barium sulphate. Although this represents a higher sensitivity (Q = 15) it has, however, been reported that Ba^{2+} ions will catalyse the non-enzymatic production of cyclic AMP and thus anomalous activity may be measured (214).

Polyacrylamide Boronate Gel columns have been used in adenylate cyclase assays (215) but the use of only one ion exchange column or only thin layer or paper chromatography may be argued to produce an assay which lacks specificity and the chosen procedure is therefore a two column elution first described by Salomon et al. (212) and more recently published in greater detail (216).

(2) Experimental Procedure

Initial separation of cyclic AMP from reaction mixtures is achieved using a Dowex AG50W-X4 (200 - 400 mesh) column eluted with water. The functional group of this cation exchange resin is a sulphonic acid which rapidly excludes highly charged anions from the column. Nucleotides are resolved by their charge content and their mobility on the resin. This first elution removes most of the ATP in the reaction mixture.

Nucleosides and heterocyclic bases are retained by the Dowex column. Cyclic AMP and residual ATP are next eluted with water directly onto neutral alumina columns which strongly adsorb multivalent anions (211). Thus, this resin will separate cyclic AMP from the nucleotide, ATP, substrate. Cyclic AMP is eluted from the alumina columns with imidazole buffer almost immediately.

[^{32}P]ATP is used as substrate in the assay and was not purified before use as it was obtained from the Radiochemical Centre, Amersham, where purification is achieved especially for adenylate cyclase assays on an ion exchange column.

[^{14}C]cyclic AMP is used as a column marker or internal standard to determine the recovery of cyclic AMP from each column, this value being used to correct for any column loss of product [^{32}P]cyclic AMP.

Although this assay system has been well documented, the column system was characterised before use to ensure that the chromatographic steps worked perfectly and to ensure the purity of the radiochemicals used.

(3) Elution Volume Determination

Elution volumes were determined by adding standards of [^{32}P]ATP and [^{14}C]cyclic AMP together with membrane protein and assay constituents to Dowex 50 columns (Fig. 2.1). At first, these columns were made as a slurry of Dowex resin and water but this was found to give irregular and irreproducibly sized columns. By drying the resin first, then weighing, columns of reproducible size were made.

(A) Dowex 50 Columns

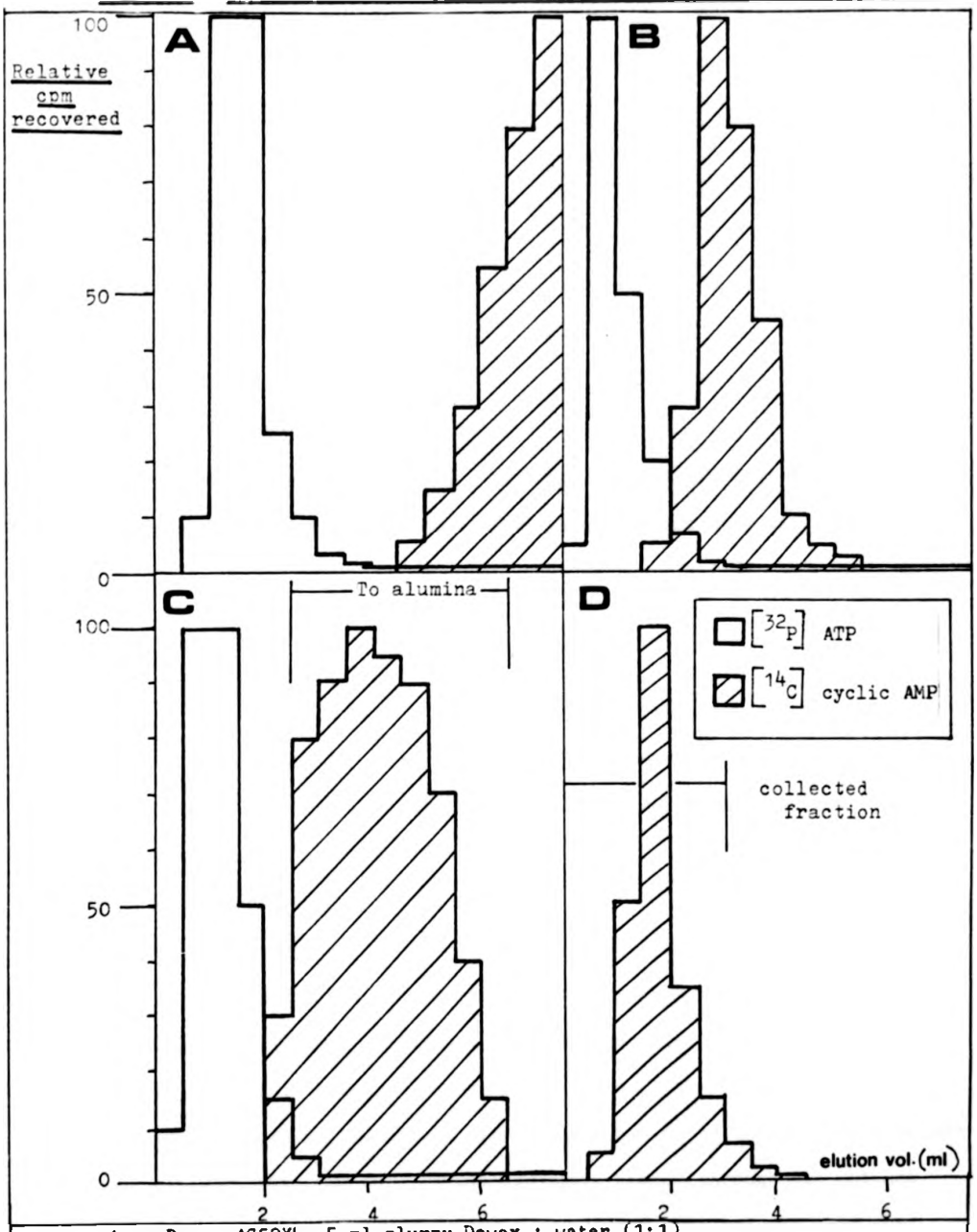
After the addition of standards to the columns, portions of water (0.5 ml) were added and collected separately in scintillation vials for counting. After each addition, the columns were allowed to drain completely with no ill-effects to the column bed. This determined the elution profile of the Dowex 50 columns (Fig. 2.1C).

(B) Neutral Alumina Columns

(i) Standards were added to Dowex 50 columns and most of the ATP removed by the addition of water (2.5 ml) as determined by the elution profile for Dowex 50 columns (Fig. 2.1C).

(ii) The Dowex 50 columns were then placed above neutral alumina columns (0.6g in stoppered Pasteur pipette) and cyclic AMP eluted from the Dowex 50 onto the alumina columns by the addition of water (4.0 ml), again as determined by the elution profile for Dowex 50 columns (Fig. 2.1C).

Fig. 2.1 Elution Profiles of Columns Used In The Adenylate Cyclase Assay



A Dowex AG50X4, 5 ml slurry Dowex : water (1:1)

B Dowex AG50X4, 2 ml slurry Dowex : water (1:1)

C Dried Dowex used as column bed (2.5 g)

D Aluminium Oxide (0.6 g) column. Elution of cyclic AMP fraction indicated from column C

Each result is averaged for two columns.

(iii) After removal of the Dowex 50 columns, portions of imidazole-Cl buffer (0.5 ml, 0.1 M, pH7.3) were run through the column and collected directly into scintillation vials for counting. This determined the elution profile for alumina columns (Fig. 2.1D). As can be seen from this profile, nearly all of the cyclic AMP was eluted in the first three ml and no radioactivity due to $[^{32}P]$ nuclide could be detected.

(4) Column Preparation

(A) Packing of Dowex 50 Columns

'Econocolumns' were packed with 2.5g of Dowex AG50W-X4 (200 - 400 mesh) which had been washed (deionised water, repeatedly) and dried (gently in a desiccator below 60°C) previously. HCl (1M) was run through each column before use (≈ 10 ml).

(B) Packing of Alumina Columns

Dry alumina (0.6 g) was added to glass wool-stoppered Pasteur pipettes. Imidazole-Cl buffer (1.0 M, pH7.3) was used to wash the columns (≈ 10 ml). Imidazole-Cl buffer (0.1 M, pH7.3) was then used similarly. The columns were then ready for use.

(C) Column Recycling

(i) Dowex 50 Columns

It was found that the best method of recycling the columns to obtain reproducible elution profiles was to first wash the columns with HCl (1.0 M, ≈ 10 ml). When this was partly drained, the column bed was resuspended by gentle agitation with a glass rod or by inverting the column 2 to 3 times. HCl (1.0 M, ≈ 10 ml) was again passed through the columns after the column bed had been allowed to reform. The columns were then ready for re-use.

(ii) Alumina Columns

Alumina columns were discarded after use and therefore new columns were prepared for each assay.

(5) Assay Cocktail

(A) ATP, Cyclic AMP and GTP

Solutions of these compounds were made and their concentrations at pH 7.0 accurately measured spectrophotometrically. ATP and cyclic AMP were measured at 259 nm., assuming a molar absorbance of 15,400 l mole Cm^{-1}. GTP was measured at 252 nm., assuming a molar absorbance of 13,700 l mole Cm^{-1}.

Small aliquots of each (<0.2 ml) were stored at $-25^{o}C$. The required amount of each was thawed and diluted to the required concentration before use.

(B) Reagents

Stock solutions were prepared of all the reagents used in the assay and stored at 0 to $4^{o}C$ (Table 2.1). These were added to a test tube, in the order listed in Table 2.1, containing the solid creatine phosphate and creatine phosphokinase, and made up to a total volume of 1.0 ml with water.

(C) $[^{32}P]ATP$

The required amount of $[^{32}P]ATP$ (2 to 8 x 10^{6} dpm per assay tube) was evaporated to dryness (this compound had been supplied in 50% ethanol solution). The appropriate amount of assay cocktail (Table 2.1), was then added to the dry test tube (10 µl per assay tube).

Activity of $[^{32}P]ATP$ = 1 µCi per µl at t = 0

= 2.22 x 10^{6} dpm

TABLE 2.1 Reagents for the preparation of assay cocktail

SOLUTION	REAGENT Conc. (mM)	µl of reagent	conc. in cocktail (mM)	Final conc. in assay (mM)
Creatine phosphate	solid	6.4 mg	25	5
Creatine phosphokinase		250 U	250 U/ml	50 U/ml
Tris acetate	1000	125	125	25
Mg acetate	1000	25	25	5
ATP	100	25	2.5	0.5
cyclic AMP	10	25	0.25	0.05
DTT	100	50	5	1
BSA	10 mg/ml	50	0.5 mg/ml	0.1 mg/ml
GTP	1	50	0.05	0.01
EGTA	100	50	5	1
[^{32}P] ATP	-	-	2-6 x 10^8 cpm/ml	2-6 x 10^6 cpm/assay

This was true for most batches of [^{32}P]ATP obtained from Amersham, however, occasionally, a batch was received of only half this activity. Approximately twice this amount was required per assay tube, and the appropriate correction factor for [^{32}P] decay also applied :

$$\text{Amount required (for n assays)} = \frac{2n}{\text{decay factor}} \ \mu l$$

(6) Assay Procedure (Step By Step)

Glass test tubes containing assay cocktail (10 µl), and test compounds (CP and derivatives) to a total volume of 25 µl were equilibrated to 30°C in a water bath (Table 2.2).

(A) The reaction was started by adding freshly-thawed and diluted plasma membrane suspension (1 to 8 µg protein) in 20 µl , preincubated for 15 minutes with H_2O (5 µl), hormone or fluoride (5 µl). Final reaction volume was 50 µl.

The reaction mixture was vortex mixed and then incubated for 15 minutes at 30°C.

(B) The reaction was then stopped by adding stopping solution (100 µl) containing unlabelled ATP (45 nM), cyclic AMP (1.3 mM), SDS (2%) and again vortex mixed.

(C) After the reaction was stopped, [^{14}C]cyclic AMP (approximately 20,000 cpm) was added carefully to each tube (50 µl).

(D) All reaction tubes were next boiled in a water bath for 3 to 5 minutes and cooled to room temperature afterwards. This procedure completely solubilised all membrane protein.

(E) At this stage in the assay procedure, the tubes may be stored for up to a few days at -20°C. After storage, test tubes were allowed to equilibrate to room temperature before use.

TABLE 2.2 Solutions used in the assay of adenylate cyclase

SOLUTION	Reagent conc. (mM)	µl of reagent	Final conc in assay (mM)
Assay Cocktail	-	10	-
†(Theophylline	33.33	15	10)
‡ACTIVATORS NaF	100	5	10
glucagon	100 nM	5 nM	10 nM
Plasma membrane	-	20	-
Total assay volume		50 µl	
Stopping solution		100 µl	

† when included in assay.

‡ fluoride or glucagon was added to the plasma membrane preparation 15 min. before the start of the assay.

CP and derivatives were included in the assay cocktail or replaced theophylline.

(F) Two types of blanks were prepared for the assay :

(i) No-enzyme blanks were prepared using Tris-DTT (Tris acetate, 10 mM, pH7.6, DTT, 1 mM) solution in place of the plasma membrane preparation.

(ii) Zero-time blanks were prepared by adding the stopping solution to the assay cocktail before the addition of plasma membrane suspension.

(G) Standards of the radiolabelled compounds were prepared and counted.

The [^{32}P]ATP solution (10 µl of a 1/100th dilution in assay cocktail) was added directly to a scintillation vial containing scintillant (20 ml) and imidazole-Cl buffer (290 µl).

Similarly, the [^{14}C] cyclic AMP solution (50 µl) was added to a vial containing scintillant (20 ml) and imidazole buffer (250 µl).

Background counts were obtained using imidazole-Cl buffer (3.0 ml) in scintillant (20 ml).

(7) Chromatography

Water (300 µl) was added to each tube (total volume 0.5 ml) before applying the samples to the Dowex 50 columns.

(A) Elution of ATP

A rack of Dowex 50 columns was placed over a collecting tray, having been washed prior to use with water. The contents of each assay tube was decanted onto the columns and allowed to drain completely. Water (2.5 ml) was then run through each column and allowed to drain into the collecting tray. This fraction contained ATP and was therefore discarded.

(B) Elution of Cyclic AMP

The Dowex 50 columns were placed above a rack of freshly prepared alumina columns and water (4.0 ml total volume, in 1.0 ml portions) was allowed to drain through both columns and discarded.

Scintillation vials containing scintillant (18 ml) were placed under each of the alumina columns and the Dowex 50 columns removed. Imidazole-Cl buffer (0.1 M, pH7.3) was run through the alumina columns and collected directly into the scintillation vials (3.0 ml total volume). This step eluted cyclic AMP.

The vials were stoppered, agitated to disperse the sample and produce a single phase solution, then counted using manually-set windows for dual label counting. Vials were counted for four minutes.

(8) Identification of Product

The identity of the radioactive product of the adenylate cyclase assay was determined by thin layer chromatography on PEI-cellulose plates in sodium acetate (60 mM, pH5.4). Spots were visualised by UV and the plates then cut into 1 cm squares for counting. These squares were left overnight in Nuclear Chicago Solubiliser (0.7 ml of a 1:5 dilution) at 40°C in order to solubilise the adsorbed compounds, before counting in scintillant. Standards were prepared by spotting onto 1 cm squares of PEI-cellulose plates and were subjected to the same procedures as the samples after TLC.

Although the [^{32}P]-labelled product of the adenylate cyclase assay system co-chromatographed with the added [^{14}C]-labelled cyclic AMP standard on the Dowex-alumina chromatographic system, further confirmation of its identity was felt to be advisable. Using this method of TLC, the ATP in the assay cocktail was shown to co-chromatograph with ATP standard (R_f = 0.12). When either the assay cocktail or the cyclic AMP fraction collected from the alumina columns was subjected to TLC (after incubation) both [^{14}C] and [^{32}P]-labelled compounds were shown to co-chromatograph with a cyclic AMP standard solution (RF = 0.25). When assay cocktail was used prior to column chromatography, the area on the plate corresponding to ATP was also observed to contain the [^{32}P]-label.

Therefore, in summary, it has been shown that the [^{32}P]-labelled product of the adenylate cyclase assay, as well as co-chromatographing with [^{14}C] cyclic AMP on the Dowex-alumina column system, also co-chromatographs with cyclic AMP in the PEI-cellulose-sodium acetate TLC system.

(9) Calculations

Two methods for calculating the activity of only one isotope in a dual labelled sample are available.

(A) Absolute dpm

In order to calculate the absolute dpm, one must determine the efficiency of counting each isotope in each of the two channels used. This can be done by accurately measuring a standard sample volumetrically

	[^{14}C] channel A	[^{32}P] channel B
[^{14}C] standard	C	C'
[^{32}P] standard	P	P'
Sample (assay)	S	S'
Background	B	B'

and then calculating the actual dpm, from the known specific activity. cpm is then obtained by counting the sample and the discrepancy between calculated and measured values (dpm and cpm respectively) may be assumed to be due to the efficiency of the machine.

$$\% \text{ efficiency} = \frac{\text{obtained cpm}}{\text{calculated dpm}} \times 100$$

The following equations can be used to calculate the absolute dpm of each isotope in a dual isotope sample :

(i) $[^{32}P]$ cpm ch A = $[^{32}P]$ activity x $[^{32}P]$ efficiency ch A

(ii) $[^{14}C]$ cpm ch A = S - $[^{32}P]$ cpm ch A

= S - ($[^{32}P]_{act.}$ x $[^{32}P]_{eff.}$ ch A)

$$\therefore [^{32}P]_{act.} \text{ (dpm)} = \frac{S'}{[^{32}P]_{eff.} \text{ ch B}} \times 100$$

$$\therefore [^{14}C]_{act.} \text{ (dpm)} = \frac{[^{14}C] \text{ cpm ch A}}{[^{14}C]_{eff.} \text{ ch A}} \times 100$$

$$= \frac{S - ([^{32}P]_{act.} \times [^{32}P]_{eff.} \text{ ch A})}{[^{14}C]_{eff.} \text{ ch A}}$$

$$= \frac{S - S' \dfrac{[^{32}P]_{eff.} \text{ ch A}}{[^{32}P]_{eff.} \text{ ch B}}}{[^{14}C]_{eff.} \text{ ch A}} \times 100$$

(B) <u>Relative cpm</u>

Absolute dpm may not necessarily be used when correcting for "channel leakage". For clarity, it may be assumed that the background counts, B and B', have already been subtracted from all counts.

(i) [^{14}C] counts in the [^{32}P] channel

$$\text{Correction factor } F = \frac{C'}{C} \times S$$

To be subtracted from cpm in ch B sample, S'

(ii) [^{32}P] Counts in the [^{14}C] Channel

$$\text{Correction factor } F' = \frac{P}{P'} \times S'$$

To be subtracted from cpm in channel A sample, S.

(iii) Cyclic AMP Recovery

This is calculated as a percentage :

$$\text{cyclic AMP recovered} = \frac{S}{C} \times 100 \quad (\%)$$

The factor for multiplying sample cpm by in order to correct for recovery is :

$$\frac{C}{S} \quad \text{which is modified to} \quad \frac{C}{S-F'}$$

(iv) Specific Radioactivity (SpR)

Where A is the concentration of ATP per assay tube in pmol.

$$SpR = \frac{P' \times 100}{A}$$

(v) Cyclic AMP Produced

This is equal to S'-F and is converted to pmol by dividing by SpR. The correction factor for % recovery is applied and the result expressed as a function of the membrane protein (E) per assay.

$$\text{cyclic AMP}_{gross} = \frac{C}{S - F'} \times \frac{(S' - F) \times 1000}{SpR \times E} \text{ (pmol/15 min/mg prot)}$$

The blank value (Z) is calculated similarly and, :

$$\text{cyclic AMP}_{net} = \text{cyclic AMP}_{gross} - Z$$

(10) Q Values

Q is a measure of the purity of [^{32}P] ATP used and provides an indication also of the performance of the chromatographic procedure.

Q values should be below 10 and typically about 4.0 for this assay.

$$Q = \frac{SpR \times E \times Z \times 10}{P'}$$

note: P' is the direct cpm of a 1/100 dilution of [^{32}P] ATP used in the assay.

Typically, Q values of 5 to 7 were obtained in these studies.

2.3 The Assay of Guanylate Cyclase Activity

(1) Introduction

Guanylate cyclase catalyses the conversion of GTP to cyclic GMP and pyrophosphate, in the presence of divalent cations. The basal activity of guanylate cyclase is dependent upon the specificity of the enzyme for manganese, Mn^{2+}, ions. This enzyme is stimulated by a wide range of physiological and non-physiological compounds (52). In these studies, using the cytosolic fraction of rat liver as the enzyme source, MNNG was used as the enzyme activator (73).

Some laboratories measuring guanylate cyclase activity have reported methods of determining the cyclic GMP formed by competitive binding assays requiring either the availability of relatively scarce antibodies (217) or a sufficiently specific lobster-tail binding-protein (218). When using radiolabelled GTP as substrate, the product cyclic GMP must be separated before estimation by liquid scintillation counting. As is the case in the adenylate cyclase assay system, many techniques for this separation have been described.

Single column chromatography using aluminium oxide with (219) or without (210) suitable preparation and dual column chromatography either on first Dowex 50 eluted with water followed by aluminium oxide (220) (221) or on first aluminium oxide followed by Dowex 1 (222) have been utilised.

(2) The Development of The Chromatographic Step

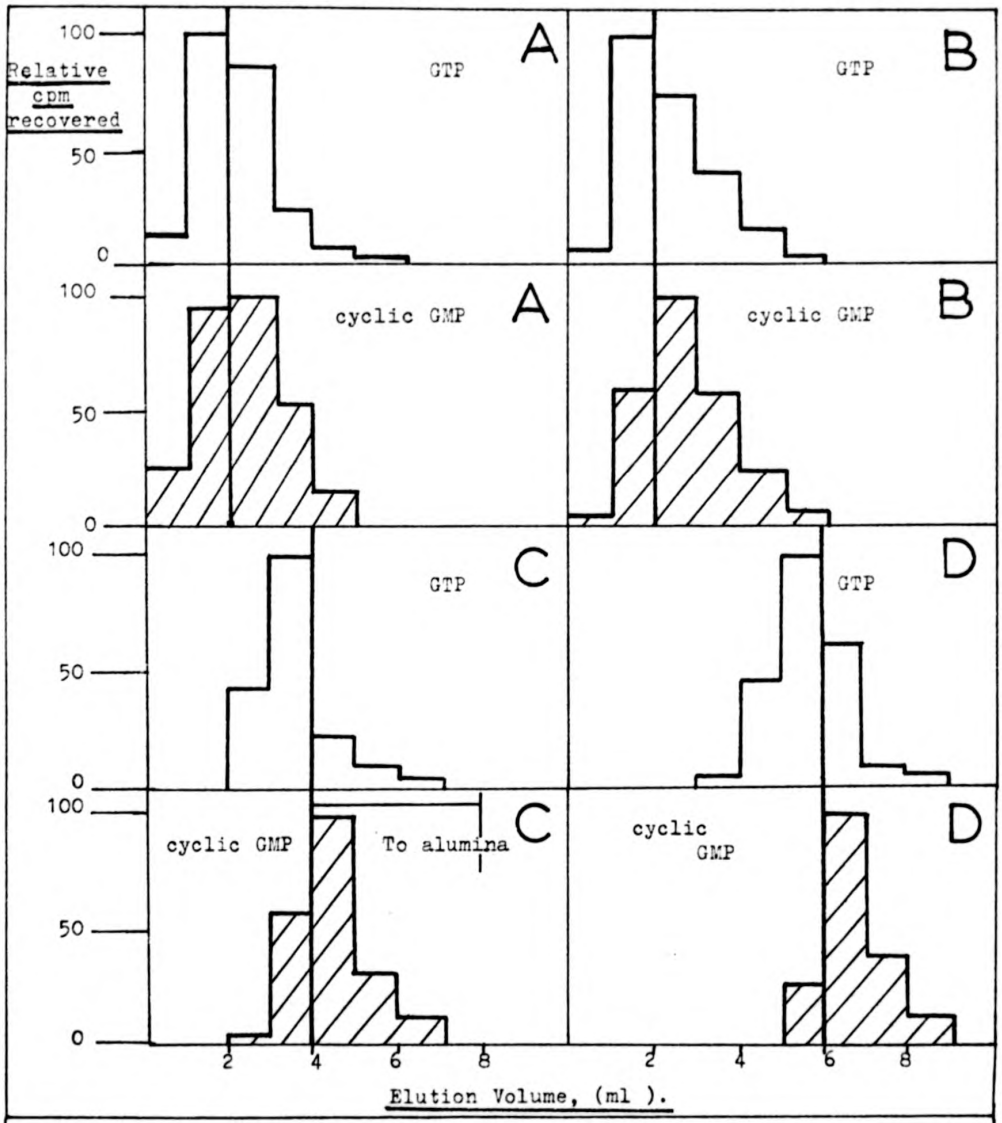
The assay method of dual chromatography on Dowex 50 followed by alumina was investigated, applying the modifications of Nesbitt *et al.* (223) and Wallach *et al.* (224) to the adenylate cyclase assay system of Salomon (216).

Elution profiles were determined by the method reported in section 2.2 (3) for the adenylate cyclase system.

The results from Dowex 50 elutions were very poor (Fig. 2.2A). No separation of GTP from cyclic GMP could be seen and, therefore, an investigation of the effect of the column bed size was carried out (Fig. 2.2). It can be seen that 'separation' is optimally carried out with a bed size of 8 cm (x 0.7 cm). This separation was, however, very poor and not as reported previously (220) (221).

Other types of Dowex resin are commercially available and two of these were investigated. Dowex 50X8, a resin with more cross-linking than

Fig. 2.2 Elution Profiles of Dowex AG50X4 (200 - 400 mesh) Eluted With Water



A 2.0 X 0.7 cm column bed

B 4.0 X 0.7 cm column bed

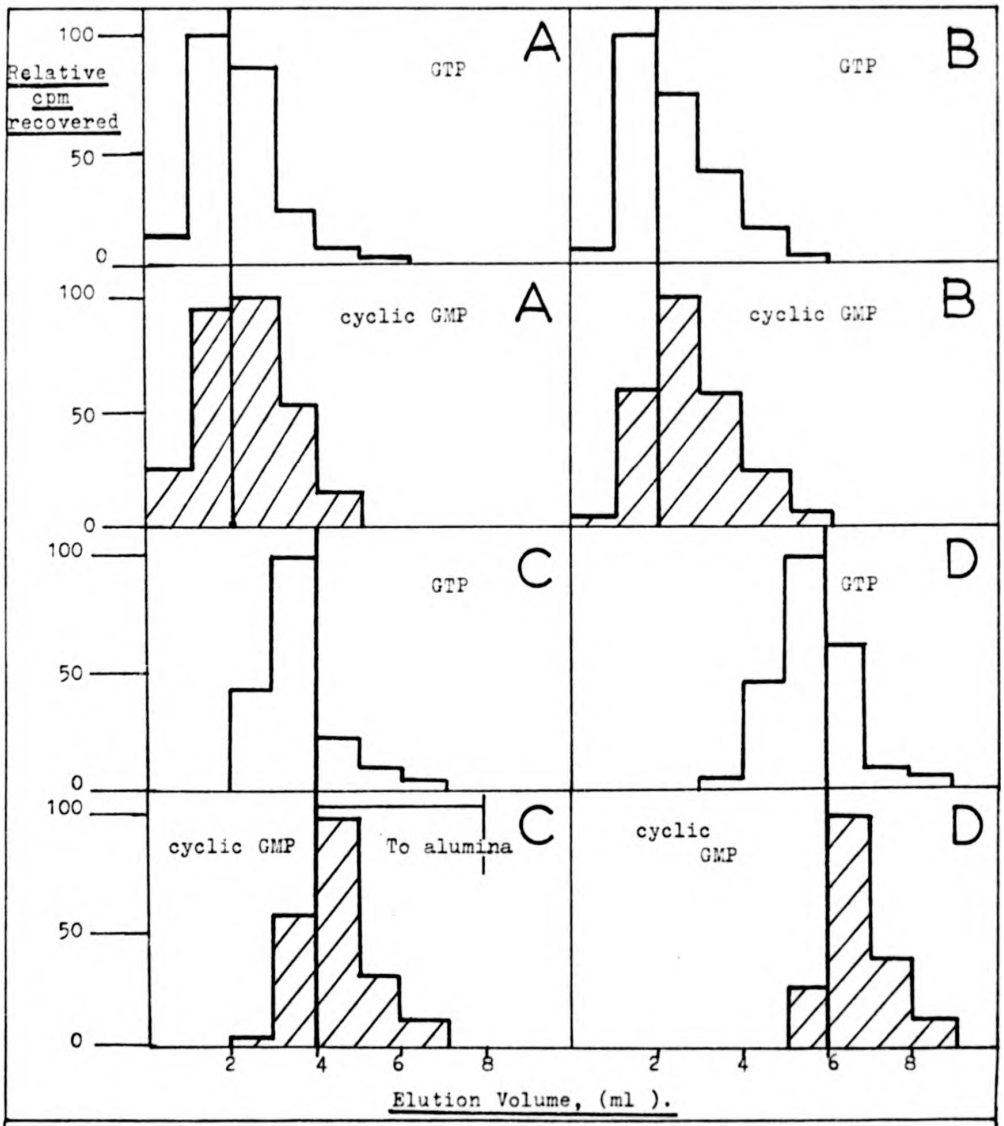
C 8.0 X 0.7 cm column bed

D 15.0 X 0.7 cm column bed

Each result is averaged for 2 columns

See text for details

Fig. 2.2 Elution Profiles of Dowex AG50X4 (200 - 400 mesh) Eluted With Water



A 2.0 X 0.7 cm column bed

B 4.0 X 0.7 cm column bed

C 8.0 X 0.7 cm column bed

D 15.0 X 0.7 cm column bed

Each result is averaged for 2 columns

See text for details

Dowex 50X4 (8% instead of 4%) gave similar results (Fig. 2.3A). Dowex 1X8 (chloride form) is an anion exchange resin and was found to give adequate separation using water as eluant (Fig. 2.3B). However, the volume containing cyclic GMP was very large (6 to 8 ml) and the columns required a long time to run (retention volume for cyclic GMP was approximately 14 ml). These findings are in agreement with those of Birnbaumer et al. who went on to modify the Dowex 50 chromatographic procedure to separate GTP and cyclic GMP to a much greater extent (225).

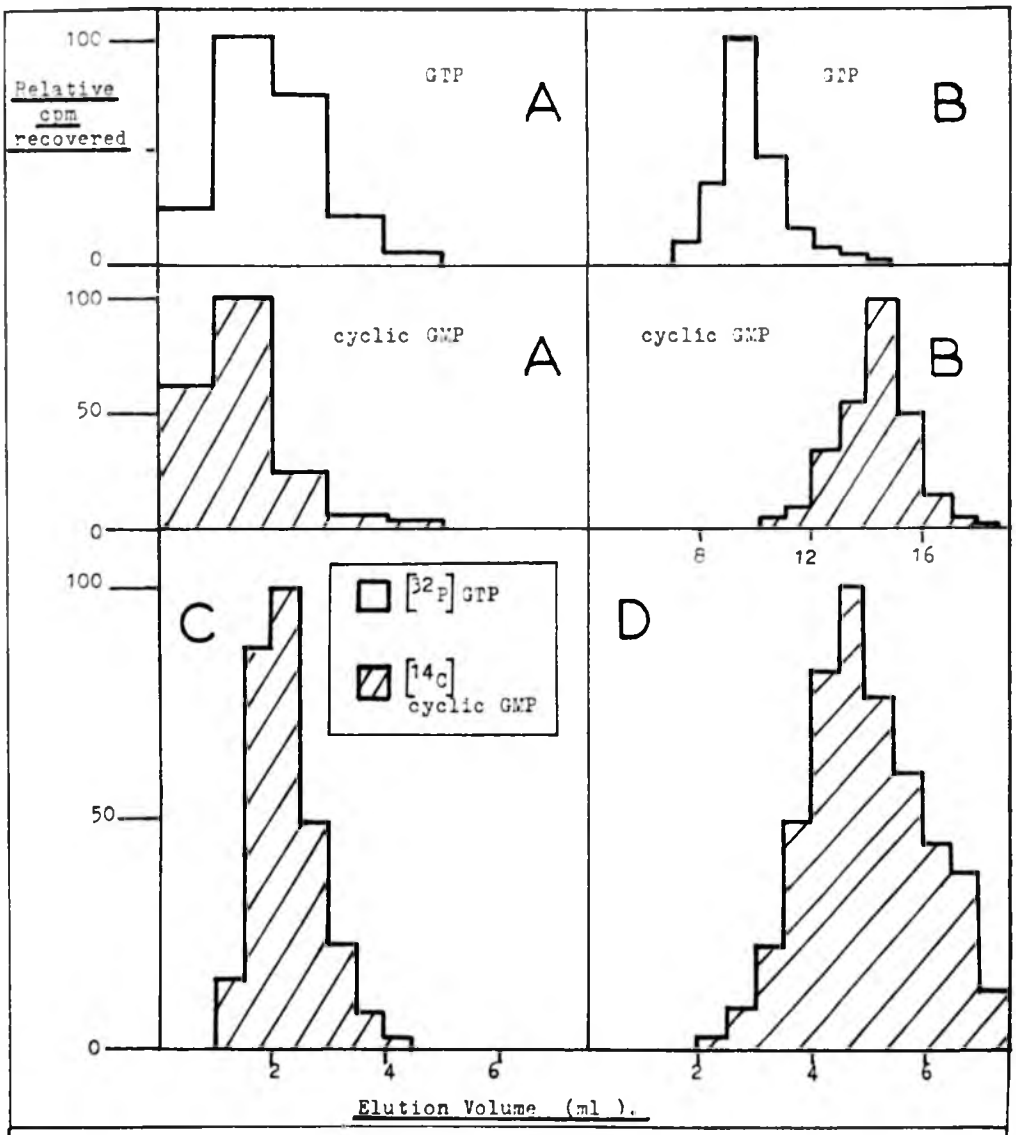
By applying the developments of Birnbaumer et al. (225) GTP and cyclic GMP were separated on Dowex 50 columns to an extent which compares favourably with the separation of ATP and cyclic AMP during this stage in the adenylate cyclase assay system (Fig. 2.4A, cf. Fig. 2.1).

The major modification introduced is the application of sample and subsequent elution of GTP from the Dowex 50 columns under acid conditions. Cyclic GMP is eluted onto alumina columns using water, the precise volumes required having been estimated from the elution profile.

Alumina elution was carried out basically as in the adenylate cyclase assay system but larger columns (1.0 g) were used, and cyclic GMP was eluted after an initial void volume of approximately 4.0 ml was first obtained. Elution profiles for alumina columns are shown for the unmodified Dowex 50 procedure (Fig. 2.3C, 2.3D) and the modified one (Fig. 2.4B).

This procedure gave consistently high recovery rates of cyclic GMP (70 to 80% recovered, compared to only 50 to 60% recovered in other systems) and very low blank values. Blank values in some systems are considerably higher, probably because of the poor separation obtained on the Dowex columns (in some systems up to 20% of the applied GTP may elute with cyclic GMP) (225).

Fig. 2.3 Elution Profiles of Columns Investigated For Application to Guanylate Cyclase Assays



A Dowex AG50X8 : 4.0 X 0.7 cm column bed.

Eluted with water.

B Dowex AG1X8 : 4.0 X 0.7 cm column bed.

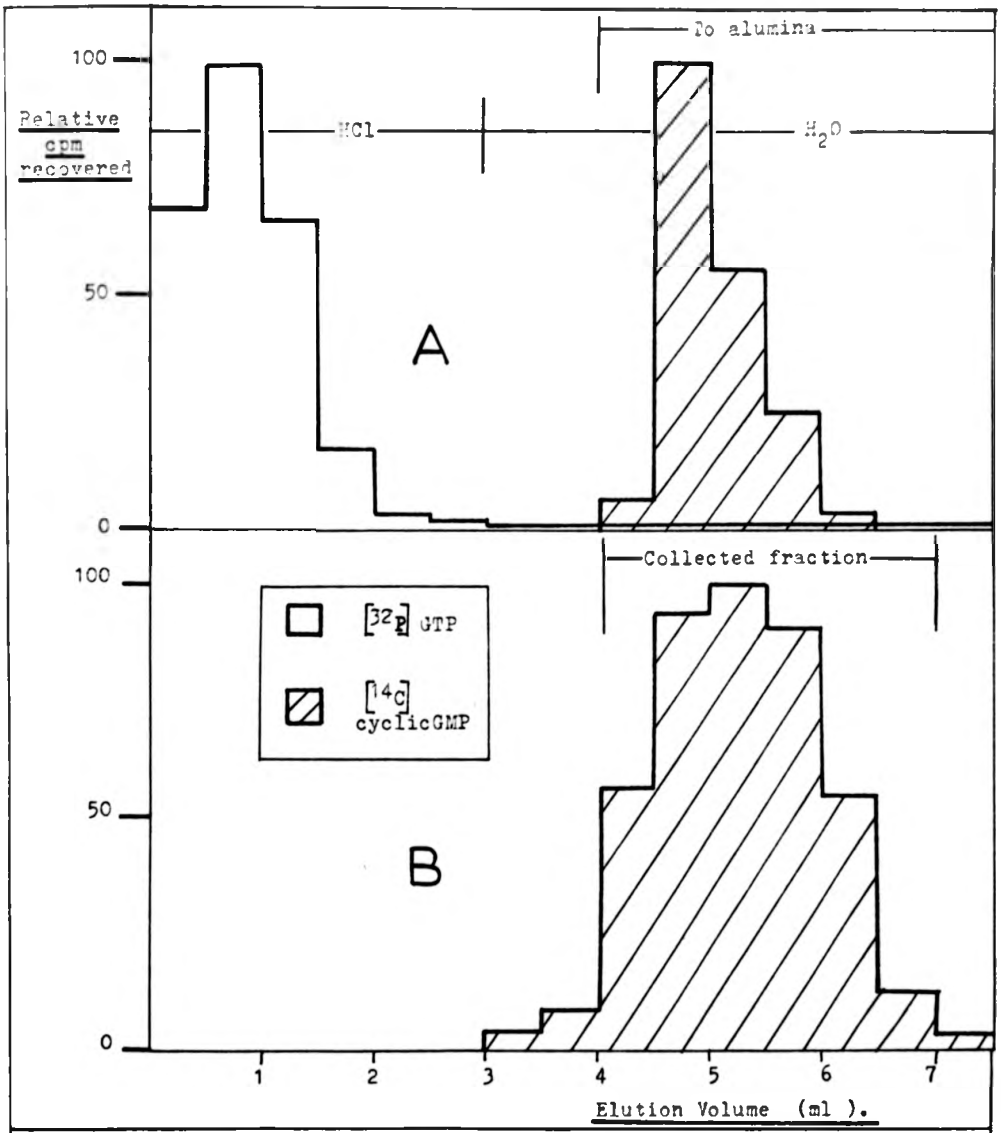
Eluted with water.

Aluminium oxide columns after application of cyclic GMP fractions from Dowex AG50X4 columns (8.0 X 0.7 cm) as shown in Fig. 2.2C.

C Eluted with Tris-Cl (0.1 M pH7.3).

D Eluted with water.

Fig. 2.4 Elution Profiles of Columns Used In The Guanylate Cyclase Assay



A Dowex AG50WX4 (200 - 400 mesh). 0.7 X 8.0 cm (3.0 ml) column bed, eluted first with HCl (50 mM) then water. The 4 ml fraction indicated containing cyclic GMP was applied to alumina columns.

B Aluminium oxide, 1g column eluted with imidazole buffer (0.1 M pH7.3) after application of cyclic GMP fraction from Dowex column. The 3 ml fraction indicated was used to collect cyclic GMP in the assay.

Each result is averaged for 2 columns.

(3) Column Preparation

Columns were prepared in precisely similar ways reported in section 2.2(4).

(4) Assay Cocktail

Stock solutions were prepared and stored as previously stated, GTP and cyclic GMP being stored in small aliquots after their concentration was accurately measured spectrophotometrically. The assay cocktail was made similarly (section 2.2(5)) but to a final concentration of 1.5 ml (Table 2.3). The appropriate volume of [^{32}P]GTP was dried under nitrogen and redissolved in the required amount of assay cocktail.

(5) Assay Procedure

Glass test tubes containing assay cocktail (50 µl) and 'test' compounds (25 µl) were equilibrated to 30°C in a water bath (Table 2.4). During studies with MNNG, the enzyme solution was diluted to the required concentration of protein with MNNG solution which had been freshly prepared. The concentration of the MNNG solution was carefully calculated to yield the desired concentration of MNNG when used to dilute the enzyme solution. The enzyme-MNNG mixture was kept in an ice bath for 30 minutes before use. A similar procedure was followed with cyclophosphamide derivatives during pre-incubation experiments.

(A) The reaction was started by the addition of the enzyme solution to the assay cocktail. This had first been appropriately diluted with either (i) buffer, (ii) MNNG or (iii) 'test' compound depending upon the experiment. 50 to 150 µg of protein were used per assay (in 25 µl). The reaction mixture was mixed thoroughly by vortex mixing and incubated at 30°C for 15 minutes.

(B) The reaction was stopped by the addition of stopping solution (100 µl) containing SDS (2%) and EDTA (30 mM, pH7.6).

TABLE 2.3 Reagents for the preparation of assay cocktail in the assay of guanylate cyclase activity

SOLUTION	REAGENT conc. (mM)	µl of reagent	conc. in cocktail (mM)	Final conc. in assay (mM)
Creatine phosphate	solid	11.52 mg	30	15
Creatine phosphokinase		150 U	100 U/ml	50 U/ml
Tris-HCl	1000	150	100	50
$MnCl_2$	400	30	8	4
GTP	60	60	2.4	1.2
cyclic GMP	90	90	5.4	2.7
Theophylline	33.33	900	20	10
BSA	10 mg/ml	30	0.2 mg/ml	0.1 mg/ml
H_2O		240		
[^{32}P]GTP			$0.4-1.2 \times 10^8$ cpm/ml	$2-6 \times 10^6$ cpm/assay

TABLE 2.4 Composition of the reaction mixture in guanylate cyclase assay

SOLUTION	VOLUME OF REAGENT (µl)
assay cocktail	50
test compounds	25
ENZYME	25
Total volume	100
stopping solution	100
[^{14}C]cyclic GMP	50
Total volume	250

(C) After the reaction had been stopped [^{14}C]cyclic GMP (approximately 20,000 cpm per assay tube) was carefully added (50 µl).

(D) All reaction tubes were boiled for 3 to 5 minutes and then cooled to room temperature. This step solubilised all protein present in each sample.

(E) As in the adenylate cyclase assay, the procedure may be interrupted at this stage and the assay tubes stored for a few days at -25°C. Stored tubes were allowed to equilibrate to room temperature before use.

(F) Suitable blanks were prepared of no-enzyme (Tris-HCl buffer in place of the enzyme solution) and zero-time (addition of stopping solution before the addition of enzyme).

(G) Standards were also prepared according to the method reported for the adenylate cyclase assay, using [^{14}C]cyclic GMP and assay cocktail containing [^{32}P]GTP.

(6) Chromatography

To each sample was added HCl (1.0 ml, 50 mM). Each tube was vortex mixed and then decanted onto previously prepared Dowex 50 columns.

(A) Elution of GTP

When the sample had drained completely, a further portion of HCl (1.0 ml, 50 mM) was applied to each column and also allowed to drain. A further portion of HCl (2.0 ml). was applied and allowed to drain, after which H_2O (1.0 ml) was also allowed to drain through each column. The eluant from this procedure contained the majority of the GTP in each sample, and was appropriately disposed of since this also contained radioactive material.

The Dowex 50 columns were placed above alumina columns and H_2O (4.0 ml) was allowed to drain completely through both columns. This eluted the cyclic GMP from the Dowex onto the alumina columns, together with residual amounts of GTP and was discarded.

(B) Elution of Cyclic GMP

Imidazole-Cl buffer (4.0 ml, 0.1 M, pH7.3) was allowed to drain through each alumina column and was discarded. A further portion of the same buffer (3.0 ml) was passed through each column and collected directly into scintillation vials containing scintillant (18.0 ml), as this contained the eluted cyclic GMP (Fig. 2.4B).

(7) Discussion

Using this method of assay, recovery of [^{14}C] cyclic GMP was consistently high (60 to 80%) and Q vaues, although seemingly quite high (20 to 100) indicate a definite advantage over other methods where high blank values are routinely obtained. Blank values also varied with the batch of [^{32}P]GTP used, a not unexpected effect, since the Radiochemical Centre, Amersham, do not apply the purification techniques used for their [^{32}P]ATP to their [^{32}P]GTP product. A purification column could have been employed but the time and effort involved were deemed to be too extravagant with regards to the small advantage to be gained.

2.4 The Measurement of Alkylating Activity By The NBP Test

(1) Introduction

During the course of this work two assays utilising NBP (γ(p-nitrobenzyl)pyridine) to measure the alkylating activity of CP and its derivatives have been used.

Friedman and Boger's modification (226) of the NBP assay for alkylating agents (227) was first used but its limitations soon became apparent. This is the most widely used chemical method for the estimation of CP and its alkylating metabolites in biological systems, because of its high sensitivity and low background. However, according to this method biological samples containing CP are deproteinised by treatment with

hydrochloric acid and boiling. This step will also hydrolyse CP to nor-nitrogen mustard (nor-HN2) and is therefore a great disadvantage. The measurement of alkylating activity after initial acid hydrolysis has been referred to as total alkylating activity, potential alkylating activity, alkylating capacity and nor-mustard-like activity. This method also produces an unstable coloured compound which must be estimated at a precise time after mixing, making the assay rather tedious and difficult particularly since estimations must be made sequentially rather than simultaneously.

The modified method first published by Morita et al. (228) is a significant improvement. Perchloric acid (2%, v/v) replaces hydrochloric acid for deproteinisation of biological samples; trichloroacetic acid, which is often used for this purpose cannot be used because it would react with NBP. Only when boiled for 20 minutes in perchloric acid will CP hydrolyse to yield nor-HN2. If the sample is kept cold (i.e. in an ice bath) then no hydrolysis will take place in the presence of perchloric acid. This provides a method for assaying, under identical conditions, the alkylating activity of CP or its derivatives directly (before or without prior acid hydrolysis) and also the potential alkylating activity (after acid hydrolysis). Furthermore, the extraction of the coloured complex into ethylene dichloride instead of ethyl acetate greatly increases the stability of the ion. When also covered with NaOH, no loss of colour is detectable for up to 3 hours. This is to be compared to a value of less than three minutes in the original assay.

A further modification to this assay has been recently published (229) but no reference to the method of Morita et al. has been made, from whom the basic assay procedure is derived. The modifications include a higher concentration of NBP in the assay, the replacement of NaOH with triethyl-

amine and the elimination of the "ethyl acetate extraction of the pigment". This is claimed to give greater stability of the pigment complex and is also claimed to be more convenient. It is difficult to see, however, why the use of copious quantities of triethylamine could possibly be considered convenient. Although this method of alkalinisation has also been previously reported by Abel et al. (230) no clear advantage can be seen. The method of Morita et al. has been followed here.

(2) Experimental Procedure

Either biological samples or solutions of pure compounds were used in the NBP test.

(I) Biological samples

The sample was first diluted 1:1 with perchloric acid (4%, v/v). This was kept in an ice bath and precipitated the protein present in the sample. Centrifugation was used to remove the protein and aliquots of the supernatant fraction taken for analysis. Aliquots were diluted to a final volume of 2.0 ml in perchloric acid (2%, v/v).

(II) Pure compounds

Pure solutions of alkylating compounds were assayed by taking aliquots and diluting to 1.0 ml with H_2O. Perchloric acid (4%, v/v) was added to a final volume of 2.0 ml. A convenient method was to use 50 µl aliquots of a 2 or 3 mM solution when using CP and its derivatives. For construction of the standard curves, using nitrogen mustard (HN2) a 1 mM solution was sufficient.

(III) Two estimations were made using the same compound or sample : the direct and the potential alkylating activity.

(A) Potential Alkylating Activity

This was measured by first hydrolysing the alkylator in perchloric acid. This was done simply by placing the prepared sample, which was contained within a stoppered test tube, in a boiling water bath for 20 minutes. After this period, the sample was removed and cooled in an ice bath.

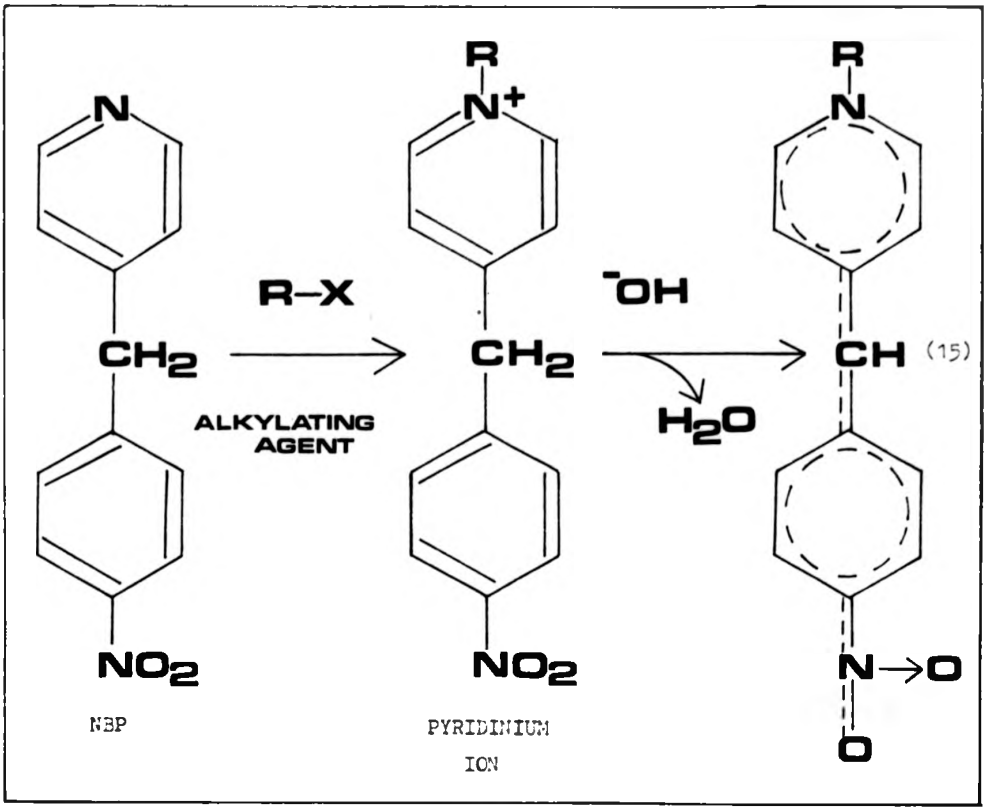
(B) Direct Alkylating Activity

This was measured by keeping the prepared sample in an ice bath ensuring that acid hydrolysis did not take place. Such samples were kept on ice while others were being acid hydrolysed.

(IV) When all samples were at ice bath temperatures ($\approx 4^{o}C$) the pH was adjusted to 4.6 by the addition of acetate buffer (pH4.6, 0.2 M)/NaOH (1N) mix (2.0 ml). This mixture had been adjusted previously so that the NaOH neutralised the perchloric acid in the sample. This was found to be best achieved by mixing 1 vol acetate buffer to 2 vol NaOH. This was not the relative proportion given by Morita et al.

(V) NBP solution (0.5 ml, 5% w/v in acetone) was added to each sample. Efficient mixing during the previous procedure (IV) was found to be essential to achieve the correct pH before addition of the NBP. During this addition the solution became an opaque white.

(VI) Each sample was next boiled for 20 minutes. This step brings about the reaction of NBP with the alkylating material to yield a pyridinium ion (reaction 15).



(VII) After boiling, the samples were cooled in ice,and acetone (2.0 ml) and ethylene dichloride (5.0 ml) added to each sample.

(VIII) NaOH (1N, 2.0 ml) was then added to each sample and the stoppered test tubes vigorously shaken. Under alkaline conditions such as these, the NBP complex (ionised) is deprotonated and the solution becomes coloured blue in the aqueous phase. On shaking, the coloured complex is extracted into the organic phase and becomes coloured pink, probably due to charge

transfer. At this stage, minimal light was allowed to reach the samples as although the colour is stable for up to 3 hours, in the dark, it was observed that the colour could be bleached by strong light, an observation not reported by Morita et al.

(IX) Samples were stored for at least 30 minutes in an ice bath and then centrifuged for 5 minutes at approximately 2500 g in a bench centrifuge. This step ensured the separation of the two phases.

(X) The upper aqueous layer was pipetted off carefully so as to not disturb the lower (pink) layer. The lower layer was then pipetted into a glass cuvette and overlaid with NaOH (1N). Careful pipetting was required as any mixing of the two layers resulted in an emulsion leading to spurious results.

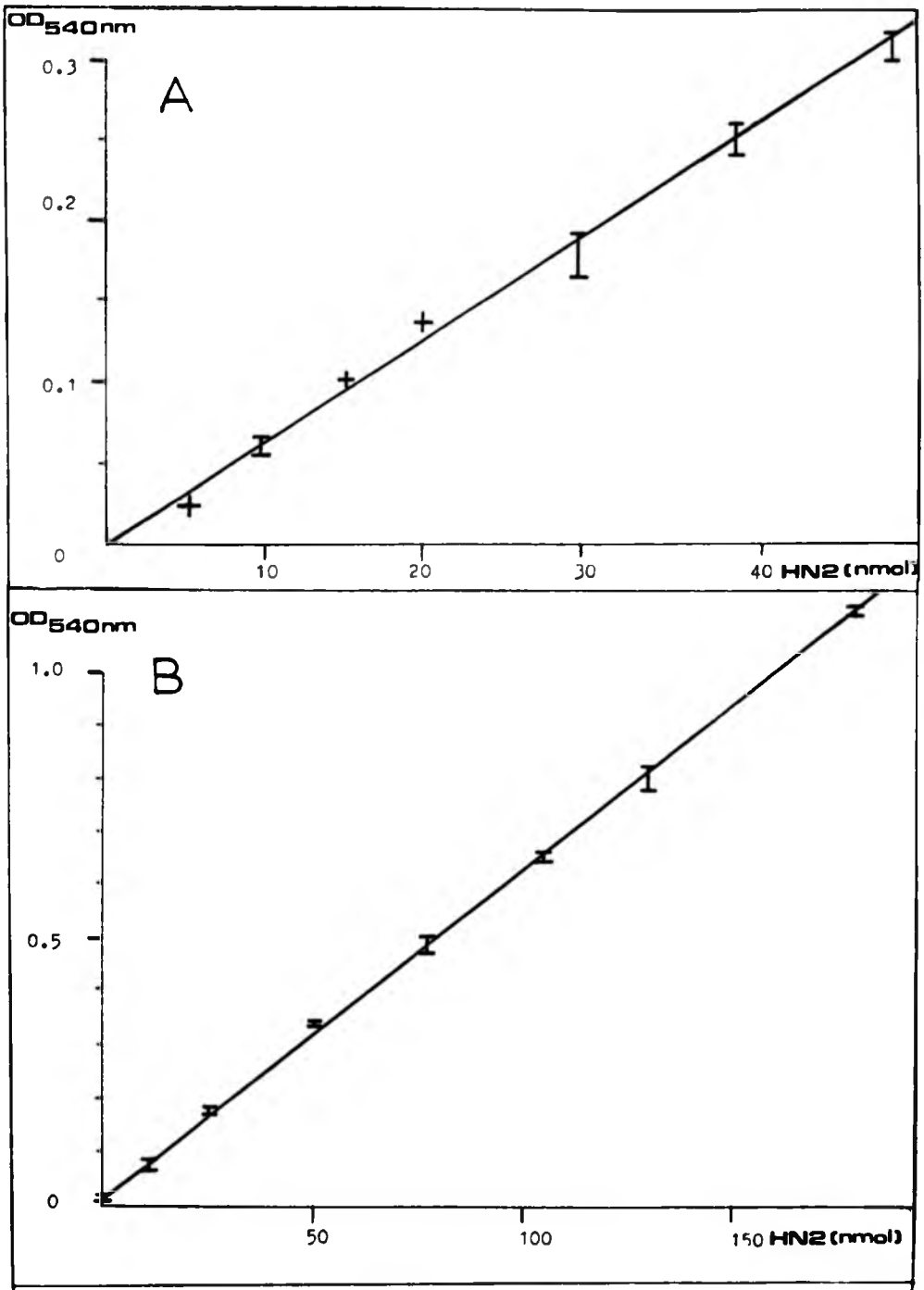
(XI) The absorbance maximum of the pink complex was measured to be 540 nm (HN2) and the sample O.D. was read at this wavelength.

(3) Standard Curve

HN2 was used to construct a standard curve using this procedure. The value obtained for alkylating activity of HN2 did not change due to acid hydrolysis and, therefore, the standard curve was constructed using the direct measurement (Fig. 2.5B).

A curve was also constructed using the method of Friedman and Boger and was found to be in good agreement with that constructed by the method of Morita et al. (Fig. 2.5A).

Fig. 2.5 Alkylating Activity Standard Curves for Nitrogen Mustard (HN2) Using NBP Test



A Method of Friedman & Boger, blank subtracted.

B Method of Morita *et al.* water used as blank.

Error bars represent minima and maxima from two separate experiments in each case.

2.5 The Ozonisation of Cyclophosphamide

(1) Introduction

Although other methods for the synthesis of activated derivatives of CP exist (231) (232) the direct ozonisation of CP was considered the simplest. Furthermore, the starting material (CP) was available in abundance.

The method of Peter *et al.* (233) produces two derivatives of CP, 4-ketocyclophosphamide (KP) and 4-hydroperoxycyclophosphamide (HP). KP is reported to be produced in about 50% yield and HP in about 40% yield. Many difficulties were encountered during the course of this synthesis but most of these were overcome by the detailed experimental procedure and useful advice given to us by Peter (234) and the later availability of HP to utilise as a standard, kindly donated by Colvin (section 2.1).

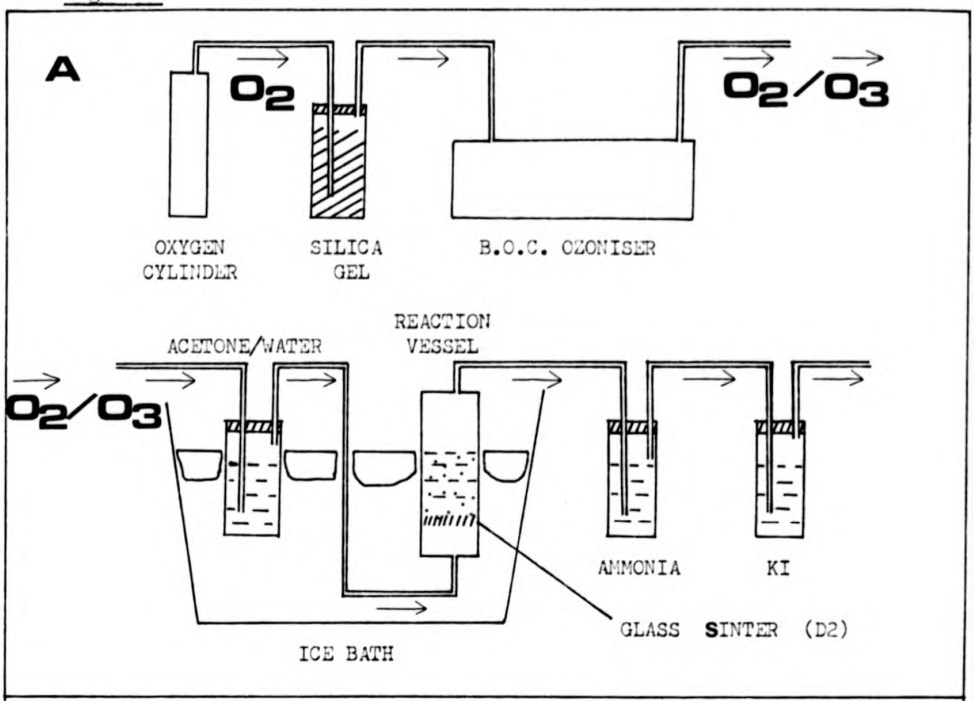
(2) Experimental Procedure

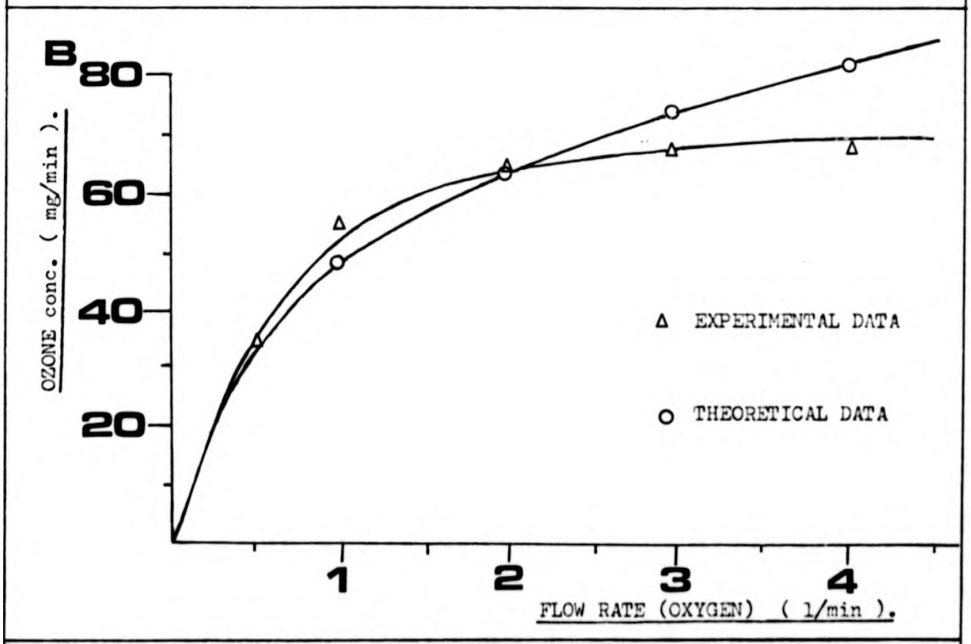
The apparatus required for this experiment is shown in Fig. 2.6A. This arrangement was arrived at after some experimentation.

(A) The Ozoniser

The ozoniser is a water-cooled device which produces a silent electric discharge through gaseous oxygen. This produces ozone (O_3) and the quantity of ozone produced can be controlled by varying the flow rate of oxygen through the device. The ozoniser was calibrated by constructing a standard curve of ozone output versus oxygen flow rate. This was done by placing acidic potassium iodide solution (5% w/v, 25 to 100 ml) in the reaction vessel and bubbling ozone through it for a measured length of time (usually judged so that the colour change observed was not too large). The iodine thus liberated was then estimated by back titration

Fig. 2.6





A The apparatus for the ozonisation of CP.

B The calibration curve for the BOC Mk II ozoniser.

Only 1 determination at each point was made.

with sodium thiosulphate (0.1 N) and the amount of ozone then calculated according to reaction 16.

$$\text{(i)}\quad O_3 + 2KI + H_2O \longrightarrow I_2 + 2KOH + O_2$$

$$\text{(ii)}\quad I_2 + 2NaS_2O_3 \longrightarrow 2NaI + Na_2S_4O_6 \qquad (16)$$

Thus for every mole of ozone, 2 moles of sodium thiosulphate will be used. Values of ozone (mg/ml) were calculated and plotted against the flow rate of oxygen (l/min) as registered on the built-in flow meter of the ozoniser (Fig. 2.6B).

This was compared to a similar curve constructed from the data supplied by the British Oxygen Company. The experimental curve demonstrates a saturation effect, probably due to ozone not being absorbed but passing through the reaction mixture at high flow rates. This had no relevance to the ozonisation, however, because the flow rate required was not on this portion of the curve. Flow rate was adjusted to give 45 mg/min of ozone (1 mmole O_3/min) or 0.8 l/min).

(B) <u>The Reaction Vessel</u>

Various reaction vessels were tried, all of which gave different values of ozone absorbed by a solution of potassium iodide within them. This was probably due to the fact that saturation of the solution was not occurring. The best vessel design was that described by Peter (234) and consisted of a glass column (21 cm x 3 cm int. dia.) with a sintered glass partition (D2). When oxygen was allowed to flow from underneath this partition, liquid could be poured into the column from above and would maintain its position above the partition. The sinter had the effect of

producing many fine bubbles of gas which percolated through the solution. Thus a large surface area was created at the gas/liquid interface.

(3) Ozonisation Of Cyclophosphamide

A scaled down version of the original synthesis (233) was used. CP (5.0 g), water (20 ml), acetone (10 ml) and hydrogen peroxide (3.3 ml) were placed in the reaction vessel while oxygen (low flow rate) was passed through it. This prevented any seepage of the reaction mixture through the sinter. The flow rate was then adjusted accordingly (to 0.8 l/min) and the reaction vessel and acetone/H_2O mixture (Fig. 2.6A) placed in an ice bath. The acetone/water mixture (1:2) was found to be necessary to prevent the precipitation of CP in the reaction vessel due to the evaporation of solvent.

The ozoniser was then turned on and ozone left to percolate through the reaction mixture for 2.5 hours. This time was found to be optimal by taking samples every 30 minutes for 6 hours and running thin layer chromatograms (section 4.2). Before being allowed to enter the fume cupboard, the gaseous extract was first allowed to pass through an ammonia solution to deactivate any alkylating material should any have been carried away from the reaction vessel and secondly through a solution of potassium iodide to remove any remaining ozone. After ozonisation oxygen was allowed to flow through the apparatus to purge the system of any ozone.

(4) Extraction of Reaction Products

After ozonisation, a precipitate of KP and some CP was removed by filtration from the reaction mixture, and acetone removed from solution by rotary evaporation at $0^{o}C$. It was essential to keep the reaction mixture cold to prevent the thermal decomposition of HP.

The mixture was left to stand in ice for 2 hours, after which a further precipitate of KP was removed by filtration. The filtrate was extracted with dichloromethane (4 x 50 ml) and the organic layer dried by the addition of molecular sieve. The organic phase was concentrated to an oily residue, under cooling with ice/water, by rotary evaporation.

Dichloromethane (8.4 ml) was added and the solution left to stand overnight at -25°C. At this stage most of the remaining KP was crystallised out and was removed from the solution by filtration. The filtrate was again concentrated to an oily residue, after the KP had been washed with dichloromethane and the washings added to the filtrate. The residue was redissolved in dichloromethane (0.5 ml) and portionwise addition of diethylether (5.0 ml) should have precipitated HP. However, the precipitation of HP from the reaction mixture is extremely difficult and it was found that the addition of diethylether served to precipitate any remaining KP in the solution. When this was removed by filtration, the remaining filtrate was only slightly contaminated with KP as determined by TLC visualised with NBP spray (section 2.14).

Chloroform extractions were performed on the filtrate and the extracts combined together and reduced to about 10 ml by rotary evaporation under ice cooling. This sample was used for column chromatography during which HP was purified.

(5) Column Chromatography

A medium pressure column chromatographic technique was employed to purify HP, which has been termed "flash chromatography" (235). A kieselgel 60 column (1" x 6") was used with ethyl acetate as eluant. This eluant was determined by prior TLC in several solvent systems. The column was pressurised using nitrogen and a flow rate of 1-2" of solvent

through the column/minute was used (approximately 20 to 40 ml/min). All chromatographic work was undertaken in a cold room (≈ 4°C).

Fractions (10 ml) were collected after application of the chloroform extract (5.0 ml). Fractions containing HP (determined by TLC) were combined and the solvent removed by rotary evaporation.

2.6 Incubation of Cyclophosphamide With Rat Hepatic Microsomes

(1) Introduction

A similar system to that described by Cohen and Jao (146), Sladek (148), and Connors *et al.* (151) was used to produce OHCP from CP by incubation of CP with rat liver microsomes. Due to the instability of OHCP, an ethoxy-CP derivative is produced during the extraction from the medium.

(2) Experimental Procedure

The incubation conditions were as described in Table 2.5.

To the substrate (0.2 ml) in a 25 ml conical flask (37°C, shaking water bath), the incubation mixture (previously kept in an ice bath) was added in three portions (i) 3 ml (ii) 3 ml (iii) 4 ml at 15 min intervals, with oxygen blown over the surface of the incubating mixture continuously. The total incubation time used was 45 min and the reaction was stopped at this time by the addition of ethanol (40 ml).

(3) Extraction of Metabolites

The addition of ethanol had two effects: (i) it precipitated the protein present in the sample, (ii) it reacted via any OHCP present to produce the 4-ethoxy-CP derivative. After the ethanol had been added, the protein was removed by centrifugation.

The supernatant fraction was reduced to 1 to 2 ml by rotary evaporation using an ice bath for cooling. The pH was adjusted to 4.0

TABLE 2.5 Additions to the microsomal incubation system for CP activation

ADDITION	CONCENTRATION
microsomes	3 ml (= 3g liver)
$NADP^+$	2.7 µmoles
Glucose 6-phosphate	55 "
$MgCl_2.6H_2O$	49.5 "
G 6-P dehydrogenase	3.5 units
Tris-HCl (0.1M, pH7.4)	7.0 ml
CP	10 µmoles

by the addition of HCl (1N) and extraction accomplished using alcohol-free chloroform (3 x 20 ml). This extract was dried using molecular sieve, and again reduced by rotary evaporation and subjected to chemical analyses primarily by NBP test (Chapter 6).

2.7 The Assay of Phosphodiesterase Activity

The method of Butcher and Sutherland (236) was used to measure the activity of cyclic AMP phosphodiesterase. This was carried out at 25°C for 30 min the latter 10 min in the presence of excess Cobra snake venom, which produces adenosine and phosphate from the 5'-AMP product of the phosphodiesterase reaction. The phosphate thus produced was assayed by the method of Fiske and Subbarow (237).

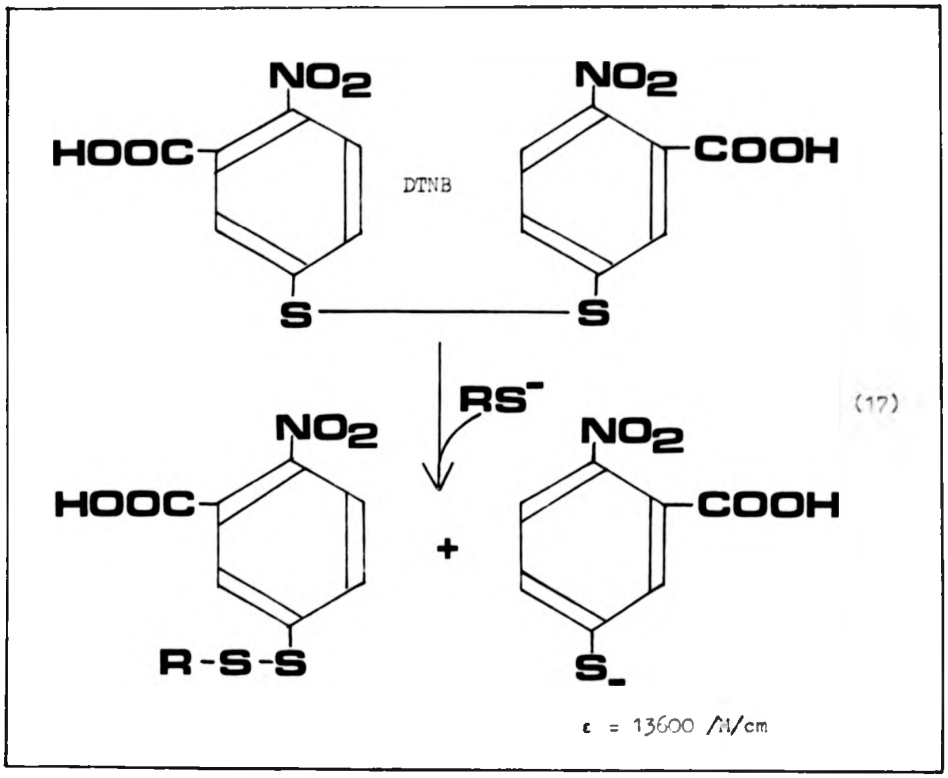
2.8 Protein Determination

The method used to determine protein concentrations in all samples was that of Lowry *et al.* (238). Colour produced is the result of : (i) biuret reaction of protein with copper ions in alkali and (ii) reduction of the phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein.

BSA (lyophilised) was used as standard and the standard curve obtained was shown to be linear with respect to BSA concentration (100 μg = 0.305 O.D.). The intensity of the blue colour was read at 680 nm and sensitivity was found to be 5 to 100 μg protein. Sample dilutions were manipulated to give a reading in the midrange of the standard curve (30 to 70 μg) before assignment of unknown protein concentrations were made.

2.9 The Assay of Free Thiol Groups By 5-5'-dithiobis(2-nitrobenzoic acid)

The method of Ellman was used to estimate thiol group concentrations (239). This involved the reduction of 5-5'-dithiobis(2-nitrobenzoic acid) by thiolate anion to yield a coloured anion (reaction 17).



Colour intensity was read at 412 nm.

Ellman's reagent was prepared in phosphate buffer (0.1 M, pH7) to a concentration of 10 mM. Tubes were prepared containing buffer mixture (H_2O : phosphate buffer, 0.1 M, pH8.0 = 4:1, 3.0 ml) Ellman's reagent (20 µl).

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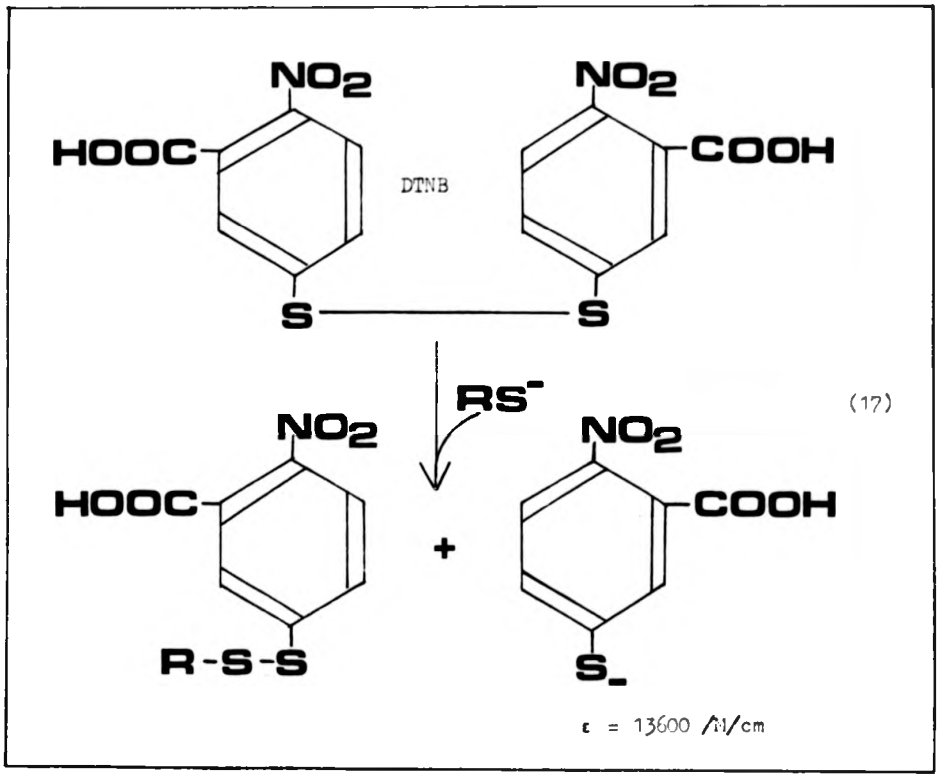


Colour intensity was read at 412 nm.

Ellman's reagent was prepared in phosphate buffer (0.1 M, pH7) to a concentration of 10 mM. Tubes were prepared containing buffer mixture (H_2O : phosphate buffer, 0.1 M, pH8.0 = 4:1, 3.0 ml) Ellman's reagent (20 µl).

CP or derivatives were incubated at 1 to 10 mM concentration with L-cysteine (10 mM) in phosphate buffer (0.1 M, pH7.0) under appropriate conditions (Chapter 9). Aliquots (50 µl) were taken at various times after the addition of compounds (to start the incubation) and added to the prepared tubes. These were usually read immediately.

Using cysteine as a standard, this assay was found to be linear with respect to concentration, between 0 and 500 nmoles (gradient = 1.33 O.D. units/µmole).

2.10 The Isolation of Rat Liver Plasma Membranes

The method of Pohl *et al.* (80) is a modification of the method of Neville (240) for producing a plasma membrane fraction from rat liver. This was used initially but found to be very difficult due to various ambiguities in the reported method. Plasma membranes were produced by this method and found to have low activities of associated marker enzymes (section 7.2). Therefore, another method, combining that of Pohl *et al.* and Emmelot *et al.* (241) was used and this method is described here.

(1) Six rats were killed by cervical dislocation and their livers removed and placed immediately in ice-cold NaCl (0.85% w/v). Typical liver weight of combined livers was 40 to 60 g.

(2) The livers were finely chopped using scissors and then gently homogenised (loose-fitting 0.6 mm teflon pestle and glass homogeniser) in approximately 8 to 10 g fractions of liver in bicarbonate buffer (30 ml, 1 mM). This was achieved by delivering 6 to 8 passes of the pestle by hand. All homogenates were pooled and made up to 800 ml with bicarbonate buffer.

(3) The homogenate was then filtered through (a) 2 layers of cheesecloth and then (b) 4 layers of cheesecloth.

(4) The filtrate was centrifuged at 500 g for 5 min and then slowly accelerated to 1,500 g which was maintained for a further 10 min. (Glass centrifuge bottles of 250 ml capacity were used in a Mistral centrifuge).

(5) The upper white layer of fat was removed. The supernatant fraction was carefully removed by pipetting. The remaining pellet of cell debris, plasma membranes and nuclear material was resuspended in 800 ml of bicarbonate buffer and re-centrifuged, following the procedure described in (4) above.

(6) Two layers were formed in the pelleted sediment which consisted of :

(i) tightly packed cell debris, etc., in a red lower layer, and

(ii) loosely packed plasma membranes in a light-tan upper layer.

(A) Most of the supernatant was pipetted off carefully and bicarbonate buffer (3.0 ml) added to each centrifuge bottle.

(B) Using a bent (90°) glass rod, the plasma membrane layer was very carefully resuspended.

(C) The resuspended plasma membranes were carefully pipetted off, leaving the pellet of nuclear and cell debris. The plasma membrane fractions were combined.

(7) The plasma membranes were re-homogenised and made up to 500 ml with bicarbonate buffer.

(8) This homogenate was centrifuged at 1,000 g for 10 min (2 x 250 ml glass bottles).

(9) After discarding the turbid supernatant, the pellet was resuspended in bicarbonate buffer (50 ml) and again centrifuged at 1,000 g for 10 min (SS34 rotor, Sorval).

(10) The pellet formed was resuspended in a small amount of bicarbonate buffer and re-homogenised. This was then added to sucrose (62 ml,

69 ± 0.1%, w/v) in a 250 ml measuring cylinder and made up to 110 ml by the addition of H_2O. The resulting solution was mixed thoroughly and adjusted to 44 ± 0.1% (w/v) sucrose by the addition of either sucrose or H_2O. This was measured accurately using a refractometer.

(11) This was distributed between 3 centrifuge tubes of a swing out rotor (25.2). Sucrose (42.3 ± 0.1% w/v) was then carefully layered on top, forming a discontinuous sucrose density gradient.

(12) The gradient was centrifuged at 107,000 g (25,000 rpm), for 150 min (L2-50 centrifuge).

(13) A small pellet was formed in each tube together with a 'float'. This floating material was carefully removed and resuspended in bicarbonate buffer by passing through a #20 guage needle (10 ml).

(14) The combined fractions were centrifuged at 12,500 rpm for 20 min in an SS34 rotor.

(15) The pellet thus formed was resuspended (by needle aspiration as in (13)) in bicarbonate buffer (3.0 ml).

(16) Aliquots (100 µl) of this suspension were stored under liquid nitrogen. These were thawed prior to their use in adenylate cyclase assays and correspond to 'crude' (not 'purified') plasma membranes.

2.11 The Isolation of Rat Liver Cytosol

A simple rat liver cytosolic fraction was prepared for experiments with guanylate cyclase.

Rat liver (prepared as in step (1), section 2.10, using only 1 rat) was homogenised in Tris-HCl (5 mM, pH7.6), and sucrose (0.25 M) then centrifuged at 750 g for 10 min. The supernatant fraction was re-centrifuged at 60,000 g (30 rotor, 26,000 rpm) for 60 min. Aliquots (200 µl) were stored under liquid nitrogen or -80°C. These were thawed prior to their use in guanylate cyclase assays.

2.12 Preparation of Rat Liver Microsomes

Male Wistar rats were fed phenobarbital (0.5 g/l) *ad libitum* in their drinking water for 10 days prior to the preparation of microsomes.

Microsomes were prepared by the method of Connors *et al.* (151). Centrifugation and initial homogenisation were performed in KCl (0.15 M) with the microsomal pellet being 'washed' at least once (i.e. with at least two high-speed centrfiguations). Final resuspension of the microsomal pellet was in Tris-HCl (0.2 M, pH7.4) to a dilution of 1 g wet weight liver per ml. Microsomes were used immediately for incubation experiments with CP.

2.13 Scintillation Counting

(1) A toluene-Triton X-100 scintillant was used containing both primary and secondary scintillators for maximum efficiency. These were PPO (5.5 g/l) and dimethylPOPOP (0.15 g/l) respectively. The emulsifying agent Triton X-100 was used to produce a stable emulsion when samples from cyclase assays were added to scintillant. The efficiency and reproducibility of counting relies on the production of such a stable emulsion and this in turn is affected by (i) sample size, (ii) salt concentration, (iii) temperature and (iv) the ratio of toluene : Triton X-100.

Sample size was usually 3.0 ml in all cases and contained imidazole-Cl buffer (0.1 M, pH7.3). The scintillation counter was refrigerated to reduce background noise from its photomultipliers and so the toluene : Triton X-100 ratio was adjusted to allow for all these factors. A 1:1 ratio (18 ml) was found to be necessary to maintain a stable emulsion. Any less than this resulted in a stable emulsion at room temperature but a two-phase system resulted when such a scintillant was placed in the cool environment of the counter (using toluene : Triton X-100 = 2:1).

(2) Using a gain of 75 (10% gain selected) the window settings of the scintillation counter were investigated throughout their range to find the best possible settings for dual isotope counting [^{32}P] and [^{14}C] of the quenched (3.0 ml imidazole-Cl buffer) samples. By selecting a window of 0 - 900, all counts due to [^{14}C] could be collected. A second window was selected 900 - ∞ within which no counts due to [^{14}C] could be detected and which could be used to measure counts due to [^{32}P]. These have been referred to as the [^{14}C] channel and the [^{32}P] channel respectively.

2.14 Thin Layer Chromatography (TLC)

(A) Production of TLC Plates

Plates for TLC (2" x 8") were produced by mixing silica gel 60 (f254) with water (30 g in 60 ml) and spread using a layer thickness of 0.02 mm after degassing the solution in a closed vessel connected to a water-powered vacuum pump. After heating to 100°C for 1 hour, the plates were cooled and stored ready for use.

Various solvent systems have been used and wherever possible, the sample was applied in the particular solvent system. Standards were run when available, the importance of this must be stressed in the case of cyclophosphamide and its derivatives since the presence of water vapour can drastically influence the Rf values obtained (234).

(B) Visualisation

Visualisation of CP and related compounds was achieved primarily by NBP reagent spray. For the hydroperoxy derivative, a peroxide spray was also employed. Iodine vapour was also used for all TLC plates before applying a spray reagent (the plates being left to decolourise before spraying). This was done by placing the TLC plate in a tank containing

solid iodine and pre-saturated with vapour. This visualised nitrogen-containing compounds.

(I) NBP Spray

This was used to visualise any alkylating material on the TLC plate. The plate was first sprayed with a solution of ethanolic NBP (5%, w/v). After heating to 100°C for 5 min (to visualise good alkylating agents) or 15 to 20 min (to visualise weakly alkylating agents) and subsequent cooling to room temperature, the plate was sprayed with ethanolic KOH (3% w/v). This method produced a temporary blue spot where alkylating material was present (157).

(II) Peroxide Spray

This spray visualised any peroxide present (263).

Spray Reagent I

KI solution (10 ml, 4%, aqueous), was mixed with acetic acid (40 ml) and a pinch of zinc powder added.

Spray Reagent II

Fresh starch solution (1%).

The zinc was filtered off and the TLC plate sprayed with solution I. Peroxides appeared as yellow spots due to iodine liberated, within 5 minutes. For better visualisation, solution II was sprayed onto the treated plate, which yielded blue spots.

2.15 The Photooxidation of Cyclophosphamide By Polymer-bound Rose Bengal

(A) Introduction

There is evidence that hydroxylation of CP by rat liver microsomes involves singlet oxygen. The ozonisation of CP to produce HP is also considered to involve this species. Sources of singlet oxygen are available for chemistry, and its photosensitised formation using various dyestuffs is the method of choice for most synthetic and mechanistic applications. The use of such systems to produce an activated CP derivative was suggested to us by Jarman (Dr M. Jarman, Chester Beatty Research Institute, London).

Sensitox is a polymer-bound Rose Bengal which is a well known UV sensitiser for the formation of singlet oxygen from the gaseous element. Sensitox II is especially developed for use in aqueous solvent systems. Immobilised Rose Bengal has been used successfully in the photooxidation of various aliphatic saturated and unsaturated compounds.

(B) Experimental Procedure

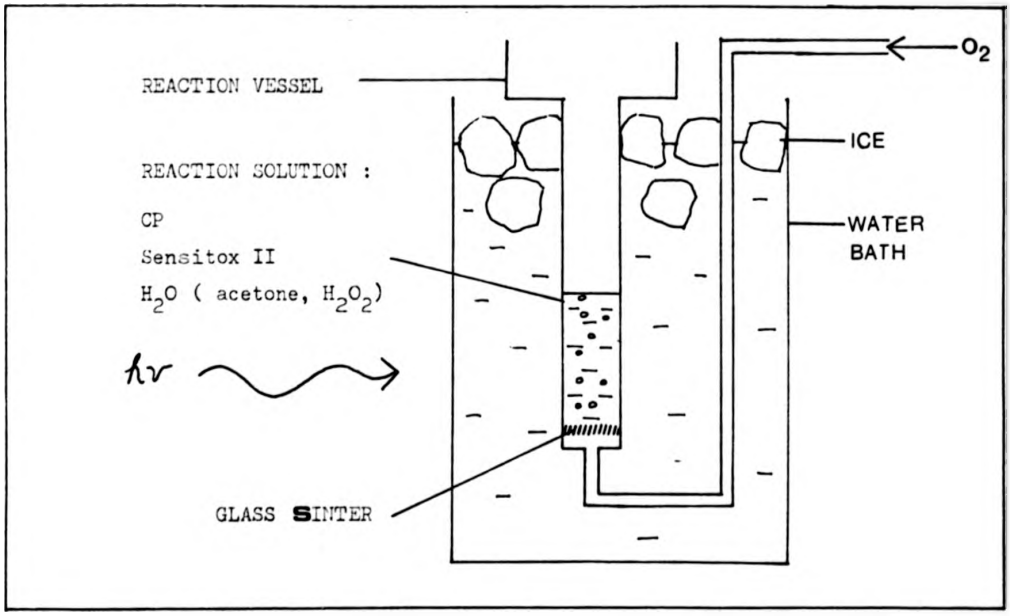
CP (60 mg) and Sensitox II (30 mg) were incubated in either H_2O (10 ml) or a H_2O/acetone (7:3) mixture in the presence or absence of H_2O_2 (0.3 ml) under oxygen aspiration as shown in Fig. 2.7.

The apparatus was immersed in an ice/water bath and illuminated for up to 6 hours with a concentrated 100W white light source. Oxygen aspiration maintained saturation of the solution and prevented the sedimentation of the Sensitox II polymer beads.

Periodically, aliquots were taken, centrifuged (bench centrifuge, 3 minutes) to remove the Sensitox II and analysed by TLC (silica gel 60, ethyl acetate) NBP test (section 2.4) or HPLC (section 2.1).

The experiment was also carried out at both low (5° to $15^{\circ}C$) and medium temperatures (30° to $35^{\circ}C$).

Fig. 2.7 The Apparatus Used To Investigate The Photooxidation of CP By Polymer-bound Rose Bengal (Sensitox II)



2.16 Safety Precautions

(1) Radioactivity

For all work involving the use of radioactive substances, the usual precautions were taken. These included working in gloves and within designated bench space, including trays, wherever possible. All cyclase assay columns were run within a large tray in case of spillage. For work involving the use of [^{32}P] nuclide, perspex screens were used which were very effective at stopping the β^- particles emitted, as determined by the use of a radioactivity monitor.

(2) Hazardous Chemicals

Many of the alkylating substances used are classified as carcinogens (HN2, HP, PM) or potential carcinogens (CP) and care was taken when using all such compounds. That such care is necessary is indicated by the study of Falck *et al.* (242). In hospitals where patients were treated with anti-cancer agents, including cyclophosphamide, not only did the urine concentrates of patients exhibit mutagenicity in an Ames test but their nurses also showed this effect.

Cyclophosphamide and 'inactive' derivatives (i.e. KP) were handled within a fume cupboard with the fans switched off. This was to prevent ingestion of any air-borne chemical.

More hazardous chemicals (HN2, PM, MNNG) were handled within a glove box. The glove box was not pressurised during use and was flushed with nitrogen, being passed through an ammonia solution (to deactivate any alkylating material) before being allowed to vent into a fume extractor after use.

All apparatus, including pipette tips, were washed in an ammonia solution before disposal or reuse. A solution of sodium thiosulphate (2M) was kept near working areas should a skin wash be necessary after an accidental spillage.

(3) Ozonisation

The ozonisation was carried out in a fume cupboard within the "dangerous operations laboratory". The entire apparatus was kept behind a screen of armour plate glass due to the explosion hazard of using ozone. Rubber tubing was avoided because of the possibility of producing explosive ozonides. All ozone was flushed from all apparatus before anyone was allowed to enter the cupboard, by flushing with oxygen (by turning off the ozoniser) for at least 10 minutes after ozonisation.

RESULTS

CHAPTER 3

THE ASSAY FOR ALKYLATING ACTIVITY BY THE NBP TEST

3.1 Introduction

The NBP test (section 2.4) is a useful analytical assay for alkylating agents. It may be used to measure the stability of a stored alkylating agent and may also help to determine which alkylating agent is present in an unknown solution by the different behaviour of an alkylating agent in the NBP test before and after acid hydrolysis.

Because of their high reactivities HN2 and nor-HN2 have been used as standards by many workers in this field.

3.2 Results and Discussion

The optical densities of the NBP complexes with various alkylating agents are shown in Table 3.1, together with HN2 equivalents as determined from the standard curve, Fig. 2.5B. The alkylation index was determined by expressing the direct reading as a percentage of the acid-hydrolysis reading (Direct/Acid-hydrolysis X 100).

Acid hydrolysis of CP derivatives is considered to cleave the molecule at the P-N bond (exocyclic) thus releasing nor-HN2 and explains the dramatic increase in alkylating activity observed in the NBP test for such compounds after acid hydrolysis (Table 3.1).

As can be seen from these results, only phosphoramide mustard and 4-hydroperoxy-CP yield values in HN2 equivalents after acid-hydrolysis, which are directly related to their true concentrations. All other compounds tested gave a much lower reading than might at first be expected.

TABLE 3.1 NBP test results for CP and derivatives

COMPOUND	λ_{max}	DIRECT READING					ACID HYDROLYSIS READING					ALKYLATION INDEX		ALKYLATING[ϕ] ACTIVITY
		O.D. 540 nm	(±)	HN2 equivalents (nmoles)	(±)	%	O.D. 540 nm	(±)	HN2 equivalents (nmoles)	(±)	%		(±)	(%)
HN2	540	0.915	0.010	148	2	100	0.935	0.015	151	3	100	97.86	2.6	100
CP	550	0.054	0.007	7	2	4	0.787	0.060	126	7	84	6.86	1.4	1.3
KP	552	0.046	0.002	6	2	4	0.710	0.050	114	6	76	6.47	0.7	1.2
PM	555	0.756	0.005	121.5	1	82	0.905	0.005	146	6	97	83.60	1.0	90
HP (acetone)		0.267	0.017	42	2	42	0.645	0.025	103	4	103	39.85	2.5	40
HP (H_2O)		0.340	0.014	54	2	54	0.517	0.065	83	8	83	66.30	1.1	-
OHCP[ϕ]		-	-	-	-	-	-	-	-	-	-	-	-	65

All measurements were made using 150 nmoles, except HP where 100 nmoles were used. % express HN2 equivalents compared to actual concentrations. All results are from 3 separate experiments, performed in triplicate. Blank values not subtracted. ϕ Data taken from ref. (172) included for comparison.

This can be explained partly by each complex possessing a slightly different molar absorbance and partly by inter-molecular P-N bond cleavage as postulated by Freidman et al. (143).

The wavelength of maximum absorbance (λ_{max}) was measured (Table 3.1) and found to vary according to the compound used, from 540 nm (HN2) to 554 nm (PM). This could have introduced a maximum error of 1.54% as the absorbance of PM measured at 540 nm (which was used for all regular alkylating assays) was found to be 98.46% of that at its λ_{max}.

CP hydrolysis at high temperatures, even in strong acid, involves competition between P-N bond cleavage leading to relase of nor-HN2 and internal alkylation leading to a series of products from which nor-HN2 cannot be released. This competition would account for the observed loss of recoverable nor-HN2 and explains the less than theoretical extent of alkylation observed when phosphoramide mustards are allowed to react with NBP. However, nothing like the reported extent could be observed.

Freidman et al. have reported that only 40% of the theoretical amount of nor-HN2 can be observed for CP after acid hydrolysis. This is clearly not the case. Morita et al. (228) have reported a value of 90% using this method of assay and as can be seen from Table 3.1, I have obtained a value of 84% relative to HN2, for CP.

This discrepancy between the measured concentration after acid hydrolysis and the true concentration in the sample would make it difficult to determine the concentrations of alkylating agents in unknown solutions accurately. This difficulty could be overcome by plotting a standard curve for each compound or by applying a correction factor, calculated from these results. However, the alkylating agent would have to be identified first and for this the direct reading is required. The direct reading is an

indication of how reactive the alkylating agent actually is, and therefore (as an analytical probe) which alkylating agent it may be. To determine this precisely, the concentration of the sample would have to be determined and the appropriate correction factor for absorbance applied.

This apparent paradox can be overcome by the use of an "alkylation index", which by dividing the direct by the acid-hydrolysis reading, applies the appropriate correction factor, and, hence, the precise concentration of alkylating agent in a solution need not be determined in order to identify it. As can be seen from Table 3.1, the values for the alkylation index correlate well with values of alkylating activity reported by Brock (172) although it was not explained how he arrived at these values. It has been assumed that they were calculated similarly to the alkylation index presented here.

It has been observed in these experiments that the initially blue alkylating agent - NBP complex formed in the aqueous layer does not always extract fully into the organic phase. The extent of extraction appears to be dependent upon the compound used. This phenomenon has been observed previously (170). The blue (aqueous phase) complex was found to have a λ_{max} of 642 nm and a small peak was observed, separate from the major absorbance maximum at 540 nm, in the extracted organic phase. The nature of this and its effect on the estimation of alkylating activity by NBP test was not determined.

An interesting effect was the different alkylation indices measured with HP, depending upon the solvent used to dissolve it prior to its estimation. When dissolved in acetone and assayed with no waiting period whilst added to aqueous solutions (section 2.4(2)iii) the alkylation index measured corresponded to the reported alkylating activity of HP. If, however, the sample of HP was first dissolved in water by gentle warming,

the alkylation index corresponded to the alkylating activity reported for OHCP. This was found to occur immediately and the alkylation index remained unchanged for up to 30 minutes during the incubation of aqueous HP solution in an ice bath. The spontaneous production of OHCP from HP in aqueous solution was suggested by Fenselau (243) and a postulated mechanism has been published. This was, therefore a very convenient method for the production of OHCP from HP and has been discussed in more detail in Chapter 10.

It should be pointed out that although the NBP test has been shown to be a useful tool for the determination of alkylating agents in solution, the results obtained may not reflect the alkylating activity of alkylating agents under physiological conditions, such as those found in *in vitro* assay systems for enzymic activity and *in vivo*.

CHAPTER 4

THE OZONISATION OF CYCLOPHOSPHAMIDE : THE PRODUCTION OF 4-HYDROPEROXY-CP (HP) AND 4-KETO-CP (KP)

4.1 Introduction

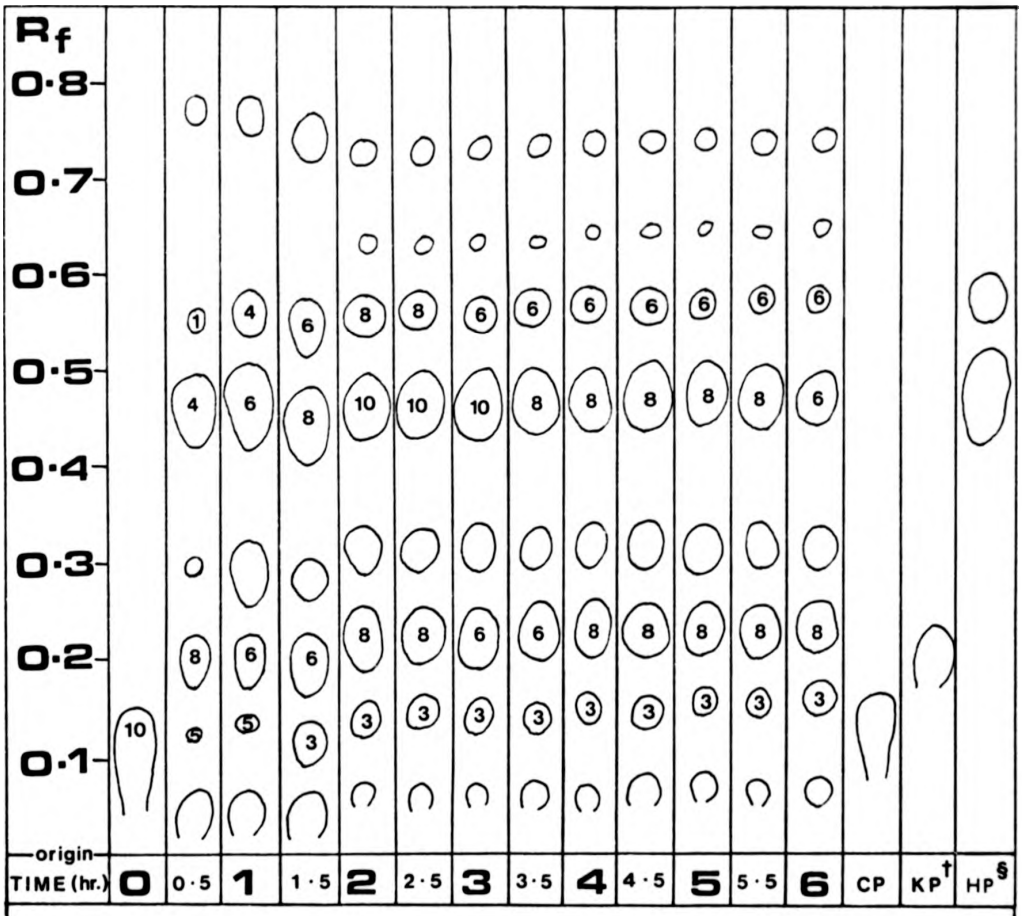
After many initial difficulties, the ozonisation of CP was found to be the most successful of the methods tried to produce an 'activated' derivative of CP. As an additional "bonus" the reaction also yields KP, the isolation of which proved to be very simple compared with that of HP. The method has been described in detail in section 2.5.

RESULTS AND DISCUSSION

4.2 The Time Course Of The Ozonisation

The ozonisation was "scaled down" from the reported procedure (5 g rather than 30 g) and it was not known whether the time required for ozonisation would have to be reduced in order to produce the maximum amount of HP. For this reason, the ozonisation was carried out for 6 hours (as reported originally by Peter *et al.* (233)) and samples were taken every 30 minutes for analysis. These samples were subjected to thin layer chromatography (TLC) after drying under air (in an ice bath) and re-dissolving in the TLC system solvent (ethyl acetate). This was done primarily to remove water which markedly affects the performance of CP and derivatives in the TLC systems leading to spurious RF values (234). The results of the TLC are shown in Fig. 4.1. As well as Rf values, the relative intensity of the spots was also recorded. For reasons discussed in Chapter 3 the intensity of the blue complexes formed with NBP cannot

Fig. 4.1 TLC Results of Samples Taken During The Ozonisation of CP



TLC was run on silica gel 60 in an ethyl acetate solvent system. Visualisation was with NBP spray, incubated for 20 minutes, at 100°C. The TLC was run at -25°C. Numbers represent the relative intensities of the spots as judged by eye. § HP sample was a gift from Colvin (section 2.1). † KP sample was from a previous ozonation experiment.

be used to distinguish the relative concentrations between different compounds. It should also be noted that a positive reaction to NBP on silica gel does not correspond to alkylating activity in solution (161). However, this method may be used to estimate the relative concentrations of the same alkylating agent in different samples. Standards were run also and were the only method employed to distinguish the relevant CP derivatives as Rf values differed greatly from those published by Peter et al. (233). The HP standard yielded two spots : since the CP molecule can have the hydroperoxy group attached to it in two positions on the C4 atom, this carbon atom becomes a chiral centre and two diastereoisomers of HP are produced.

The TLC shows that ozonisation was quite rapid : many alkylating agents were produced within the first 30 minutes. The number and type of alkylating agent produced appears to remain constant after 2 hours, and no change in Rf values or the number of spots was observed after this time. There was, however, a change in the relative concentrations of the compounds of interest. CP appeared to decrease over the period of ozonisation but did not disappear completely, even after 6 hours. A decrease was expected since CP is the precursor of the derivatives produced but its persistence was puzzling. KP appeared in quite reasonable yield in all samples and toward the latter half of the ozonisation procedure was found as a precipitate in the reaction mixture. This was confirmed by TLC which showed that KP together with some CP was precipitated from the solution after 3 hours ozonisation. The concentration of HP, however, could be seen to increase to a maximum after 2.5 hours ozonisation after which its concentration decreased. This pattern was followed by both stereoisomers. An interesting observation, however, was that the relative concentration of the fast-

running isomer appeared to be less than that of the slow-running one. This was also the case with the HP sample supplied by Colvin and which was utilised as a standard. The relative concentration was judged by the relative intensity of the spots and also by the fact that the slow-running isomer produced a much larger spot.

4.3 NBP Test

An NBP assay was also run on the samples taken at 1 hour intervals during ozonisation. The results are presented in Table 4.1.

(1) Optical Density (O.D)

The sample size for the acid hydrolysis measurement was smaller than that used for the direct measurement. For this reason, a correction factor was applied to the O.D for acid hydrolysis and these are therefore larger than actually measured spectrophotometrically.

(2) Alkylation Index

It is apparent from these results that the alkylation index is approaching that of 4-hydroxy-CP (OHCP) rather than that of HP. The reason for this is the conversion of HP to OHCP in aqueous solution during the NBP assay. This occurred during the 20 minute period whilst waiting for acid hydrolysis to take place in other samples (section 2.4 and Chapter 3) and the direct measurement samples were left to stand in an ice bath.

(3) Concentration Of Alkylating Agents In The Reaction Mixture

The concentration alkylating agents has been calculated from the acid hydrolysis readings. As can be seen from Table 3.1, CP and OHCP (HP in aqueous solution) both gave the same value relative to HN2 (and therefore their concentration) after acid hydrolysis. KP, however, gave only

TABLE 4.1 NBP test on samples taken during the ozonisation of CP

TIME (hr)	NBP TEST O.D. 540nm DIRECT	ACID	CONCENTRATION (%)	ALKYLATION INDEX	% CONVERSION
0	0.14	2.2	100	6.36	- 0.51
1	0.60	2.2	100	27.20	35.20
2	0.66	2.2	100	30.00	40.00
3	0.80	2.1	95	38.09	51.00
4	0.70	1.3	59	53.80	47.60
5	0.75	1.1	50	57.69	43.70
6	0.79	1.1	50	60.70	46.30

90.4% of this value and this effect could not be taken into account in these calculations, resulting in a maximum error of 4.7%, as only 50% of the starting material was expected to yield KP.

The concentration of alkylating agents in the reaction solution has been shown to decrease markedly after 3 hours, due to the precipitation of KP and some CP.

(4) % Conversion Of CP to HP

The alkylation indices of CP and KP were virtually identical (Table 3.1). It has been assumed therefore, for the purposes of this estimation, that only two alkylating indices contributed to the index measured in samples from the reaction mixture. (i) that of CP and KP and (ii) that of HP. The fact that many other unidentified alkylating substances were present, as shown by TLC, Fig. 4.1, has been ignored, although these may have contributed up to 10% of the reaction mixture composition and their effect on the alkylating assay unknown, the values for % conversion should therefore be read as a guide rather than in absolute terms.

The following equations were used to calculate conversion :

let x = concentration of CP (and KP)

y = concentration of HP

A = total concentration

$= x + y$

$= [CP] + [KP] + [HP]$

N = alkylation index

$$= \frac{\text{direct reading}}{\text{acid hydrolysis reading}} \times 100$$

(from Table 3.1) : $N(CP + KP) = 6.66$

$N(HP, H_2O) = 65$

% conversion of HP from CP :

$$\frac{6.66x}{A} + \frac{65y}{A} = N$$

$$6.66x + 65y = A\,N$$

$$6.66A - 6.66y + 65y = A\,N$$

$$y = \frac{A\,N - 6.66A}{58.34}\ \%$$

This equation was used to measure % conversion in Table 4.1, substituting values of concentration (A) and alkylating index (N).

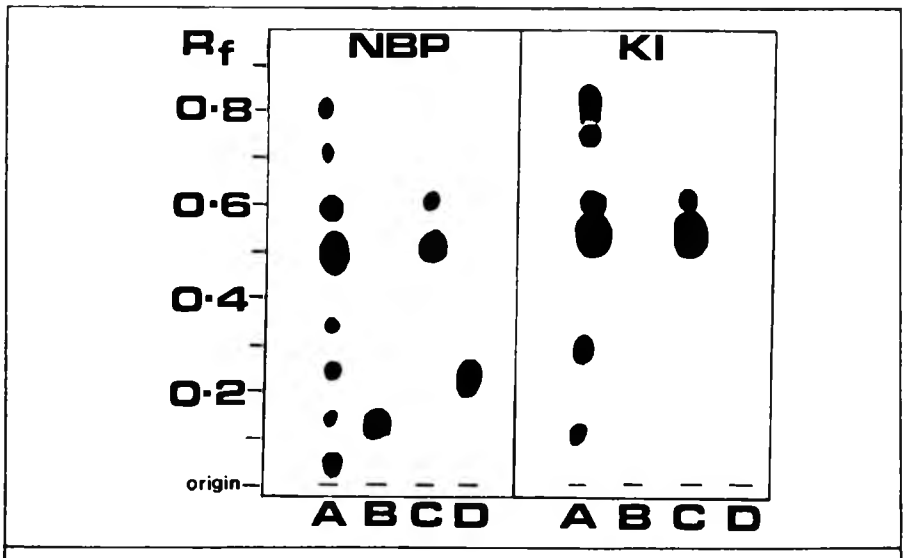
A trend can be identified in these estimations and reflects the results observed from the TLC system. The conversion increases to a maximum after 3 hours ozonisation and then decreases.

4.4 Ozonisation Conditions

From the results presented here, it was decided to use an incubation time of 2.5 hours for the ozonisation of CP. This allowed for the maximum production of HP and very little KP was found to be precipitated up to this ozonisation time. The production of such a precipitate is thought to occur only after the production of maximal amounts of HP, when the reaction then goes predominantly toward KP production, probably at the expense of HP (234). Such reaction would explain the observed decrease in HP concentration during long ozonisation times.

The TLC results of the oily residue after 2.5 hours ozonisation have been presented in Fig. 4.2.

Fig. 4.2 Thin Layer Chromatograms of Ozonisation Reaction Products



TLC system was silica gel 60, ethyl acetate run at -25°C.

Visualisation was by either NBP spray reagent to identify alkylating material or KI spray to identify peroxides.

A - ozonisation mixture after 2.5 hours ozonisation

B - CP, **C** - HP **D** - KP

4.5 Purification of KP

KP was filtered from the reaction mixture at several stages (section 2.5). Those samples, which were contaminated least as determined by TLC, were combined and recrystallised from boiling chloroform. The major contaminant was CP, which crystallised much later and at a lower temperature than KP. Substantial quantities of purified KP were obtained by filtration of the crystals formed from the recrystallisation system when it had reached room temperature.

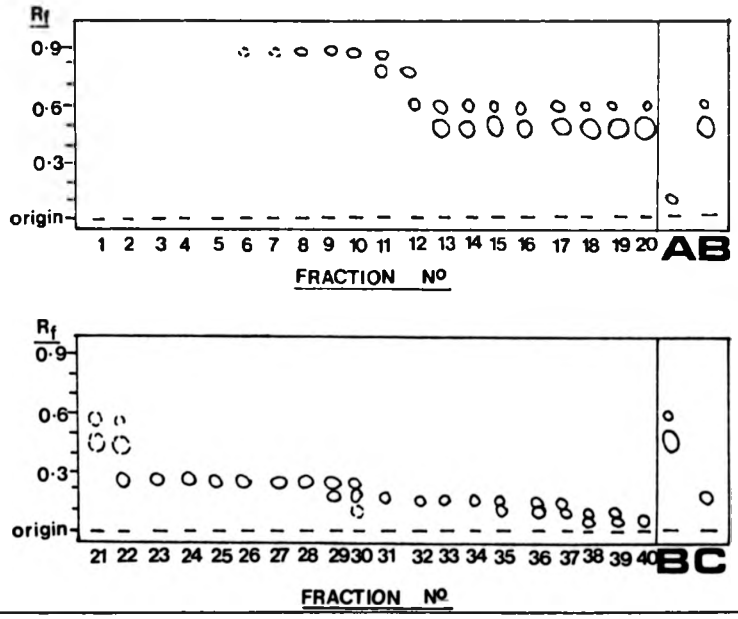
4.6 Purification of HP

As can be seen from the results presented in Fig. 4.2, more than one compound was produced by CP ozonisation which was both alkylating (NBP positive) and a peroxide (KI positive). KP could also be seen as a contaminant. HP was identified by employing a standard.

This extract was applied to a flash chromatography column (section 2.5) and the 10 ml fractions collected were reduced in volume by evaporation of solvent and applied to TLC plates which were run in the same solvent system as those in Fig. 4.2. Because there were so many samples, the TLC plates were run over the short dimension of 2". The results have been presented in Fig. 4.3.

It can be seen from these results that a good separation was achieved using this method and all samples containing only HP were pooled together (samples 13 to 21, Fig. 4.3) and the solvent removed by rotary evaporation under ice cooling. The remaining solid was stored at -25°C and further characterised by various techniques and shown to be HP.

Fig. 4.3 Thin Layer Chromatograms of Ozonisation Products After 'Flash' Chromatography



The TLC system was silica gel 60, ethyl acetate run at -25°C. Visualisation was by NBP spray, incubated for 20 minutes. Each fraction number represents a collected 10 ml. fraction off the 'flash' chromatography column which had been further concentrated by evaporation of solvent before application to the TLC plate.

A - CP, **B** - HP, **C** - KP

CHAPTER 5

THE CHARACTERISATION OF CP AND ITS DERIVATIVES

5.1 Introduction

Before their use in enzyme assay systems, it was felt necessary to test the purity of all derivatives, particularly those produced by ozonisation, the nature of which had to be confirmed. Several physical techniques were used as well as a chemical test (Chapter 3) which also supplied evidence as to the identity of the compounds.

5.2 Melting Point Determination

Table 5.1 shows the measured melting points of various compounds together with reported values from the literature. It can be seen that there is good agreement between measured and reported values.

Two of the latter melting points, however, differ significantly from all others, both reported and determined. The melting point for CP reported by Garcia-Blanco *et al.* (245) is quite low. This corresponds to the value for $[^2H_{10}]$CP reported by Cox *et al.* (185) and also to values obtained with early preparations of CP (144).

The other value to differ is that of HP determined by Struck *et al.* (247). In this case, the difference in M.P. reported is due to the unstable character of HP. On heating, HP darkens and then explosively decomposes. Struck *et al.* have noted that the recorded melting point can be manipulated by regulating the rate of heating the sample. Other values thus obtained include 108.1°C at 10°/min, 93.4°C at 2°/min and 71.4°C at 0.2°C/min. This lowering of the melting point is most probably due to the premature

TABLE 5.1 Melting points of CP and derivatives

COMPOUND	MEASURED M.P. (°C)	REPORTED M.P. (°C)	Ref.
CP monohydrate	47 - 48	47 - 49	(144)
		40 - 41	(245)
KP	148 - 149	148 - 149	(164)
HP	106 - 107	107 - 108	(231)
		102 - 103	(246)
		131 - 133	(247)
PM	103 - 107	106 - 107	(248)
cyclohexylammonium salt			

decomposition of HP before the melting point has been reached. No such difficulties were encountered under the conditions used for these determinations.

5.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

(1) [^{1}H]NMR

Proton NMR spectra have been presented in Fig. 5.1, together with the structures of the compounds tested. The assignment of peaks is shown in Table 5.2, together with integrals. Assignment was made for CP using the data of Egan *et al.* (249) and for HP using the data of Takamizawa *et al.* (231) Struck *et al.* (247) and Van der Steen *et al.* (246). The NMR spectra are in good agreement with the literature values.

Confirmation of the HP peak produced by the hydroperoxy group (OOH) was made by observation of its disappearance after the sample was shaken with a few drops of deuterium oxide (D_2O).

Assignment of peaks for KP was made by comparison with the other spectra. Only the peak due to the C_5 protons is significantly different from the other peak assignments (cf CP or HP) and this is brought about by the highly electronegative keto oxygen atom at the C_4 position, causing a downfield shift in the C_5 proton signal.

The spectrum of PM was complicated by the cyclohexylammonium moiety in the upfield region, but the peaks characteristic of the chloroethyl groups were easily identified.

Fig. 5.1 [^{1}H]NMR of Cyclophosphamide and Derivatives. Room temperature, deuterated acetone solvent

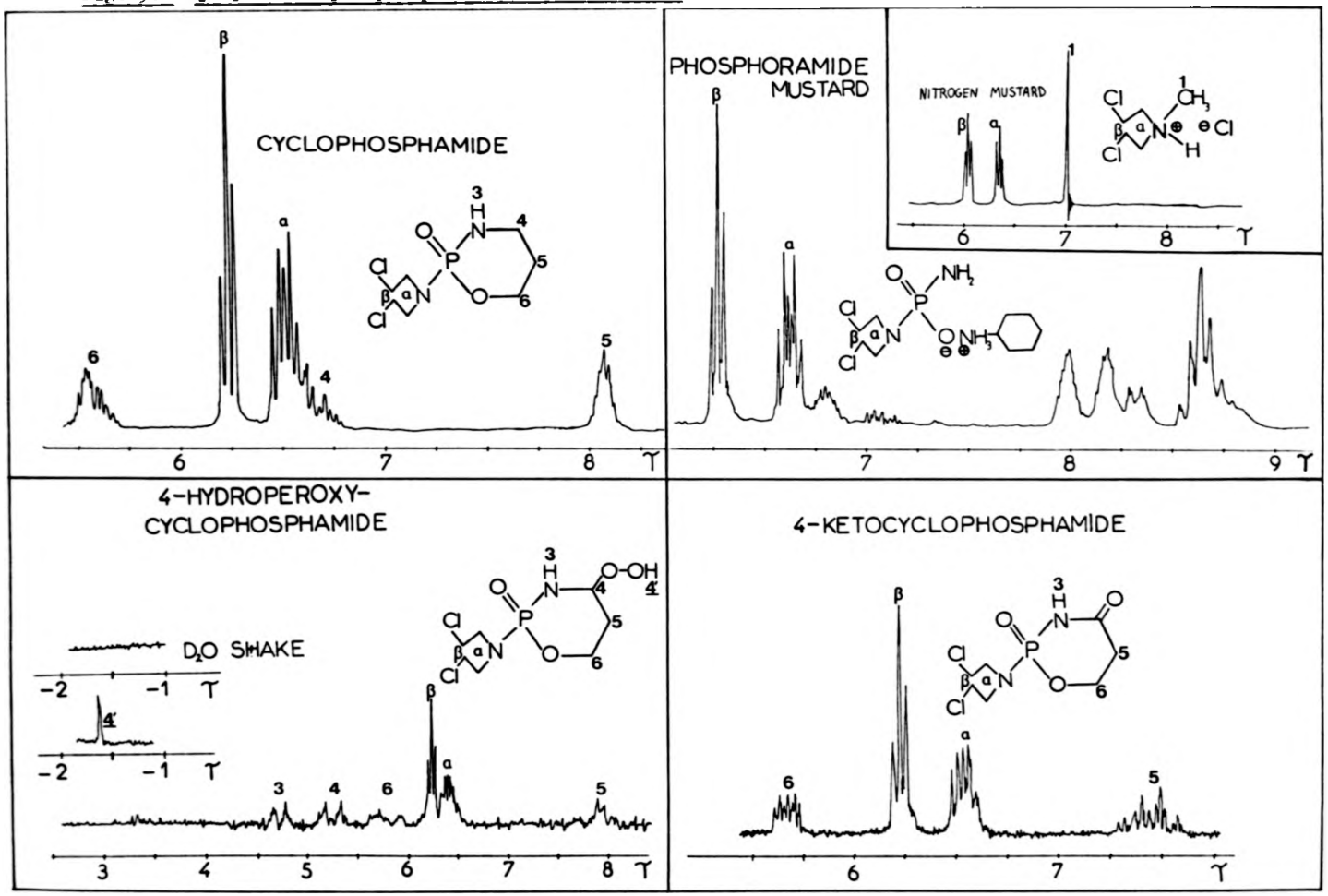


TABLE 5.2 Assignment of peaks for [^{1}H]NMR

GROUP	CHEMICAL SHIFT (τ) CP	HP	KP
OOH		- 1.64,s,(1H)	
NH		4.58 - 4.85 (1H)	
C_4H		5.10 - 5.39 (1H)	
C_6H_2	5.44 - 5.70 (2H)	5.58 - 6.00 (2H)	5.59 - 5.63 (2H)
$C\beta\beta'H_2$	6.23,t, (4H)	6.24,t,(4H)	6.24,t,(4H)
$C\alpha\alpha'H_2$	6.43 - 6.52 (4H)	6.28 - 6.58 (4H)	6.45 - 6.64 (4H)
C_4H_2	6.52 - 6.80 (2H)		
C_5H_2	7.96 - 8.21 (2H)	7.76 - 8.00 (2H)	7.28 - 7.65 (2H)

Except where stated otherwise, all peaks were multiplets (s = singlet, t = triplet). Figures in brackets are the integrals for the peaks.

(2) [^{13}C]NMR

The data obtained and the assignment of peaks for [^{13}C]NMR has been presented in Table 5.3. These are in agreement with the published data of Egan and Zon (249) and Struck *et al.* (247). Splitting of some peaks was observed due to the phosphorus atom of the oxazaphosphorinane ring.

5.4 Infrared Spectroscopy

IR spectroscopy of KP (Mull) revealed a strong band at 1695 cm^{-1}, which is indicative of an amide carbonyl group and is in agreement with the findings of Hill *et al.* (164). This band was not observed in the IR spectrum of CP. No other IR spectra were recorded.

5.5 Mass Spectrometry (MS)

(1) Introduction

Field desorption MS has been used most widely for the study of CP and its derivatives (250). This method was not, however, available and electron-impact MS was utilised to gather as much information as possible. The interpretation of the spectra was simplified by the fact that the isotopic chlorine atoms ^{35}Cl and ^{37}Cl differ in their abundance by a factor of 3:1.

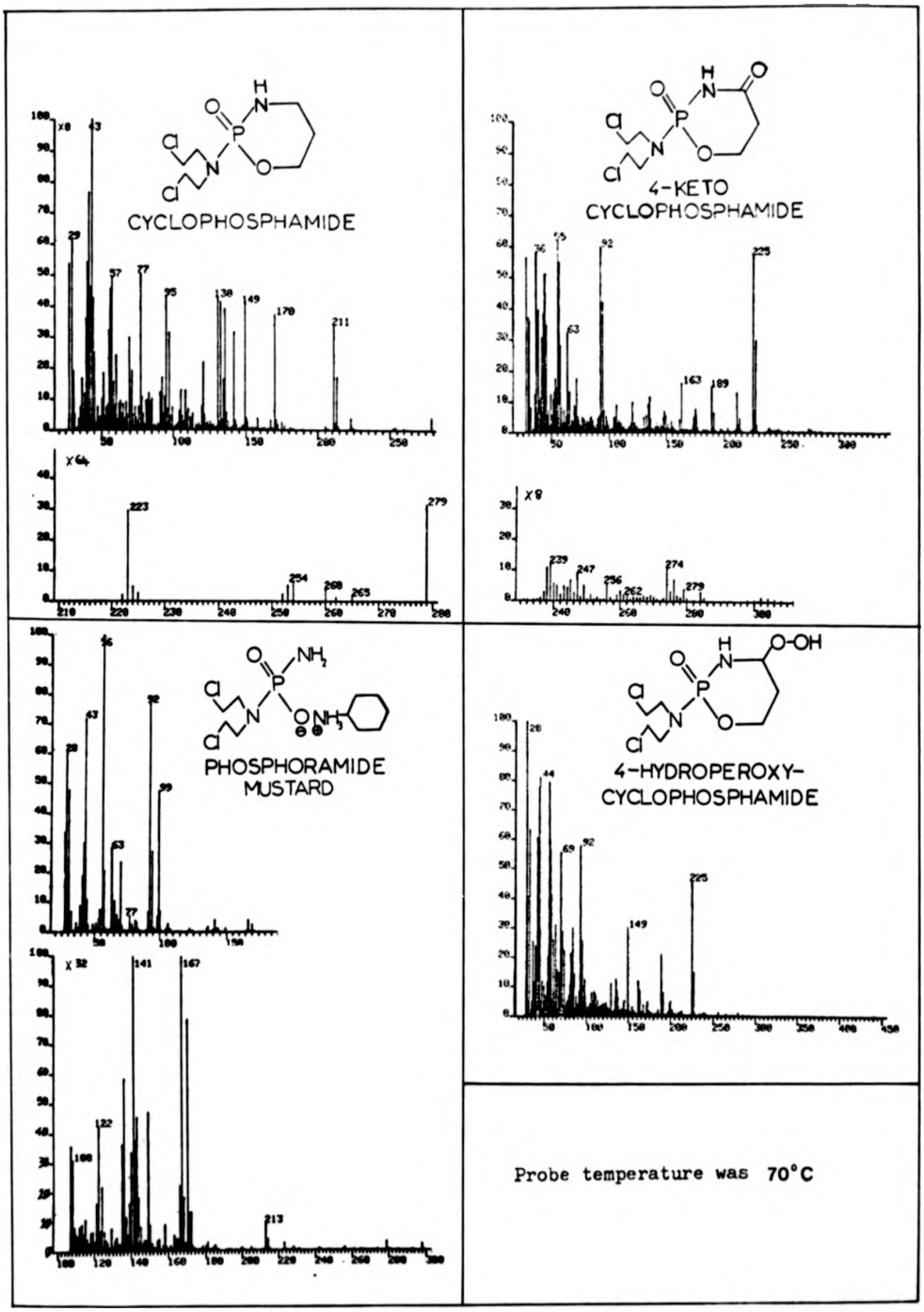
(2) Phosphoramide Mustard

The spectrum of PM (Fig. 5.2) showed only traces of ions around its molecular weight of 220. A characteristic fragmentation pattern of nitrogen mustard compounds is the loss of a CH_2Cl group and this was observed by the presence of a small peak at m/e 171. Nor-nitrogen mustard may be observed as the small peak at m/e 141 and the rest of the spectrum consisted basically of that observed for nitrogen mustards, including the

TABLE 5.3 Assignment of peaks for [^{13}C]NMR spectroscopy

COMPOUND	CHEMICAL SHIFT (δ)				
	C_4	C_5	C_6	Cββ'	Cαα'
CP^{D_2O}	41.00	25.28	69.57	42.18	47.54
$KP^{acetone-d_6}$	170.42	33.79	62.85	42.57	49.39
$HP^{acetone-d_6}$	86.65	27.77	63.12	41.67	49.05

Fig. 5.2 Mass Spectra of CP and Derivatives



characteristic fragmentation pattern m/e 92, 63/65, 56 (250). The peak observed at m/e 99 was probably due to the cyclohexylamine moiety.

(3) Cyclophosphamide

The largest mass observed in the spectrum of CP was due to the monohydrate, molecular weight 279. A very small peak was seen at m/e 265 due to the formation of an $(M + 1)^+$ molecular ion which is unusual in electron impact MS. The loss of the CH_2Cl group resulted in the peak at m/e 211/213 (2:1). All other peaks were not relevant to the determination of CP.

(4) 4-ketocyclophosphamide

The molecular ion peak (M^+) for KP could be observed at m/e 274 but it was the characteristic loss of CH_2Cl which produced the spectrum's base peak at m/e 225/227 (2:1) (164). The fragmentation pattern characteristic of nitrogen mustard was also present.

(5) 4-hydroperoxycyclophosphamide

HP has a low chemical stability and this was demonstrated by the difficulty encountered in obtaining a good electron impact MS spectrum. Conditions were optimised to prevent the decomposition of HP whilst the mass spectrum was taken. The decomposition of HP was observed by its decolouration.

Among other fragmentation pathways, the loss of H_2O and H_2O_2 to yield KP and CP respectively has been observed in field desorption MS to give peaks at m/e 274 and 258 by Przybylski *et al.* (250).

In the spectrum recorded (Fig. 5.2) no molecular ion peaks could be seen except at very high amplitude when many other random peaks also appeared. The loss of H_2O to yield KP and subsequent loss of CH_2Cl

resulted in the observed peak at m/e 225/227 (3:1) which is characteristic of KP. Most abundant fragment ions were unspecific with regard to the structural determination of this compound.

5.6 High Performance Liquid Chromatography (HPLC)

Table 5.4 shows the retention times obtained for compounds using HPLC (section 2.1 (5)). Although conditions were different, these recorded times are in agreement with reported data, with regard to relative retention times.

CP and KP gave single peaks whereas HP was found to give two peaks. The sample of HP supplied by Colvin (section 2.1) and also produced by ozonisation, was also found to exhibit this effect. It was expected that HP would do so on the basis of its performance on TLC (Chapter 4). However, the peak which has been tentatively attributed to the HP diastereomer did not appear with the longer retention time than CP as suggested by the reported data (234). This peak coincided with that found for KP which was confirmed by co-chromatography of the HP and KP samples. Measurements indicated that the purified HP sample was composed of 2% of this material, as was the supplied sample. This estimation was made only after a linear relationship between peak areas and concentration of sample was confirmed, using CP as standard (linearity was found betwen 6 and 60 µg. CP and areas were reproducible to ± 2%).

TABLE 5.4 Retention times of compounds by HPLC

	RETENTION TIMES (minutes)	
FLOW RATE (ml/min)	1	2$^{\phi}$
COMPOUND		
KP	3.58 ± 0.11	2.70
HP	4.11 ± 0.23	3.64
(diastereomer)	(3.58)	(6.57)
CP	4.80 ± 0.12	4.86

Reverse phase column with $MeOH/H_2O$ (2:3 v/v) eluant, refractometric detection was used in both cases. ϕ Measured using data supplied by Peter (234).

CHAPTER 6

MICROSOMAL HYDROXYLATION OF CYCLOPHOSPHAMIDE

6.1 Introduction

The incubation of CP with rat liver microsomes was investigated as a method for obtaining 4-hydroxycyclophosphamide (OHCP). The method of Connors *et al.* (151) was followed closely, and produces a more stable derivative of OHCP, 4-ethoxy-CP, by the addition of ethanol to OHCP in aqueous solution. The formation of alkylating material during the incubation was followed by the application of the NBP test (section 2.4 and Chapter 3).

RESULTS AND DISCUSSION

6.2 Incubation

Several experiments were performed to investigate the formation of OHCP from CP in the microsomal system (section 2.6). A representative experiment has been presented here.

Table 6.1 shows the results of the NBP test before and after the incubation period, and an increase in the direct reading can be seen, leading to an increase in the alkylation index (see Chapter 3).

If it is assumed that only CP and OHCP contribute to the measured alkylating activity after incubation, then the conversion of CP to OHCP can be calculated (see section 4.3). Using the values reported in Table 6.1, the calculated conversion is 35.10% (substituting A = 100 in the equation presented in section 4.3(4)). This was found to vary from 30 to 60% and no two experiments gave the same result. The only variable

TABLE 6.1 NBP test results from a typical microsomal incubation of cyclophosphamide

	NBP test (HN2 equivalents)		
	DIRECT READING	ACID HYDROLYSIS READING	ALKYLATION INDEX
BEFORE INCUBATION	0.75 ± 0.05	12.0 ± 1.0	6.25
AFTER INCUBATION	3.80 ± 0.05	14.0 ± 2.0	27.14

between such experiments was the microsomal preparations from rat liver (section 2.12). One experiment was performed using microsomes which had been stored in liquid nitrogen previously and were found to show very little activity, whereas the same preparation when used immediately after its preparation had produced the results shown in Table 6.1. For this reason microsomes were prepared prior to each experiment and used immediately. It must be concluded that variations in the performance of microsomes was due either to the preparation procedure or to variations in the animals used. The preparation of microsomes was carried out under identical conditions each time and so the latter possibility would seem the most likely. This is particularly likely when it is considered that no control was imposed on the dose of phenobarbital received by each animal via its drinking water. Phenobarbital was used because it has been shown to increase the activity of the microsomal fraction of rat liver towards CP activation, by induction of liver microsomal enzymes, by as much as 5-fold (146). Better reproducibility may have been attained by strict control over the dosage of phenobarbital to each animal, i.e. by administration by intra-peritoneal injection.

Using the results from Table 6.1, the increase in total alkylating activity can be calculated (146):

$$\text{Total alkylating activity} = \frac{\text{DIRECT NBP, (HN2 equiv.)}}{\text{nmoles of CP used in the NBP test.}} \times \text{total nmoles of CP in the incubation.}$$

$$\text{BEFORE INCUBATION} = \frac{0.75}{16.85} \times 10787.5 = 480.15$$

$$\text{AFTER INCUBATION} = \frac{3.80}{6.85} \times 10787.5 = 2432.70$$

INCREASE IN ACTIVITY = 1952.63 nmoles HN2 eq./3g.liver/45 min.

= 0.86 µmoles HN2 eq./ g.liver/hr.

In an experiment where 30% conversion was calculated, an increase in alkylating activity of 0.68 µmoles HN2 eq./ g.liver/hr. was found. Similarly, for the experiment where the highest conversion of 60% was calculated, an increase in alkylating activity of 1.32 µmoles HN2 eq./ g. liver/hr. was found.

These results are in good agreement with that of Cohen and Jao (146), who have found an increase of 0.60 µmoles HN2 eq./ g.liver/hr. using slightly different incubation conditions and different rat species. Cohen and Jao have reported a sex difference in the ability of rat liver microsomes to activate CP, by as much as 3-fold. For this reason, male rats were used. It is possible that species differences also occur. Furthermore, these workers have also shown that the concentration of NADPH in the incubation medium can effect the activity of the microsomes. They have reported a 10-fold change in activity (0.18 to 1.95 µmoles HN2 eq./ g.liver/hr.) due to a 10-fold difference in NADPH concentration (0.3 to 3.0 mM). In these experiments, NADP and an NADPH-regenerating system were used.

6.3 Extraction of The Metabolites

After extraction of the microsomal incubation medium (section 2.6) the extract was subjected to TLC and the results are shown in Table 6.2. This TLC system was used by Connors *et al.* (151) who utilised radio-labelled CP and found four radioactive areas on the TLC plate after

TABLE 6.2 TLC of CP and microsomal incubation extraction material

	Rf values
CP	0.46
EXTRACT	0.00, 0.46, 0.98

TLC was on silica gel 60 in a chloroform/ethanol (19:1 v/v) solvent system at 4°C. Visualisation was by NBP spray

incubation, with different Rf values to these findings. This is probably due to water vapour (section 4.2) (234). By running a CP standard, it was possible to identify the area on the plate in the extract chromatogram corresponding to this compound (Rf = 0.46). By comparing these results to those of Connors et al. (151) it is possible to assign the compound at Rf = 0.98 as the "fast-running" isomer of the 4-ethoxy-CP derivative. The "slow-running" isomer coincides with the spot due to CP. NBP-positive substances at the origin of the chromatogram probably consist of breakdown products such as monochloroethyl-CP and PM.

6.4 Instability of Products

Attempts to isolate the product at Rf = 0.98 by 'flash' chromatography (section 2.5(5)) were hampered by the unexpected instability of the extracted metabolites. Yellowing of activated CP derivatives has been used previously as an index of their decomposition (143) and this was observed in the chloroform extracts. Although it was found quite possible to isolate the product of Rf = 0.98, it was found impossible to concentrate it by reduction of the volume (ice cooling, rotary evaporator) before yellowing of the sample was observed.

CHAPTER 7

THE EFFECT OF CYCLOPHOSPHAMIDE AND ITS DERIVATIVES ON ADENYLATE CYCLASE ACTIVITY

7.1 Introduction

The effects of alkylating agents on the activity of enzymes involved in cyclic AMP degradation and expression of biological activity have been noted (section 1.11). Cyclic AMP phosphodiesterase is inhibited (141) and cyclic AMP-dependent protein kinase is activated (134) by such agents. The effect of the alkylating agent cyclophosphamide on the cyclic AMP-synthesising enzyme has been studied.

7.2 Plasma Membrane Characterisation

Plasma membranes were first produced by the method of Neville (240) modified by Pohl *et al.* (80). This preparation was found to have low activity with respect to (i) 5'-nucleotidase, a marker enzyme for plasma membranes, and (ii) adenylate cyclase (Table 7.1). Furthermore, it was found that although phosphodiesterase activity was not detectable in this preparation by using the assay method of Butcher and Sutherland (section 2.7) addition of the potent phosphodiesterase inhibitor, theophylline, greatly enhanced the apparent activity of adenylate cyclase (Fig. 7.1A).

Due to the low enzyme activities associated with this preparation, it was abandoned in favour of a technique using the combined methods of Emmelot *et al.* and Neville (section 2.10). The initial low speed centrifugations and plasma membrane separation were carried out according to the method of Emmelot *et al.* (241) and the final discontinuous sucrose

TABLE 7.1 Enzyme activities associated with various fractions isolated during the production of plasma membranes

	5'-Nucleotidase U(mg protein)$^{-1}$	Adenylate cyclase pmoles/15 min/mg protein
Plasma membranes		
(1) Neville	0.160	40.85 ± 6.48
(2) Neville/Emmelot	0.540	253.96 ± 10.93
(a) Whole homogenate	0.062	36.92 ± 6.91
(b) Cell debris	0.130	76.27 ± 10.42
(c) Membrane pellet	0.004	48.33 ± 9.83

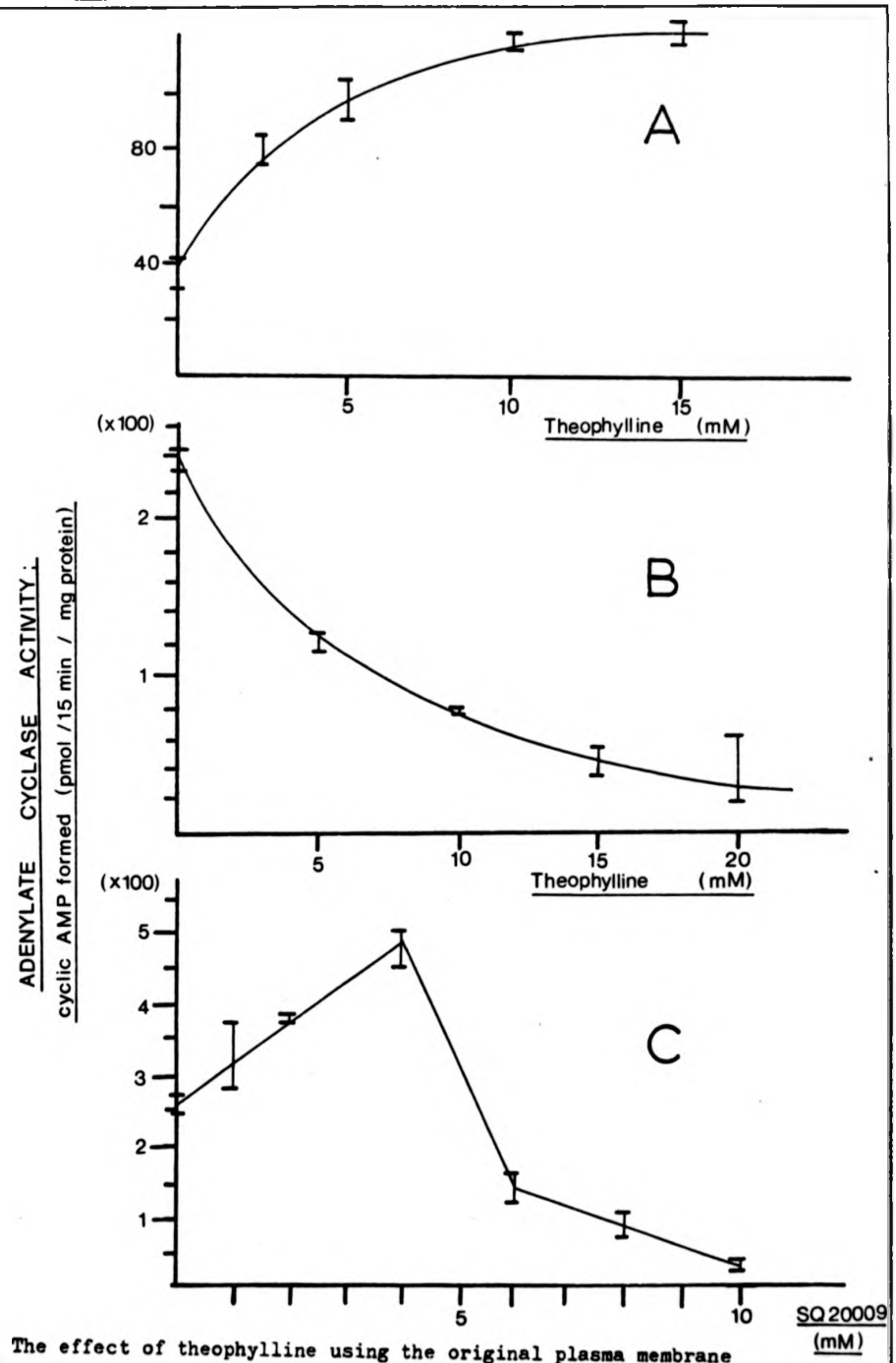
5'-nucleotidase activity was measured by the method of Widnell and Unkless (251) and expressed as μmoles of inorganic phosphate formed per minute (U). Phosphate was measured by the method of Ames *et al.* (252).

(a) Whole homogenate was prepared from step 4, section 2.10.

(b) Cell debris was prepared from step 5, section 2.10.

(c) Membrane pellet was prepared from step 13, section 2.10.

Fig. 7.1 The Effect of Phosphodiesterase Inhibitors On Adenylate Cyclase Activity



A The effect of theophylline using the original plasma membrane preparation of Neville (see text)

B The effect of theophylline

C The effect of SQ20009

Error bars represent the difference between duplicates in one experiment

density gradient separation of "crude plasma membranes" was carried out according to the method of Neville (240). This preparation showed greatly increased enzyme activities (Table 7.1) and was used for the following studies on adenylate cyclase.

7.3 The Effect of Phosphodiesterase Inhibitors On Adenylate Cyclase Activity

Since theophylline was found to increase the measured activity of adenylate cyclase in crude plasma membranes prepared by the method of Neville (Fig. 7.1A) its effects were investigated using the improved plasma membrane preparation. Fig. 7.1B shows the inhibitory effect that was observed.

Although this was an unexpected result considering the previous results obtained with theophylline, it is very similar to the effects observed by Sheppard (253). Measuring adenylate cyclase activity in rat erythrocyte ghosts, Sheppard observed between 24 and 42% inhibition by 20 mM theophylline. This effect was only apparent when phosphodiesterase was not a contributory factor in the measurement of adenylate cyclase activity.

The effect of another potent phosphodiesterase inhibitor, SQ20009, was also investigated. As can be seen from Fig. 7.1C, SQ20009 increased the measured activity of adenylate cyclase in concentrations up to 4.0 mM. After this concentration was reached, a precipitate was found to form when SQ20009 and Tris buffer were present in the assay medium. This may explain the drop in measured adenylate cyclase activity above 4.0 mM SQ20009 (Fig. 7.1C).

Because of the effects observed with phosphodiesterase inhibitors it was decided not to include them in the assay system for routine adenylate

cyclase assays. According to the reasoning of Sheppard (253) such inhibitors are not necessary and the 'cold trap' arrangement first developed and used by Weiss and Costa (254) seems perfectly adequate to deal with any reduction in cyclic AMP due to phosphodiesterase activity. It has not been considered necessary to include phosphodiesterase inhibitors when this arrangement is used (216).

7.4 Characterisation of The Adenylate Cyclase Assay

The assay of adenylate cyclase in rat liver plasma membranes has been well characterised in the past (15) and for this reason there seemed to be no necessity to characterise the system with respect to Mg^{2+} ions, pH and ATP concentration, these being taken from the data of Pohl *et al.* (80).

Table 7.2 shows the effects of EDTA, EGTA and calcium ions on the activity of adenylate cyclase. It has been shown that in the presence of Mg^{2+} ions, Ca^{2+} ions are inhibitory in concentrations above 0.1 mM (80). This has been corroborated and may explain the effect of the chelating agents EDTA and EGTA in the assay system. EDTA was first introduced into the adenylate cyclase assay system by Pohl *et al.* (80) and has been used since as a 'standard' addition by many workers.

If the effects observed with the chelating agents is due to the chelation of endogenous Ca^{2+} ions then the increased effectiveness of EGTA over EDTA can be explained, since EGTA has a greater specificity for Ca^{2+} ions than does EDTA. EGTA was therefore included in the assay medium for all routine assays.

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TABLE 7.2 The effect of calcium ions and chelating agents on adenylate cyclase activity

ADDITION	ADENYLATE CYCLASE ACTIVITY cyclic AMP formed : pmol/15 min/mg protein
none	256.41 ± 32
EGTA (1 mM)	440.31 ± 8
EDTA (1 mM)	371.62 ± 20
$CaCl_2$ (1 mM)	47.69 ± 6

7.5 Linearity of The Assay

Before further experiments were performed, it was felt necessary to demonstrate the linearity of the adenylate cyclase assay with respect to time and protein concentration. It was necessary to perform all routine assays under conditions which corresponded to the linear portions of such curves. Fig. 7.2 shows the results of time course and protein concentration studies in the absence or presence of EGTA (1 mM). It can be seen that the assay was linear for up to 20 minutes when 15 μg of protein were used and linear for up to 20 μg of protein when assayed for 15 minutes. It can also be seen that although EGTA increased the measured activity, no effect on linearity with respect to time or protein was observed.

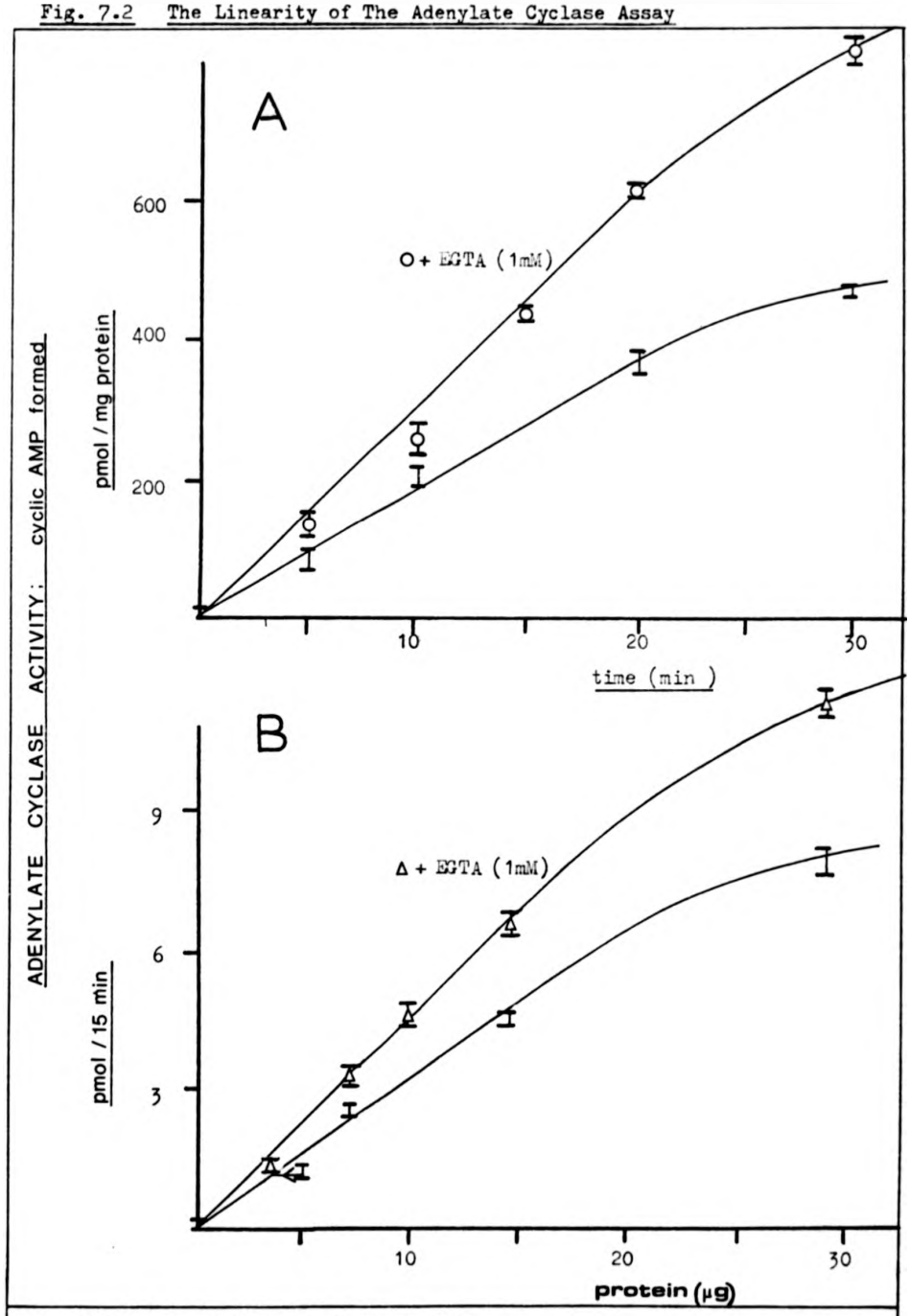
Loss of linearity may have been due to the fact that liver plasma membrane contains a potent ATPase (255) and this may effect the assay even in the presence of the creatine phosphate-creatine phosphokinase ATP-regenerating system.

7.6 The Effect of CP And Its Derivatives On Basal Adenylate Cyclase Activity

The effect of CP and its derivatives on basal adenylate cyclase activity in the presence or absence of EGTA is shown in Table 7.3. Of those compounds tested in the absence of EGTA, only the metabolite extract from a microsomal incubation of CP had any significant effect.

The microsomal incubation and extraction of metabolites was performed on the same day as the adenylate cyclase assay due to the observed instability of the activated compound (Chapter 6). The alkylation index for this extract was 40.58 which corresponds to a 59.3% conversion of CP to OHCP (section 4.3(4)). It has been calculated, using the data from the NBP test, that the chloroform extract, totalling 18 ml contained 6.21

Fig. 7.2 The Linearity of The Adenylate Cyclase Assay



A With respect to time of incubation

B With respect to concentration of protein

Error bars represent the difference between duplicates in one experiment.

TABLE 7.3 The effect of CP and derivatives on basal adenylate cyclase activity. I

Addition	Concentration (mM)	ADENYLATE CYCLASE ACTIVITY cyclic AMP formed : pmol/15 min/mg protein -EGTA		+EGTA	
none	-	242.66 ± 10	(1.00)	440.77 ± 5	(1.00)
CP	5	237.64 ± 21	(0.98)	462.97 ± 11	(1.04)
KP	5	249.36 ± 23	(1.03)	426.98 ± 31	(0.97)
HP	1	-		361.63 ± 6 [P < 0.01]	(0.82)$^{\phi}$
PM	5	253.72 ± 4	(1.04)	455.67 ± 4	(1.02)
HN2	5	-		460.72 - 9	(1.04)
Metabolites					
(OHCP)	2.76	155.72 ± 9 [P < 0.01]	(0.64)$^{\phi}$		
(CP)	1.89				

$^{\phi}$Figures show a significant difference from the control in the Student's t test.

Figures in brackets represent the ratio of activity between assay and control.
All Figures represent triplicate results from one experiment.

μmoles of OHCP, assuming that only CP and OHCP (or its ethoxy derivative) were present in the solution. 4.0 ml of this extract was evaporated to dryness (rotary evaporator under ice cooling) and the residue redissolved in water (200 μl). An aliquot (20 μl) of this was used per assay tube in the adenylate cyclase assay. For a final assay volume of 50 μl, this corresponded to a concentration of 2.76 mM for OHCP.

CP was also present in this sample at a concentration of 1.89 mM. CP, however, had no effect on adenylate cyclase activity alone (Table 7.3) and the result must be regarded as being solely due to the presence of OHCP.

This result was confirmed by the behaviour of HP in the assay system. In solution HP spontaneously forms OHCP and the inhibition observed for both metabolites and HP is therefore due to the same compound, OHCP. In the presence of EGTA, HP was the only derivative tested to show any significant effect on adenylate cyclase activity (Table 7.3). Furthermore, a dose-response relationship was also observed (Table 7.4, Fig. 7.5) between HP concentration and inhibition of activity. None of the other compounds tested showed any significant effect on basal activity at concentrations up to 15 mM. HP produced an inhibition of approximately 50% at 5.0 mM concentration (Table 7.4).

7.7 The Effect of CP And Its Derivatives On Stimulated Adenylate Cyclase Activity

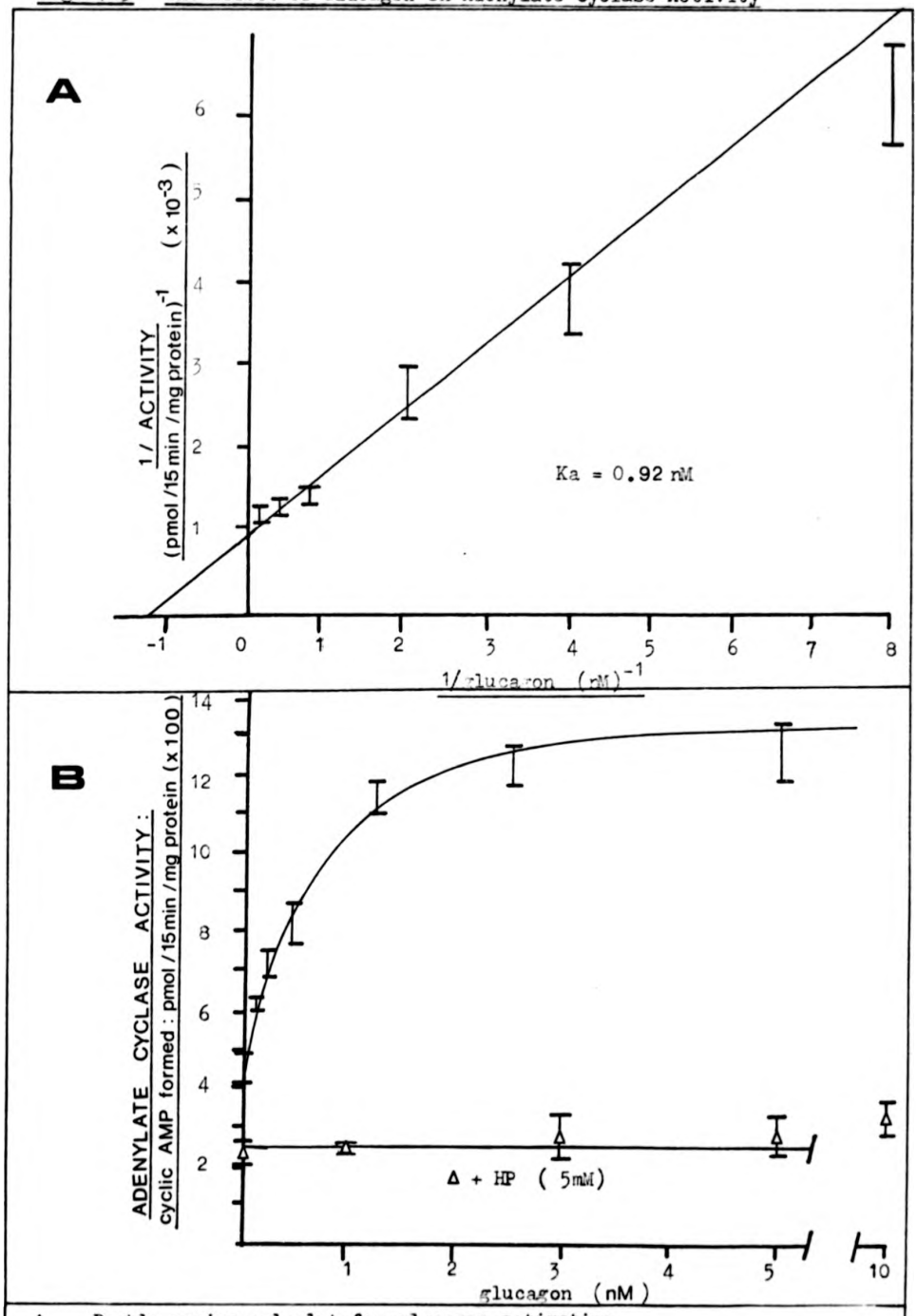
Table 7.5 shows the effects of glucagon (3 nM) and fluoride (3 mM) on adenylate cyclase activity. This concentration of glucagon corresponds to a saturating level (of glucagon receptors) as demonstrated by the dose-response curve for glucagon, Fig. 7.3B. Although this represents

TABLE 7.4 The effect of cyclophosphamide and its derivatives on basal adenylate cyclase activity. II

CONCENTRATION (mM)	ADENYLATE CYCLASE ACTIVITY cyclic AMP formed : pmol/15 min/mg protein							
	CP		KP		HP		PM	
0	431.2 ± 5	(1.00)	444.4 ± 10	(1.00)	462.0 ± 8	(1.00)	418.0 ± 21	(1.00)
1	-		-		398.2 ± 4 [P < 0.001]	(0.86)$^{\phi}$	-	
2.5	409.2 ± 15	(0.95)	431.2 ± 8	(0.97)	-		506.0 ± 4	(1.21)
3	-		-		330.0 ± 5 [P < 0.001]	(0.71)$^{\phi}$	-	
5	448.8 ± 8	(1.04)	442.0 ± 10	(0.99)	247.2 ± 8 [P < 0.001]	(0.54)$^{\phi}$	475.1 ± 15	(1.14)
10	465.5 ± 14	(1.08)	418.0 ± 11	(0.94)	-		478.5 ± 22	(1.14)
15	419.0 ± 6	(0.97)	400.0 ± 10	(0.90)	-		426.7 ± 13	(1.02)

$^{\phi}$Figures show a significant difference from the control in the Student's t test. Figures in brackets represent the ratio between activities in the assay to the control. All figures represent duplicates in one experiment except for those shown for HP which represent duplicate results from two separate experiments.

Fig. 7.3 The Effect of Glucagon On Adenylate Cyclase Activity



A Double reciprocal plot for glucagon activation

B Glucagon activation in the presence or absence of 4-hydroperoxycyclophosphamide (HP)

Error bars represent the standard deviation of quadruplicate measurements from two separate experiments

maximal stimulation of the enzyme, the observed effect, a 2.95-fold increase over the basal enzyme activity, is low compared with that reported by some laboratories. Pohl et al. (80) for example, have reported an 11.1-fold increase using 5.71 μM glucagon. This group have also reported that half-maximal stimulation with glucagon occurred at 4 nM.

Fluoride (3 mM) represents a non-saturating concentration (Fig. 7.4B). Again, however, these findings disagree with those reported by Pohl et al. and others.

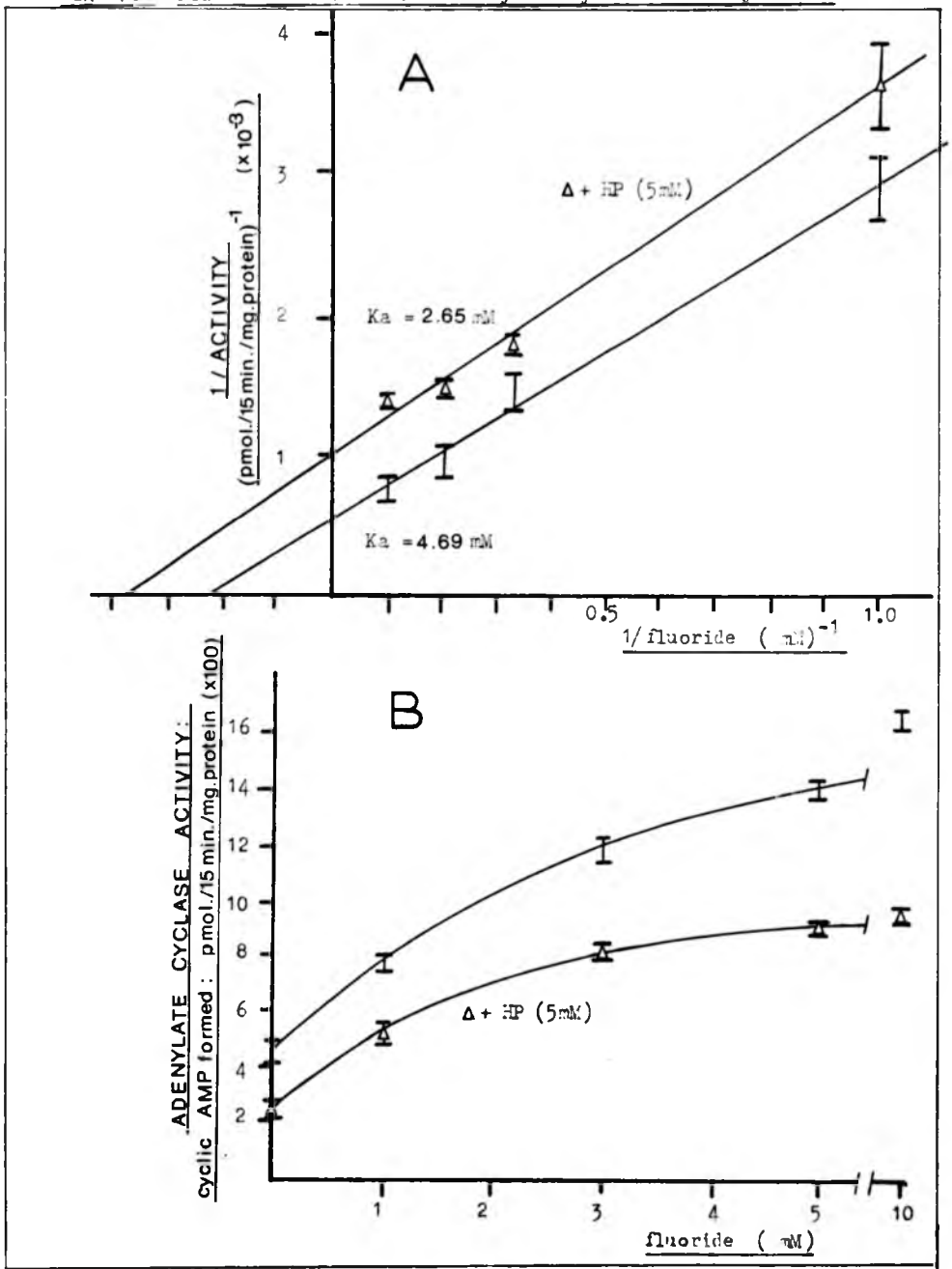
Fluoride (3 mM) causes a 2.68-fold increase over the basal level and 10 mM fluoride has been found to increase basal activity by 3.69-fold. Pohl et al. (80) have reported a 6.4-fold increase for 10 mM fluoride but only a 1.6-fold increase at 3 mM fluoride. However, Marinetti et al. (256) have reported inhibition by fluoride, activation by calcium ions and inhibition by insulin as well as a much reduced sensitivity to glucagon, in rat liver plasma membranes.

(1) Effect On Glucagon-Stimulated Activity

Table 7.5 shows that, of the compounds tested, only HP showed any significant effect on adenylate cyclase activity when stimulated by glucagon. The resultant, inhibited, activity is not significantly different from the HP inhibited basal activity reported in Table 7.4.

Fig. 7.3 shows the effect of varying the glucagon concentration in the absence or presence of HP (5 mM). In the absence of HP, it demonstrates the characteristic 'saturation' effect and the association constant, Ka, of glucagon for its receptor can be calculated from the intercept of the double reciprocal plot, after basal activity has been subtracted, Fig. 7.3A, and was found to be 0.92 nM. In the presence of HP (5 mM, Fig. 7.3B) all sensitivity for glucagon was lost, with no significant difference between any of the points shown.

Fig. 7.4 Fluoride Stimulation of Adenylate Cyclase Activity In The



A Double reciprocal plot

B Direct plot

Error bars represent the standard deviation between duplicate measurements from three separate experiments

TABLE 7.5 The effect of CP and its derivatives on glucagon and fluoride stimulated adenylate cyclase activity

ADDITIONS (5 mM)	ADENYLATE CYCLASE ACTIVITY cyclic AMP formed : pmol/15 min/mg protein			
	+ GLUCAGON (3 nM)		+ FLUORIDE (3 mM)	
none	1309.06 ± 28	(1.00)	1191.67 ± 50	(1.00)
CP	1222.50 ± 27	(0.93)	1185.00 ± 10	(0.99)
KP	1315.24 ± 55	(1.00)	-	
HP	274.51 ± 63 [P < 0.05]	(0.21)$^{\phi}$	852.61 ± 20 [P < 0.1]	(0.72)$^{\phi}$
PM	1322.12 ± 53	(1.01)	1217.50 ± 32	(1.02)

$^{\phi}$Figures show a significant difference from the control in the Student's t test. Figures in brackets represent the ratio of activity in the assay to the control. All figures represent duplicates from one experiment.

(2) Effects On Fluoride-Stimulated Activity

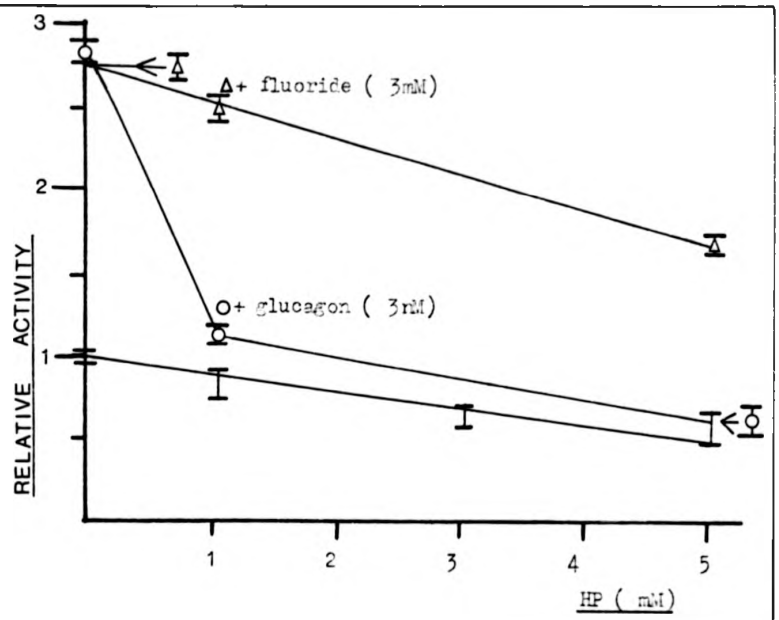
As for glucagon-stimulated activity, Table 7.5 shows that of the compounds tested, only HP showed any significant effect on fluoride-stimulated activity. The inhibition observed, however, still represents an increase over the basal activity of the enzyme.

Fig. 7.4B shows the effect of varying fluoride ion concentration on adenylate cyclase activity in the absence or presence of HP (5 mM). It can be seen that although HP inhibits the activity of the stimulated enzyme, fluoride ion still evokes response in a dose-dependent manner in the presence of HP. The association constants, Ka, of fluoride for its receptor have been calculated from the intercepts of the double-reciprocal plots in Fig. 7.4A (after subtraction of basal activity). The Ka for fluoride in the absence of HP was found to be 4.69 mM and this is almost halved in the presence of 5 mM HP to 2.65 mM. Behaviour such as this is often regarded as evidence of 'uncompetitive' inhibition. However, because of the complexity of the adenylate cyclase enzyme system, one must be cautious in such analyses.

7.8 Effect of 4-hydroperoxy-CP On Adenylate Cyclase Activity

Fig. 7.5 shows the effect of HP on basal, glucagon and fluoride stimulated adenylate cyclase activity. Although very few data points are present, it can be seen that a dose-response relationship exists between between cyclase activity and HP concentration in all circumstances. Whereas the response appears to be linear for both basal and fluoride-stimulated activity, HP has a much more 'pronounced' effect on glucagon-stimulated activity at low concentrations, resulting in a non-linear dose-response relationship. At a concentration of 5 mM HP, the glucagon-stimulated activity coincides with that of HP inhibited basal activity as shown in Fig. 7.3B for different concentrations of glucagon.

Fig. 7.5 The Effect of 4-hydroperoxycyclophosphamide (HP) On Adenylate Cyclase Activity In The Presence Or Absence Of Glucagon Or Fluoride



Error bars represent the standard deviation between duplicate measurements from two separate experiments

CHAPTER 8

THE EFFECT OF CYCLOPHOSPHAMIDE AND ITS DERIVATIVES ON GUANYLATE CYCLASE ACTIVITY

8.1 Introduction

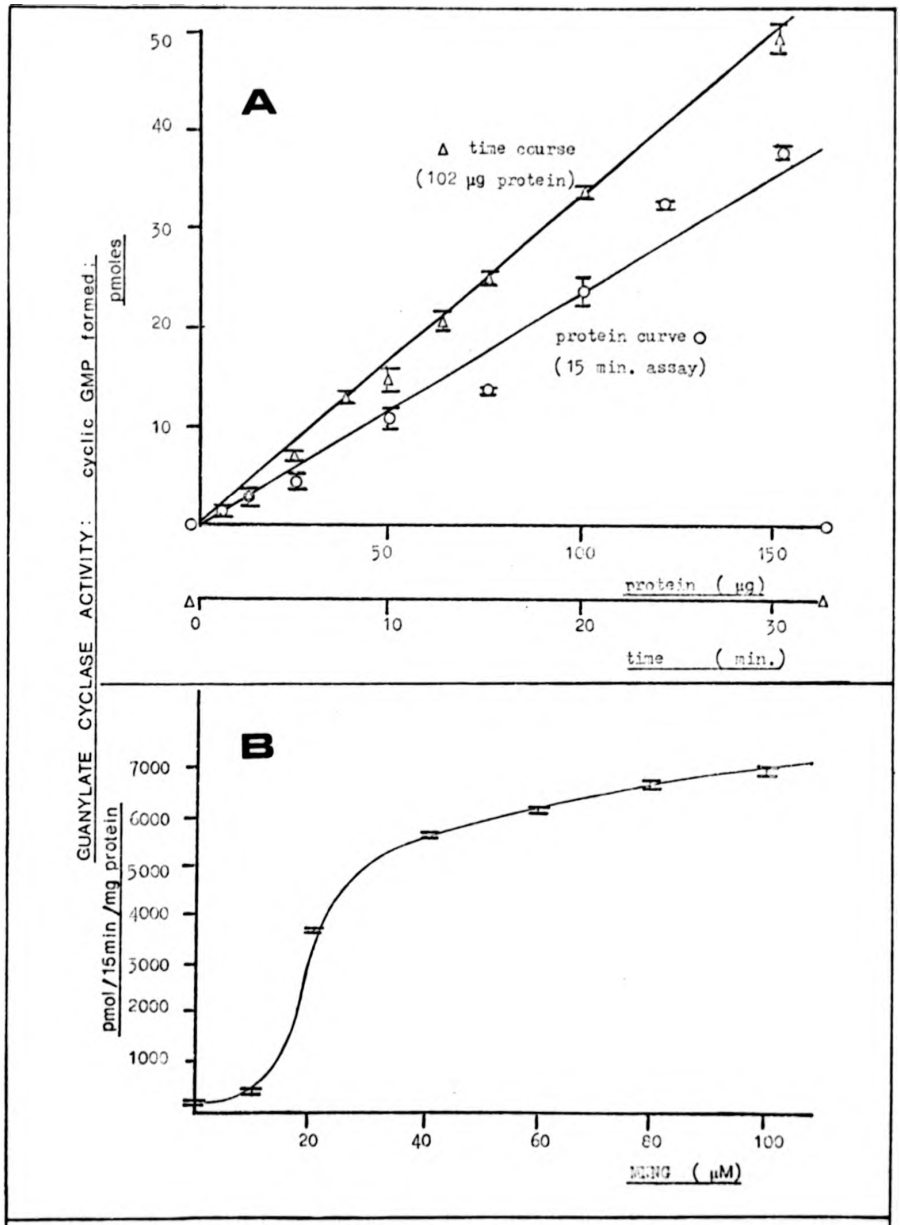
Alkylating agents have been reported to produce a small elevation in intracellular cyclic GMP levels (257) and the inhibitory effects of many anti-tumour agents, including alkylating agents, on carcinogen-stimulated guanylate cyclase activity have been noted (section 1.11). CP has been shown to depress the stimulation induced by NNPG (121).

The effects of activated CP derivatives on both basal and carcinogen (MNNG)-stimulated guanylate cyclase activity have been studied.

8.2 Linearity of The Guanylate Cyclase Assay

Fig. 8.1 shows the linearity of guanylate cyclase activity with respect to time (at a constant protein concentration) and to protein concentration (at a constant incubation time). As can be seen, no deviation from linearity was observed for up to at least 150 μg protein (using a 15 minute incubation time) and for up to at least 30 minutes (using 102 μg protein). Routinely, 102 μg protein and an incubation time of 15 minutes were used for each assay. This produced a basal activity of 253.22 ± 5.0 pmol cyclic GMP formed/15 minutes/mg protein.

Fig. 8.1 Guanylate Cyclase Activity



A Effect of time of incubation and concentration of protein

B Effect of MNNG concentration

Error bars represent duplicates in one experiment.

8.3 Effect of MNNG On Guanylate Cyclase Activity

MNNG is the most potent guanylate cyclase activator known and the dose-response relationship is shown in Fig. 8.1B.

DeRubertis and Craven (73) have reported a 36-fold increase in guanylate cyclase activity using 1 mM MNNG in the presence of 1-methyl-3-isobutylxanthine a powerful inhibitor of phosphodiesterase. In these studies 100 µM MNNG has been observed to stimulate cyclase activity by 28-fold.

For studies on the effects of CP and its derivatives on MNNG-stimulated activity, 40 µM MNNG was selected. Although this concentration is not quite maximal with regard to enzyme stimulation (and the enzyme is not, therefore, fully 'saturated') the activity does not fall on the steep part of the sigmoidal curve, Fig. 8.1B.

8.4 The Effect Of CP And Its Derivatives On Basal Guanylate Cyclase Activity

Table 8.1 shows the effects of CP and its derivatives on basal guanylate cyclase activity. This represents the activity of the enzyme in the presence of Mn^{2+} ions. The only effects observed were evoked by HP and PM. HP was found to inhibit cyclase activity by nearly 50% at a concentration of 30 mM. PM was found to stimulate the enzyme by up to 40% at 30 mM concentration but this effect was not observed in the pre-incubation studies, discussed in the following paragraphs. It should be noted, therefore, that during these experiments the alkylating agents were present in the assay cocktail for no longer than five minutes before the start of the assay by addition of enzyme solution (liver cytosol).

TABLE 8.1 The effect of cyclophosphamide and its derivatives on basal guanylate cyclase activity. I.

COMPOUND	GUANYLATE CYCLASE ACTIVITY cyclic GMP formed : pmol/15 min/mg protein CONCENTRATION (mM) 0		10		30	
CP	250.19 ± 5	(1.00)	248.18 ± 8	(0.99)	225.27 ± 12	(0.90)
KP	255.23 ± 6	(1.00)	252.65 ± 6	(0.99)	266.39 ± 6	(1.04)
HP	253.01 ± 5	(1.00)	223.40 ± 4 [P < 0.01]	(0.88)$^{\phi}$	135.17 ± 6 [P < 0.001]	(0.53)$^{\phi}$
PM	252.52 ± 6	(1.00)	279.14 ± 8 [P < 0.01]	(1.11)$^{\phi}$	350.36 ± 10 [P < 0.001]	(1.39)$^{\phi}$
HN2	250.38 ± 5	(1.00)	248.64 ± 6	(0.99)	249.81 ± 7	(0.99)

$^{\phi}$Figures show a significant difference from the control in the Student's t test. Figures in brackets represent the ratio between activities in the assay to the control. Results are from two separate experiments performed in duplicate.

None of the other compounds tested showed any significant effect on basal cyclase activity. This corroborates the results of Vesely and Levey (121) who found no action of CP on basal guanylate cyclase activity at concentrations of up to 53 mM.

Table 8.2 shows the results of pre-incubation studies on basal cyclase activity.

The first column (a) in Table 8.2 was derived similarly to results presented in Table 8.1, except that the test compounds were pre-incubated in the assay cocktail for 30 minutes. The final concentration of test compounds was 10 mM, hence the concentration during incubation was 33.33 mM. Apart from the fact that basal enzyme activity was apparently reduced (although this is not a significant difference from previously measured activity as determined by the Student's t test) two major differences were observed. Firstly, PM did not appear to stimulate the cyclase enzyme and secondly, the inhibitory effect of HP was more pronounced, being 31% compared with 12% reported in Table 8.1.

The fact that PM did not appear to stimulate the enzyme may have been due to its removal from the cocktail solution by reaction with assay cocktail constituents or by its breakdown in aqueous solution (Chapter 9).

The increased effectiveness of HP as an inhibitor may have been due to similar reasons. The breakdown of HP, however, produces PM which would be expected to stimulate the enzyme (Table 8.1). The formation of OHCP from HP has been discussed in Chapters 9 and 10, and is considered to have been complete before the addition of HP to the cocktail. Enhancement of inhibition may therefore have been due to the unique binding properties of OHCP thiol or alcohol groups of compounds present in the assay cocktail. It is unclear how this might affect the assay to increase the observed inhibition.

TABLE 8.2 The effect of CP and its derivatives on basal guanylate cyclase activity. II

ADDITION	GUANYLATE CYCLASE ACTIVITY cyclic GMP formed : pmol/15 min/mg protein			
	to cocktail (a)		to enzyme (b)	
none	216.88 ± 5	(1.00)		
CP	202.60 ± 24	(0.93)	198.65 ± 1	(0.92)
KP	254.50 ± 11	(1.17)	213.95 ± 7	(0.99)
PM	158.25 ± 18	(0.73)	175.79 ± 3 [P < 0.1]	(0.81)$^{\phi}$
HP	149.50 ± 4 [P < 0.05]	(0.69)$^{\phi}$	50.88 ± 6 [P < 0.05]	(0.23)$^{\phi}$
HN2	223.29 ± 8	(1.03)	206.52 ± 6	(0.95)

Compounds were added to either cocktail or enzyme solutions to yield a 10 mM final concentration in the assay. (a) compounds in the cocktail were incubated for 30 minutes at 30°C. (b) compounds in the enzyme solution were incubated for 30 minutes at 4°C. $^{\phi}$Figures show a significant difference from the control in the Student's t test. Figures in brackets represent ratio of test:control. Results from one experiment only.

TABLE 8.2 The effect of CP and its derivatives on basal guanylate cyclase activity. II

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	to cocktail (a)		to enzyme (b)	
none	216.88 ± 5	(1.00)		
CP	202.60 ± 24	(0.93)	198.65 ± 1	(0.92)
KP	254.50 ± 11	(1.17)	213.95 ± 7	(0.99)
PM	158.25 ± 18	(0.73)	175.79 ± 3 [P < 0.1]	(0.81)$^{\phi}$
HP	149.50 ± 4 [P < 0.05]	(0.69)$^{\phi}$	50.88 ± 6 [P < 0.05]	(0.23)$^{\phi}$
HN2	223.29 ± 8	(1.03)	206.52 ± 6	(0.95)

Compounds were added to either cocktail or enzyme solutions to yield a 10 mM final concentration in the assay. (a) compounds in the cocktail were incubated for 30 minutes at 30°C. (b) compounds in the enzyme solution were incubated for 30 minutes at 4°C. $^{\phi}$Figures show a significant difference from the control in the Student's t test. Figures in brackets represent ratio of test:control. Results from one experiment only.

The second column (b) of Table 8.2 shows the effect of pre-incubating the test compounds with enzyme. Since the final concentration was 10 mM, the concentration of compounds during pre-incubation was 40 mM. The enzyme and test compounds were pre-incubated for 30 minutes at 4°C before their addition to the assay cocktail to start the reaction. Again, there are two important differences to be noted. Firstly, PM appeared to inhibit the enzyme by 19% and secondly, the inhibition produced by HP was increased to 77%.

The effects observed for PM are contradictory to those presented in Table 8.1. It is unlikely that under incubation conditions at 4°C for 30 minutes, PM would break down and therefore it may be assumed that the enzyme pre-incubation study reflects the 'true' action of PM upon the enzyme. However, the recorded results were the results from only one experiment in the case of the pre-incubation studies. It can be seen that the inhibition observed by PM during pre-incubation with enzyme (column (b), Table 8.2) was apparently a higher specific activity than that recorded for PM pre-incubated with assay cocktail (column (a), Table 8.2). Application of the Student's t test to these results suggested that although the assay cocktail pre-incubation results for PM appeared to reflect a higher inhibition of enzyme activity than pre-incubation with enzyme, they were not of statistical significance. This result may shed some doubt on the findings for pre-incubation of PM with enzyme. More data would have to be obtained to clarify the situation regarding pre-incubation of PM.

The increased effectiveness of HP to inhibit the enzyme probably reflects an action of HP directly upon the enzyme. Therefore, the inhibition was increased due to both an increase in time and concentration.

8.5 The Effect of CP And Its Derivatives On MNNG-stimulated Guanylate Cyclase Activity

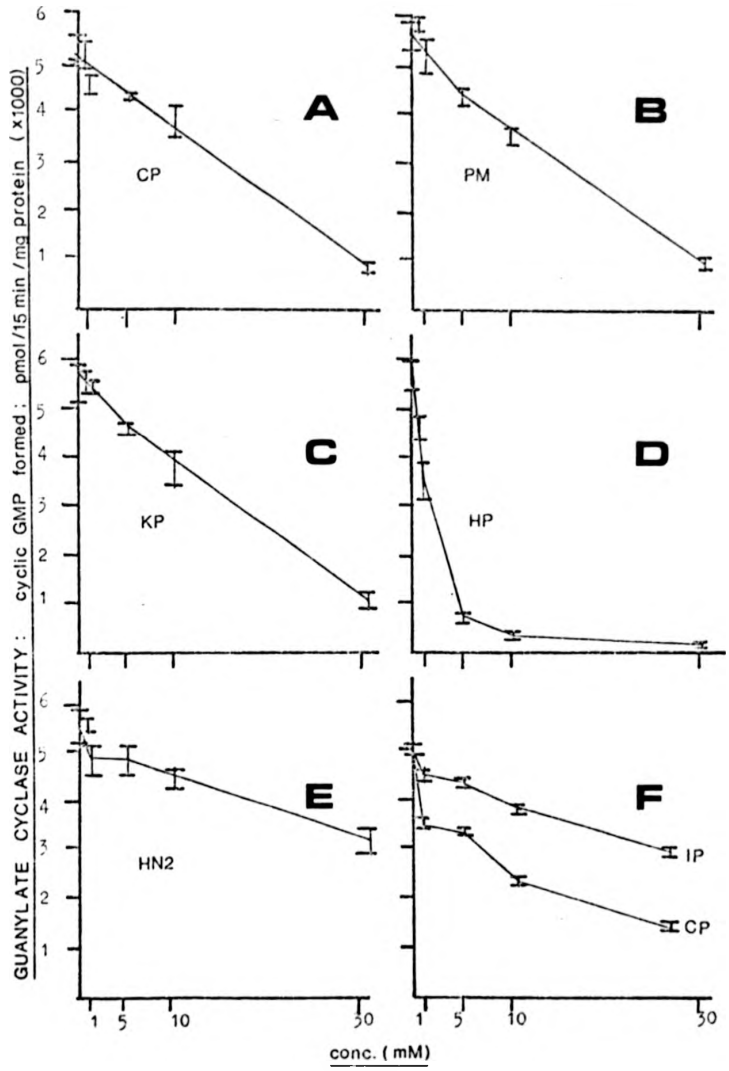
The effect of MNNG on guanylate cyclase activity has been presented in Fig. 8.1B. Fig. 8.2 shows the inhibitory effects of CP and its derivatives on this activity. The dose-response curve demonstrate a non-linear relationship which is very similar to that reported by Vesely and Levey (121) whose data have been redrawn in Fig. 8.2F for CP and isophosphamide inhibition of NNPG-stimulated guanylate cyclase activity.

It is apparent from these results that HP was the most effective and HN2 the least effective agent used, in inhibiting MNNG-stimulated activity. CP, KP and PM all showed very similar curves intermediary between HP and HN2. Some indication of their relative effectiveness may be given by the concentration required to reduce the stimulated activity by half. Such values are presented below :

CP	18.20 mM
PM	16.72 mM
KP	17.70 mM
HP	1.97 mM
HN2	>30 mM

These figures have been deduced from the data shown in Fig. 8.2 but it should be noted that the curves are not linear and, therefore, calculations relying on the extrapolation of data must be considered approximations only. However, it can be clearly seen that HP is between 8 to 9 times more effective than either CP, KP or PM.

Fig. 8.2 Effect of Cyclophosphamide And Derivatives On MNNG-stimulated Guanylate Cyclase Activity



A to E : In the presence of 40 μM MNNG. Test compounds were present in the assay cocktail, MNNG was pre-incubated with enzyme.

F : Redrawn using data from ref. (121). Stimulation is with NNPG (1 mM) and enzyme assayed for 10 min at 37°C.

Error bars represent standard deviation of results from two separate experiments performed in duplicate.

Table 8.3 shows the results of pre-incubation studies on MNNG-stimulated activity. To produce the results presented in Fig. 8.2 MNNG was pre-incubated with enzyme (4°C for 30 minutes) before its addition to the assay cocktail containing the test compounds. Column (c) (Table 8.3) was produced similarly but the test compounds were pre-incubated in the assay cocktail (30 minutes at 30°C). No differences between these results and those in Fig. 8.2 can be seen. This is in contrast to the results observed on basal enzyme activity (section 8.4).

A difference was observed, however, if the MNNG was incubated together with the test compound in the assay cocktail (30 minutes at 30°C) as seen in column (a) (Table 8.3). HP was found to be 4 times more effective and both CP and KP approximately 1.5 times more effective. Differences in activity observed could have been due to reactions of nitrogen mustards with MNNG or cocktail constituents. It is possible that HP, CP or KP may have also prevented the production of nitric oxide from MNNG or the interaction of this compound with the enzyme. These three compounds, of those tested, are the only ones which possess an oxazaphosphorinane ring.

When pre-incubated with the enzyme first (column (b)) a significant difference was observed for HP. This was similar to the effect observed on basal activity (section 8.4) HP inhibited the enzyme to a much greater extent. This can only reflect a direct action of HP on the enzyme. The greatest effect observed occurred when both MNNG and the test compounds were incubated with the enzyme column (d). All alkylating agents used, with the exception of HN2 showed a significantly increased effectiveness under these conditions compared to pre-incubation in assay cocktail alone, column (c). HP produced a 28-fold decrease in activity and PM a 5-fold decrease in activity under these conditions.

TABLE 8.3 The effect of CP and its derivatives on MNNG stimulated guanylate cyclase activity

ADDITION	GUANYLATE CYCLASE ACTIVITY cyclic GMP formed : pmol/15 min/mg protein			
	(a) to MNNG in cocktail		(b) to enzyme solution	
none	5254.11 ± 239	(1.00)		
CP	2762.14 ± 75	(0.55)	5884.00 ± 320	(1.16)
KP	2433.84 ± 5	(0.48)	3205.30 ± 670	(0.63)
PM	3824.41 ± 212	(0.76)	3890.74 ± 125	(0.77)
HP	115.54 ± 3	(0.02)	26.63 ± 1	(0.005)
HN2	4376.71 ± 365	(0.87)	4442.11 ± 200	(0.88)
	(c) to assay cocktail		(d) to enzyme in MNNG	
none	5234.24 ± 103	(1.00)		
CP	4237.13 ± 106	(0.81)	1355.85 ± 31	(0.26)
KP	4130.50 ± 23	(0.79)	2610.40 ± 231	(0.50)
PM	3588.85 ± 215	(0.69)	609.16 ± 129	(0.12)
HP	508.19 ± 127	(0.10)	18.70 ± 1.7	(0.003)
HN2	4565.27 ± 384	(0.87)	4324.01 ± 228	(0.83)

Test compounds were added to yield a final concentration of 10 mM, MNNG was added to yield a final concentration of 40 μM.

Results represent data from only one experiment performed in duplicate.

The most likely explanation of these results is (i) a direct effect of alkylating agent with enzyme, or (ii) a reaction of alkylating agent with MNNG, probably with nitric oxide produced from MNNG.

Pre-incubation studies, therefore, have suggested that CP and KP may act by an action with MNNG, possibly acting as centres or reaction with nitric oxide, and that HP apparently acts by a direct effect upon the enzyme. Several effects have been observed by pre-incubation;

(1) There was no significant difference in inhibition of MNNG-stimulated activity when the alkylating agents were pre-incubated with the assay cocktail.

(2) HN2 showed no significant difference in its effectiveness, whether it was pre-incubated with enzyme, MNNG or both enzyme and MNNG. Inhibition observed with this compound was 13.75 $\pm$ 2.2%.

(3) All other compounds were most effective when pre-incubated with both enzyme and MNNG. This may suggest that the inhibitory effects are brought about by a mechanism preventing MNNG stimulation.

(4) PM did not increase in effectiveness in any pre-incubation system other than with both enzyme and MNNG. Inhibition by PM was effectively increased from 26 $\pm$ 4.3% to 88 $\pm$ 2.5%.

(5) Although the results for CP were quite inconsistent (no inhibition observed in one set of data) this compound and KP appeared to be most effective when pre-incubated with MNNG.

(6) HP appeared to be more effective when pre-incubated with enzyme. Inhibition increased from 90 to 98% on pre-incubation with MNNG, to 99% on pre-incubation with enzyme and to 99.7% when pre-incubated with both MNNG and enzyme.

CHAPTER 9

THE BEHAVIOUR OF CYCLOPHOSPHAMIDE AND ITS DERIVATIVES IN SOLUTION : EFFECTS OF THIOL-CONTAINING COMPOUNDS ON ALKYLATING ACTIVITY

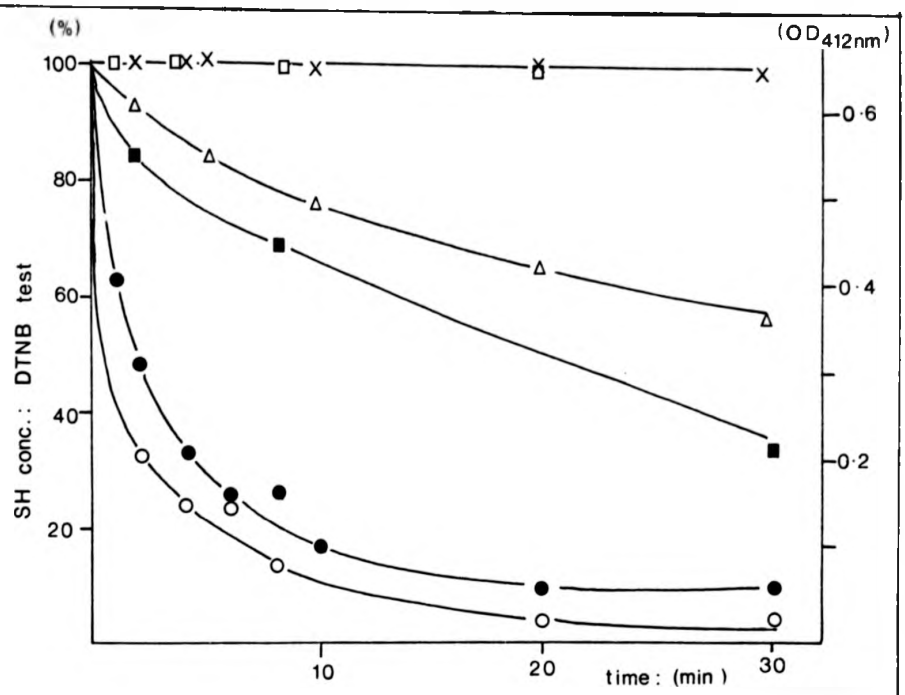
9.1 Introduction

The reaction of 4-hydroxycyclophosphamide (OHCP) with thiol (SH) groups has been noted (section 1.8) and has been studied previously by measurement of the decrease in titratable SH groups during incubation with thiol-containing compounds (169) and of the stabilising effect of such compounds on its alkylating activity. In this chapter, an account has been given of the behaviour of CP and its derivatives in solution.

9.2 Effect of Alkylating Agents On Measureable Thiol Groups

The results of incubation studies of CP and its derivatives with cysteine (section 2.9) are shown in Fig. 9.1. Within the time course of the experiment, neither CP nor KP had any effect on the concentration of free thiol groups in buffered solution. PM, however, produced a decrease in the concentration of free thiols, in a time-dependent manner. HP was found to exhibit the greatest effect, reducing the concentration by 50% in approximately 4 minutes at 30°C. The rate of this observed reduction in free thiol groups was shown to be reduced by either (i) decreasing the temperature of incubation (ii) decreasing the molar ratio of HP : cysteine. At a molar ratio of 1 : 10 the rate appeared to be reduced to zero, equalling that measured for CP, KP or cysteine alone. The data presented for HP appear to have little in common with the published data for OHCP

Fig. 9.1 Decrease In Titratable SH Groups By CP Derivatives



Except where stated otherwise, cysteine (10 mM) was incubated with test compound (10 mM) in phosphate buffer (0.1 M, pH7.0) at 30°C.

EFFECT OF :

X CYSTEINE ALONE

X CYCLOPHOSPHAMIDE (CP) or

X 4-KETOCYCLOPHOSPHAMIDE (KP)

Δ PHOSPHORAMIDE MUSTARD (PM)

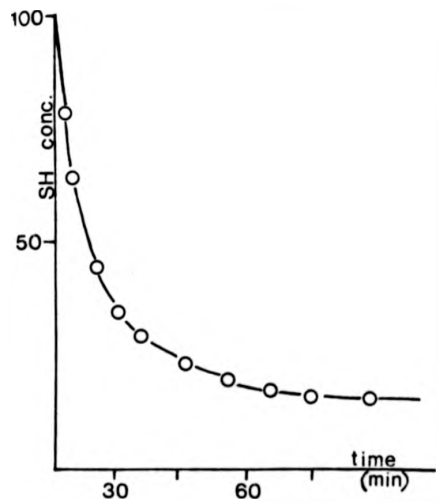
4-HYDROPEROXY-CP (HP) :

○ 37°C

● 30°C

■ 4°C

□ 30°C, 1 mM



Data for 4-HYDROXYCYCLOPHOSPHAMIDE, from ref.(169).

Results are from one experiment in each case, performed in triplicate

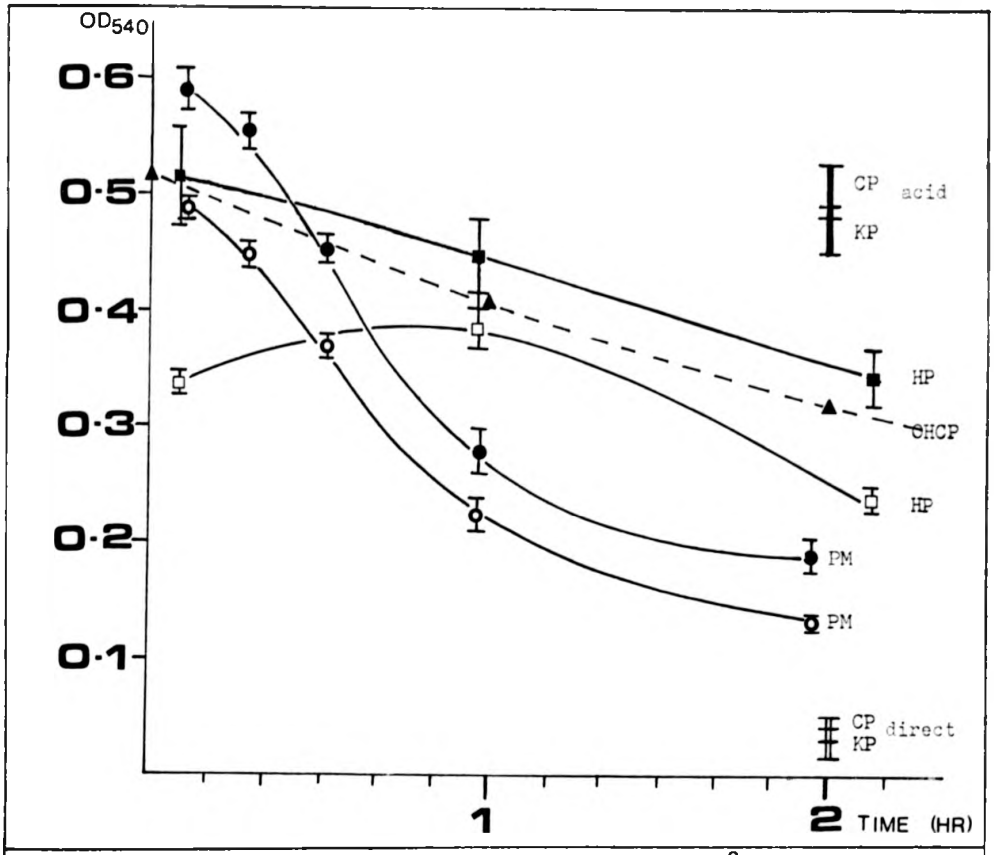
(Fig. 9.1). The observed reaction of HP with cysteine was both faster and more pronounced, than the published data suggest for OHCP and cysteine.

9.3 Behaviour of Alkylating Agents In Buffered Solution

By using the NBP test (section 2.4) the alkylating activity (both before and after acid hydrolysis) was determined. No detectable difference in this activity was observed for CP or KP during a two hour incubation in phosphate-buffered solution, Fig. 9.2. PM showed a decrease in both direct and acid hydrolysed alkylating activity. Although both curves (Fig. 9.2) show a sigmoidal relationship of activity with respect to time, they are not apparently proportional to each other. This had the effect of increasing the alkylation index (the ratio of direct : acid hydrolysed activity) which reached a maximum after approximately 70 minutes incubation. After this time the two curves appear to diverge (thus decreasing the alkylation index). A similar effect has been observed for HP, Fig. 9.2. The total or acid-hydrolysed activity decreased gradually, following approximately the decrease in alkylating activity reported for OHCP (169) (Fig. 9.2). The direct activity, however, increased to a maximum after 50 to 60 minutes incubation and then declined. The effect of this is an increase in the alkylation index from 65.3 (measured 5 minutes after addition of HP to buffer) to 86.6 after 54 minutes incubation.

There are a number of chemical transformations which may take place during incubation of PM or HP in buffered solution. For PM the most likely is hydroxylation of the chloroethyl groups by replacement of the chlorine atoms. This process is quite probably responsible for the observed decreases in alkylating activity of both PM and HP. HP may be capable of various other transformations. The observed initial alkylation

Fig. 9.2 Effect of Time On Alkylating Activity During Incubation In Buffer.

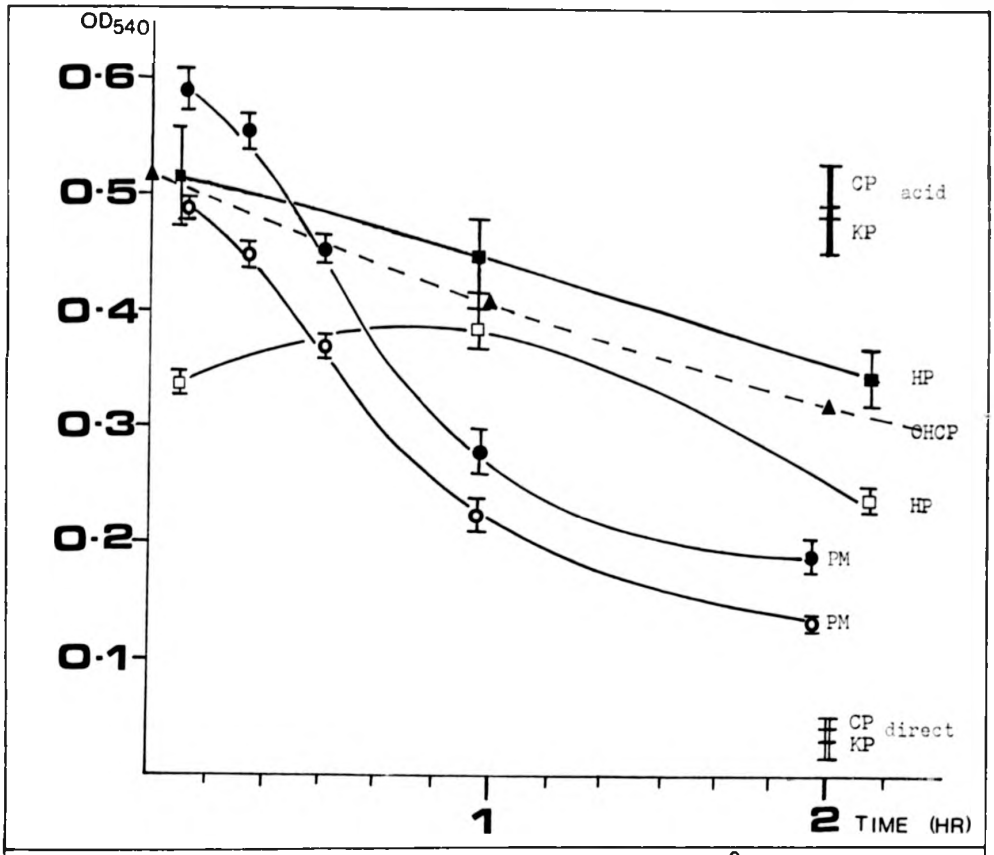


Incubation in phosphate buffer (0.1 M, pH7.0) at 30°C. Open figures represent readings before and closed figures readings after acid hydrolysis.

- ● PHOSPHORAMIDE MUSTARD (PM).
- □ 4-HYDROPEROXYCYCLOPHOSPHAMIDE (HP)
- △ 4-HYDROXYCYCLOPHOSPHAMIDE (OHCP) Plotted from data obtained from ref. (169)

All compounds used at a concentration of 2 mM. 100 nmoles assayed for alkylating activity by the NBP test, section 2.4. Results are from one experiment performed in triplicate. CP and KP showed constant activities (indicated) from 5 min to 2 hr curves omitted for clarity.

Fig. 9.2 Effect of Time On Alkylating Activity During Incubation In Buffer.



Incubation in phosphate buffer (0.1 M, pH7.0) at 30°C. Open figures represent readings before and closed figures readings after acid hydrolysis.

o PHOSPHORAMIDE MUSTARD (PM).

□ 4-HYDROPEROXYCYCLOPHOSPHAMIDE (HP)

Δ 4-HYDROXYCYCLOPHOSPHAMIDE (OHCP) Plotted from data obtained from ref. (169)

All compounds used at a concentration of 2 mM. 100 nmoles assayed for alkylating activity by the NBP test, section 2.4. Results are from one experiment performed in triplicate. CP and KP showed constant activities (indicated) from 5 min to 2 hr curves omitted for clarity.

index supports the hypothesis that HP spontaneously produces OHCP in aqueous solution and the close similarity of the decrease in acid hydrolysed activity for HP to OHCP (Fig. 9.2) provides further evidence. OHCP is capable of further degradation to more reactive intermediates (section 1.14) and this may account for the observed increase in directly measured alkylating activity.

9.4 Effect of Thiol Groups On Alkylating Activity

Table 9.1 shows the effect of incubating CP and its derivatives with cysteine on their alkylating activity. Although PM is the only compound to show a significant difference in alkylating activity after incubation, it must be borne in mind that section 9.3 described the effect on alkylating activity of incubation of these compounds under identical conditions but in the absence of cysteine. The results presented in Fig. 9.2 must, therefore, be used as the control for this experiment. It can be seen from the results that CP and KP showed no significant change in alkylating activity when incubated either with or without cysteine. Although PM lost alkylating activity after 2 hours incubation in phosphate buffer alone (Fig. 9.2) the loss of activity observed in the presence of cysteine appeared much greater (Table 9.1). This may be explained by the fact that PM is the only active alkylating agent tested, under the conditions of the incubation, and that alkylation of the thiol compound, cysteine, may be responsible for this loss in activity. This is further supported by the observed loss of thiol groups during such incubation (section 9.2).

TABLE 9.1 The effect of incubation with cysteine on alkylating activity

COMPOUND	ALKYLATING ACTIVITY			
	BEFORE INCUBATION		AFTER INCUBATION	
	DIRECT	ACID	DIRECT	ACID
CP	0.032	0.510	0.027	0.500
KP	0.025	0.470	0.024	0.465
HP	0.345	0.530	0.320	0.510
PM	0.495	0.580	0.052	0.080

Alkylators were incubated at equimolar concentration with cysteine (2 mM) in phosphate buffer (0.1 M, pH7.0) for 2 hours at 30°C. Alkylating activity was measured by NBP test (section 2.4) before (DIRECT) and after (ACID) acid hydrosysis, and expressed in O.D. units measured at 540 nm. Triplicate measurements were made and errors were within 5%.

Although HP showed no significant difference in alkylating activity during incubation with cysteine (Table 9.1) comparison with Fig. 9.2 shows that the alkylating activity has been stabilised in the presence of cysteine, a finding reported previously for OHCP (169). This effect correlates well with the observed disappearance of thiol groups during incubation (section 9.1).

CHAPTER 10

DISCUSSION

10.1 The Production of An 'Activated' Derivative of Cyclophosphamide

For studies of the mechanism of action of CP, especially those studies involving tissue or cell homogenates, it is essential to use an active form of CP unless the homogenate contains the necessary enzymes required for the conversion of CP to the 'primary' hydroxylated metabolite. Three methods of synthesis of such activated species were investigated. All methods involved the use of CP as starting material, which was available in abundance. Of these three methods, ozonisation of CP was the most successful.

(1) The Photooxidation Of CP (section 2.15)

The liver microsomal system which hydroxylates CP *in vivo* has been shown to generate singlet oxygen ($^1O_2$) (258). Furthermore, $^1O_2$ is thought to be formed in the reaction mixture of CP ozonisation and there is some evidence for this species forming HP upon reaction with CP (233). Photooxidation was attempted as a method of producing $^1O_2$ in the presence of CP, in the hope that a hydroxylated or hydroperoxylated derivative might be formed. The results were extremely disappointing. No change in the composition of the reaction mixture could be detected by NBP test (a measurement of alkylating activity), TLC or HPLC. The CP remained completely unchanged throughout the incubation procedure for up to 6 hours. Alteration in composition of the reaction mixture or in temperature had no effect.

Interestingly, the Rf value of CP obtained on TLC (silica gel 60, ethyl acetate, -25°C) was found to be dependent upon the solvent of

application. When applied in acetone, a high Rf value (0.92) was obtained and when applied in H_2O a low value (0.35) was observed. This was probably due to the presence of H_2O on the TLC plate when an aqueous application solvent was used. Thorough drying of the sample after application to the TLC plate might have prevented this effect but the risk of causing thermal decomposition of the sample was considered to be too great.

(2) Microsomal Activation of CP (section 2.6 And Chapter 6)

The microsomal hydroxylation of CP by the mixed function oxidase system of rat liver was rather more successful. This method has been used in the past (146) (148) (151) and information from these sources was used (a) to produce microsomes of the highest activity (using male rats fed with phenobarbital before utilising their livers for the production of microsomes) and (b) to produce a more stable derivative of CP than OHCP, by 'trapping' the OHCP produced as its ethoxy derivative. This was achieved simultaneously with deproteinisation upon the addition of ethanol to the reaction mixture (section 6.2).

It was found possible to identify both CP and the 'fast-running' diastereoisomer (151) of 4-ethoxy-CP by TLC. Other, possibly decomposition products, i.e. PM, were also observed. The presence of such compounds made it important that purification was carried out before use in enzyme studies.

It was possible to isolate the fast-running product relatively easily by flash chromatography. However, its instability proved to be too great for effective purification, concentration and subsequent characterisation by this route.

(3) Ozonisation of CP (section 2.5 and Chapter 4)

Ozonisation of CP was the most successful of the methods tried, many initial difficulties being overcome. Although many products are formed (Fig. 4.2) the eventual availability and use of a sample of HP as a standard, made identification of product by TLC quite simple. It was not possible to identify HP unequivocally by published Rf values because of the inconsistency of these. Variation is thought to be due to the presence of water vapour (234) and the effect of application solvent upon Rf value bears this out (section (1), above).

Of the many products formed during ozonisation most were shown to be alkylating agents (by visualisation with NBP spray) and some of these were also shown to be peroxides (by visualisation with KI). Although there were so many products, the subsequent isolation of HP after prior removal of most KP, was successfully achieved by the application of flash chromatography. Some success has been reported by the use of fractional crystallisation but this is exceptionally difficult even when the process of 'seeding' with crystals of HP is employed. The technique currently employed by other workers in this field for purification of products is preparative HPLC (234). Silica gel column chromatography has been previously reported for the isolation of HP from reaction mixtures, after the Fenton oxidation of CP, containing CP, KP, HP and the 4-hydroperoxy-CP-anhydrodimer (247) but the use of pressure columns in particular has not. The major advantage of this technique in this context is one of time-saving. This is particularly important in view of the instability of the product at temperatures above -25°C. Once the flash chromatography column had been characterised and the elution profile for HP known, chromatography took less than 30 minutes at 4°C. Concentration of the product by solvent evaporation *in vacuo* at 0°C did not affect the physico-chemical properties of the product.

Peter *et al.* (233) have suggested the following reactions for the formation of products during ozonisation (reaction 18) but this does not

$$\begin{array}{llllll} (1) & CP & + & O_3 & \longrightarrow & 4\text{-keto-CP} \quad + \quad {}^1O_2 \\ (2) & CP & + & {}^1O_2 & \longrightarrow & 4\text{-hydroperoxy-CP} \end{array} \qquad (18)$$

correlate with the observation that increased ozonisation reaction time results in a greater yield of KP at the expense of HP (234).

(4) Characterisation (Chapter 5)

Using various physical techniques (and the NBP test, Chapter 3) it was possible to show that various parameters measured agreed with the published data and that the samples of CP, KP and PM were chemically pure. The use of a standard sample of HP was essential due to certain discrepancies found between measured and reported properties. This has been found, particularly, for the material present in both supplied (by Colvin) and synthesised samples which has been assumed to be the diastereoisomer of HP (Fig. 1.13). NMR and melting point data suggest that the sample synthesised is indeed HP. Contamination of the sample may have appeared in the NMR spectra and would probably have reduced the melting point from that observed. TLC and HPLC both revealed two products in this sample, however. Peter *et al.* (233) have reported that one of the diastereoisomers produced is less stable than the other and this may result in a lower yield of this product. This has allowed the discrimination between the isomers in the chromatographic systems. By HPLC, the fast-running isomer has been measured to comprise only 2% of the total sample (section 5.6). By TLC, the fast running isomer has been estimated to be in lower

concentration than the other, by colour intensity and spot size when visualised with NBP spray (Fig. 4.1). Although the HPLC system was unable to resolve KP from the fast-running HP isomer, it was possible to easily differentiate between these compounds by TLC.

The chromatographic data reported by Peter et al. (233) (234) suggests a larger difference in the physico-chemical properties of HP and its diastereoisomer than has been observed. This would not, perhaps be expected where no large groups are involved in the structure of the compound (Fig. 1.13). The sample donated by Colvin (see section 2.1(1)) was produced in his laboratory by ozonisation of CP as reported here. The sample produced in our laboratory was found to be identical to that of Colvin's, by melting point, TLC, HPLC, mass spectroscopy and alkylating activity. The performance of the less stable diastereoisomer in these samples in TLC and HPLC systems does not agree with reported data which suggests much larger differences between isomers than has been observed.

10.2 The Conversion of HP to OHCP

The NBP assay for alkylating activity has been shown to be capable of differentiating between certain alkylating agents by their activity in the assay before and after acid hydrolysis and the reasons for this have been discussed (Chapter 3). Brock (172) has provided data which shows that it is possible to discriminate between HP and OHCP using this method. No details of his method were given, however, and it has been assumed, therefore, that the quantity he has termed "% alkylating activity" was derived in the same way as that which I have termed the "alkylation index" (section 3.2).

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Using this method, evidence for the spontaneous conversion of HP to OHCP has been presented (Chapter 3). This evidence has been provided by the different alkylation indices observed for HP dependent upon the solvent in which the sample had been dissolved. In non-aqueous, organic solvent, i.e. acetone, a lower activity (Alkylation Index = 39.85) was recorded than when HP was dissolved in aqueous solution (Alkylation Index = 66.3). These values correlate well with those reported by Brock for HP (40%) and OHCP (65%) respectively. This result has been interpreted as the deoxygenation of HP in aqueous solvent, yielding OHCP. A possible mechanism for this reaction has been published by Benckhuysen et al. (244) and involves the production of hydroxyl radicals from HP (reaction 19). The involvement of one molecule of water per molecule of HP explains why this reaction does not occur in non-aqueous solvent systems.

Further evidence for this conversion has been provided by the behaviour of HP in solution (Chapter 9). It has been shown that the decrease in alkylating activity (after acid hydrolysis) observed over two hours

$$\begin{array}{lcl} \text{CP-O-OH} & & \text{CP-O} \quad + \quad \text{-OH} \\ \text{CP-O} \quad + \quad H_2O & & \text{CP-OH} \quad + \quad \text{OH} \\ \hline \text{CP-O-OH} & & \text{CP-OH} \quad + \quad \text{OH} \\ \hline\hline \end{array} \qquad (19)$$

in aqueous solution (buffered) for HP follows closely the reported rate for OHCP (Fig. 9.2). Furthermore, the stabilisation of HP alkylating activity by equimolar cysteine in buffered solution also agrees with data for OHCP (Table 9.1). This effect is brought about by the derivitisation to sulphido-compound of OHCP via the postulated imino-

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phosphamide intermediate (section 1.18). Evidence for this reaction is further supported by the observed reduction in the amount of free thiol groups in the presence of HP (Fig. 9.1). This latter observation also provides some conflicting evidence. The extent of sulphydryl "removal" and the rate of reaction does not agree with the published data for OHCP (Fig. 9.1) (169). The observed effect of HP was more rapid and effective (i.e. removed more free thiol groups from solution) than that reported for OHCP. It may well be that there is some involvement of hydroxyl radicals in this behaviour of HP towards thiol-containing compounds, as these would not be present in solutions of OHCP produced by other means. Triphenylphosphine in equimolar concentration to HP has been extensively used as a method for converting HP to OHCP (154) and has been reported to give OHCP in only 40% yield. However, the spontaneous conversion in aqueous solution reported here is now generally accepted and has been used in previously published experiments (154) (157) (199) (243).

Given the formation of OHCP from HP, there is no reason to suppose that further conversions will not occur. Tautomerisation of OHCP to AP followed by β-elimination of acrolein to yield PM is quite possible, considering the instability of OHCP (154). The production of PM from HP in solution has been suggested (199) to explain the observed similarity of action of these two compounds in long time course (48 hr) experiments. The observed increase in alkylation index of HP in solution (Fig. 9.2) may be evidence for the existence of derivatives formed from OHCP. At the maximum value recorded, the alkylation index reached 84.78 after 46 minutes incubation in phosphate buffer. This compares favourably with the alkylation index observed for PM (83.60).

10.3 Behaviour of The Alkylating Agents In Solution (Chapter 9)

Whereas CP and KP were shown to undergo no changes in buffered aqueous solution, both PM and HP have been found to exhibit different alkylating activities during incubation. The possibility of conversion of HP to other derivatives with higher alkylating activity has been discussed and this may be responsible for the observed rise in activity with time. Decreases observed in alkylating activity were most probably due to hydrolysis of the compounds. In the presence of thiol-containing compounds the measured decrease in PM alkylating activity is most probably attributable to both hydrolytic products and alkylation of the thiol (section 9.4). It has been observed that the direct and acid-hydrolysed alkylating activity do not vary in direct proportion. This may be explained by the formation of new compounds, possibly by hydrolysis, which have different alkylating indices. Hydrolysis would be expected to decrease the total alkylating and potential alkylating material (as measured after acid hydrolysis) and would also decrease the 'true' or direct alkylating activity.

During the time of incubation with enzyme in such studies (15 minutes) very little change in alkylating properties can be seen to have occurred. Greater changes may be expected for longer incubation times such as those used in the pre-incubation studies with guanylate cyclase activity. Voelcker *et al*. (157) have also shown that the spontaneous β-elimination of acrolein from OHCP (and therefore the production of PM) is reduced by 70% in the presence of Mg^{2+} or Ca^{2+} ions. This effect is relevant to the incubation conditions used for enzyme studies.

10.4 Adenylate Cyclase (Section 2.2 and Chapter 7)

(1) Characterisation

The observed activity of adenylate cyclase in rat liver plasma membranes was slightly lower than reported levels (approximately 90% compared to recorded activity (80)). Difficulties were encountered with preparing the membranes from rat liver and the method of preparation may be responsible for this discrepancy (section 2.2). Stimulated enzyme activity using either sodium fluoride or glucagon was also slightly less than reported values, however, the fact that stimulation did occur suggested that the adenylate cyclase system was intact within the membranes. The specific activity observed would be a function of the purity of the membrane preparation, and a low cyclase : protein ratio could be obtained if membranes were contaminated with other proteins. A second possibility is loss of or inactivation of some cyclase during membrane preparation. The assay for the standard 'marker' enzyme for plasma membranes, 5-nucleotides, indicated that the subcellular fractionation technique employed, did in fact isolate plasma membranes, as the specific activity for this enzyme was equal to reported values (Table 7.1). However, the effects of the phosphodiesterase inhibitor SQ20009 on adenylate cyclase activity may demonstrate that the preparation was contaminated with small amounts of phosphodiesterase, although another inhibitor of this enzyme, theophylline, did not corroborate this and it was not considered necessary to include such a compound in the routine assay (Fig. 7.1). Purification of the membranes by sucrose density gradient centrifugation has been reported (240) but highly purified membranes have been shown to possess low specific activity of adenylate cyclase (80) and this procedure was not, therefore, carried out.

(2) The Effect of Cyclophosphamide And Its Derivatives On Adenylate Cyclase Activity (Chapter 7)

Of the derivatives tested (CP, KP, HN2, PM and HP) only HP was found to evoke any response on the adenylate cyclase activity. No effects were observed on basal or stimulated (sodium fluoride or glucagon) activity in the presence of the other four compounds. Although CP and KP are chemically unreactive, PM and HN2 may be considered to be active alkylating agents under the cyclase assay conditions. It is important to note, therefore, that alkylating activity *per se* does not determine the effectiveness or activity of CP derivatives in the cyclase assay. HP, it has been noted, produces OHCP, the 'primary' metabolite of microsomal activation, under appropriate conditions, and it is considered that the latter compound is present in the enzyme assay incubation. Both HP and OHCP are inactive alkylating agents under the conditions of the assay, and the effects observed are not, therefore, considered to be due to the alkylating activity of these compounds.

(3) Effects of HP On Basal Adenylate Cyclase Activity

HP was shown to inhibit basal (Mg^{2+}) adenylate cyclase activity by 50% at approximately 5 mM concentration. This inhibition of activity exhibited a linear dose-response relationship with respect to the concentration of HP (Fig. 7.5). Although the inhibition observed at 1 mM HP was quite small (18%) it was shown to be a significant difference by the Student's t test (Table 7.3).

Although microsomal activation *in vitro* and subsequent metabolite extraction was found to be difficult (Chapter 6 and section 10.1) a freshly prepared extract was employed in this assay. Information from the NBP test on this sample suggested that it contained OHCP at a

concentration of 2.76 mM (the 4-ethoxy-CP derivative of OHCP produced during extraction by this method yields OHCP spontaneously in aqueous solution in a similar fashion to HP). The same sample was also shown to inhibit basal cyclase activity by approximately 36%. Although the estimation of OHCP concentration may be subject to error due to certain assumptions made in the calculation (section 4.3) it can be seen that these values of concentration and enzyme activity (or inhibition) correlate quite well with the inhibition observed with HP (Fig. 7.5). Further, discrepancy between these values in comparison to the results obtained for HP may be accounted for by the fact that EGTA was not included in the reaction cocktail when the microsomal extract was used (Table 7.3).

Inhibition of basal activity implies that OHCP interacts with, in some way, the catalytic component itself, and may involve the Mg^{2+}-binding (probably the N) component also (section 1.4). Reaction by alkylation must be ruled out, but OHCP possesses chemical reactivity of an unusual type (section 1.18). This reactivity is unique to OHCP (although similar binding can occur with AP). OHCP has the ability to bind reversibly to thiol or alcohol-containing compounds via an iminophosphamide intermediate, with which it is in equilibrium. Apart from this reaction, the only other possible interaction for OHCP involves its suggested structural similarity to cyclic AMP (137). Physico-chemical effects such as membrane permeation may also be involved (see section 10.4(6)).

(4) Effects of HP On Sodium Fluoride-Stimulated Adenylate Cyclase Activity

HP inhibits the sodium fluoride-stimulated activity of adenylate cyclase (Fig. 7.4). Inhibition is only partial (as is inhibition of basal activity) and sodium fluoride is therefore still an effective activator in the presence of HP, although the degree of activation is proportionately lower. Increasing HP (inhibitor) concentration decreased the enzyme activity in the presence of sodium fluoride (activator) (Fig. 7.5) in a linear dose-response relationship similar to that observed for HP and basal activity.

F^- ion activation is mediated by the N (nucleotide binding) component of adenylate cyclase which then associates more closely with the catalytic unit and by an unknown mechanism this stimulates cyclase activity (Fig. 1.6). During inhibition by HP this mechanism still appears to be operating since sodium fluoride is still effective as an activator ligand. This may be seen as evidence that HP does not exert its effect by action on the N-component, but on the catalytic unit. At least, HP cannot be acting at the level of F^- ion binding or N-C component coupling.

From a kinetic point of view, analysis of the observed data is extremely difficult due to several factors : (1) The adenylate cyclase enzyme is a complex unit of functional sub-components (2) Two activator ligands are present in this experiment (Mg^{2+} ion and F^- ion) (3) Enzyme activity is absent without Mg^{2+} ion but is still present without F^- ion (at a lower velocity) (4) The inhibition observed with HP is only partial. Many models of Enzyme-Activator(s)-Substrate-Inhibitor interactions seem to satisfy the sparse evidence for such modelling presented in Fig. 7.4. Of the apparently more probable cases, those involving the binding of inhibitor to an enzyme-activator and/or enzyme-activator-

substrate complex appear to be consistent with the results. To ascertain more precisely the mechanism of action of HP, studies should be made with varying substrate concentrations, in the presence or absence of HP and/or activators. This would enable one to discriminate between sites of action for HP. Because of considerations of time and expense, this could not be done.

(5) Effect of HP On Glucagon-Stimulated Adenylate Cyclase Activity

The stimulation of cyclase activity evoked by glucagon was completely abolished by HP at 5 mM concentration (Fig. 7.3). Increasing HP concentration decreased the enzyme activity in the presence of glucagon rapidly, and in a non-linear dose-response relationship (Fig. 7.5).

At 5 mM concentration, the inhibited adenylate cyclase activity in the presence of glucagon was not significantly different from the HP inhibited basal activity. Several possible sites of HP-cyclase interaction may be postulated. Glucagon acts by binding to the receptor (R) component (section 1.4) and the stimulatory action on the catalytic unit is mediated by the nucleotide binding protein (N component) which must bind GTP to exert any effect on the C unit during glucagon stimulation (Fig. 1.4).

If HP were to act on the catalytic unit alone, as suggested for basal and fluoride-stimulated cyclase inhibition, it would be difficult to explain the observed effects of HP on glucagon-stimulated activity. In this case, HP probably acts at a second site also. HP may act at the level of glucagon binding, R-N interaction, GTP binding to N or RC-N interaction. The latter case is unlikely in view of the results for fluoride stimulation in the presence of HP, unless the N-C interaction is somehow different during glucagon stimulation.

Further experiments would be necessary to determine the site of action of HP during glucagon stimulation. Binding studies of glucagon and GTP have been used to investigate adenylate cyclase component interactions (15) and similar studies in the presence of HP would help to elucidate and discriminate between proposed mechanisms of action.

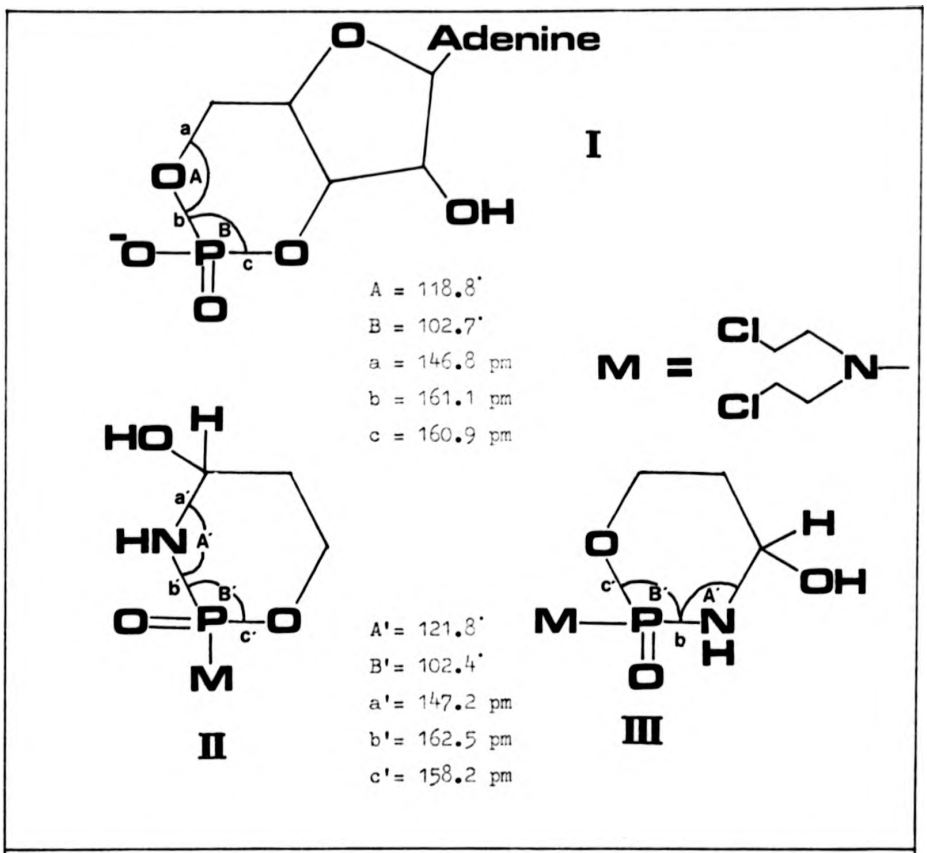
(6) Postulated Mechanisms of Action of HP

In view of the fact that only HP of all the alkylating agents tested had any effect on adenylate cyclase activity, it may be assumed that its inhibitory actions were brought about by a property unique to this compound. In this respect, the further conversion of HP to chemically reactive compounds may be important.

The conversion of HP to OHCP has been discussed (section 10.2) and it is considered that the latter compound is present in enzyme incubations. The production of free radicals during this process is one possible mechanism for HP action due to the high reactivity of such species. Both CP-O· and ·OH radicals are thought to be produced (244) (reaction 19) and either of these could conceivably attack either enzyme molecules or assay cocktail constituents. This mechanism seems unlikely due to the pre-incubation of HP before its addition to the assay ($4^{o}C$, H_2O) and the usually short half life of free radicals.

OHCP exhibits both structural and functional properties which may be involved in its (and, therefore HP) action. Tisdale (137) has suggested a structural similarity between cyclic phosphate moieties of cyclic AMP and cyclophosphamide (section 1.11) Fig. 10.1. This evidence does not appear entirely convincing, especially since the proposed structural congruity is shared between all CP derivatives which possess an intact oxazaphosphorinane ring (these include CP, KP, HP, OHCP and related

Fig. 10.1 Bond Distances And Angles Of The Cyclic Phosphate Moiety Of Cyclic AMP And 4-Hydroxycyclophosphamide



I Cyclic AMP

II 4-hydroxy-CP (OHCP) drawn as proposed by Tisdale (137)

III OHCP redrawn to re-orientate the OH group

sulphido-derivatives of OHCP). Because this property is not unique to the active species (the OHCP derivative) it cannot, alone explain the observed effects specific to HP or OHCP. However, recognition of this portion of the molecule by the active site of an enzyme may serve to bring the derivatives into close proximity with the enzyme molecule, where other properties of the derivative might act to alter the activity of the enzyme.

The functional, thiol (sulphydryl group) and alcohol (hydroxyl group) binding property of OHCP have been discussed and this is a highly probable mechanism for HP action. Binding to protein thiol or alcohol groups could well interfere with the further binding of ligands (glucagon, GTP, F^- ion, Mg^{2+}ion, ATP in the case of adenylate cyclase) or interfere with component interactions. Binding of OHCP to thiols may also be a method for bringing the molecule into close proximity to the enzyme. Although OHCP is not an active alkylating agent, and appears to be unreactive when bound as a sulphido derivative, it may be possible that under the right conditions, release of active PM would occur from OHCP in the close proximity of the enzyme. The alkylating properties of PM would in this case be responsible for the inhibitory effects of HP. Such a hypothesis must also explain why PM itself is ineffective. It may be postulated that the site of alkylation of the enzyme which leads to its inhibition is somehow inaccessible to PM. Binding of OHCP to enzyme, by whatever mechanism, would thus serve to introduce alkylating material into the immediate neighbourhood of the enzyme.

Although it seems likely that OHCP exerts its tumour-specificity through selective permeation of cancer cells (section 1.18) and that this may be achieved via a carrier mechanism, permeation of OHCP into the liver plasma membrane may be of importance to its action on adenylate cyclase activity.

Further conversion of OHCP is very likely. Tautomerisation to AP is reported to result in an equilibrium favouring OHCP (157). This tautomerisation may be important as AP has been shown to be a thiol-reactive compound, producing semi-mercaptal derivatives which may be capable of cyclisation to mercapto derivatives of OHCP (157) (233). β-elimination of acrolein from AP yields PM. The formation of PM may not be responsible for the effects of HP, since PM alone has no effect on cyclase activity.

10.5 Guanylate Cyclase (Section 2.3 And Chapter 8)

(1) Effect of CP And Its Derivatives On Basal Activity

Guanylate cyclase basal (Mn^{2+}) activity was found to be close to reported values. Different CP derivatives were found to affect basal activity in different ways; (1) HP was found to inhibit basal activity by nearly 50% at 30 mM concentration (50% inhibition was estimated to occur at approximately 32 mM concentration) (2) PM was found to stimulate basal activity by nearly 40% at 30 mM concentration (50% stimulation was estimated to occur at approximately 36 mM concentration). None of the other compounds tested (CP, KP or HN2) had any significant effect on guanylate cyclase activity (Table 8.1).

The inhibitory effectiveness of HP was increased when it was pre-incubated with assay cocktail before addition of enzyme, the observed inhibition increased from 12 to 31% at a concentration of 10 mM HP (Table 8.2). Several explanations are possible. For example, the effect could be due to reaction of OHCP with assay constituents which contain thiol or hydroxyl groups. Presuming that HP itself is not inhibitory and that inhibition of cyclase activity is dependent upon the conversion of HP to OHCP, pre-incubation may conceivably increase conversion of HP to OHCP

or even favour tautomerisation of OHCP to AP. This explanation assumes that conversion of HP to OHCP was not complete before addition to the assay cocktail or that formation of AP from OHCP enhances the inhibitory effect. An explanation of the mechanism of action of HP on guanylate cyclase may be as follows: guanylate cyclase has been shown to possess and require vicinal thiol groups for full expression of its activity (section 1.8(iii)),(70) binding of OHCP or AP to these groups would most likely inhibit the enzyme.

A third possible mechanism is that some other product of HP incubation in assay cocktail produces the enhanced inhibition (Chapter 9). One possibility here is that HP (OHCP or AP) may "remove" necessary compounds from the assay cocktail or that the products of such reactions are themselves inhibitory but this seems unlikely in view of the nature of the cocktail constituents (Table 2.4).

Pre-incubation of HP with enzyme solution showed that inhibition does not apparently involve assay constituents since inhibition under these conditions was greatly enhanced. Inhibition increased to 77% (Table 8.2) when HP was pre-incubated with enzyme. This is probably due to a combined effect of time and concentration. By extrapolation of previous data (Table 8.1) increasing concentration to 40 mM would not be expected to produce the observed inhibition. The favoured mechanism of action on basal cyclase activity is the reaction of OHCP or AP with protein thiol groups. Increased incubation time would allow more thiols to be bound to OHCP or AP if the reaction equilibrium was reached only slowly.

When PM was pre-incubated with assay cocktail, the stimulation observed originally was abolished (Table 8.2). Incubation of PM under such conditions reduces its alkylating activity by hydrolytic reactions (Chapter

9) but alkylating activity per se is not apparently a prerequisite for effecting activity (as in the case of adenylate cyclase activity) since HN2 had no effect. Reaction of PM with assay cocktail constituents is a possibility. Pre-incubation of PM with enzyme produced an inhibition of cyclase activity, in complete contradiction of previous results. There would appear to be two possible explanations for this result. The first is that low concentrations of PM are stimulatory whereas high concentrations are inhibitory. The loss of any action after pre-incubation in assay cocktail would therefore be due to hydrolysis of PM with a resultant loss of alkylating activity. In view of the results presented in Table 8.1 this explanation seems very unlikely since the action of PM would have to change from stimulatory (1.39-fold) at 30 mM to inhibitory (0.81-fold) at 40 mM (as used for enzyme incubation studies to yield a final concentration of 10 mM). The second explanation assumes that the hydrolysis product of PM is inhibitory whereas PM itself is stimulatory. However, since pre-incubation with assay cocktail would be expected to produce more of the hydrolysis product due to the raised temperature (30°C cf. 4°C during enzyme pre-incubation) this explanation does not fit the results. Inhibition of the enzyme by a PM hydrolysis product would not explain all of the observed effects, although inhibition by an intermediate of PM hydrolysis might. Such an intermediate could be the product of hydrolysis of just one chloroethyl group. Why there should be such variation in the effects of PM depending on the assay conditions is completely unknown.

(2) Effect of CP And Its Derivatives On MNNG-Stimulated Guanylate Cyclase Activity

MNNG stimulated guanylate cyclase activity by a maximum of 28-fold at 100 μM concentration (Fig. 8.1B) and exhibited a sigmoidal dose-response

relationship. It is considered that this compound acts on the cyclase enzyme via the spontaneous formation of nitric oxide radical which is a potent stimulator (71), the mechanism of action of which may involve the free thiol groups of the enzyme (section 1.8(vi)). All of the alkylating agents tested were shown to inhibit MNNG-stimulated activity. The extent of inhibition varied between compounds, relative effectiveness being in the order : HN2 CP KP PM HP (Fig. 8.3). HP was nearly 10 times as effective as either, CP, KP or PM. The dose-response relationship for these compounds was found to be non-linear and very similar to that reported for CP and isophosphamide inhibition of NNPG-stimulated activity (Fig. 8.3F) (121).

The mechanism of the inhibition observed cannot be related to the alkylating activity of the derivatives; both reactive and unreactive compounds showed an inhibitory effect. The superior effectiveness of HP may have been due to the action of OHCP or AP on enzyme thiol groups especially in view of this compounds effects on basal activity.

Pre-incubation studies (Table 8.3) suggest that CP and KP may exert their effects by interaction with MNNG and that HP exerts its effects on the enzyme molecule predominantly. PM was shown to exert greater inhibition when pre-incubated with both MNNG and enzyme, whereas HN2 was found to show no difference in the level of inhibition whether it was pre-incubated or not.

Inhibition of MNNG-stimulated activity may be produced by interaction with NO·(nitric oxide radical), and its action on the enzyme. It may be possible that the CP derivatives used are preferential substrates for NO· attack, thus preventing its reaction with enzyme. The only objection to this hypothesis is the difference between the concentration of MNNG (and

therefore NO·) and nitrogen mustard derivatives required for inhibition of its effects. MNNG was present in only 40 μM concentration whereas partial inhibition (50%) required approximately 17 mM concentration (CP, KP, PM) a difference of over 400-fold in favour of CP derivatives.

(3) Radicals

The results of HP with guanylate cyclase activity may reflect the fact that conversion of HP to OHCP has already been completed before addition of HP solution to the enzyme assay. The generation of OHCP is thought to generate hydroxyl radicals. Hydroxyl radicals have been shown to be a potent stimulator of guanylate cyclase activity (section 1.8(vii)). Since HP produced inhibition of the enzyme either (i) OHCP is in competition with stimulatory hydroxyl radicals or (ii) no hydroxyl radicals are present and OHCP only is affecting the enzyme.

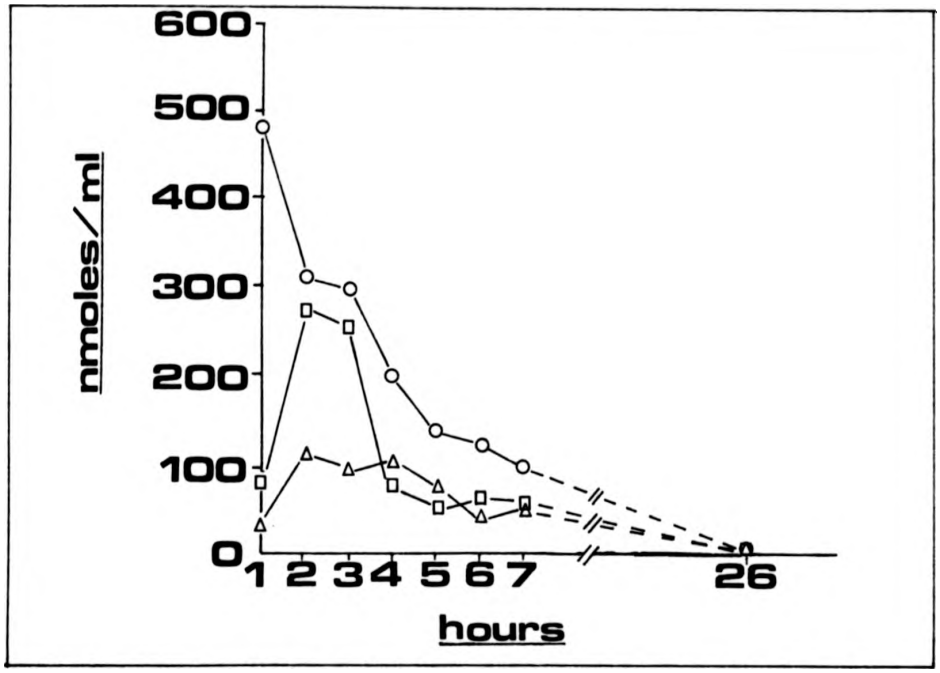
10.6 Relevance To Cyclophosphamide Therapy

The physiological concentrations of CP and its derivatives in CP-treated patients have not yet been fully determined. Fig. 10.2 shows the changes in concentration of CP, PM and nor-HN2 in the plasma of a patient receiving CP chemotherapy.

The maximum plasma concentration of CP reached is 0.5 mM, nor-HN2 0.3 mM and PM 0.1 mM. Jardine *et al.* (174) have reported a large variation in these levels between patients. In some cases the levels of nor-HN2 peaked at 0.5 to 0.6 mM, similar to that of CP. PM plasma concentration, however, has not been reported above 0.1 mM.

Quantitative estimation of CP metabolites possess several problems but some attempts have been made. Successful attempts to measure the

Fig. 10.2 Plasma Levels of CP (O) PM (Δ) And Nor-HN2 (□) For A Patient Receiving A 1 Hour Infusion of Cyclophosphamide (75 mg/kg). From Ref (174)



primary activated metabolite OHCP or AP have been reported. Conversion of OHCP to 4-(S-benzyl)mercapto-CP by reaction with benzyl mercaptan followed by separation of this derivative by thin layer chromatography was shown to be possible (259). Fenselau et al. (155) have characterised AP by its derivatisation to the cyanohydrin derivative followed by gas chromatography-mass spectrometry. The authors of these papers suggest that these techniques could be used to quantitate OHCP and AP levels in plasma but, unfortunately, no results of such studies have been presented.

The concentration of these metabolites is possibly increased in urine. Averaged results for urine collected over 4 hour periods from patients receiving CP treatment, suggest that CP has a maximum concentration of 2.5 mM (8 to 12 hours after start of CP infusion) and nor-HN2 peaks at 3.97 mM (12 to 16 hours after start of infusion) (174). PM concentration apparently remains low in urine, peak concentration being 0.21 mM (12 to 16 hours after start of infusion). Its higher reactivity at acid pH values would make this compound a very reactive metabolite in urine (Table 1.6) which may possibly prevent its detection.

The plasma concentration of OHCP may be equivalent to that of PM. Cells are not likely to experience concentrations higher than 0.1 mM unless metabolites were selectively concentrated. This may well be the case for OHCP since the oncostatic specificity of CP is considered to be exerted at least in part by the specific permeation of tumour cells by OHCP. The concentration of OHCP within such cells has not been measured and is difficult to estimate.

It must be remembered that the effects observed on the cyclase enzymes were exhibited by normal tissue. There is no reason at present to assume that cyclase enzymes of tumour cells would respond differently.

10.7 Concluding Remarks

Although the mutogenic, carcinogenic and (probably) anti-tumour effects of cyclophosphamide metabolites are undoubtedly the manifestation of some effect of these agents on the cell nucleus (damage to DNA in particular) the fact that possibly active compounds must first reach the nucleus will of necessity bring them into contact with cytoplasmic constituents. Binding of CP derivatives has been observed to be quite indiscriminate and involves the binding of DNA, RNA and protein, cross-linking of these has also been reported (196) (204) (195). Alkylating agents have been shown to effect cytoplasmic proteins (Chapter 1) and of particular interest here are the effects observed on phosphodiesterase, protein kinase and cyclic nucleotide binding protein (section 1.11). Guanylate cyclase is a predominantly cytoplasmic protein (section 1.8(ii)) and it may, therefore, be considered inevitable that during CP chemotherapy this enzyme would be subjected to contact with low concentrations of CP metabolites.

Increased binding to cytoplasmic constituents has been observed for OHCP and is thought to occur because of sulphydryl binding (180) and this fact may be of particular relevance to guanylate cyclase, since free sulphydryls are required for full expression of activity (section 1.8(iii)) (10.5).

Even before CP metabolites can reach the cell cytoplasm, they must come into contact with the outer cell membrane. Metabolites are probably in contact with the cell surface at concentrations not dissimilar from the reported plasma levels (section 10.6). On penetration of the membrane (whether carrier-dependent or not) and upon entering the cell, the metabolites undoubtedly must come into contact with membrane-bound proteins.

The fact that activated CP metabolites can affect membrane-bound proteins has been demonstrated by the denaturation of cytochrome P-450 by acrolein and OHCP (260). Effects of alkylating agents on outer membrane protein components has also been demonstrated. The trifunctional nitrogen mustard, HN3, has been shown to cross-link many of the membrane proteins of the human erythrocyte as well as cytoplasmic constituents (261). HN2 has also been shown to exert effect on membrane-bound ATPase and is a potent inhibitor at extremely low concentrations (262). These results demonstrate the inevitability of adenylate cyclase, a membrane-bound enzyme, coming into contact with CP metabolites.

To produce a significant difference in the activity of the cyclase enzymes (say 50%) it has been found that relatively high concentrations of CP metabolites are required. Only HP affected adenylate cyclase and a concentration of 5 mM was required for a significant difference in its activity to be affected (50% inhibition). Guanylate cyclase was inhibited by HP only at very high concentrations (30 mM) and stimulated by PM to a lesser extent. It is unlikely, therefore, that these effects would be of any significance to the anti-tumour action of CP. Unless selectively concentrated, OHCP would be present in concentrations 50-fold less than that required to inhibit adenylate cyclase and 300-fold less than that required to effect guanylate cyclase in this way. This assumes that OHCP does not exceed the maximum concentration observed for PM in plasma (0.1 mM). Cyclic nucleotide metabolism is not therefore likely to be altered by a change in the synthesis of nucleotides during CP chemotherapy. Cyclic nucleotide metabolism is most likely going to be altered by changes in the degradation of cyclic AMP, while synthesis remains unaffected.

Guanylate cyclase is stimulated by many chemical carcinogens (section 1.8) and under such stimulated conditions is inhibited by all CP derivatives tested at lower concentrations than those required to inhibit basal activity. Of these, HP was found to be most effective and produced 50% inhibition at approximately 2 mM, the difference between this and possible physiological concentrations of OHCP being 20-fold. It seems unlikely, therefore, that cyclic GMP metabolism would be altered significantly by changes in the activity of this enzyme during CP therapy.

Concentrations of metabolites may be increased during treatment of nonlymphocytic leukaemia before bone-marrow transplantation, when large doses of CP are given (143). PM has been used in clinical trials alone, and would presumably therefore be present in quite high concentration. Local perfusion of organs by active CP metabolites has been suggested but clinical use of HP has not been forthcoming due to the effects of hydroxyl radicals which would be formed during production of OHCP from this compound. If local perfusion was carried out with HP, high concentrations would presumably be used and the effects upon the cyclase enzymes observed here, particularly on adenylate cyclase, may become significant.

Of the CP derivatives tested, OHCP (generated from HP) appears to have a uniquely high reactivity with both adenylate and guanylate cyclase. In further studies on potential targets for activated CP derivatives, there must be included comparisons of OHCP and PM action on the target system examined. Although PM is now considered the active alkylating metabolite of CP, the ultimately therapeutically important form of CP may well turn out to be OHCP.

APPENDIX I

THE STUDENT'S t TEST

(1) Definition of symbols used

X = value of each datum

N = number of data

$\bar{X}$ = mean

$\Sigma x^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{N}$

df = degrees of freedom

$= (N_1 + N_2 - 2)$ when $N_1 = N_2$

$= N-1$ when $N_1 = N_2$

(2) Calculation of S_{DX} (standard error of difference between the means)

When $N_1 \neq N_2$

$$S_{DX} = \sqrt{\frac{\Sigma x_1^2 + \Sigma x_2^2}{N_1 + N_2 - 2}\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}$$

When $N_1 = N_2$

$$S_{DX} = \sqrt{\frac{\Sigma x_1^2 + \Sigma x_2^2}{N(N-1)}}$$

(3) Calculation of t

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_{DX}}$$

APPENDIX I

THE STUDENT'S t TEST

(1) Definition of symbols used

X = value of each datum

N = number of data

$\bar{X}$ = mean

$\Sigma x^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{N}$

df = degrees of freedom

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$= N-1$ when $N_1 = N_2$

(2) Calculation of S_{DX} (standard error of difference between the means)

When $N_1 \neq N_2$

$$S_{DX} = \sqrt{\frac{\Sigma x_1^2 + \Sigma x_2^2}{N_1 + N_2 - 2}\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}$$

When $N_1 = N_2$

$$S_{DX} = \sqrt{\frac{\Sigma x_1^2 + \Sigma x_2^2}{N(N-1)}}$$

(3) Calculation of t

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_{DX}}$$

(4) The t table is used to find the t value for a given degree of freedom. Reading across the t values for a given degree of freedom, the value next lowest to that calculated (above) will give the probability of significant difference from the column heading of the table.

Table of t values for use in the t test

df	.1	.05	.01	.001
1	6.314	12.706	63.657	636.619
2	2.920	4.303	9.925	31.598
3	2.353	3.182	5.841	12.941
4	2.132	2.776	4.604	8.610
5	2.015	2.571	4.032	6.859
6	1.943	2.447	3.707	5.959
7	1.895	2.365	3.499	5.405
8	1.860	2.306	3.355	5.041
9	1.833	2.262	3.250	4.781
10	1.812	2.228	3.169	4.587
11	1.796	2.201	3.106	4.437
12	1.782	2.179	3.055	4.318
13	1.771	2.160	3.012	4.221
14	1.761	2.145	2.977	4.140
15	1.753	2.131	2.947	4.073
16	1.746	2.120	2.921	4.015
17	1.740	2.110	2.898	3.965
18	1.734	2.101	2.878	3.922
19	1.729	2.093	2.861	3.883
20	1.725	2.086	2.845	3.850

Table Continued.....

df	.1	.05	.01	.001
21	1.721	2.080	2.831	3.819
22	1.717	2.074	2.819	3.792
23	1.714	2.069	2.807	3.767
24	1.711	2.064	2.797	3.745
25	1.708	2.060	2.787	3.725
26	1.706	2.056	2.779	3.707
27	1.703	2.052	2.771	3.690
28	1.701	2.048	2.763	3.674
29	1.699	2.045	2.756	3.659
30	1.697	2.042	2.750	3.646
40	1.684	2.021	2.704	3.551
60	1.671	2.000	2.660	3.460
120	1.658	1.980	2.617	3.373
∞	1.645	1.960	2.576	3.291

APPENDIX II

LINEAR REGRESSION

(1) Definition of symbols used

X	=	x	data coordinate
Y	=	y	data coordinate
$\bar{X}$ or $\bar{Y}$	=		mean values
σ	=		standard deviation

(2) Calculation of the gradient (m)

$$m = \frac{\dfrac{\sum_{i=1}^{n} X_i Y_i}{n} - \bar{X}\bar{Y}}{\sigma X^2}$$

(3) Calculation of the intercept (b)

$$b = Y - m\bar{X}$$

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