PRODUCTION AND ACTION OF LOCAL MEDIATORS IN THE RAT GASTRIC MUCOSA.

by

James Francis Brown

Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM
March 1994.

UNIVERSITY OF ASTON IN BIRMINGHAM

PRODUCTION AND ACTION OF LOCAL MEDIATORS IN THE RAT GASTRIC MUCOSA

by

JAMES FRANCIS BROWN

A thesis submitted for the degree of DOCTOR OF PHILOSOPHY - 1994

This study concerns the production and action of the local mediators nitric oxide (NO) and prostaglandin E_2 (PGE_2) in the rat gastric mucosa. The major objectives were: (i) to determine which mucosal cell type(s) contained NO synthase activity, (ii) to establish the functional role(s) of NO in the gastric mucosa and (iii) to investigate regulation of gastric PGE_2 production.

Gastric mucosal cells were isolated by pronase digestion coupled with intermittent calcium chelation and were separated by either density-gradient centrifugation or by counterflow elutriation. The distribution of Ca^{2+}-dependent NO synthase activity, measured via the conversion of [^{14}C]-L-argmine to [^{14}C]-L-citrulline, paralleled the distribution of mucous cells in elutriated fractions. Pre-treatment of rats with lipopolysaccharide caused the induction of Ca^{2+}-independent NO synthase in the elutriator fractions enriched with mucous cells.

Incubation of isolated cells with the NO donor isosorbide dinitrate (ISDN) produced a concentration-dependent increase in the guanosine 3',5'-cyclic monophosphate (cGMP) content which was accompanied by a concentration-dependent increase in release of immunoreactive mucin. Intragastric administration of ISDN or dibutyryl cGMP *in vivo* increased the thickness of the mucus layer overlying the gastric mucosa.

The NO donor S-nitroso-N-acetylpenicillamine (SNAP) produced a concentration-dependent inhibition (IC_{50} 247 μM) of histamine-stimulated aminopyrine accumulation, a measure of secretory activity, in cell suspensions containing >80% parietal cells. SNAP increased the cGMP content of the suspension but did not decrease cellular viability, glucose oxidation or adenosine 3',5'-cyclic monophosphate content. The inhibitory effect of SNAP was observed in permeabilised cells stimulated with ATP and was stereospecifically blocked by preincubation with Rp-8-bromoguanosine 3'5'-monophosphorothioate, which inhibits activation of cGMP-dependent protein kinase.

Stimulation of PGE_2 release by bradykinin in a low density cell fraction, enriched with parietal cells and devoid of vascular endothelial cells and macrophages, involved a bradykinin B_1 receptor.

In summary, NO synthase activity is probably present in gastric mucous epithelial cells. NO may promote mucus secretion by elevation of cGMP. NO donors inhibit acid secretion at a specific site and their action may involve cGMP. The bradykinin B_1 receptor is involved with PGE_2 production in the gastric mucosa.

Key words: Gastric mucosa, nitric oxide, gastric secretions, guanosine 3',5'-cyclic monophosphate, prostaglandin E_2.

For my families,
especially my late father,
my guide and inspiration.

"Believe nothing, O monks, merely because you have been told it...or because it is traditional, or because you yourselves have imagined it. Do not believe what your teacher tells you merely out of respect for the teacher. But whatsoever, after due examination and analysis, you find to be conducive to the good, the benefit, the welfare of all beings--that doctrine believe and cling to, and take it as your guide."

- Gautama Buddha, Indian philosopher (536?-483? B.C.)

ACKNOWLEDGEMENTS

I would sincerely like to thank both of my supervisors Dr P.J. Hanson and Dr B.J.R. Whittle for their extremely helpful discussion and guidance throughout the course of this work. Thanks Peter and Brendan.

Thanks are also due to Melvin Gamble and Brian Burford for their help with intravenous administration of agents and also Alan Richardson for his help with blood pressure measurements. I also wish to thank all my friends and colleagues at Aston and Wellcome for making my stay at both institutions so very enjoyable, especially Professor Barry Tepperman for his unprecedented humour, wit, social intercourse and practical advice.

Finally I would like to thank my families for their support and encouragement throughout all of my studies, especially my wife Emma for her tolerance with me during the writing of this thesis.

CONTENTS

LIST OF FIGURES, PLATES AND TABLES.

FIGURES

PLATES

TABLES

ABBREVIATIONS

The following, non-standard abbreviations were used throughout this work.

ANOVAR	Analysis of variance
cAMP	adenosine 3',5'-cyclic monophosphate
cGMP	guanosine 3',5'-cyclic monophosphate
Db-cAMP	N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate.
Db-cGMP	N^2,2'-O-dibutyrylguanosine 3':5'-cyclic monophosphate.
d.p.m.	disintegrations per minute
EC_{50}	concentration of an agent causing a half-maximal stimulatory effect
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
Fig.	Figure
G-kinase	guanosine 3',5'-cyclic monophosphate-dependent protein kinase.
h	hour(s)
Hb	oxyhaemoglobin
IC_{50}	concentration of an agent causing a half maximal inhibitory effect
i.p.	intraperitoneal
ISDN	isosorbide dinitrate
i.v.	intravenous
kDa	kilodalton (dalton=1/12th of the mass of 1 atom of nuclide ^{12}C)
LNMMA	N^G-Monomethyl-L-arginine
LPS	lipopolysaccharide (from *E. coli.*, serotype 0111:B4)
min	minute(s)
M&B 22948	1,4-Dihydro-5-(2-propoxyphenyl)-1,2,3-triazolo[4,5-*d*] pyrimidin-7-one (Zaprinast)
NO	nitric oxide
NOS	nitric oxide synthase
8-pCPT-cGMP	8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphate
PDE	phosphodiesterase
PGE_2	Prostaglandin E_2
Rp-8-Br-cGMPS	Rp-8-bromoguanosine-3',5'-cyclic monophosphorothioate
r.p.m.	revolutions per minute
s	second(s)
SEM	standard error of the mean
SNAP	S-nitroso-N-acetylpenicillamine
Sp-8-Br-cGMPS	Sp-8-bromoguanosine-3',5'-cyclic monophosphorothioate

Chapter One
GENERAL INTRODUCTION

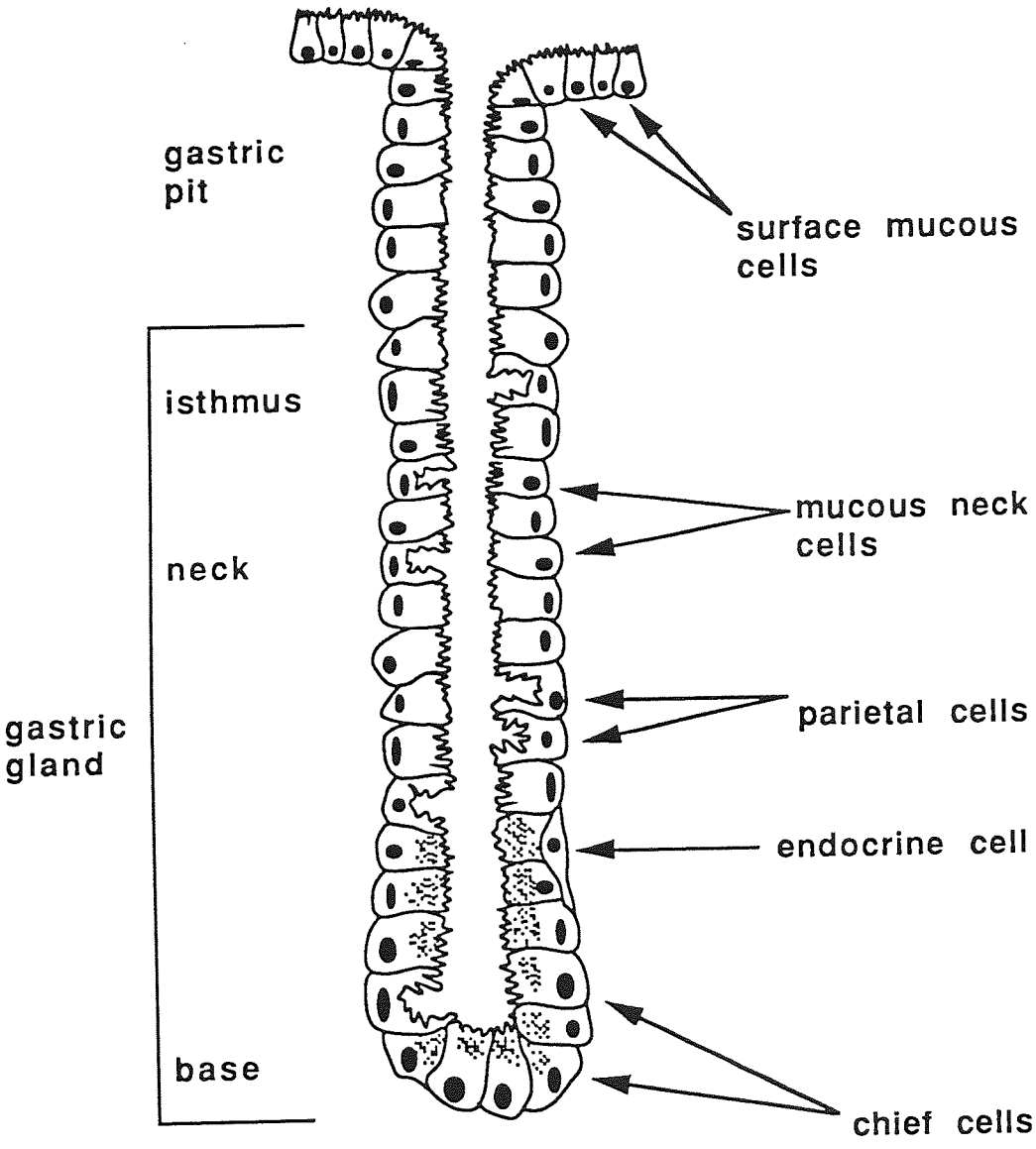
Gastric and duodenal ulceration are major diseases in many areas of the world. Current drug therapy for the treatment of gastric ulceration is effective in the short term, but the relapse rate following cessation of treatment is unsatisfactorily high. Identification of novel targets for potential therapeutic intervention that may provide a far more effective long term maintenance of the integrity of the gastric mucosa is therefore of considerable importance. Recently, the biological status of nitric oxide (NO) has evolved from being an environmental pollutant to a regulator of cellular function and much of this thesis relates to the production and actions of this local mediator in the gastric mucosa. In the present chapter, a general overview of the anatomy of the gastric mucosa and a review of the role of NO in signal transduction processes in mammalian cells is presented. Additional work on the production of another local mediator, prostaglandin E_2 (PGE_2) in the gastric mucosa is introduced in chapter 6.

1.1 ANATOMY OF THE GASTRIC MUCOSA.

1.1.1 Gross gastric morphology.

The mammalian stomach of most non-ruminant animals forms an expanded region of the gastrointestinal tract between the oesophagus and duodenum. Ingested food is temporarily stored and partially digested prior to leaving the stomach and entering the duodenum. The rat stomach can be broadly divided into two regions. The oesophagus opens into the non-glandular area, termed the forestomach or cardiac region which is only involved in food storage and which is covered by a keratinised stratified squamous epithelium. The glandular region of the rat stomach is the corpus which consists of epithelial cells from which mucus and bicarbonate are secreted, and numerous tubular invaginations termed the gastric glands (Fig. 1.1), from which acid (which may help to sterilise the food) and pepsinogen (which is converted to pepsin at low pH) are secreted. In the rat an antral glandular region also exists between the fundus and duodenum, which can be identified by the anatomy of its characteristic deep pyloric glands.

Figure 1.1 Diagrammatic representation of a gastric gland from a mammalian stomach (not to scale).



1.1.2 Cell types of the gastric mucosa.

1.1.2.1 Mucous cells.

The mammalian stomach has two types of cells which contain high molecular mass mucous glycoprotein termed mucin: (i) surface mucous cells and (ii) mucous neck cells.

The surface mucous cells cover the luminal surface of the glandular epithelium and also extend into the pit and isthmus of the glands (Fig. 1.1). The cells form a simple columnar epithelium, joined by belt-like tight junctions which prevent or restrict the passage of large and small molecules across the epithelial sheet and which restrict the exchange of apical membrane components and ions with those in the basolateral membrane (Farquhar & Palade, 1963). The intracellular organisation of the surface mucous cell is typical of exocrine secretory cells, with the rough endoplasmic reticulum, golgi complex, mitochondria and nucleus usually concentrated in the basal and lateral cytoplasm. The secretory granules containing mucin are stored in the apical cytoplasm, and in surface mucous cells, carbohydrate specific stains such as the periodic acid/Schiff (PAS) reagent intensely stain the apical cytoplasm.

In addition to surface mucous cells, the gastric mucosa also consists of mucous neck cells which are present in the neck and isthmus region of the glands where a gradual transition from surface to neck cells occurs (Fig. 1.1). The cytoplasm of the mucous neck cell is essentially similar to that of the surface mucous cell. Staining of the apical cytoplasm with periodic acid/Schiff is similar in both types of mucous cell. However, alcian blue staining at low pH is more intense for mucous neck cells than for surface mucous cells which may be a consequence of a greater proportion of sulphated (acidic) mucin in the mucous neck cells (Spicer *et al.*, 1978). Both types of mucous cell are lost into the lumen of the stomach either by mechanical sloughing or cellular degeneration (Lee & Leblond, 1985). Cells are continually replaced by proliferation of multipotent endodermal stem cells which are capable of differentiating into any of the specialised cell-types in the gastric epithelium (Williams, 1977). Replacement of mucous neck cells takes

approximately one week whereas replacement of surface mucous cells usually occurs within three days (Hunt & Hunt, 1963).

1.1.2.2 Parietal cells.

The most distinctive and characteristic cell of the gastric mucosa is the gastric parietal cell, so named because it often bulges outward from the walls of the gastric glands. The parietal cell is responsible for secretion of HCl into the gastric lumen which may act to sterilise food prior to digestion. Parietal cells are most numerous in the neck or isthmus of the gastric gland but rarely in the gastric pits or the luminal surface (Fig. 1.1) and are always found in close proximity to mucous neck cells (Ito, 1987).

Parietal cells are large oval to pyramidal cells measuring up to 25 μm in diameter which stain characteristically with acidic dyes, such as eosin, although they are not truly acidophilic and staining is not indicative of secretory activity (Graumann, 1965). The most conspicuous structures in parietal cells are the intracellular canaliculi and the abundant, large mitochondria which may account for approximately one third of the cytoplasmic volume (Helander *et al.*, 1986). The secretory canaliculi are poorly developed in the resting cell, but profound morphological changes become apparent as early as three minutes following exposure of the resting cell to secretagogues, a process described in more detail in chapter 5 (Forte *et al.*, 1981).

The properties of the secretory membrane are probably unique since it is exposed to extremely high concentrations of HCl (around 0.16 M). Histochemical studies have revealed the presence of glycoconjugates around the region of the apical membrane which are thought to be important in the protection of the parietal cell (Sato & Spicer, 1980), although these glycoconjugates have also been proposed to represent the β-subunit of the proton-pump (Callaghan *et al.*, 1990).

1.1.2.3 Chief cells.

The chief or peptic cell is a typical protein secreting exocrine cell, similar in morphology to the pancreatic acinar cell except that it synthesises, stores and secretes pepsinogen rather than pancreatic enzymes. Chief cells are predominantly found in the base of the gastric gland although their distribution varies in different regions of the glandular mucosa, but they are most abundant in the corpus region of the stomach (Blum *et al.*, 1971). The zymogen granules, which contain pepsinogen, are usually numerous in the apical cytoplasm and are released by exocytosis or merocrine secretion (Ito & Winchester, 1963).

1.1.2.4 Endocrine cells.

Another group of minor cell types which are present in the gastric mucosa are endocrine cells of which up to nine different types have been described. Some features of these cells are summarised in Table 1.1.

Table 1.1 Endocrine cells of the gastric mucosa

Compiled by Grube & Forssmann, (1979).

1.1.2.5 Other cell types.

The cells discussed thus far form the vast majority of cell types which constitute the normal gastric mucosa. However, there are additional cell types that have been described and are consistent features of this epithelium. These include intraepithelial

lymphocytes, globular leukocytes and caveolate cells. The functions of these cells is unclear although they may play an immunological or sensory role in response to changes in the epithelial or luminal environment (Ito, 1987).

Macrophages are widespread throughout the body (Van Furth, 1980) and are also present in large numbers throughout the lamina propria of the gastrointestinal tract, including the stomach (Hume *et al.*, 1984). Macrophages are able to release factors which are potentially beneficial to gastric mucosal defence, such as prostaglandins (see section 6.1.3 for discussion of functions). Prostaglandins are produced by macrophages in response to various stimuli, including phagocytosis of sulphated polysaccharides such as zymosan (Hsueh *et al.*, 1979) and receptor mediated stimulation, for example, by bradykinin (Chen *et al.*, 1989). However, macrophages also possess the capacity to secrete factors which amplify tissue injury, such as lysosomal enzymes, superoxides and nitrogen containing oxides (Stuehr & Marletta, 1985). Thus although activated macrophages may release gastroprotective agents they may also exacerbate tissue injury.

1.2 EXISTING LOCAL MEDIATORS IN THE GASTRIC MUCOSA.

Regulation of gastric function can be contributed to by a number of chemical messengers which are produced and act locally within the gastric mucosa. These transmitter substances can be colloquially termed "local mediators" and include histamine, somatostatin, eicosanoids such as PGE_2, bradykinin and transforming growth factorα. Part of this thesis is concerned with the effects of bradykinin on PGE_2 production and consequently these two mediators are discussed in more detail in Chapter 6 and only mentioned briefly here.

1.2.1 Histamine.

Histamine has a direct stimulatory effect upon acid secretion via interaction with histamine H_2-receptors located on the parietal cell basolateral membrane (Batzri *et al.*, 1982). The source of histamine involved in the stimulation of acid

secretion in the rat gastric mucosa is thought to be the enterochromaffin-like cells (Andersson *et al.*, 1990). The enterochromaffin-like cells have receptors for both acetylcholine and gastrin, with activation of these receptors resulting in the localised release of histamine (Hellander & Keeling, 1993; Håkanson & Sundler, 1991). Hence, the control of histamine release is under both neuronal and hormonal control.

1.2.2 Somatostatin.

Somatostatin is a potent inhibitor of acid secretion yet the mechanisms involved in this effect remain unclear. Somatostatin has been demonstrated to inhibit the secretory response to pentagastrin and cholinergic innervation *in vivo* (Sanders & Soll, 1986) and histamine-stimulated aminopyrine accumulation (an index of acid secretion, see chapter 5) *in vitro* (Batzri & Dyer, 1981). The release of somatostatin in the gastric mucosa may be controlled by adrenergic innervation and by gastrin stimulation of somatostatin-like immunoreactive cells (Soll *et al.*, 1984) whereas cholinergic influence results in attenuation of adrenaline and gastrin-stimulated somatostatin release (Yamada *et al.*, 1984).

1.2.3 Eicosanoids.

The eicosanoids, namely PGE_2, have numerous functions in the gastric mucosa including inhibition of acid secretion, stimulation of mucus release and regulation gastrointestinal blood flow (Soll, 1980b; Bickell & Kauffman, 1981; Main & Whittle, 1973). A more detailed discussion of the production and action of PGE_2 in the gastric mucosa is presented in sections 5.1.5.1, 6.1.2 and 6.1.3.

1.2.4 Bradykinin.

Bradykinin is produced in response to tissue injury via the action of kallikreins on kininogens (Proud & Kaplan, 1988). Bradykinin receptors on endothelial cells have been reported to increase eicosanoid synthesis (Sung *et al.*, 1988), thus, during gastric injury when localised production of bradykinin will occur, this may result in a localised increase

in prostaglandins. A more detailed discussion of the production and action of bradykinin is presented in section 6.1.4.5.

1.2.5 Transforming Growth Factorα (TGFα).

Expression of TGFα and TGFα/EGF-receptor mRNA have been detected in the gastric mucosa of guinea pig, rat, dog and humans (Beuachamp *et al.*, 1989). TGFα has been reported to inhibit acid secretion in the stomach (Lewis *et al.*, 1990) possibly by interacting with the TGFα/EGF-receptor on parietal cells although the precise mechanism by which TGFα mediates its inhibitory effect are unclear.

1.3 BIOLOGY OF NITRIC OXIDE: A NOVEL LOCAL MEDIATOR.

Until recently, nitric oxide (NO) was regarded as being a noxious environmental pollutant, present in such unsavoury concoctions as tobacco smoke, car exhaust fumes and smog. Destroyer of ozone and suspected carcinogen, this gas had a bad reputation. However, more recent evidence has shone a more favourable light upon NO and identified it as being involved in the mediation of numerous physiological processes. It is now widely accepted that the biological effects of NO and endothelium-derived relaxing factor are indistinguishable and that endothelium-derived relaxing factor is indeed NO (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). This section aims to discuss the mechanisms involved in the production of NO and also its role as a mediator of signal transduction, and aims to set the scene for the bulk of the experimental work in this thesis.

1.3.1 Isozymes of nitric oxide synthase.

In mammalian cells, NO is enzymatically formed from a terminal guanidino-nitrogen of the amino acid L-arginine (Palmer *et al.*, 1988) by a family of nitric oxide synthases (NOS; EC 1.14.23) (Bredt *et al.*, 1990). All NOS enzymes which have been identified yield L-citrulline as a co-product of the reaction and were originally classified into three distinct groups according to their subcellular localisation and requirements for

free Ca^{2+} (see Table 1.2). This classification has been supported by the recent cloning and expression of the corresponding genes (Bredt *et al.*, 1991; Nakane *et al.*, 1993; Marsden *et al.*, 1992). Other ways of classification were to subdivide the enzymes according to whether their expression was constitutive or inducible, by the relative potency of analogues of L-arginine as inhibitors of enzyme activity. NOS-I and III are constitutively expressed and more potently inhibited with N^{ω}-nitro-L-arginine than N^{G}-monomethyl-L-arginine (L-NMMA), whereas NOS-II is expressed following induction by cytokines or bacterial endotoxin and is more readily inhibited by N^{G}-methyl-L-arginine than N^{ω}-nitro-L-arginine (Schmidt *et al.*, 1993). The expression of NOS-II is inhibited by pre-administration of glucocorticoids, which may be an important component of their therapeutic action (Rees *et al.*, 1990). Induction of NOS-II activity is evident in the rat ileum at approximately 3 h after administration of endotoxin with maximal activity being observed at approximately 6 h, and a return to control activity at approximately 18 h after treatment (Salter *et al.*, 1991).

1.3.2 Structure of nitric oxide synthase.

All isoforms of NOS are homodimers of subunits ranging from 130 to 160 kDa molecular weight (Schmidt *et al.*, 1991). The N-terminal half contains binding sites for NADPH, FAD and FMN and displays approximately a 30% sequence homology to NADPH-cytochrome-P_{450} reductase, which is the only other mammalian enzyme that contains both flavin nucleotides (Schmidt *et al.*, 1993; see Fig. 1.2). Other possible domains within the NOS enzyme are for binding of haem, tetrahydrobiopterin, calmodulin and of course, L-arginine. Dimerisation of NOS, a prerequisite for L-arginine turnover, is a tetrahydrobiopterin-dependent process with exogenous tetrahydrobiopterin having to be added to purified enzyme preparations for L-arginine conversion to L-citrulline to occur (Stuehr *et al.*, 1991a).

Table 1.2 Characteristics of NOS isoforms.

NOS ISOFORM	I	II	III
Major Source	Brain	Macrophages	Endothelium
Calculated molecular mass (kDa)[1]	160	130	133
V_{max} (μmol/mg protein/min)	0.3-3.4	0.9-1.6	0.015
Soluble fraction (% of total)	90-100%	85-90%	5%
REGULATION OF EXPRESSION			
Constitutive	Yes	No[2]	Yes
TNF, INF, IL-1	Suppress	Induce	Suppress
Glucocorticoids	No effect	Inhibit induction	No effect
CALMODULIN SITE			
Exogenous calmodulin required for activity	Yes	NR[3,4]	Yes
Ca^{2+}, EC_{50} (μM)	0.2-0.4	No dependence	0.3
Calmodulin, EC_{50} (nM)	1-70	Irreversible binding[3]	3.5
L-ARGININE SITE			
L-Arginine, K_m (μM)	2-4.3	2.3-2.8	2.9
Nitro-arginine, IC_{50} (μM)	0.9	212[5]	0.2
Methyl-arginine, IC_{50} (μM)	1.6	7.4[5]	0.9
HAEM SITE			
CO	Inhibits	Inhibits	NR
NO	Inhibits	Inhibits	NR
POST-TRANSLATIONAL MODIFICATIONS			
Phosphorylation	Ser/Thr	NR	NR

Notes:

[1] Deduced from amino acid sequence.

[2] Except a type II NOS which is constitutively expressed in smooth muscle cells of the colon and vas afferens of the kidney (Schmidt *et al.*, 1993).

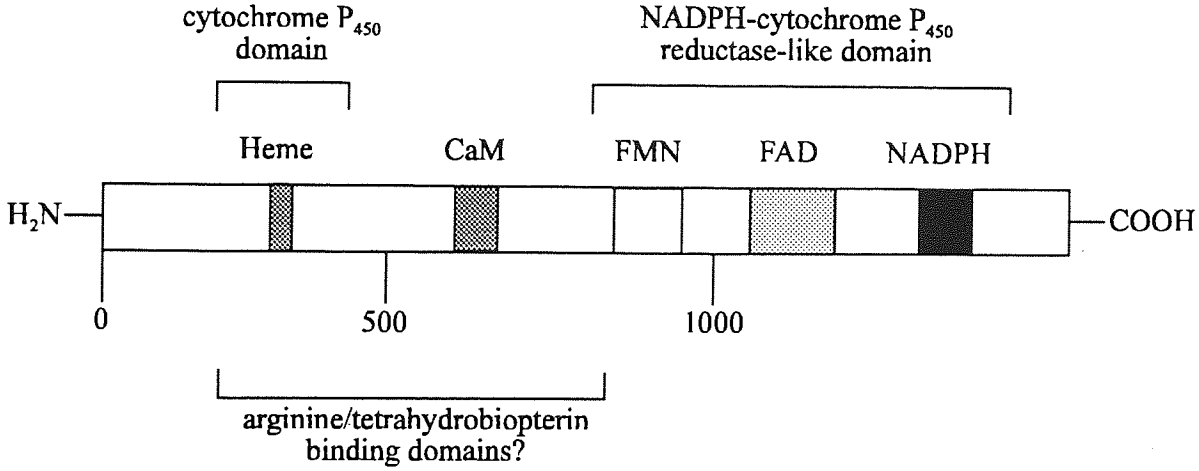
[3] Calmodulin may be a subunit of NOS from macrophages (Cho *et al.*, 1992)

[4] Liver inducible NOS-II is calmodulin dependent (Evans *et al.*, 1992)

[5] Values obtained with partially pure enzyme preparations (Gross *et al.*, 1990).

Abbreviations: TNF, tumour necrosis factor-α; TGF, transforming growth factor-β; INF, interferon-γ; IL, interleukin; NR, not reported.

Figure 1.2 **Protein domains and binding sites of nitric oxide synthase.**



Key:

CaM	= Calmodulin
FMN	= Flavin mononucleotide
FAD	= Flavin adenine dinucleotide
NADPH	= Nicotinamide adenine dinucleotide phosphate

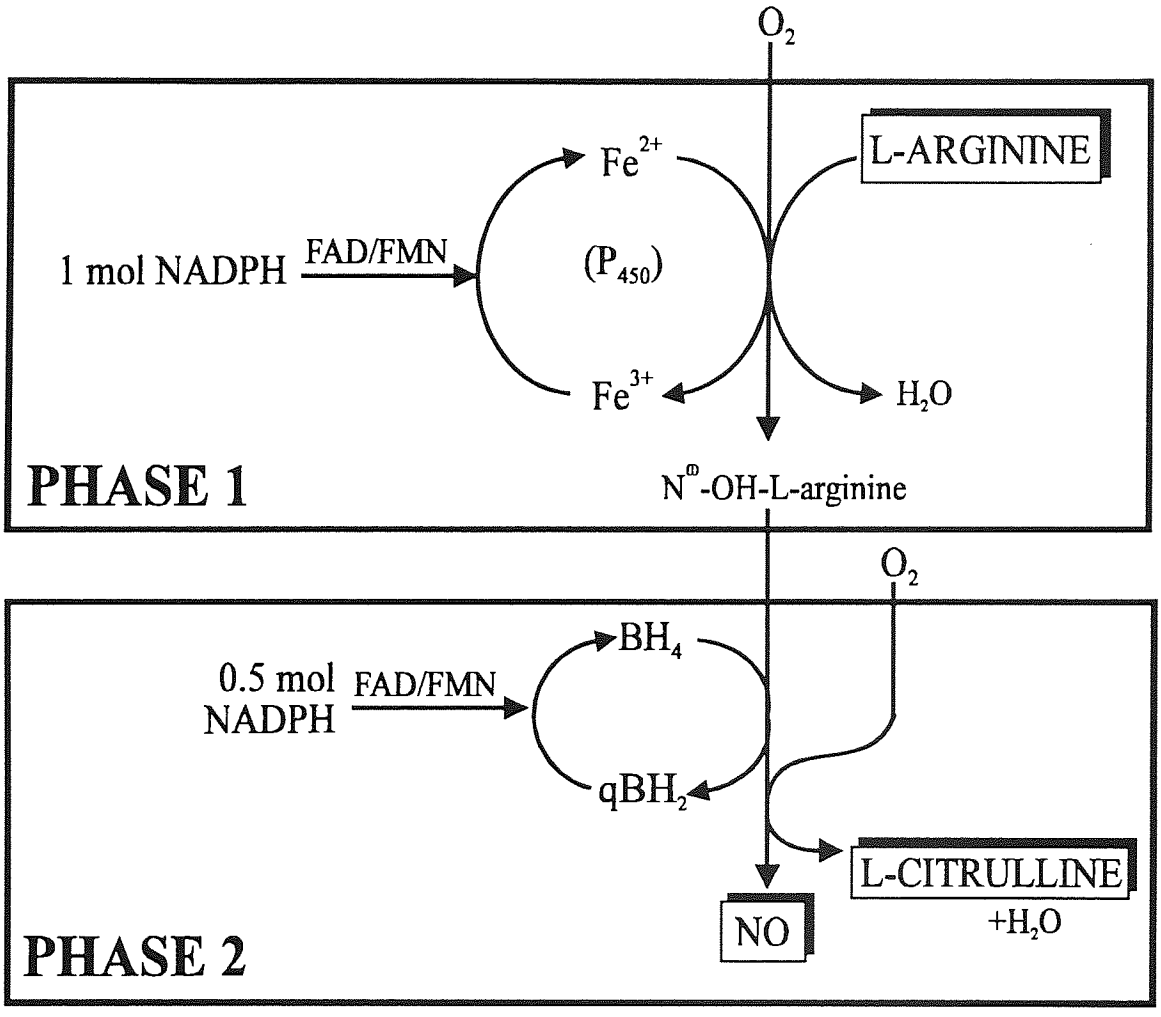
1.3.3 Nitric oxide biosynthesis.

The precise end product of NO catalysis is still unclear since most methods used for determining NOS activity involve the measurement of stable end products such as nitrite and nitrate which are formed in amounts which are equimolar to L-citrulline formation (Stuehr *et al.*, 1991b). However, when NO production is measured by selective methods such as gas-phase chemiluminescence without prior sample treatment, only trace amounts of NO have been detected in relation to total L-arginine turnover (Mayer *et al.*, 1990). This probably reflects the high reactivity of the NO free radical since NO may be stabilised by or react with free thiols, free iron (Myers *et al.*, 1990) and thiol and iron-sulphur containing proteins (Sobala *et al.*, 1991) to produce secondary NO adducts and complexes such as NO^+ and NO^-, some of which may not be detectable as free radicals (Stamler *et al.*, 1992). Nevertheless, conversion of L-arginine to L-citrulline has been demonstrated to correlate with the production of NO measured spectrophotometrically via the conversion of oxyhaemoglobin to methaemoglobin (Knowles *et al.*, 1990) and L-citrulline formation is now regarded as a standard method for measuring NOS activity.

The general reaction catalysed by NOS is presented in figure 1.3. Four cofactors (haem, FMN, FAD and tetrahydrobiopterin) and two co-substrates (O_2 and NADPH) participate in the reaction. The first stage of the reaction involves hydroxylation of L-arginine to N^{ω}-OH-L-arginine which requires 1 mol NADPH and molecular oxygen and is performed by the cytochrome P_{450} region of the enzyme (Stuehr *et al.*, 1991b). The second phase involves oxidation of N^{ω}-OH-L-arginine to L-citrulline and NO and requires a further 0.5 mol NADPH and is absolutely dependent upon tetrahydrobiopterin (Schmidt *et al.*, 1992a). A further stage of the reaction may involve recycling of quinone-dihydrobiopterin to tetrahydrobiopterin by quinone-dihydrobiopterin reductase which may be closely related to the NADPH-diaphorase domain of NOS, and requires a further 0.5 mol NADPH (Hope *et al.*, 1991; Schmidt *et al.*, 1992a). The recycling of biopterin cofactors is uncoupled in the presence of the soluble nitroblue tetrazolium, an artificial electron acceptor, which is converted by an NADPH-diaphorase reaction to the insoluble

and visible formazan (Scherer-Singler *et al.*, 1983). The NADPH-diaphorase activity is remarkably resistant to various tissue fixatives providing a convenient histological method for localisation of NOS activity (Hope *et al.*, 1991). However, care must be taken when considering this method for determining the distribution of NOS activity since NOS represents only a fraction of the total cellular NADPH-diaphorase activity in macrophages, brain and vascular endothelium (Tracey *et al.*, 1993).

Figure 1.2 **Diagrammatic representation of the reaction catalysed by nitric oxide synthase**



KEY:

NADPH	Nicotinamide adenine dinucleotide phosphate
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
BH_4	Tetrahydro biopterin
qBH_2	Quinoid-dihydrobiopterin

1.3.4 Regulation of nitric oxide synthase activity.

All NOS isoforms have conserved amino acid sequences for calmodulin binding (see table 1.2 for summary). However, both NOS-I and NOS-III require elevated intracellular concentrations of Ca^{2+} prior to activation via the binding of a Ca^{2+}/calmodulin complex (Bredt & Snyder, 1990). At resting intracellular Ca^{2+} concentrations (<100 nM) both NOS-I and NOS-III are inactive and calmodulin free, whereas during periods of elevated intracellular Ca^{2+} concentrations (>450 nM) the enzymes bind calmodulin and become active. NOS-III however, binds calmodulin alone with great affinity and is thus Ca^{2+}-independent and fully active at resting intracellular Ca^{2+} concentrations (Cho *et al.*, 1992). Furthermore, NOS-II activity is neither enhanced by exogenous Ca^{2+}/calmodulin, nor inhibited by chelators of divalent cations or drugs which inhibit calmodulin binding (Stuehr *et al.*, 1991a; Yui *et al.*, 1991). Hence, once expressed, NOS-II is constantly active with termination of activity probably being determined by the ability of the enzyme to avoid proteolysis.

From the predicted amino acid sequence of NOS-I, possible phosphorylation sites have been identified (Bredt *et al.*, 1991) suggesting that the soluble constitutively expressed enzyme as well as being regulated by changes in intracellular Ca^{2+} concentrations may also be regulated by phosphorylation. These suggestions were supported by Nakane *et al.*, (1991), who demonstrated that brain NOS activity was inhibited following phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II but increased following phosphorylation by protein kinase C. Furthermore, in mouse striatal neurones N-methyl-D-aspartic acid receptors stimulated a specific NOS which was not activated by non-N-methyl-D-aspartic acid receptors or by high intracellular Ca^{2+} concentrations, but was dependent upon activation of protein kinase C (Martin *et al.*, 1992). These results demonstrate that NO biosynthesis may be subject to complex regulation not only by Ca^{2+}/calmodulin and co-factors, but also by phosphorylation.

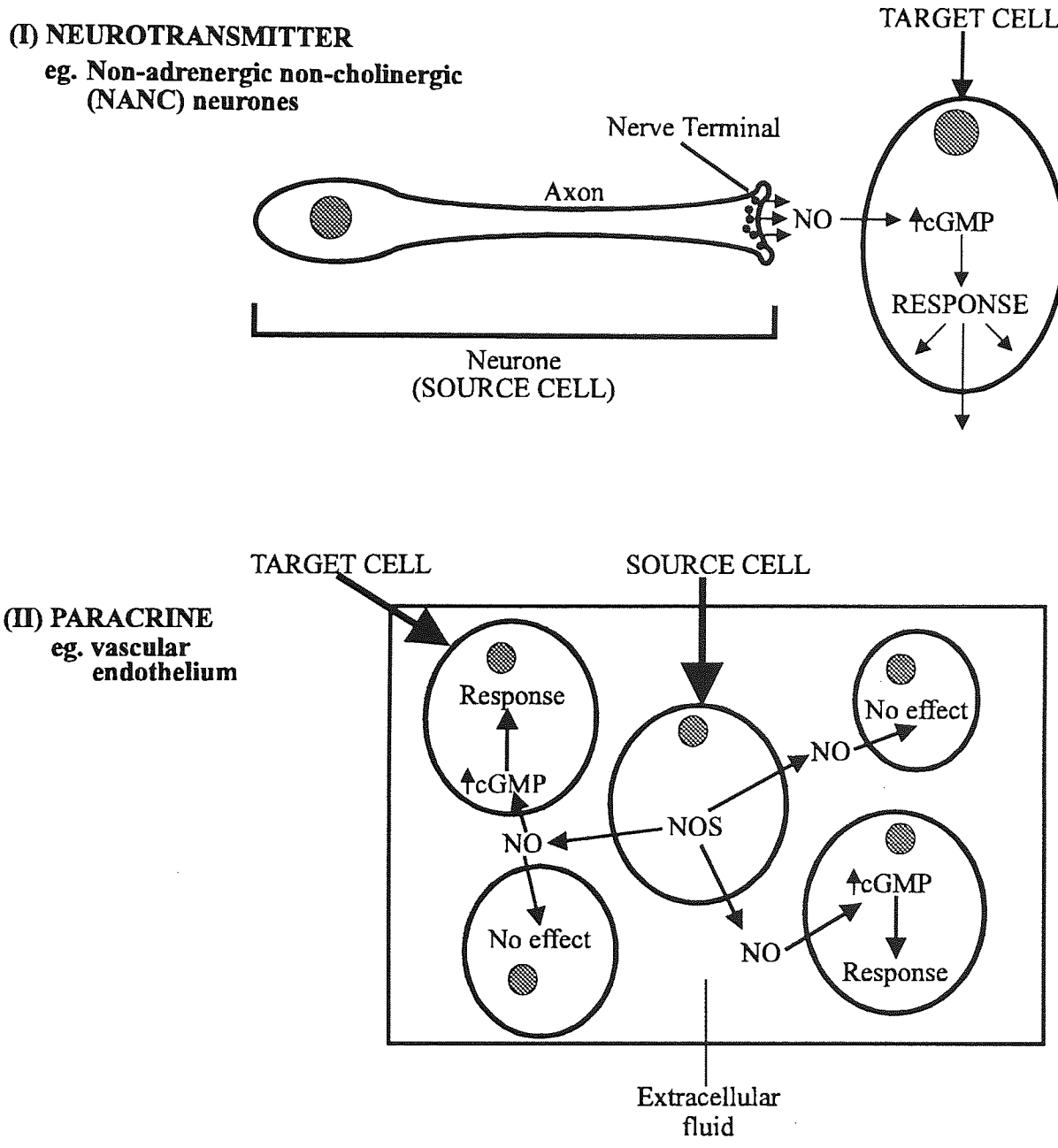
1.4 BIOLOGICAL EFFECTS OF NITRIC OXIDE.

Following its synthesis, NO has been demonstrated to mediate numerous and diverse biological processes (see table 1.4 and section 1.6 for a summary). Once synthesised NO can act as either a cytotoxic agent (as discussed below in section 1.4.1), a neurotransmitter or as a paracrine agent or intracellular messenger (Fig. 1.4), depending upon the quantity released, the duration of release and tissue in question (Moncada *et al.*, 1991b). The criteria for classification of NO as a neurotransmitter have been critically tested and most of them have been fulfilled with the exclusion of transmitter storage (Stark & Szurszewski, 1992). The role of NO as a paracrine agent is evident in vascular tissue, NO synthesis occurring in the endothelium with the target being vascular smooth muscle cells (Palmer *et al.*, 1988). Furthermore, a function as an intracellular messenger has been demonstrated in platelets, since they do not seem to transfer endogenous NO to other platelets or cells but modulate their own ability to aggregate (Moncada *et al.*, 1991).

1.4.1 Cytotoxicity.

A prolonged release of NO can be achieved via induction of NOS-II activity in cells responsive to inducing agents such as bacterial lipopolysaccharide (LPS) and cytokines. This induction was originally reported in activated macrophages exposed to LPS or interferon-γ, with enzyme activity being reported as NADPH-requiring and independent of Ca^{2+} (Hibbs *et al.*, 1987; McCall *et al.*, 1991). However, a wider occurrence of this enzyme is now apparent since i.p. administration of LPS to rats, rabbits and guinea-pigs resulted in a widespread tissue distribution of Ca^{2+}-independent NOS-II activity (Salter *et al.*, 1991). In the macrophage, induced NOS-II activity has been associated with cytotoxicity, since coincubation of activated macrophages with tumour cells resulted in inhibition of DNA synthesis and aconitase activity in the target cell (Granger *et al.*, 1980; Kilbourn *et al.*, 1984). Furthermore, inhibition of aconitase activity has also been reported in activated macrophages themselves (Drapier & Hibbs, 1988). However, the role of induced NOS-II activity in other cell types is unclear,

Figure 1.4 Potential biological roles of NO.



although a cytotoxic action is indicated since prolonged exposure to exogenous NO produced an increase in the DNA mutation rate of a human lymphoblastoma cell line (Nguyen *et al.*, 1992), and a decrease in cell viability of isolated rat intestinal epithelial cells (Tepperman *et al.*, 1993).

1.4.2 Activation of guanylate cyclase.

At low concentrations, the main if not sole effect of NO is activation of the soluble form of the haem-centred metallo-protein, guanylate cyclase (EC 4.6.1.2), which converts guanosine 5'-triphosphate to cyclic guanosine 3',5'-monophosphate (cGMP) (Moncada *et al.*, 1991a). The signal transduction process is initiated by the binding of NO to haem and consequent disruption of the haem Fe^{2+} axial ligand, whereas the process is terminated upon re-establishment of the haem Fe^{2+} axial ligand as the labile NO-haem complex decomposes with the liberation of NO_2^- and higher oxides of nitrogen (Fig. 1.5; Ignarro, 1991).

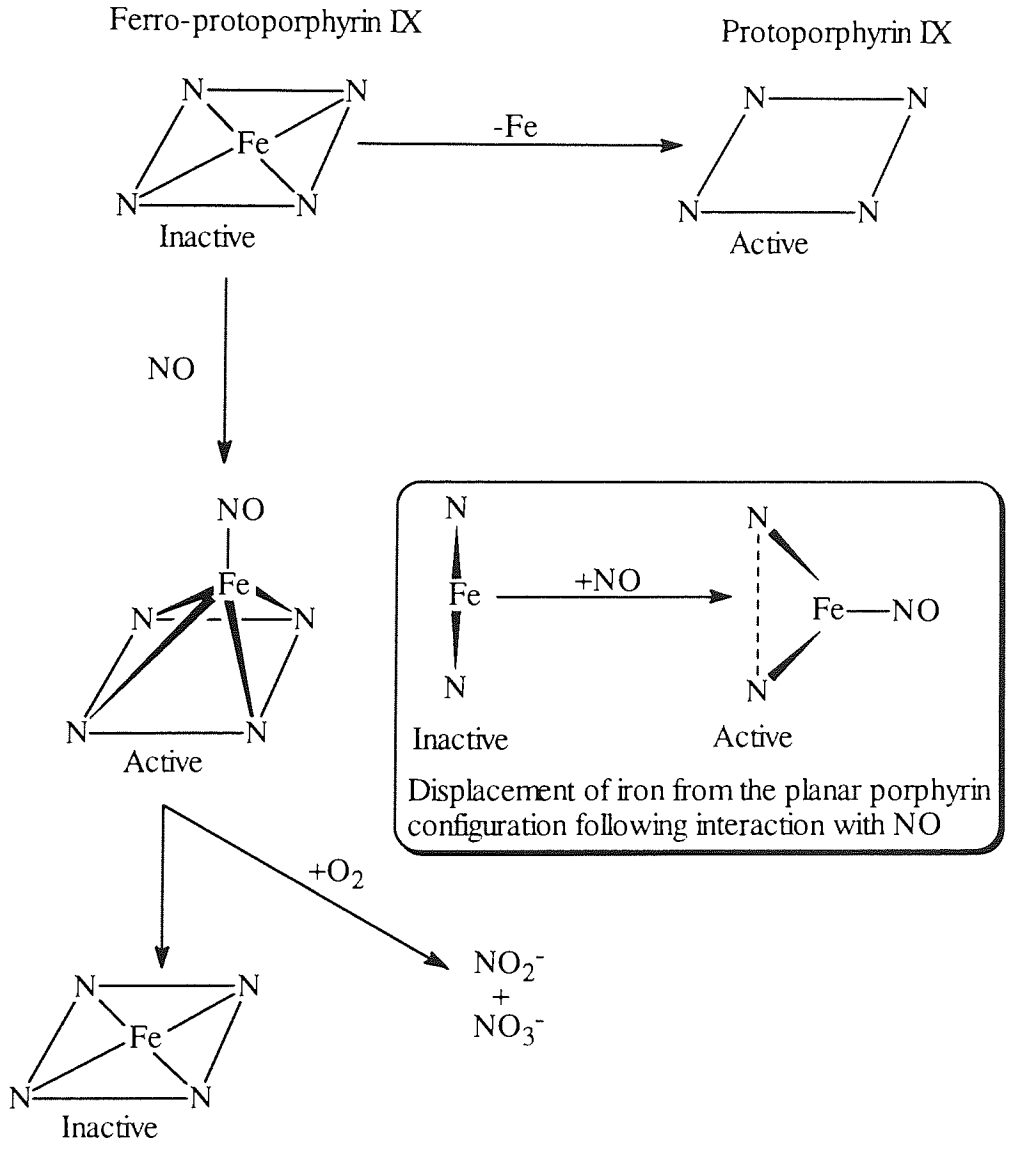
1.5 INTRACELLULAR ACTIONS OF cGMP.

Intracellular targets, or receptors for cGMP can be divided into three main groups: (i) channel proteins, (ii) phosphodiesterases and (iii) cGMP-dependent protein kinases (G-kinase).

1.5.1 Regulation of ion channels.

One such channel protein is located in photoreceptor cells of the rod outer segment of the retina (Cook & Kaupp, 1986). Decreased binding of cGMP to the channel protein produces closure of cation channels in the photoreceptor cell plasma membrane resulting in hyperpolarization.

Figure 1.5 **Interaction of NO with the protoporphyrin ring of guanylate cyclase.**



1.5.2 Regulation of phosphodiesterase (PDE) activity.

Several phosphodiesterases bind cGMP at sites distinct from their catalytic centre. One PDE in this group is the cGMP-stimulated PDE (Type II), which is activated at submicromolar concentrations of cGMP (Thomas *et al.*, 1990) and contains an allosteric site for cGMP binding and catalytic centre for cAMP and cGMP hydrolysis. The binding of cGMP to the purified cGMP-stimulated PDE from brain, heart and adrenal cortex produces a 3 to 10 fold activation of cAMP hydrolysis, but at higher concentrations of cGMP, cAMP hydrolysis is inhibited in a competitive fashion since the

V_{max} for cGMP hydrolysis is similar to cAMP (Thomas & Cornwell, 1993). A cGMP inhibited cAMP phosphodiesterase (Type III) is present in heart and other tissues (Harrison *et al.*, 1986). The cGMP-specific cGMP-binding phosphodiesterase (Type V_A) found in lung and platelets (Beavo & Reifsnyder, 1990) also has an allosteric site which binds cGMP, however cGMP binding at the allosteric site has not been reported to alter the kinetic properties of the enzyme (Thomas *et al.*, 1990). The rod outer segment PDE (ROS PDE), which is coupled by transducin to rhodopsin in the retina and is responsible for the photon-induced lowering of cGMP mentioned in section 1.5.1 (Wolfe *et al.*, 1987) is probably another member of this class and is designated type V_{B1}.

1.5.3 Regulation of cGMP-dependent protein kinase activity.

Further effector proteins for cGMP include cGMP-dependent protein kinases (G-kinase). A number of iso-enzymes have been described (Hofmann *et al.*, 1992). In most tissues, mammals express a homodimeric enzyme (type I) although a monodimeric isoform (type II) has been demonstrated in rat intestinal brush border (De Jonge, 1981). The type I enzyme has a native molecular mass of approximately 154 kDa whereas the monomeric type II enzyme is of a mass of approximately 86 kDa (Lincoln *et al.*, 1988; De Jonge, 1981). Forms of the type I G-kinase have also been reported termed Iα and Iβ which differ only in their amino terminal part of the protein (Wernett *et al.*, 1989; Sandberg *et al.*, 1989). The amino acid terminus plays an important role in the regulation of catalytic activity of G-kinase since the K_a values for cGMP required to activate isozymes Iα and Iβ are 0.1 and 1.2 μM respectively (Ruth *et al.*, 1991). The catalytic domain of G-kinase is highly conserved from mammal to insect and is the closest homologue to cAMP-dependent protein kinase with approximately 70% sequence homology (Takio *et al.*, 1984).

A major focus over the past few years has been to establish the mechanism by which cGMP mediates the relaxation of hormonally-contracted smooth muscle. cGMP, via activation of G-kinase, inhibits agonist-induced Ca^{2+} elevation (Felbel *et al.*, 1988;

Cornwell & Lincoln, 1989). Several mechanisms have been described to account for the decrease in intracellular free Ca^{2+} in smooth muscle involving either the prevention of Ca^{2+} entry or the stimulation of its efflux across the plasma membrane, or alternatively inhibition of its release from or stimulation of its sequestration into, the endoplasmic reticulum (Furukawa *et al.*, 1991; Cornwell *et al.*, 1991). Table 1.3 clearly demonstrates the vast array of potential substrates for G-kinase I, although the physiological roles of this enzyme still remain unclear.

1.5.4 Other intracellular actions of cGMP.

Still other mechanisms have been proposed for the effects of cGMP, and these include inhibition of GTP-binding protein function (Hirata *et al.*, 1990) and inhibition of phospholipase C activation (Rapoport, 1986). These mechanisms may contribute to the lowering of intracellular Ca^{2+} concentrations during agonist-induced relaxation of smooth muscle but they would have little effect on depolarised cells since phospholipase C activation is not involved in the mobilisation of intracellular Ca^{2+} in depolarised cells (Lincoln & Cornwell, 1993).

Table 1.3 **Potential substrates for cGMP-dependent protein kinase I.**

NAME	TISSUE	SIZE (kDa)	PROPOSED FUNCTION	REFERENCE
cGMP kinase I (auto-phosphorylation)	lung	75	decreases K_a for cAMP	Hofmann & Flockerzi, 1983.
G-substrate	cerebellum	23	phosphatase inhibitor	Aswad & Greengard, 1981.
Phospholamban	smooth muscle	11	stimulates Ca^{2+} (SP) ATPase	Saracevic *et al.*, 1989.
240-kDa protein	smooth muscle	240	stimulates Ca^{2+} (CaM) ATPase	Yoshida *et al.*, 1991.
Vasodilator-stimulated phosphoprotein (VASP)	platelets	46	inhibits $\uparrow Ca^{2+}$ concentration by phospholipase C?	Reinhard *et al.*, 1992.
Vimentin	neutrophils	57	proliferation and degranulation	Wyatt *et al.*, 1991.
cGMPB-PDE	lung & platelets	95	unknown	Thomas *et al.*, 1990.
Substrate G0	smooth muscle	250	unknown	Casnellie *et al.*, 1979.
Substrate G1	smooth muscle	130	unknown	Saracevic *et al.*, 1990
Substrate G2	smooth muscle	85	unknown	Casnellie *et al.*, 1979.
Substrate G3	smooth muscle	75	unknown	Casnellie *et al.*, 1979.

Key:
CaM = Calmodulin,
cGMPB-PDE = cGMP-binding phosphodiesterase (Type V),
SP = sarcoplasmic.

1.6 PHYSIOLOGICAL ROLES OF NITRIC OXIDE.

The tissue and species distribution of both Ca^{2+}-dependent (type I) and Ca^{2+}-independent (type II; induced) soluble NOS activity has been described in various tissues and species (Salter *et al.*, 1991). When this study commenced the significance of these findings was unclear and precise physiological roles, except in the control of vasodilatation were poorly defined. However, over the past few years numerous

functions for all three NOS isoforms have been proposed, ranging from neutrophil degranulation to the regulation of renal function (Wyatt *et al.*, 1991; Romero *et al.*, 1992).

Table 1.4 **Summary of the source, proposed functions and mechanism of signal transduction of NO in various tissues and cells.**

TISSUE/ CELL	NOS	FUNCTION	MECHANISM OF ACTION	REFERENCE
Cerebellum	I	motor co-ordination	G-kinase-I, phosphorylation of G-substrate, ↓phosphatase 1 & 2A activity	Bredt *et al.*, 1990. De Camilli *et al.*, 1984.
NANC neurones	I	gastrointestinal motility	G-kinase-I, may modulate Ca	Sanders & Ward, 1992.
Heart[1]	I/II	negative inotropic effect	G-kinase-I, inhibits slow inward Ca^{2+} flux.	Méry *et al.*, 1991.
Kidney	I	↑ diuresis and renin secretion	G-kinase-I ↓opening of amiloride-sensitive Na^{+} channel	Light *et al.*, 1990. Romero *et al.*, 1992.
Adrenal gland	I	catecholamine secretion	unknown	Palacios *et al.*, 1989. O'sullivan & Burgoyne, 1990.
Mast cells	I	modulation of histamine release	unknown	Salvemini *et al.*, 1990
Platelets	I	inhibition of aggregation	G-kinase-I, phosphorylation of VASP (Table 1.3)	Radomski *et al.*, 1990a. Reinhard *et al.*, 1992.
Neutrophils	I	migration, degranulation	G-kinase-I, phosphorylation of vimentin	Schmidt *et al.*, 1989. Wyatt *et al.*, 1991.
Macrophages	II	cytotoxicity	DNA mutations, inhibition of aconitase	Hibbs *et al.*, 1987. Nguyen *et al.*, 1992.
Vascular endothelium	III II	relaxation of vascular smooth muscle, endotoxic shock	G-kinase-I, reduces intracellular Ca^{2+} concentration as for type-III?	Förstermann *et al.*, 1991. Lincoln & Cornwell, 1993. Radomski *et al.*, 1990b.

Notes:

1. Source of NO is unclear although NOS-I has been demonstrated in neonatal rat cardiac myocytes and NOS-II activity was induced with pro-inflammatory cytokines (Balligand *et al.*, 1993; Schulz *et al.*, 1992)

Table 1.4 only represents a proportion of all tissues which exhibit NO forming ability, since several cells able to produce NO have as yet no described physiological function associated with NO synthesis. The majority of known physiological processes are mediated via the type-I isoform of NOS and although these tissues normally express only one isoform of NOS, induction of NOS-II activity has been reported in a number of these tissues following exposure to LPS or cytokines (Salter *et al.*, 1991). However, the precise physiological significance of these findings is unclear, although induction of NOS-II activity in vascular endothelial cells is believed to be a major contributor to the often fatal hypotension observed during endotoxic shock (Radomski *et al.*, 1990b).

1.7 ROLE OF NITRIC OXIDE IN THE GASTROINTESTINAL TRACT.

When this study commenced very little information was available regarding the role of NO in the gastrointestinal tract. However, NO produced by NOS-I had been reported to be involved in NANC-mediated relaxation of gastrointestinal smooth muscle (Bult *et al.*, 1990) and induced NOS-II activity had been implicated in mediating gastric damage in endotoxin pre-treated rats (Boughton-Smith *et al.*, 1990). Furthermore, inhibition of endogenous NO synthesis by bolus i.v. injection of L-NMMA in rats chronically pre-treated with capsaicin (which destroys sensory neurones and depletes their neuropeptide content) resulted in severe necrotic lesions after challenging the mucosa with 0.15 M HCl (Whittle *et al.*, 1990). The extent of mucosal damage was enhanced in animals co-administered indomethacin with L-NMMA, suggesting an important interaction between NO, prostanoids and sensory neuropeptides in the maintenance of gastric mucosal integrity.

1.8 AIMS OF THIS INVESTIGATION.

Following the detection of a Ca^{2+}-dependent form of NOS in the rat gastric mucosa (Whittle *et al.*, 1991), the major aims of this work were:

1. To determine which cell-type(s) of the gastric mucosa are responsible for the production of NO,

2. To establish the functional role(s) of NO in the gastric mucosa and,

3. To investigate the regulation and source of gastric PGE_2 production in response to stimulation with bradykinin.

Chapter Two

GENERAL METHODOLOGY

2.1 PREPARATION OF ISOLATED CELLS.

2.1.1 Preparation of an everted stomach sac.

A fed male Wistar rat (200-300g body weight) was anaesthetised by an i/p injection of sodium pentobarbitone (SAGATAL® May & Baker) at a dose of 60 mg/kg body weight. A mid-line incision was made to expose the stomach. The oesophagus was ligated and the stomach removed by transecting above the ligature and also across the duodenum (Fig 2.1). The animal was immediately sacrificed by puncturing the diaphragm.

The stomach was then washed in ice-cold saline (0.9% w/v NaCl) prior to tying a ligature at 1/3 of the distance form the pylorous to the glandular/non-glandular border. The non-glandular region was then removed and a blunt glass rod inserted from the pyloric end to evert the stomach (Fig. 2.1). The everted stomach was then rinsed briefly in ice-cold saline to remove any adherent food before being tied off to form an everted stomach sac. The sac was then inflated by introducing a pronase solution containing 1000 PUK/ml pronase dissolved in medium A (composition given in Table 2.1), via a 26-gauge needle.

2.1.2 Preparation of a crude gastric mucosal cell suspension.

The term "crude" shall be used throughout this work to refer to an unfractionated cell suspension that contains approximately 20% parietal cells and 30% mucous cells with the majority of the remainder being enterochromaffin cells (Chen *et al.*, 1990). The isolation method was similar to that of Trotman & Greenwell (1979), which is a modification of the method devised by Lewin *et al.* (1974). Usually four everted stomach sacs were transferred to a 125 ml plastic bottle containing 40 ml medium A (Table 2.1) and were incubated for 30 min at 37°C in a shaking water bath (60 cycles/min) with continuous gassing of the medium with 95%O_2/5%CO_2. Following the 30 min incubation the sacs were removed from medium A, blotted dry on filter paper (Whatman No. 1, Whatman, Maidstone) and were transferred to 20 ml medium B in a round plastic beaker of 50 ml capacity which was sealed with laboratory film (Whatman, Maidstone) and the air space above the medium gassed with 95%O_2/5%CO_2.

The sacs were continually stirred with a magnetic stirring bar rotating at approximately 100 r.p.m. in medium B (Table 2.1) for 30 min at ambient temperature. Cells released into medium B were filtered through nylon mesh (150 μm pore size; Sericol Group Ltd., London) into 13 ml polystyrene centrifuge tubes and were centrifuged at 125 g for 7 min at 15°C. Following centrifugation the supernatant was discarded and the cell pellet was gently resuspended in 10 ml of fresh medium B by repeatedly drawing into, and expelling the suspension form a plastic transfer pipette (L.I.P. Ltd., Shipley). The suspension was stored in a 50 ml plastic conical flask at 37°C with continuous shaking (140 cycles/min) with gassing of the air space above the suspension as previously described. The incubation of the sacs in medium A followed by harvesting in medium B and subsequent filtration, centrifugation and resuspension in fresh medium B was repeated twice, after which the isolated cell suspensions were pooled, centrifuged for 7 min at 125 g and 15°C and resuspended in the required incubation medium.

2.2 FRACTIONATION OF CRUDE ISOLATED CELL SUSPENSIONS.

Two methodologies were used to fractionate the crude cell suspension.

2.2.1 Separation by density gradient centrifugation.

Iso-osmotic Percoll® was prepared by mixing 1 volume of 10 X concentrated Eagle's Minimum Essential Medium with 9 volumes of Percoll® and adding 20 mM $NaHCO_3$. A pellet of crude cells was resuspended in 28 ml of medium C (Table 2.1), 4.5 ml of suspension was transferred to 10 ml high speed centrifuge tubes (MSE) containing 3 ml of iso-osmotic Percoll® which had previously been gassed with 95%O_2/5%CO_2 for 15 min at ambient temperature and the pH corrected to 7.4 with HCl. The cell suspensions were then centrifuged at 30,000g_{av} for 13 min at 4°C using a 20° angle rotor (MSE Superspeed 50, MSE, Crawley).

Centrifugation produced two bands of cells on the density gradient, a high density band of density approximately 1.059 g/ml and a low density band of cells of density

approximately 1.029 g/ml. Both cell fractions were removed in a 1.5 ml volume from each tube and were transferred to 12.5 ml polypropylene test tubes. The volume in each test tube was made up to 10 ml with medium B' (Table 2.1) and the cells were gently resuspended before being centrifuged at 125g for 7 min at 15°C. The supernatant was discarded and the cells were resuspended in a further 10 ml of medium B' and centrifuged at 125g as before. This process was repeated once more to ensure the removal of any remaining Percoll®. The cell fractions were then resuspended in the appropriate experimental medium.

Figure 2.1 Preparation of an everted fundic sac from the rat stomach.

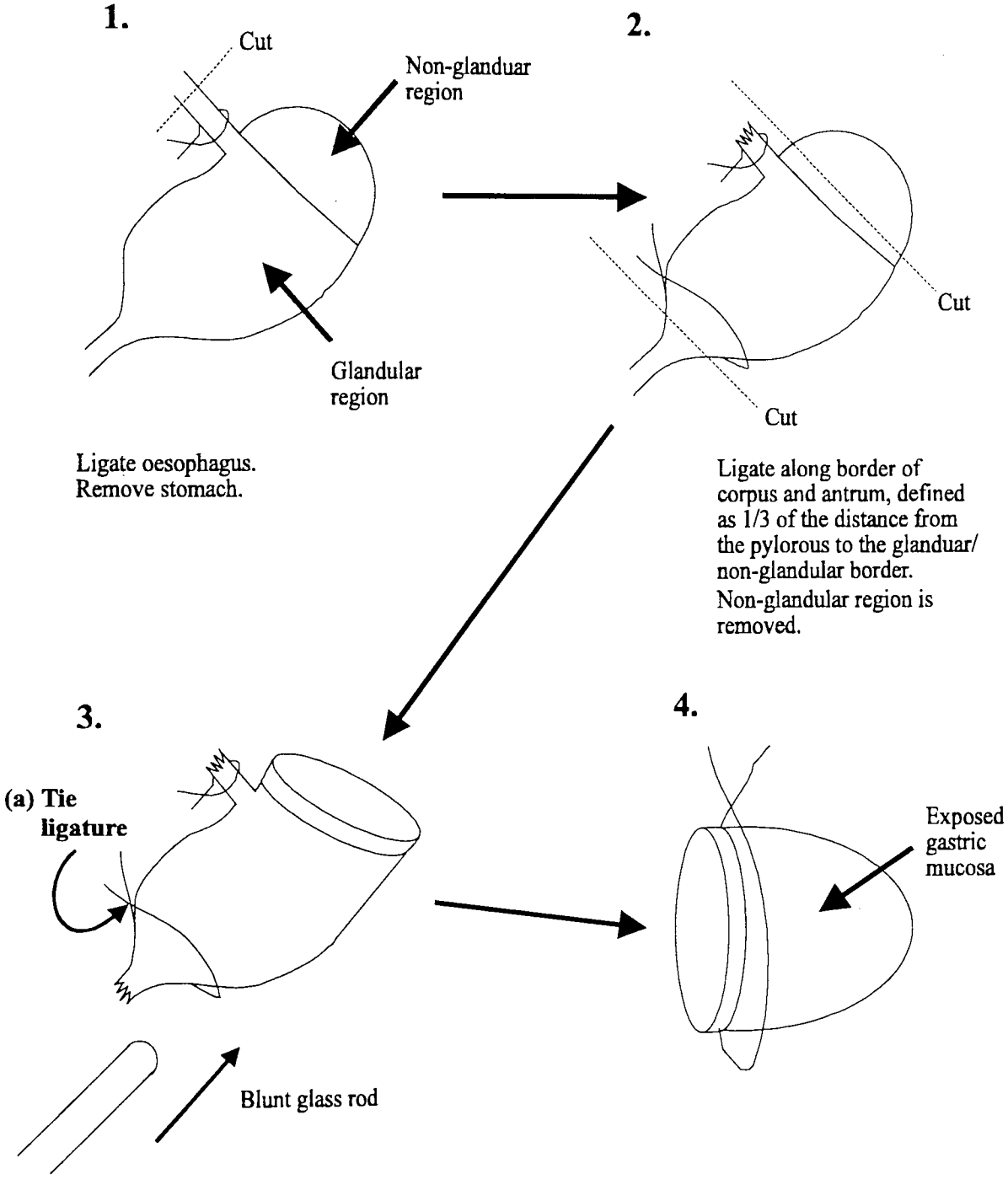


Table 2.1 **Composition of the solutions used in the isolation, separation and incubation of gastric mucosal cells.**

The following additions were made to Eagle's Minimum Essential Medium (See Appendix A2) containing 25 mM $NaHCO_3$ and 20 mM HEPES.

Medium	Additions
A	EDTA (2 mM) Soyabean trypsin inhibitor (0.1 mg/ml) Dextran (30 mg/ml)
B	Bovine serum albumin fraction V (30 mg/ml)
B'	Bovine serum albumin fraction V (1 mg/ml)
C	EGTA (3 mM) Dithiothreitol (0.5 mM)

2.2.3 Separation by counterflow centrifugation.

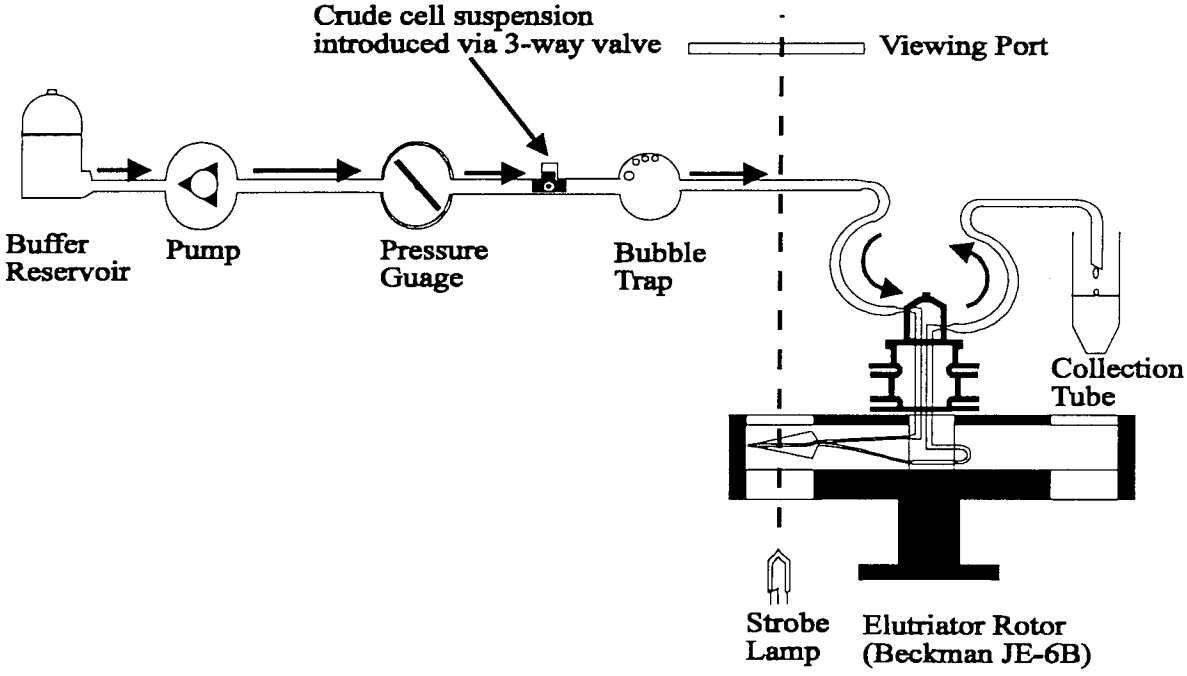
The method for elutriation was similar to that described by Schepp *et al.* (1990). Crude gastric mucosal cells were resuspended in 10 ml of Hanks Balanced Salt Solution (HBSS), containing 20 mg/ml BSA and 2.5 mg/ml pancreatin. The cell suspension was stirred at 90 r.p.m. on ice at 4°C using a magnetic follower for 15 min. Following this, the cell suspension was filtered through cheese cloth before being centrifuged for 7 min at 125 g and 15°C. The resulting pellet was then resuspended in 10 ml of HBSS containing 20 mg/ml BSA, filtered through cheese cloth and finally filtered through nylon mesh (62 μm pore size) to remove any cell clumps. A 0.5 ml aliquot of the suspension was added to 50 ml of HBSS containing 5 mg/ml BSA and was stored on ice until completion of the elutriation. The elutriator rotor (Beckman JE-6B; Fig. 2.2) was filled with HBSS containing 5 mg/ml BSA, care being taken to ensure that all air bubbles were removed by inverting the rotor several times whilst HBSS containing 5 mg/ml BSA was pumped through the rotor at a flow rate of 50 ml/min. The remainder of the cell suspension was loaded at a flow rate, rotor speed and temperature of 18 ml/min, 2,500 r.p.m. and 4°C respectively, via a 3-way stop-cock, into the loading reservoir. The elutriation medium (HBSS containing 5 mg/ml BSA) was then diverted through the loading reservoir (via the 3-way stop-cock) to transfer cells to the elutriation chamber of the rotor. When the loading reservoir was void of cells, the stop-cock was closed so that the flow of medium bypassed the loading reservoir. The cells in the elutriation chamber were allowed to equilibrate for 2 min whilst being washed at a high flow rate and rotor speed, before collecting the fractions according to the parameters given in Table 2.2.

Following elutriation, each fraction (including the non-elutriated one) was centrifuged for 10 min at 200 g and 4°C, and the pellets of cells were resuspended in the appropriate experimental medium.

Table 2.2 **Rotor speeds and solution flow rates for separation of isolated rat gastric mucosal cells by counterflow centrifugation.**

FRACTION	FLOW RATE (ml/min)	ROTOR SPEED (r.p.m.)
WASH	20	2400
F_1	20	2300
F_2	20	2100
F_3	29	2000
F_4	40	2000
F_5	58	2000

Figure 2.2 **Essential aspects of the elutriator system.**



2.2.4 Determination of cell viability.

Cell viability was routinely determined for crude cell suspensions and cells separated by both density gradient and counterflow centrifugation. The structural integrity of the cell membrane, used as an index of viability, was measured by the ability of cells to exclude the dye trypan blue. An aliquot of cells (20 µl) was mixed with an equal volume of trypan blue (4 mg/ml) dissolved in physiological saline (0.9% w/v NaCl). An overestimate of cell viability may be obtained in the presence of

high concentrations of BSA above 10 mg/ml (Seglen, 1976) or if the contact time between the cells and the dye is inadequate (Elliot, 1979). Cell viability was therefore assessed in either B' or HBSS containing 5 mg/ml BSA. Cells were counted using a haemocytometer (E. Leitz, Wetzlar) by light microscopy (x400 magnification), and on each occasion at least 200 cells were counted. If less than 85% of the cells in the preparation excluded trypan blue then it was not used for experiments.

2.3 MEASUREMENT OF THE cGMP CONTENT OF CELL SUSPENSIONS.

2.3.1 Extraction of cGMP from frozen cell suspensions.

The cGMP was extracted from frozen samples by addition of ice-cold ethanol to produce a final concentration of 65% (v/v). The samples were allowed to thaw before being vortexed for 10 s. The solution was allowed to settle prior to removal of the supernatant. The remaining precipitate was washed with 1 ml ice cold 65% (v/v) ethanol and the supernatant was pooled with the first wash. The combined washes were centrifuged at 2000 g for 15 min at 4°C before the supernatant was transferred to 3 ml polypropylene tubes. The combined extracts were evaporated to dryness under a stream of nitrogen gas at 60°C.

2.3.2 Assay for cGMP content by enzymeimmunoassay.

The dried extracts were reconstituted in 100 µl of assay buffer (Amersham cGMP Enzymeimmunoassay Kit) consisting of 0.05 M sodium acetate buffer, pH 5.8, containing 0.02% (w/v) bovine serum albumin and 0.005% (w/v) thimerosal. Standards were prepared by the serial dilution of a 10.24 pmol/ml stock solution of cGMP standard with an equal volume of assay buffer to produce standards of 512, 256, 128, 64, 32, 16, 8, 4 and 2 fmol/50 µl. A 100 µl aliquot of each standard was transferred to 3 ml polypropylene tubes to which 10 µl of acetylating reagent, consisting of 1 volume acetic acid to 2 volumes triethylamine was added. Each tube was vortexed immediately following addition of the acetylating reagent. Similarly, 10 µl of acetylating reagent was

added to the tubes containing 100 µl of the reconstituted sample, and the tubes were vortexed immediately.

The assay protocol is given in table 2.3. Plates were emptied by inverting over a sink, shaking three times and blotting on a wad of absorbent paper. Wash buffer consisted of 0.01 M phosphate buffer, pH 7.5, containing 0.05% Tween 20. Enzyme substrate was 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide with incubation for 30 min at ambient temperature and constant shaking on a microtitre plate shaker running at 90 cycles/min. The 1.0 M sulphuric acid was added to halt colour development prior to measuring the absorbance at 450 nm using a microtitre plate reader (Anthos 2001, Anthos Labtec Instruments, Austria).

Table 2.3 **Enzyme immunoassay protocol for acetylated cGMP measurement.**

(All volumes are in microlitres)

ADDITIONS TO WELLS

	Substrate blank	**Non-specific binding (NSB)**	**Zero standard (B_0)**	**Standards**	**Samples**
Buffer	-	150	-	-	-
Antiserum	-	-	100	100	100
*Standard	-	-	50	50	-
*Sample	-	-	-	-	50

*From acetylated tubes

Cover plate, incubate at 3-5°C for 2 hours

Peroxidase conjugate	-	100	100	100	100

Cover plate, incubate at 3-5°C for 60 minutes.

Empty plate, wash all wells four times with wash buffer (400 µl on each occasion).

Substrate	200	200	200	200	200

Cover plate, incubate at ambient temperature (15-30°C) for exactly 30 minutes while shaking.

1.0 M sulphuric acid	100	100	100	100	100

Shake to mix contents and determine absorbance at 450 nm

2.3.3 Calculation of results.

The absorbance of blank wells was automatically subtracted from all other wells by the plate reader. All absorbance measurements were performed in duplicate. The percentage bound for each standard and sample was calculated using the following relationship:

$$\%B / B_0 = \frac{(\text{Absorbance of standard or sample} - \text{Absorbance NSB})}{(\text{Absorbance } B_0 - \text{Absorbance NSB})} \times 100$$

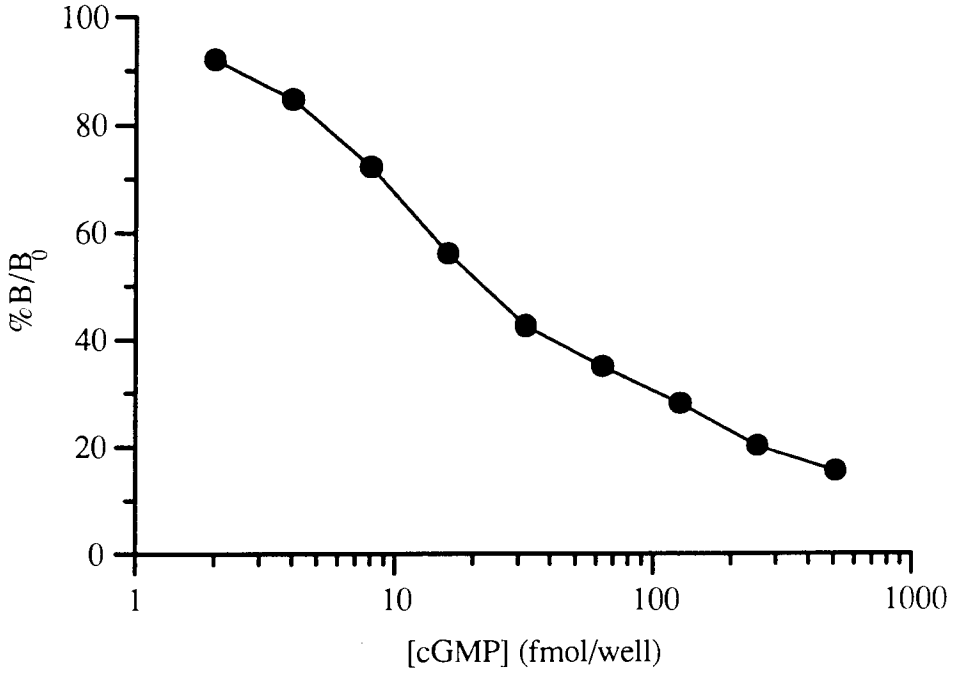
The standard curve was generated by plotting the percentage B/B_0 as a function of the $\log_{10}$ cGMP concentration. Typical absorbance values are shown in Table 2.4 and the resulting curve is presented in Figure 2.3.

Table 2.4 Typical assay data for cGMP enzyme immunoassay.

Standard (fmol/well)	*Absorbance at 450 nm	Mean-NSB	$\%B/B_0$
512	0.198 0.195	0.197	15.54
256	0.263 0.251	0.257	20.28
128	0.375 0.342	0.359	28.33
64	0.423 0.470	0.447	35.24
32	0.525 0.559	0.542	42.78
16	0.710 0.720	0.715	56.39
8	0.934 0.904	0.919	72.53
4	1.086 1.066	1.076	84.93
2	1.160 1.174	1.167	92.11
0	1.250 1.284	1.267	-
NSB	0.000 0.000		

Note: * Blank absorbance of 0.223 has been subtracted prior to tabulation.

Figure 2.3 **A typical standard curve for the enzyme immunoassay for cGMP.**



Data are obtained from a single experiment.

2.4 MEASUREMENT OF THE cAMP CONTENT OF CELL SUSPENSIONS.

The protocol for the measurement of the cAMP content of cell suspensions was almost identical to the procedure used to measure the cGMP content described above in section 2.3 with the following exceptions:

i) The volume of assay buffer used to reconstitute the dried extract described in section 2.3.2 was increased to 110 µl in experiments where both cAMP and cGMP were measured, 100 µl being used for the cGMP assay and the remaining 10 µl being used to measure the cAMP content with the volume of the cAMP sample being made up to 100 µl with assay buffer,

ii) The volume of substrate (Table 2.3) was 150 µl for the cAMP assay compared with 200 µl for the cGMP assay,

iii) The incubation period with substrate was for 30 min for the cAMP assay and 60 min for the cGMP assay and,

iv) The range of the assay for acetylated samples of cAMP was between 2 and 128 fmol/well whereas the cGMP assay measured between 2 and 512 fmol/well.

2.5 PREPARATION OF SCHIFF REAGENT.

Schiff reagent was prepared by dissolving 1 g of basic fuchsin (BDH, Poole) in 100 ml boiling water to which 20 ml 1 M HCl was added. The solution was then allowed to cool to 50°C before addition of 300 mg activated charcoal. The solution was shaken for 5 min before removal of the charcoal by filtration through filter paper (No. 1, Whatman, Maidstone). The charcoal addition and filtration was repeated once more and the resulting solution was stored in an amber glass bottle at room temperature until required. Immediately before use 6 ml Schiff reagent was incubated with 0.1 g sodium metabisulphite at 37°C until a yellow colour was obtained (usually 2-3 h).

Chapter Three

DISTRIBUTION OF NITRIC OXIDE SYNTHASE ACTIVITY IN THE RAT GASTRIC MUCOSA

3.1 INTRODUCTION

The presence of calcium-dependent and soluble nitric oxide synthase (NOS-I) in gastric mucosal homogenates was firstly demonstrated by Whittle *et al.* (1990) using a spectrophotometric determination of NO production from its interaction with oxyhaemoglobin. A major aim of this section of work was to investigate the distribution of this constitutive NOS activity between cell types in the rat gastric mucosa by using both density gradient and counterflow centrifugation (elutriation) to fractionate the cells. An additional aim was to establish whether pretreatment of animals with lipopolysaccharide (LPS) could induce additional NOS activity in the gastric mucosa and to examine its distribution and effect on cell viability.

3.2 METHODOLOGY

3.2.1 Preparation of cell homogenates.

Cells from the rat corpus mucosa were isolated as previously described in section 2.1 to produce a crude cell suspension. The crude fraction was then resolved into either high and low density fractions by Percoll density gradient centrifugation (2.2.1) or into fractions F_1 to F_5 by counterflow elutriation (2.2.2). In certain experiments, animals were pretreated with either LPS (*E. coli* serotype O111:B4, 3 mg/kg i.v.), or dexamethasone (2 mg/kg i.p.) 1 h prior to the administration of LPS. Both agents were dissolved in sterile physiological saline at concentrations of 3 mg/ml and 2 mg/ml for LPS and dexamethasone respectively. Following administration of LPS, animals were left for 4 h with food and water available *ad libitum*. Animals were then sacrificed and the cells prepared as previously described (2.1; 2.2.1 and 2.2.2).

Cell pellets were resuspended in homogenisation buffer (pH 7.4), consisting of 10 mM HEPES, 0.32 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 10 µg/ml soyabean trypsin inhibitor, 10 µg/ml leupeptin, 2 µg/ml aprotinin and 100 µg/ml phenylmethyl sulphonyl fluoride at a cell concentration of between 0.2 and 1.3×10^8cells/ml, (routinely, a cell concentration of 1×10^8cells/ml was used), before being transferred to 1.5 ml micro-centrifuge tubes. The resulting suspensions were then frozen

in liquid nitrogen before being allowed to thaw at 37°C. This process of freezing and thawing was repeated twice more prior to centrifugation at 10,000g and 4°C for 20 min (Eppendorf Chillspin Microcentrifuge). The supernatant was removed and stored on ice for no longer than 10 min, before estimation of enzyme activity.

3.2.2 Estimation of NOS activity.

3.2.2.1 Preparation of activated cation exchange resin (DOWEX AG 50-W8).

In order to ensure that all sites on the exchange resin are available to bind positively charged species, the resin requires activation. To 50 g of DOWEX AG 50-W8, 200 ml of 1 M NaOH was added and the suspension was mixed thoroughly by gentle swirling of the container and left for 1 h to allow the DOWEX to settle. The aqueous layer was carefully decanted off before a further 200 ml of 1 M NaOH was added, the suspension mixed as before and left to stand for a further 1 h. The aqueous layer was again decanted off before the addition of 300 ml distilled water. The suspension was mixed as before and left to stand for a further 1 h before the water was decanted off and 300 ml of fresh distilled water was added. The addition of distilled water was repeated until the DOWEX suspension was approximately pH 6.5. The water was then decanted off and a 1:1 (v/v) suspension of DOWEX with distilled water was prepared. The container was covered with sealing film and the DOWEX stored at room temperature until required.

3.2.2.2 Assay for NOS activity.

Conversion of the substrate [U-^{14}C]-L-arginine to the NOS co-product, which would include, [^{14}C]-L-citrulline, was used to provide an index of the NOS activity in cytosolic cell fractions. A 20 µl aliquot of homogenate supernatant (prepared as described in 3.2.1), was transferred to 1.5 ml micro-centrifuge tubes containing 50 µl of a substrate solution which had been pre-incubated at 37°C for 5 min. The final concentrations of constituents in the assay system were: 30 mM potassium phosphate, 150 µM $CaCl_2$, 0.7 mM $MgCl_2$, 15 µM [U-^{14}C]-L-arginine (700,000 d.p.m./ml),

0.7 mM NADPH and 7 mM L-valine (added to inhibit possible arginase activity; Knowles *et al.*, 1990). Certain micro-centrifuge tubes also contained final concentrations of either EGTA (1 mM) or L-NMMA (1-100μM) to allow definition of enzyme activity. Furthermore, blank control tubes containing substrate were also incubated with 20 μl of homogenisation buffer to determine blank radioactivity (i.e. residual arginine not adsorbed to resin). The cell homogenate supernatant and substrate solutions, or blank tubes, were incubated for 10 min at 37°C, after which time 500 μl of a 1:1 suspension of DOWEX (activated as described in 3.2.2.1) in distilled water was added, to bind any unconverted [^{14}C]-L-arginine followed by addition of 1 ml distilled water. The DOWEX was allowed to settle before 975 μl of supernatant was removed, transferred to 20 ml polyethylene scintillation vials and 5 ml of scintillation fluid (Optiphase HiSafe II) added to the scintillation vial. Product formation was determined by liquid scintillation counting using a Packard (Tri-Carb® 1500/TR) liquid scintillation counter (see Appendix A3.).

3.2.2.3 Calculation of nitric oxide synthase activity.

The radiolabelled products formed at the end of the incubation was used as an index of the NOS activity in the cell homogenate which was calculated from the formula:

$$\text{Product formation (nmol / min / } 10^6 \text{ cells)} = \frac{\text{d.p.m.} > \text{background}}{\text{SpA}} \text{ X } \frac{\text{Vol 1}}{\text{Vol 2}} \text{ X } \frac{50}{\text{t}}$$

Where:

d.p.m.>background	= d.p.m. of sample - d.p.m. in absence of enzyme (blank),
SpA	= specific activity of [^{14}C]-L-arginine substrate,
Vol 1	= total volume of liquid phase (μl)
	i.e. total volume of additions = 1570 μl
	volume of solid dowex = 250 μl
	∴ total volume of liquid phase = 1320 μl,
Vol 2	= volume removed for counting (i.e. 975 μl),
50	= correction factor to convert to activity/ml of homogenate,
t	= incubation time (min)

Total NOS activity is defined as product formation which is inhibitable by the presence 100 μM L-NMMA. This is a standard method of data expression (Moncada *et al.*, 1991) as the arginine analogue selectively inhibits NOS, hence any radiolabelled products formed in its presence can be regarded as "background noise" which will also include any arginine not adsorbed by the resin.

3.2.3 Characterisation of cell-types in cell fractions separated by counterflow elutriation.

3.2.3.1 Preparation of cells and staining with periodic acid Schiff reagent.

Cells from the rat corpus mucosa were isolated as described in section 2.1.2 and separated into fractions F_1 to F_5 by counterflow elutriation as described in section 2.2.3. Cell pellets were resuspended in an appropriate volume of medium B' (see table 2.1 for composition), usually 1 ml, although if a low cell yield was expected ($<10^7$ cells), the volume for resuspension was decreased to 0.5 ml. A 100 μl aliquot was removed and transferred to a clean, dry glass microscope slide by using a cytospin centrifuge to form a thin and even smear of cell suspension. The cytospin smear was then fixed in 37% formaldehyde and 95% ethanol (1:9 v/v) for 10 min followed by a 5 min wash under gently running tap water. Slides were then immersed in periodic acid solution (1% w/v) for 20 min after which time they were again washed under tap water for 5 min. Finally the slide was immersed in Schiff's reagent (prepared as described in section 2.5) for 20 min and rinsed in tap water for a further 10 min, as above, before application of a glass cover-slip which was sealed in position with clear nail varnish.

3.2.3.2 Identification of cell types.

Following staining with periodic acid Schiff reagent, the proportions of mucous epithelial and parietal cells was determined. Mucous cells were identified by their positive staining with periodic acid Schiff, whereas parietal cells were identified by their large size (diameter >12 μm) concentric nucleus and dense granular cytosol.

3.2.4 Presentation of results.

When the data are presented as "NOS ACTIVITY", the d.p.m. of the enzyme in the presence of 100 μM L-NMMA has already been subtracted from the results prior to plotting. Whereas, if the data are presented as "PRODUCT FORMATION" the d.p.m. in the presence of 100 μM L-NMMA has **NOT** been subtracted from the results prior to plotting. "Ca^{2+}-INDEPENDENT NOS ACTIVITY" is NOS ACTIVITY corrected for the activity in the presence of 1 mM EGTA. In **ALL CASES**, blank d.p.m. values will have been subtracted from sample d.p.m. data prior to calculation of activity or formation of products. The formulae used to calculate the aforementioned data are presented below:

$$\text{NOS ACTIVITY} = \left((U_{dpm} - UB_{dpm}) - (L-NMMA_{dpm} - L-NMMAB_{dpm})\right) x1.415x10^{-3}$$

$$\text{PRODUCT FORMATION} = (U_{dpm} - UB_{dpm}) x1.415x10^{-3}$$

$$Ca^{2+}\text{I NOS ACTIVITY} = \left((EGTA_{dpm} - EGTAB_{dpm})\right) - \left(L-NMMA_{dpm} - L-NMMA_{dpm}\right) x1.415x10^{-3}$$

Where:

U_{dpm}	= d.p.m. of uninhibited enzyme,
UB_{dpm}	= d.p.m. of uninhibited blank,
$L\text{-}NMMA_{dpm}$	= d.p.m. of enzyme in the presence of 100 μM L-NMMA,
$L\text{-}NMMAB_{dpm}$	= d.p.m. of blank in the presence of 100 μM L-NMMA,
Ca^{2+} I	= Ca^{2+}-independent,
$EGTA_{dpm}$	= d.p.m. of enzyme in the presence of 1 mM EGTA,
$EGTAB_{dpm}$	= d.p.m. of blank in the presence of 1 mM EGTA,
$1.415x10^{-3}$	= correction factor from calculation in section 3.2.2.3

3.3 RESULTS & DISCUSSION

3.3.1 Constitutive NOS activity.

3.3.1.1 Distribution and assay of enzyme activity in crude, high and low density cell fractions.

Cells were resolved into two zones on the self-forming Percoll® gradient. The low density fraction centred on a density of 1.029 g/ml and was enriched with parietal cells (84±2% compared with 20±1% in the crude preparation). The high density fraction centred on a density of 1.059 g/ml and was depleted of parietal cells (11±2%).

Significant ($P<0.05$ by paired Student's t-test) product formation was demonstrated in both crude (cells not separated on the density gradient), and high density cell fractions, with no detectable activity in the low density fraction (Fig. 3.1). The enzyme activity in the high density fraction was significantly ($P<0.001$ by ANOVAR and Dunnett's test) greater than that observed in the crude preparation, hence this cell fraction was used to validate the assay and to characterise the enzyme and establish whether or not the enzyme activity was indeed attributable to NOS.

NOS activity was linearly related to the number of cells ($0.2\text{-}1.4\times10^6$ cells) used to prepare the homogenate (Fig. 3.2) and also linearly related to time (Fig. 3.3), thereby justifying measurement of activity after a 10 min incubation and also the use of cell fraction homogenates containing different cell concentrations.

No significant difference was observed (ANOVAR, $P>0.05$) between the blank d.p.m. values for EGTA (1 mM) or L-NMMA (100 µM) when compared to the uninhibited blank, with d.p.m. values of 1372±64, 1325±50 and 1370±58 (number of experiments, n=9) for uninhibited, EGTA and L-NMMA blanks respectively. It was therefore possible to directly subtract product formation in the presence of 100 µM L-NMMA from the uninhibited product formation to convert the data to NOS activity. This correction method for converting product formation to NOS activity will now be used for all subsequent data unless stated otherwise.

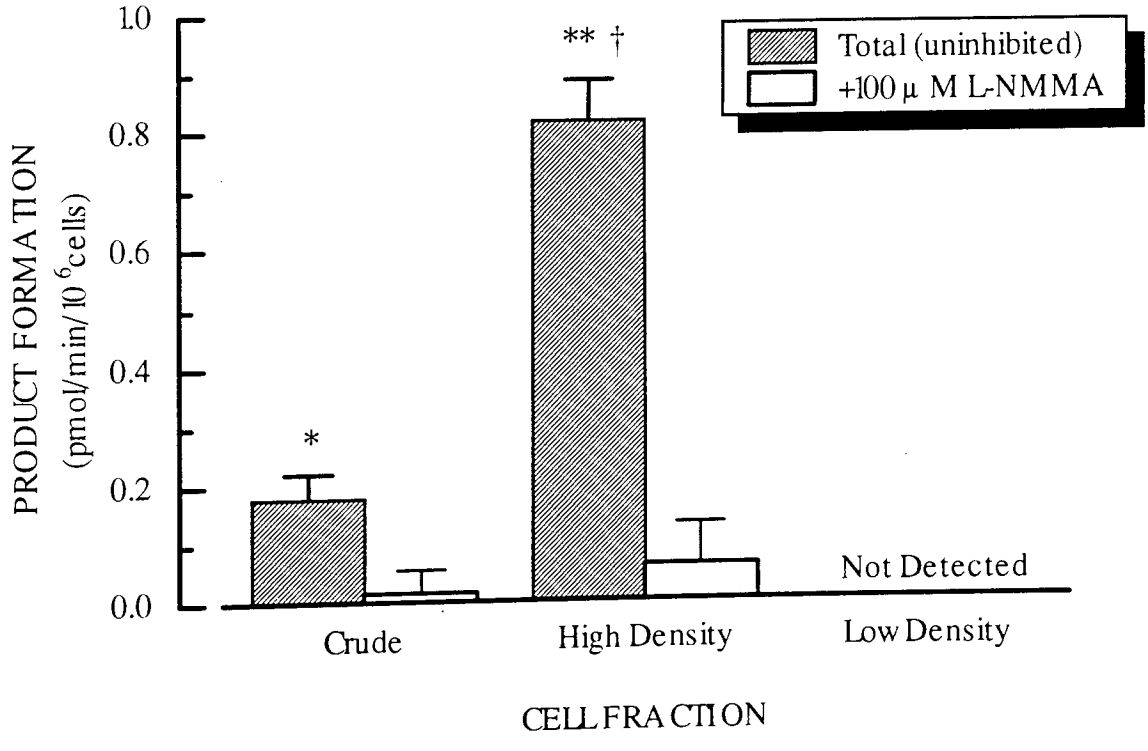
3.3.1.2 Characterisation of enzyme activity.

In the presence of EGTA (1 mM), product formation was significantly reduced by 97±3% (n=8, P<0.01, ANOVAR and Dunnett's test; Fig. 3.4). Furthermore, L-NMMA (1-100μM), caused a concentration-dependent decrease in both product formation and enzyme activity (Figs 3.4, 3.5) with an IC_{50} of 4.2μM (generated by the computer program FIT; Barlow, 1983). L-NMMA (100μM) significantly inhibited product formation by 91±3% (n=8, P<0.01 by ANOVAR and Dunnett's test), while the enantiomer D-NMMA inhibited by only 20±1% (n=4). In the presence of L-NMMA or D-NMMA (25μM), enzyme activity was inhibited by 70±2% (n=8, P<0.01 by ANOVAR and Dunnett's test) and 6±4% (n=4) respectively (Fig. 3.4).

These results firstly suggest that the enzyme activity is Ca^{2+}-dependent due to its sensitivity to the Ca^{2+}-chelator EGTA, and secondly, inhibition by the arginine analogue NMMA is stereospecific as the D enantiomer was without effect at a concentration of 25 μM. The minor inhibitory activity of D-NMMA at 100 μM could reflect a 1% contamination with the active L-enantiomer. Spectrophotometric assay of NOS activity in homogenates of whole gastric mucosa from rat (Whittle *et al.*, 1991) gave an activity of 2.1±0.4 nmol/min/g tissue and an IC_{50} of 38±9 μM for L-NMMA which compares with 0.340±0.084 nmol/min/g wet weight cells and an IC_{50} of 4.2 μM in crude cell extracts measured via [^{14}C]-L-citrulline formation. The conversion of cell concentration to g wet weight is achieved through the observation that $1x10^6$ crude rat gastric mucosal cells have a dry weight of 0.158 mg and that the wet weight to dry weight ratio is 3 (Hatt, 1988). The discrepancy between the two values for NOS activity and IC_{50} for L-NMMA probably arise from the higher concentration of arginine used in the spectrophotometric assay. NOS in the radio-chemical assay may not be fully saturated due to the lower concentration of arginine (required to ensure a high specific activity) in the assay system. This lower concentration of arginine will also produce a lower IC_{50} for L-NMMA since it is a competitive inhibitor of NOS (Palmer & Moncada, 1989).

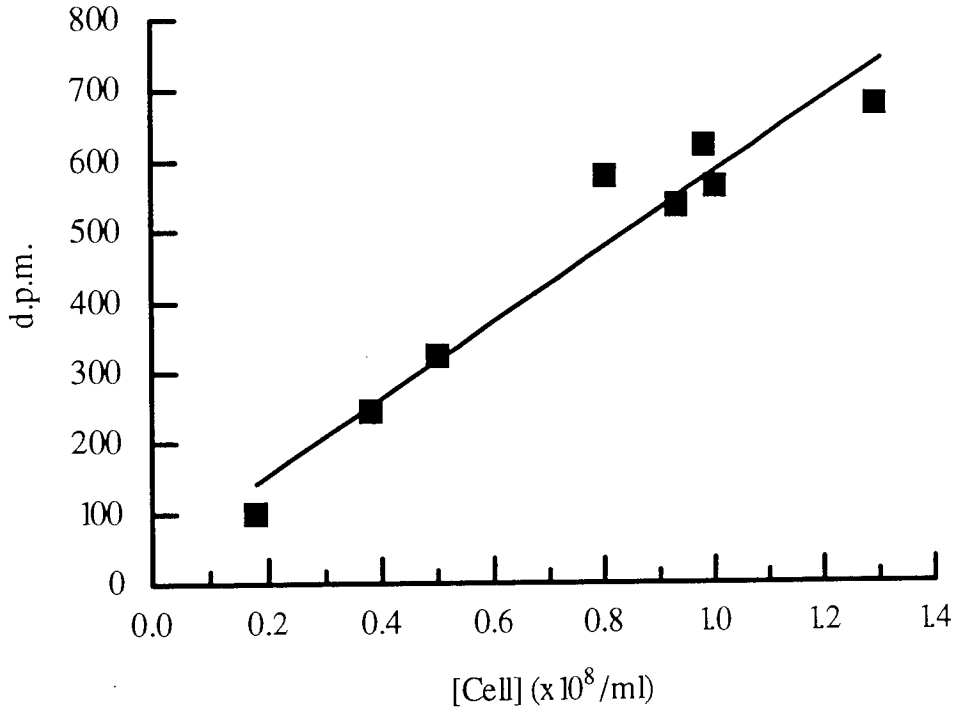
Although the enzyme under investigation is indeed likely to be NOS, which isoform of the enzyme is present remains to be established. Förstermann *et al.* (1991) have suggested that six possible isoforms of NOS exist which are characterised by their cofactor requirement, cellular localisation and method of regulation. The enzyme present in the gastric mucosa under these conditions is likely to belong to the type I or type III group, as type II is only expressed following exposure to endotoxin or cytokines, and type III is particulate.

Figure 3.1 **Distribution of product formation in cell fractions of gastric mucosal cells separated by density gradient centrifugation.**



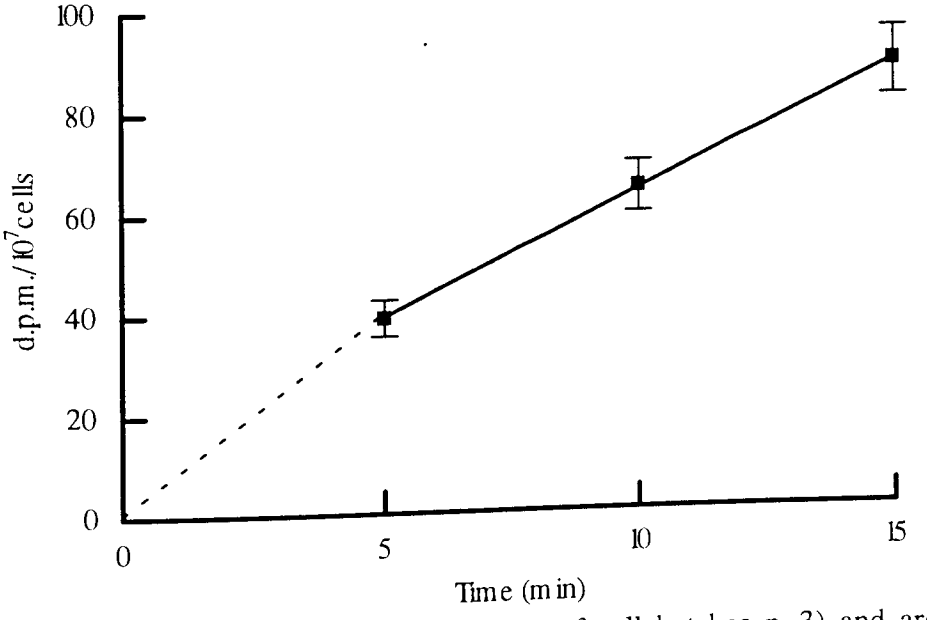
Results are presented as mean±SEM (number of cell batches n=7, 13 and 4 for crude, high and low density respectively) where **P<0.01 and *P<0.05 for difference from product formed in the presence of 100 μM L-NMMA by Student's t-test for paired data and †P<0.001 for difference from crude total product formation by ANOVAR and Dunnett's test.

Figure 3.2 **Relationship between product formation (d.p.m.) and the cell concentration used to prepare the homogenate.**



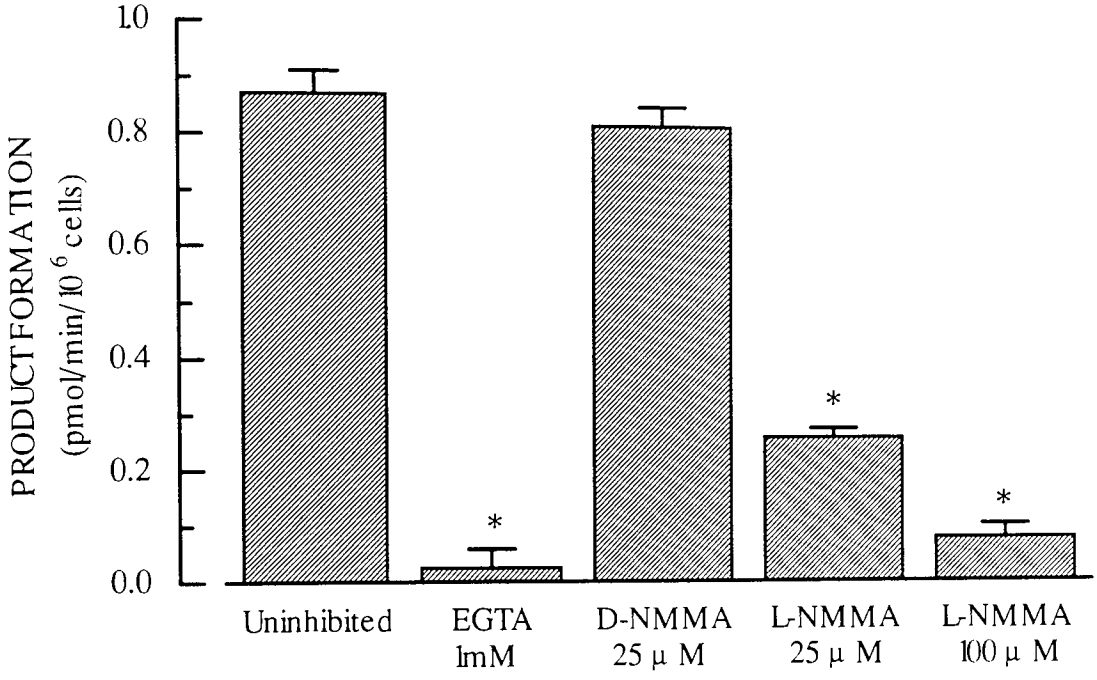
Results are obtained from separate batches of high density gastric mucosal cell homogenates separated by density gradient centrifugation. Each d.p.m. value has been corrected for blank d.p.m. (i.e. activity in the presence of 100 μM L-NMMA). The equation of the regression line is y=45.52+551.2x with a correlation coefficient of 0.97.

Figure 3.3 **Time dependency of production of radiolabelled products.**



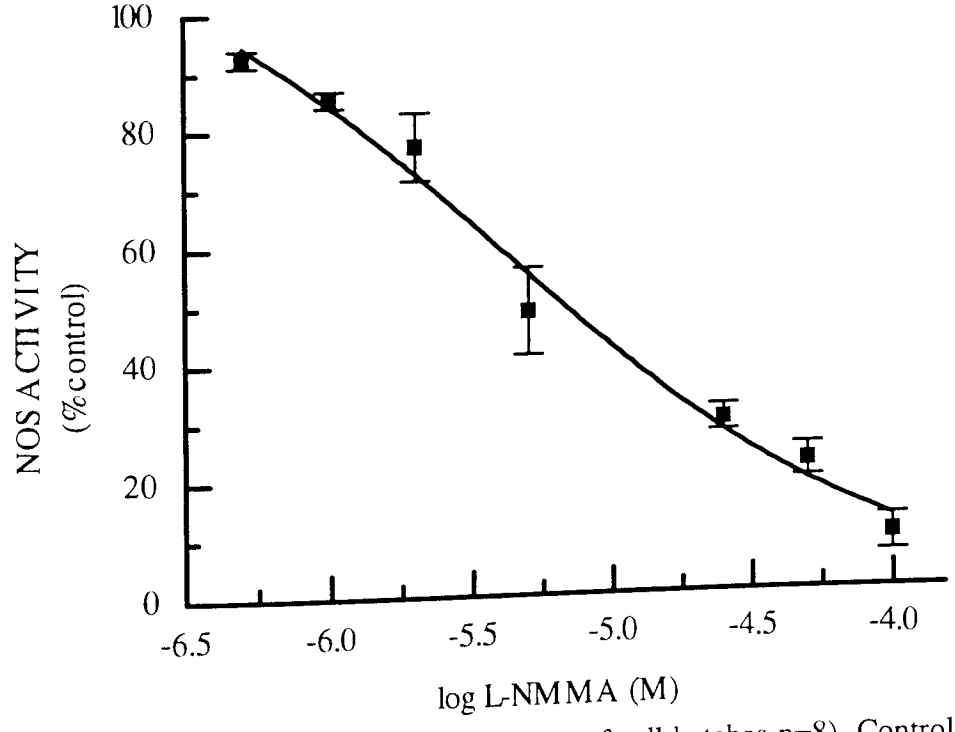
Results presented as mean±SEM (number of cell batches n=3) and are corrected for activity in the presence of L-NMMA (100 μM). The equation for the regression line is y=14.56+4.96x with a correlation coefficient of 0.999.

Figure 3.4 **Sensitivity of the enzyme in an homogenate of high density cells to EGTA, D-NMMA and L-NMMA.**



Results are presented as mean±SEM (number of cell batches n=8, 8, 4, 8 and 8 for uninhibited through to 100 μM L-NMMA respectively), where *P<0.01 for difference from uninhibited activity by ANOVAR and Dunnett's test.

Figure 3.5 **Effect of L-NMMA on the conversion of [U-^{14}C]-L-arginine by homogenates of high density cells prepared from the rat gastric mucosa.**



Data are presented as mean±SEM, (number of cell batches n=8). Control activity in the absence of inhibitor was 0.88±0.03 pmol/min/10^{6}cells.

3.3.1.3 Distribution of NOS activity in cells separated by counterflow elutriation.

Crude gastric mucosal cells were separated according to size by counterflow elutriation into five fractions, termed F_1 to F_5. Fraction 1 and 2 consisted of cells of small diameter previously identified by Chen *et al.*, (1990), as endothelial cells and macrophages, whereas fraction 3 was enriched with mucous cells (61±1%, n=4) and fraction 5 was enriched with parietal cells (78±1%, n=10). Fraction 4 had previously been characterised by Schepp *et al.*, (1990a) (and personal communication) and contained 50% chief cells, with the remainder consisting of parietal and mucous cells in roughly equal proportions (Figs 3.6 A & B and Plates 3.1, 3.2,.3.3, 3.4 and 3.5).

The distribution of nitric oxide synthase in these fractions followed reasonably well that of the proportion of mucous cells (Figs 3.6 B & C). No NOS activity could be detected in the small cells of fractions 1 and 2, with maximal activity being located in fraction 3. NOS activity was also found in fraction 4 although this could reflect the activity of mucous cells which constitute approximately 25% of this elutriator cell fraction. Furthermore, no significant ($P>0.05$ by Student's t-test for paired data) NOS activity was detected in fraction F_5, the parietal cell rich fraction, which supports observations from Percoll® density gradient separation that NOS is not present in parietal cells.

3.3.2 Induced nitric oxide synthase activity.

Nitric oxide synthase activity was measured in homogenates of gastric mucosal cells isolated from control (untreated) animals, and animals which had been administered LPS (3 mg/kg i.v.), 4 h prior to cell isolation (induced). In certain experiments animals were pre-dosed with dexamethasone (2 mg/kg i.p.) 1 h prior to the administration of LPS.

Plate 3.1 Magnified view (x125) of gastric mucosal cells in fraction 1, separated by counterflow centrifugation (section 2.2.3) and fixed and stained as described in section 3.2.3. Bar represents 10 μm.

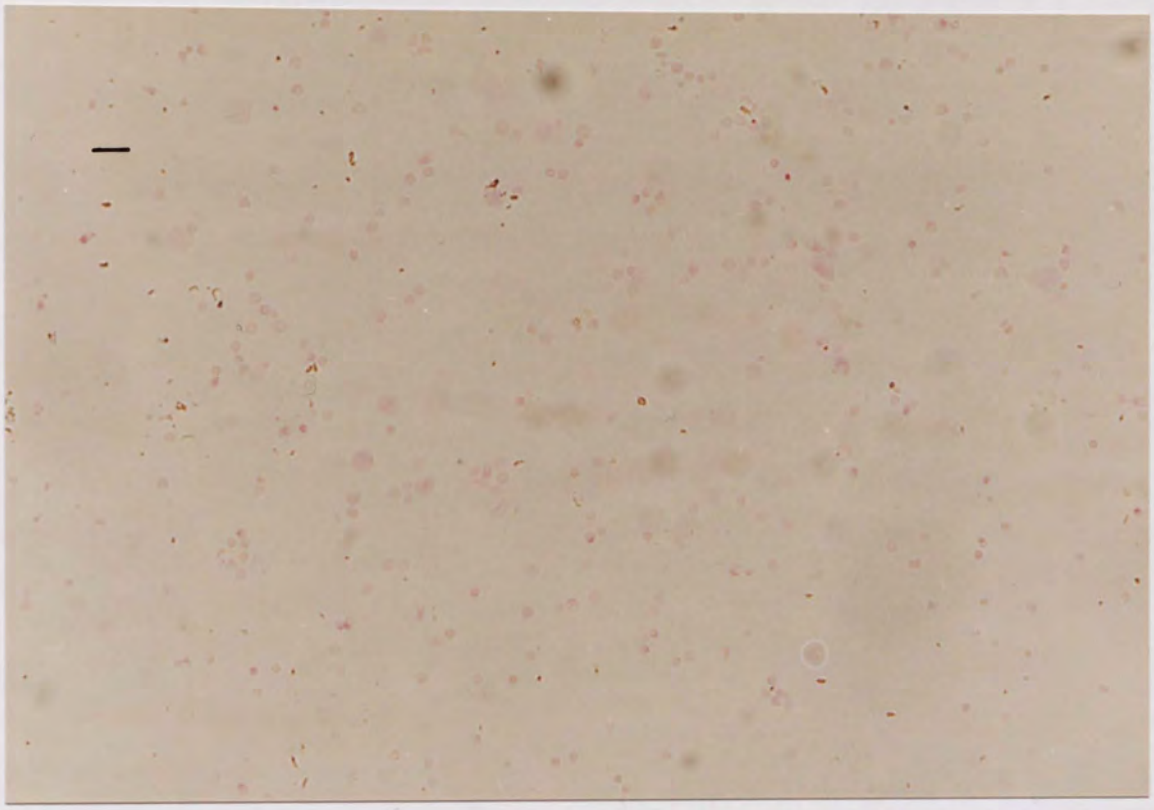


Plate 3.2 Magnified view (x125) of gastric mucosal cells in fraction 2, separated by counterflow centrifugation (section 2.2.3) and fixed and stained as described in section 3.2.3. Bar represents 10 μm.

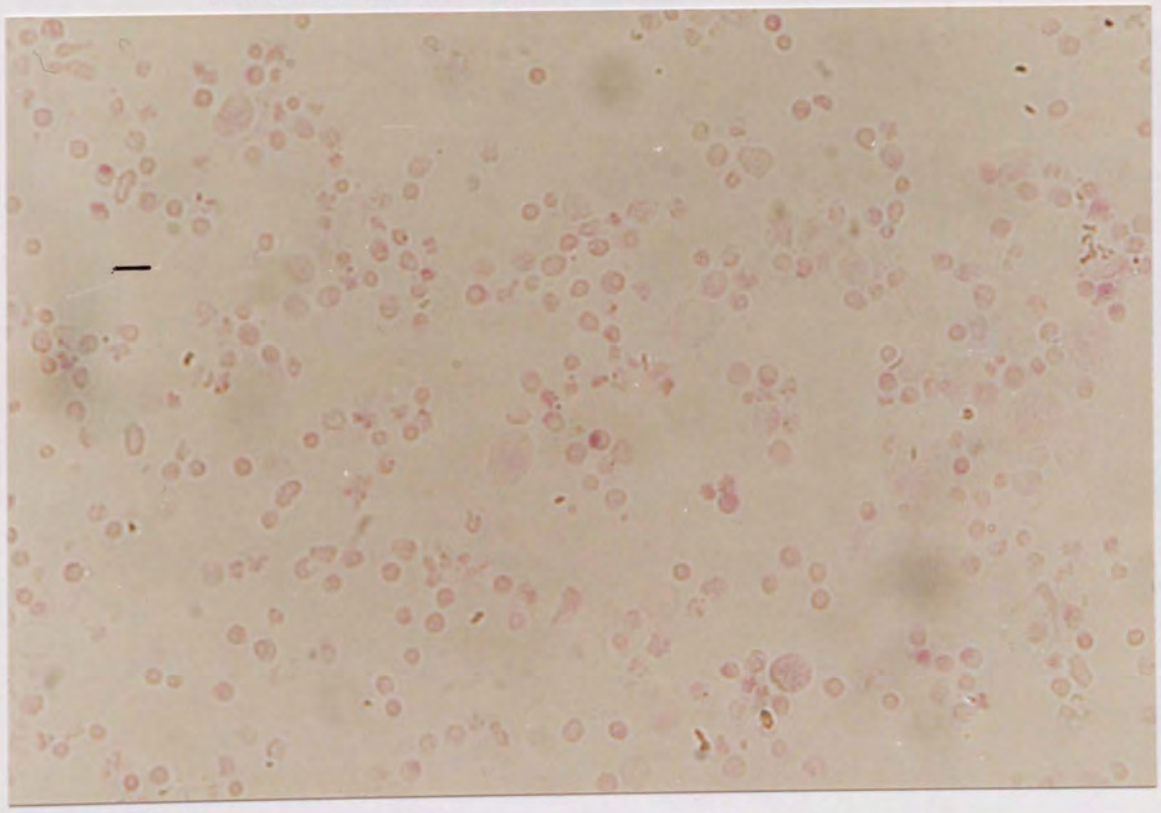


Plate 3.3 Magnified view (x125) of gastric mucosal cells in fraction 3, separated by counterflow centrifugation (section 2.2.3) and fixed and stained as described in section 3.2.3. Bar represents 10 μm, P=parietal cell and M=mucous cell.

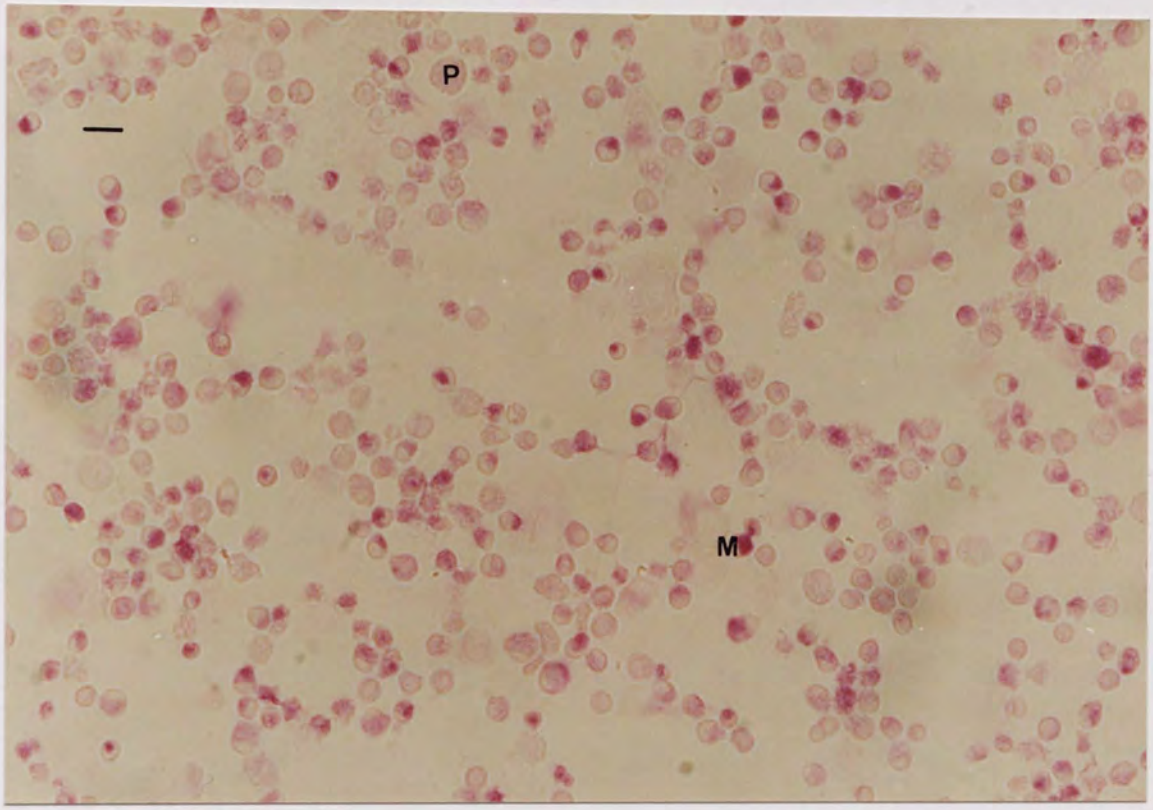


Plate 3.4 Magnified view (x125) of gastric mucosal cells in fraction 4, separated by counterflow centrifugation (section 2.2.3) and fixed and stained as described in section 3.2.3. Bar represents 10 μm, P=parietal cell and M=mucous cell.

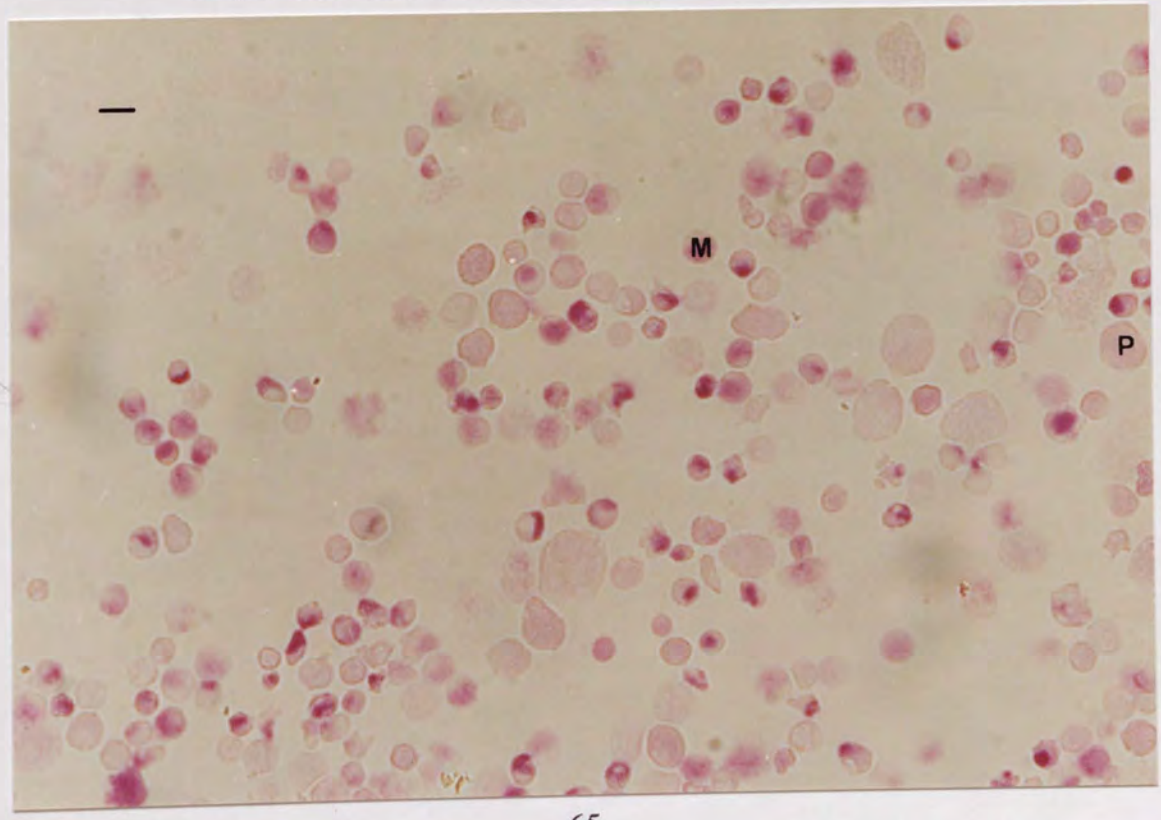


Plate 3.5 Magnified view (x125) of gastric mucosal cells in fraction 5, separated by counterflow centrifugation (section 2.2.3) and fixed and stained as described in section 3.2.3. Bar represents 10 μm, P=parietal cell and M=mucous cell.

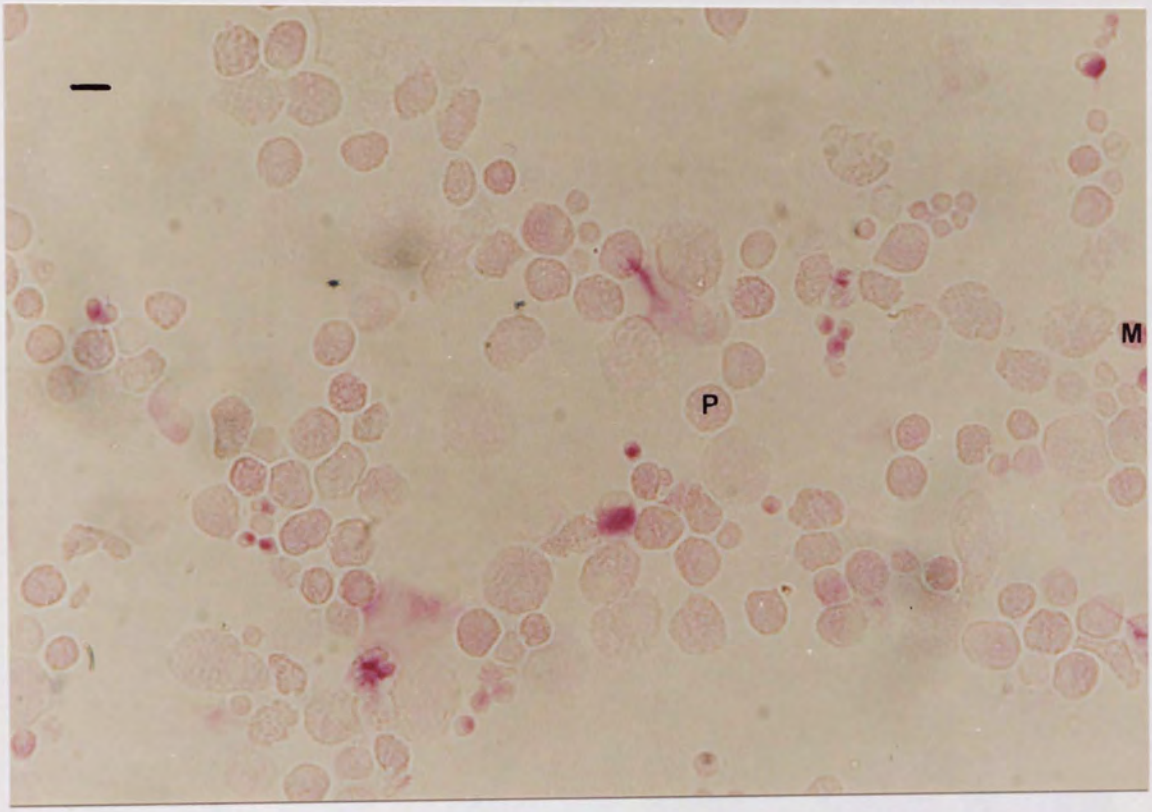
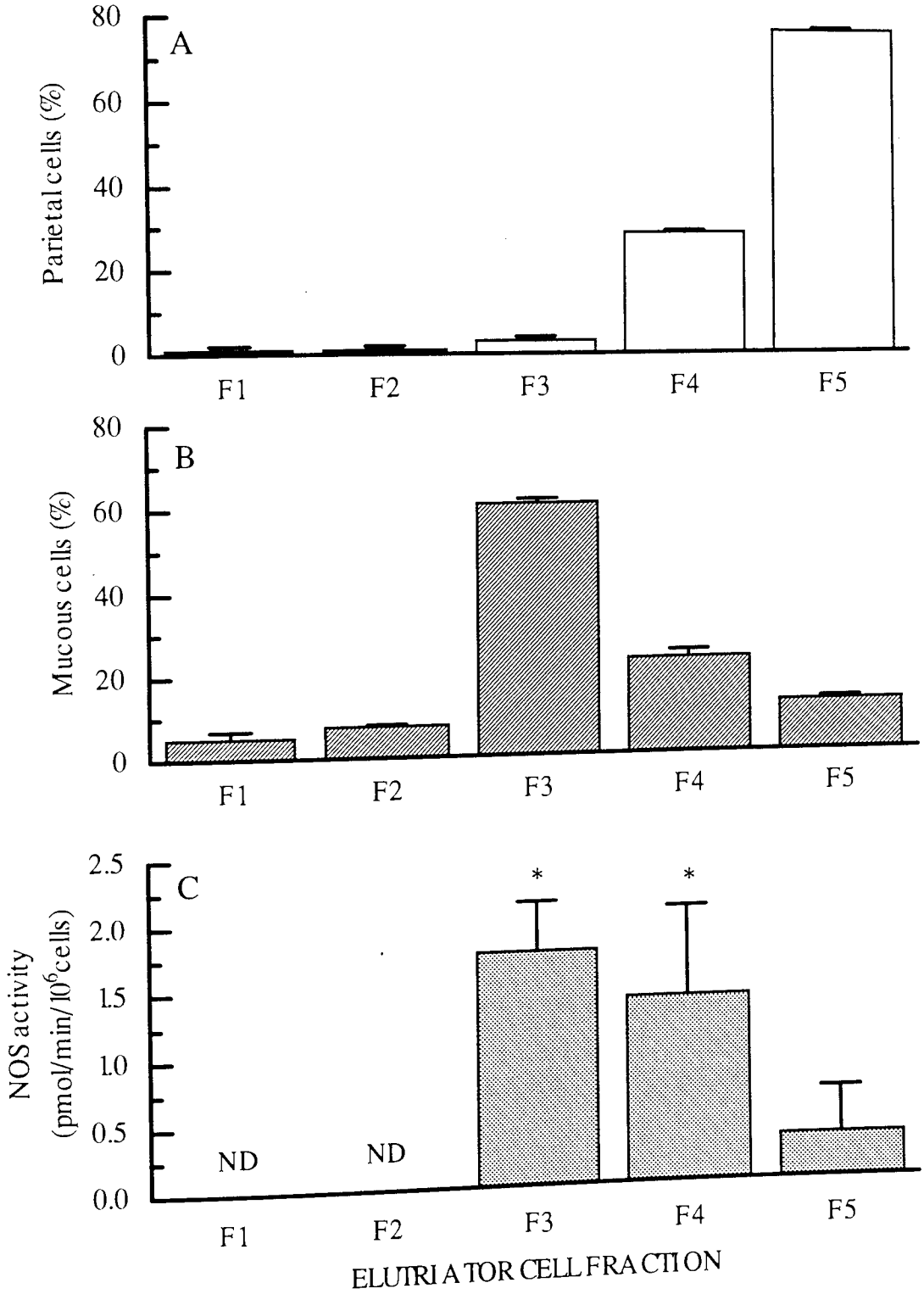


Figure 3.6 **Distribution of parietal cells (A), mucous cells (B) and NOS activity between gastric mucosal cell fractions separated by counterflow centrifugation.**



Data are presented as mean±SEM (number of cell batches n=10, 4 and 4-6 for panels A, B and C respectively), where *P<0.01 for difference from activity in the presence of 100 μM L-NMMA by Student's t-test for paired data. ND denotes no detectable NOS activity.

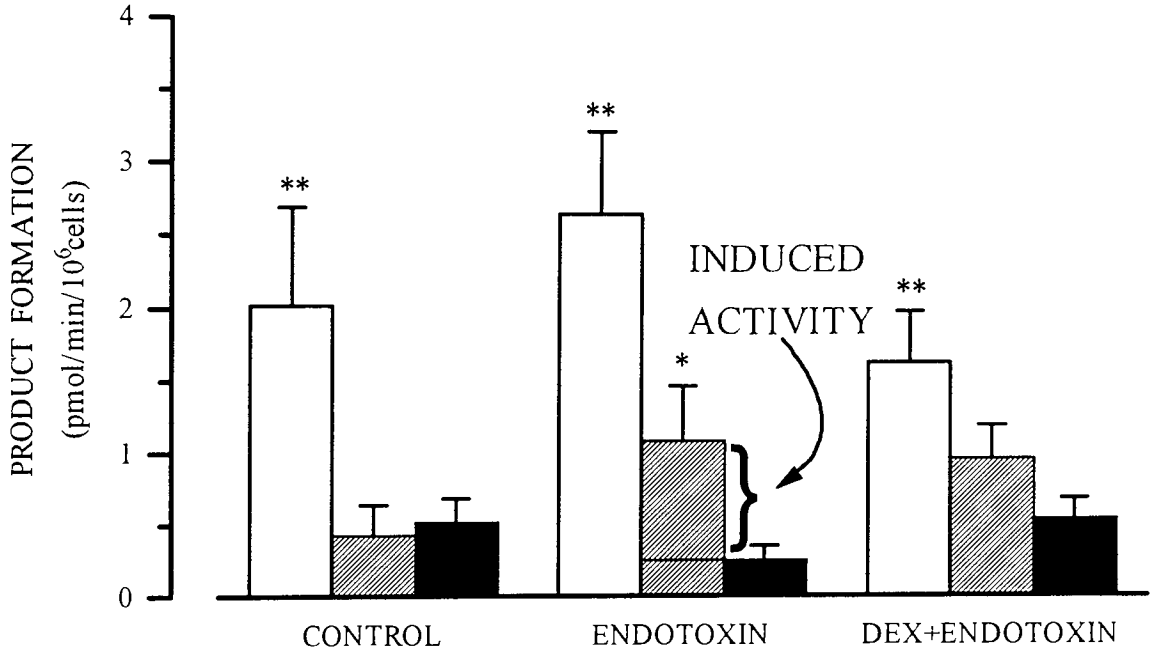
3.3.2.1 Characterisation of induced nitric oxide synthase activity.

Treatment with LPS resulted in the presence of significant ($P<0.05$ by ANOVAR and Dunnett's test) NOS activity in homogenates of crude gastric mucosal cells which was not affected by the addition of 1 mM EGTA (Fig. 3.7). This effect of LPS was abolished if the cells were isolated from animals which had been pretreated with dexamethasone prior to administration of LPS (Fig. 3.7). These results indicate that a classical induction of NOS-II activity has occurred in the crude gastric mucosal cell preparation since the total NOS activity is no longer totally Ca^{2+}-dependent and this effect could be abolished by pretreatment with glucocorticoids. Ca^{2+}-independent NOS was originally described in macrophages activated by exposure to cytokines or LPS (Haushildt *et al.*, 1990). Induction of macrophage NOS was inhibited by pretreatment with glucocorticoids such as dexamethasone (Di Rosa *et al.*, 1990). Glucocorticoids also inhibit induction of NOS-II activity in other tissues such as the vascular endothelium (Radomski *et al.*, 1990b) where a direct effect of glucocorticoids has been established as steroid receptor antagonism abolishes the inhibitory effects of dexamethasone and hydrocortisone. Thus, since dexamethasone inhibited the effect of LPS in gastric mucosal cells, the Ca^{2+}-independent NOS activity observed following LPS treatment is attributable to induction of NOS-II activity (Fig. 3.7).

3.3.2.2 Distribution of induced NOS activity.

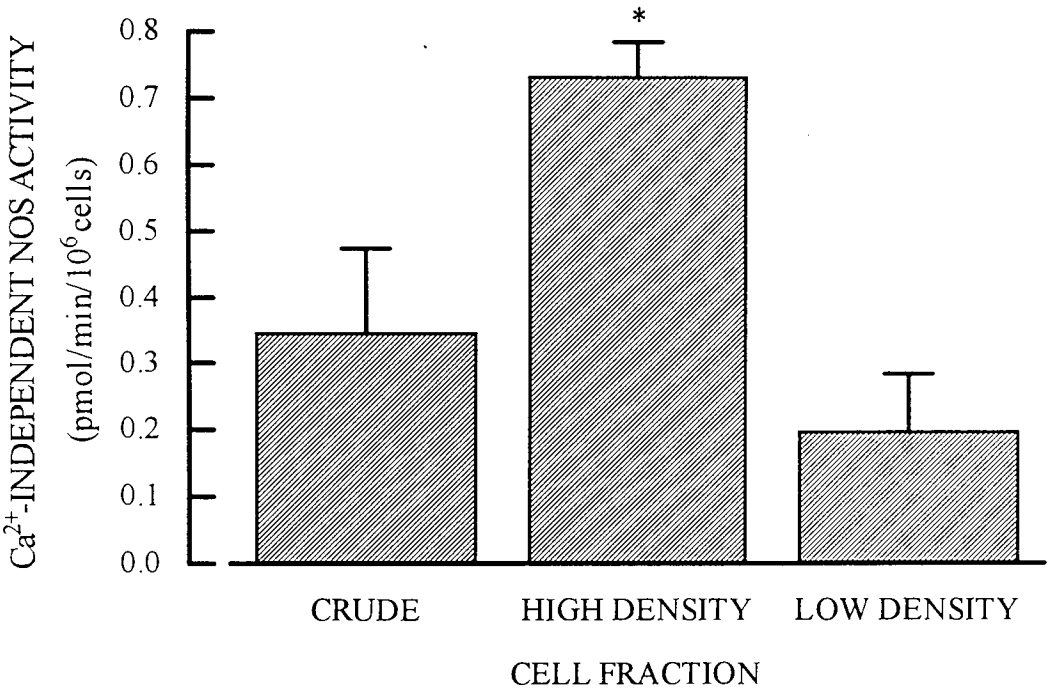
Crude cell suspensions isolated from animals injected intravenously with endotoxin (3 mg/kg) and separated by Percoll® density gradient centrifugation were assayed for NOS activity in the presence of 1 mM EGTA. Ca^{2+}-independent NOS in the high density fraction was significantly greater than activity in the crude and low density cell fractions ($P<0.01$ by ANOVAR and Dunnett's test; Fig. 3.8). These results suggest that induction of NOS activity does not occur in parietal cells which were enriched four-fold in the low density fraction (see Section 3.3.1.1).

Figure 3.7 **Effect of LPS alone and LPS following pretreatment with dexamethasone, on NOS activity in the supernatant of crude gastric mucosal cell homogenates.**



Product formation for uninhibited enzyme ( ), and product formed in the presence of 1 mM EGTA( ) and 100 μ M L-NMMA( ).
LPS (3 mg/kg i.v) was administered 4 h prior to cell isolation and dexamethasone (2 mg/kg iv.) was administered 1 h prior to administration of LPS.
Results are presented as mean±SEM (number of cell batches n=7, 8 and 4 for control, endotoxin and dexamethasone plus endotoxin groups respectively). Where ** $P<0.01$ and * $P<0.05$ for difference from values obtained in the presence of L-NMMA (100μ M) by analysis of variance and Dunnett's test.

Figure 3.8 **Effect of LPS on the distribution of Ca^{2+}-independent NOS activity of gastric mucosal cells separated by density gradient centrifugation.**

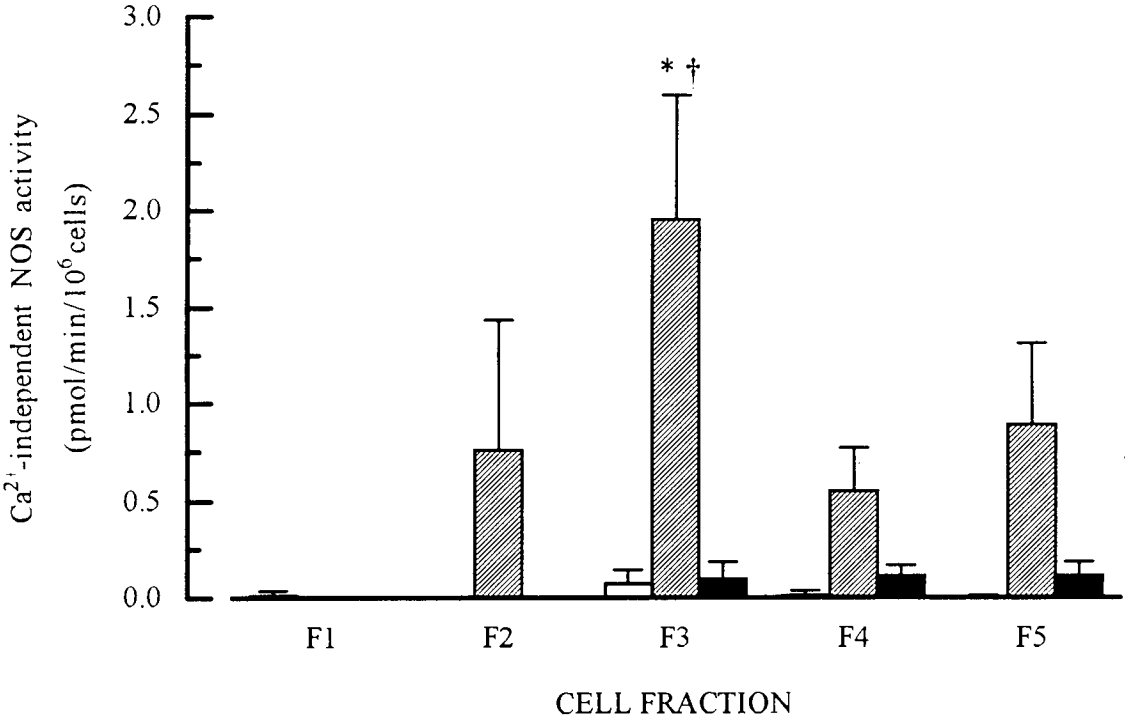


LPS (3 mg/kg i.v.) was administered 4 h prior to cell isolation.
Results are presented as mean±SEM (number of cell batches n=5), where *P<0.05 for difference from both crude and low density fractions by ANOVAR and a Newman-Keuls test (see Appendix A3).

The distribution of Ca^{2+}-independent NOS activity in cell fractions separated by counterflow centrifugation and isolated from animals injected with LPS (3 mg/kg i.v.) 4 h prior to cell isolation was compared with that of cells from control rats or rats injected with dexamethasone (2 mg/kg i.p.), 1 h prior to administration of LPS (Fig. 3.9). Enzyme activity determined in the presence of 1 mM EGTA was significantly (P<0.05 by ANOVAR and Newman-Keuls test) greater than control and LPS plus dexamethasone only in fraction 3. Some preparations appeared to show induced activity to be present in fractions 2, 4 and 5 form LPS treated rats, although the results were not consistent. The lack of consistent activity in the small cells of fractions 1 and 2 is somewhat surprising since fractions 1 and 2 of canine gastric mucosal cells separated by counterflow centrifugation were identified as being primarily macrophages (Chen *et al.*, 1990) which produce considerable amounts of NO following activation by endotoxin or cytokines

(Hibbs *et al.*, 1987). Macrophages and endothelial cells may therefore have been "lost" during the isolation and fractionation procedure. The significant induced activity demonstrated in fraction 3 suggests that induced, along with constitutive NOS activity may be predominantly localised to a mucous cell rich fraction.

Figure 3.9 **Effect of LPS and dexamethasone pretreatment on the distribution of Ca^{2+}-independent NOS activity.**



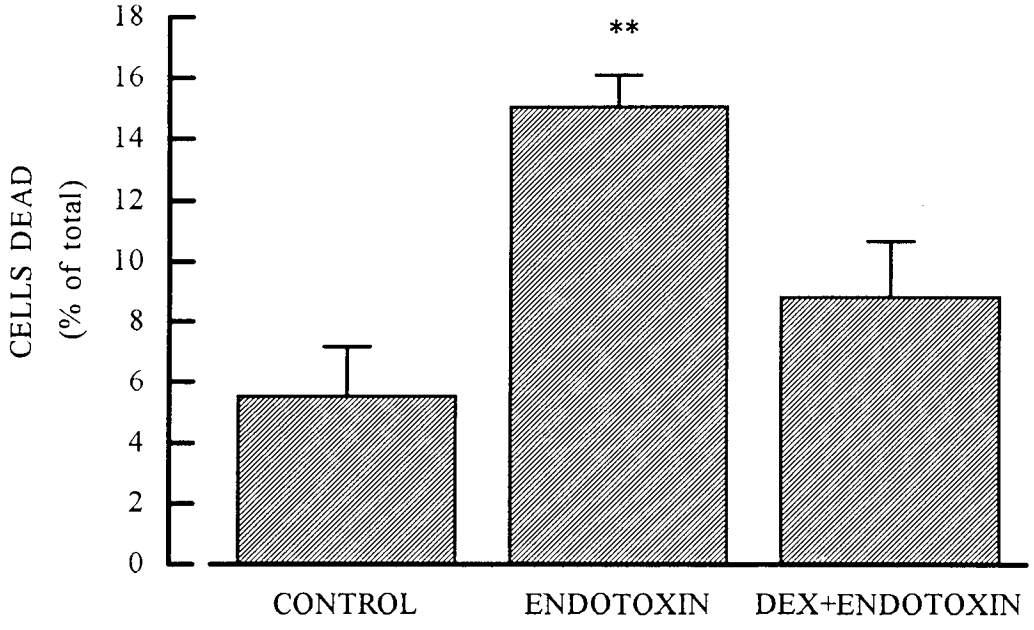
Endotoxin (3 mg/kg i.v.) was administered 4 h prior to cell isolation and dexamethasone (2 mg/kg i.p.) was administered 1 h prior to administration of endotoxin.
Results presented as mean±SEM (n=4-5) for control( ), endotoxin ( ) and dexamethasone plus endotoxin( ) treated rats, where *P<0.05 for difference from control, and †P<0.05 for difference from dexamethasone plus endotoxin treated rats by ANOVAR and a Newman-Keuls test.

3.3.2.3 Effect of induction on cellular viability.

Induction of NOS activity in a crude cell fraction (Fig. 3.9) was associated with an increase in the percentage of cells unable to exclude the dye trypan blue (Fig. 3.10). Furthermore, this increase was abolished in cells isolated from animals which had been pre-treated with dexamethasone prior to induction with endotoxin (Fig. 3.10).

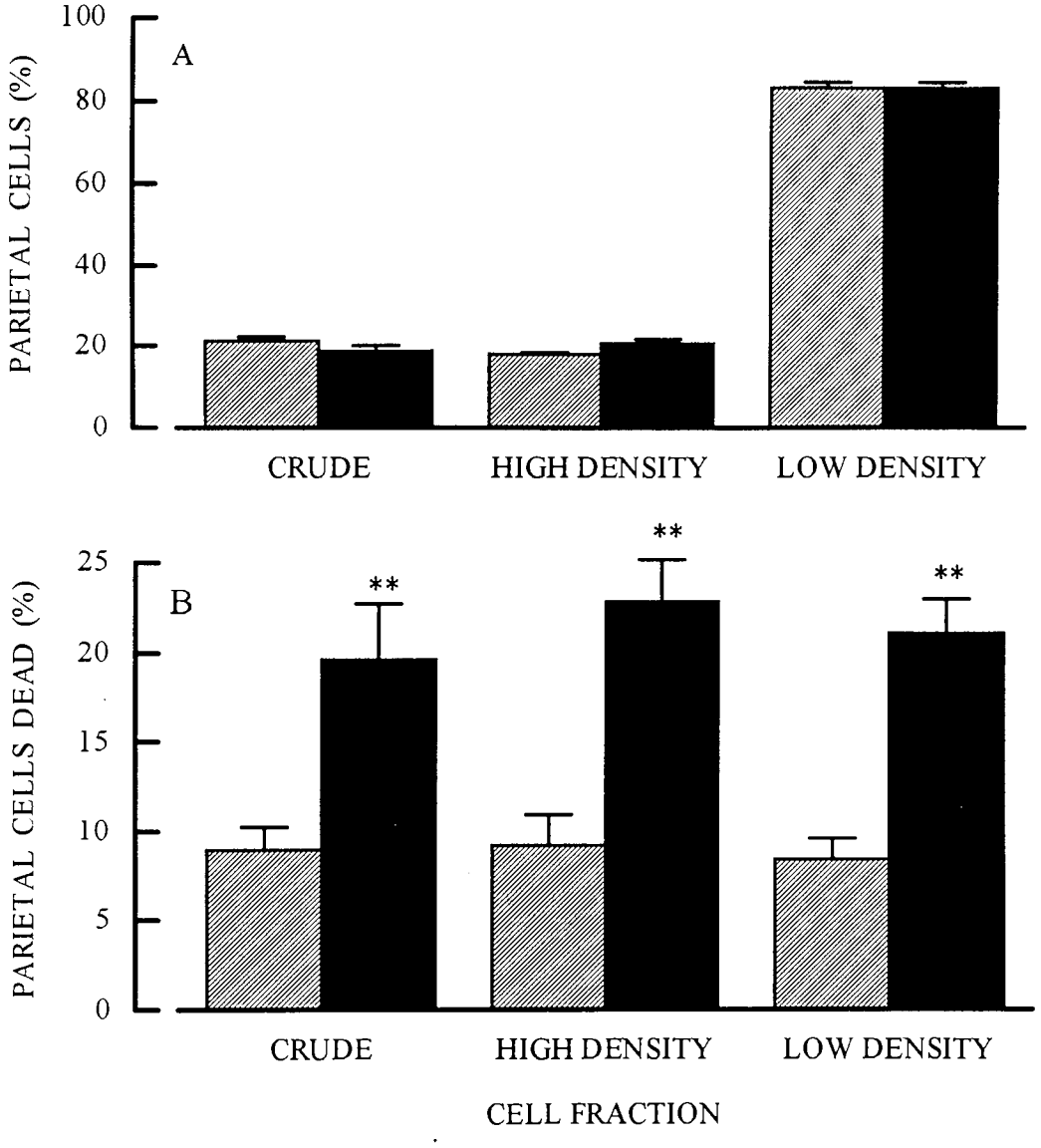
In cell fractions isolated from endotoxin treated animals and separated by Percoll® density gradient centrifugation, no affect upon the distribution of parietal cells was observed (Fig. 3.11 A). However, a significant increase in the proportion of dead parietal cells was observed in all fractions ($P<0.01$ by Student's t-test for paired data). These findings suggest that although parietal cells do not contain either form of NOS they are damaged by prolonged exposure to NO produced by other cells. The physiological or pathological significance of which is unknown.

Figure 3.10 **Effect of LPS and dexamethasone plus LPS on the percentage of total dead cells in crude gastric mucosal cell suspensions.**



LPS (3 mg/kg i.v.) was administered 4 h prior to cell isolation and dexamethasone was administered 1 h prior to LPS.
Results are presented as mean±SEM (number of cell batches n=4), where **P<0.01 for difference from control viability by ANOVAR and Dunnett's test.

Figure 3.11 **Effect of LPS treatment on the proportion of parietal cells (A) and parietal cell viability (B).**



LPS (3 mg/kg i.v.) was administered 4 h prior to cell isolation. Results are presented as mean±SEM (number of cell batches n=4 for control(▨) and endotoxin(■) pretreated animals). Where ** $P<0.01$ for difference from control by Student's t-test.

3.4 GENERAL DISCUSSION.

The significance of a soluble, Ca^{2+}-dependent form of NOS in the rat gastric mucosa is at present unclear, although *in vivo* biosynthesis of NO by the gastric mucosal circulation has been implicated in the regulation of gastric mucosal blood flow (Pique *et al.*, 1989). It is thus possible that NO produced by the gastric mucosa may be involved in the physiological regulation of gastric blood flow. Endogenous NO has also been demonstrated to interact with sensory neuropeptides and prostanoids in the gastric mucosa, as depletion of all mediators by administration of L-NMMA, capsaicin and indomethacin respectively, resulted in severe haemorrhagic necrosis following intra-gastric instillation of acid saline (Whittle *et al.*, 1990). These findings suggest a gastroprotective role for endogenous NO in modulating gastric mucosal integrity. Furthermore, since the intracellular effects of NO are predominantly mediated via activation of the soluble form of guanylate cyclase and subsequent elevation of intracellular cGMP concentrations (Moncada *et al.*, 1990), NO may be directly involved in the regulation of mucus or bicarbonate secretion, the latter having been proposed as a cGMP-dependent process (Flemstrom, 1987).

As with the constitutively expressed and Ca^{2+}-dependent isozyme of NOS, NOS-I, the significance of an inducible and Ca^{2+}-independent form of NOS, NOS-II, in a mucous cell rich fraction of gastric mucosal cells is also unclear. NOS-II activity is often associated with inflammation and immune cells (Moncada *et al.*, 1991b) but has also been observed in trinitrobenzenesulphonic acid-induced colitis in rats (Boughton-Smith *et al.*, 1992). In the gastric mucosa, induction of NOS-II and subsequent sustained production of NO may occur during inflammatory episodes in diseases such as gastritis, which is thought to be caused by bacterial colonisation of the stomach by campylobacter pylori (Rathbone *et al.*, 1986). Since NOS-II is expressed following exposure to LPS, bacterial colonisation of the stomach may result in enzyme induction. Under these circumstances a prolonged release of NO may be cytotoxic, since prolonged exposure to NO can result in DNA damage (Nguyen *et al.*, 1992) and inhibition of metabolic enzymes such as aconitase (Hibbs *et al.*, 1988). NO could therefore influence

either bacterial growth, or inhibit parietal cell function, since parietal cells have a high aconitase activity due to the enormous energy requirements of acid secretion (Sachs, 1987). However, further work is required to elucidate the overall significance of the findings presented in this chapter.

3.5 SUMMARY

1. Product formation was linearly related to time and the concentration of cells used to prepare cell homogenates.
2. Inhibition of product formation by the arginine analogue N^G-monomethyl arginine was both concentration- dependent and stereospecific with an IC_{50} of 4.2μM for the L-enantiomer.
3. Nitric oxide synthase was localised to a mucous-cell rich fraction of gastric mucosal cells separated by counterflow elutriation.
4. An inducible form of nitric oxide synthase was present in cell fractions isolated from animals injected intravenously with endotoxin (3 mg/kg) 4 h prior to cell isolation.
5. Induction of nitric oxide synthase activity could be abolished by intraperitoneal injection of dexamethasone (2 mg/kg) 1 h prior to injection of endotoxin.
6. Induction was associated with a decrease in cell viability, with a profound decrease in parietal cell viability.

Chapter Four

EFFECTS OF NITRIC OXIDE DONORS AND CYCLIC NUCLEOTIDE ANALOGUES ON MUCIN RELEASE FROM PREPARATIONS *IN-VITRO* AND *IN-VIVO*.

4.1 INTRODUCTION.

Chapter 3 described the distribution of NOS activity between cells isolated from the rat gastric mucosa with enrichment of NOS in a cell fraction containing a high proportion of mucous cells. The aim of this section was to determine whether NO might be involved in the regulation of mucus release from gastric mucosal cells *in vitro* or in controlling the mucus gel thickness *in vivo*.

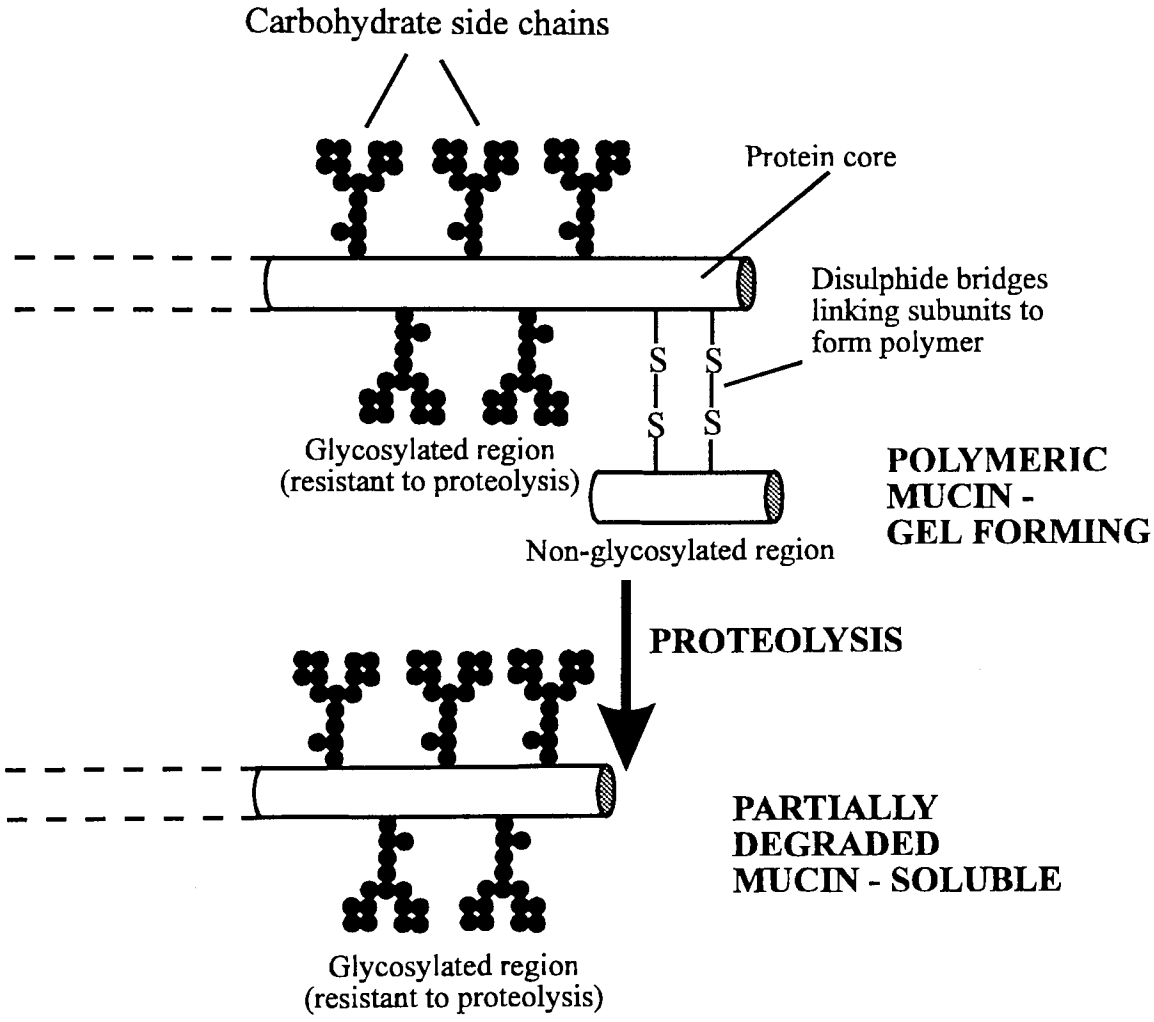
The morphology of the gastric mucous cell has been outlined in Chapter 1 and the introduction to this chapter deals only with the structure, physiological roles and secretion of gastric mucus.

4.1.1 Structure of mucus.

Three phases of mucus have been identified: firstly there are presecreted intracellular stores of mucus, secondly mucus exists as a water-insoluble gel which adheres to the epithelial cell surfaces forming a protective barrier against attack by pepsin and acid, and finally there is a soluble phase that can be removed with the luminal contents by washing the mucosal surfaces (Allen *et al.*, 1989).

The principal gel-forming components of mucus secretions are large molecular weight glycoproteins or mucins. Despite large variations between tissues in the structure of mucins, a number of compositional features are shared and serve to characterise mucous glycoproteins (Neutra and Forstner, 1987). Several glycoprotein subunits are joined together by disulphide bridges to form a polymer (Snary *et al.*, 1970). Each glycoprotein subunit is composed of a central protein core with many carbohydrate side-chains attached which consist of several sugar residues, many of which are negatively charged due to the presence of sulphate ester and sialic acid residues. Glycosylation of the protein core protects the protein backbone from enzymatic cleavage. However, the regions where the glycoprotein subunits are joined by disulphide bridges are not heavily glycosylated, and hence these regions are prone to proteolytic cleavage which results in the formation of proteolytically degraded mucin which is soluble (Allen *et al.*, 1989; Fig 4.1).

Figure 4.1 **Diagrammatic representation of the structure of gastric mucin indicating the site of proteolysis and subsequent breakdown of the mucin polymer (adapted from Allen *et al.*, 1989).**



Intracellular production of mucin is achieved through the ribosomal synthesis of core peptides and glycosyl transferase enzymes to form sugar-nucleotides. The endoplasmic reticulum and Golgi complex add N-acetylgalactosamine to serine or threonine residues in the sugar-nucleotide chain with final processing in the Golgi to produce the oligosaccharide cores. The cores are then elongated one sugar residue at a time to form a backbone before the terminal sugars are added. Finally, other post-translational modifications are made such as sulphation and polymerisation to complete the macromolecule. Presecreted mucins are packaged in supranuclear secretory vesicles and are ready for migration to the cell apex for subsequent secretion (Neutra and Forstner, 1987).

To date six genes coding for mucins have been described termed MUC1-MUC6 (Toribara *et al.*, 1993) Not all MUC genes may actively be responsible for mucin production under normal physiological conditions. Thus MUC1 and MUC3 do not localise to cells which histochemically contain mucin (Ho *et al.*, 1993) and little has been published about MUC4. However, of the three mucins localised to chromosome 11p15, MUC2 is almost certainly a secreted mucin and probes to MUC6 mRNA react only with gastric epithelial and mucous neck cells in the stomach and occasional goblet cells in the remainder of the gastrointestinal tract and trachea, all of which are mucin secreting cells (Toribara *et al.*, 1993). Secreted polymeric mucins are all of very high molecular mass (substantially>1 million Daltons; Forstner & Forstner, 1985). Thus, complete sequencing of the cDNA for these mucins will enable more precise estimates of subunit size and the manner in which they are assembled into polymers.

4.1.2 Physiological role of gastric mucus.

There are four proposed functions of gastric mucus. Firstly, soluble mucin helps lubricate ingested food during the digestive process. Secondly, the adherent insoluble mucus gel layer acts as a physical barrier to attack by pepsin, impeding its access to the epithelium(Allen *et al.*, 1984b). Thirdly, the adherent gel layer acts as a mixing barrier where acid diffusing from the lumen is neutralised by bicarbonate secreted by the epithelium (Flemstrom & Garner, 1982) which results in a pH at the epithelial cell surface approaching neutrality, except when the luminal pH falls below 1.4 (Ross *et al.*, 1981; Wallace, 1989). The second and third functions are often referred to jointly as the "mucus-bicarbonate barrier". Finally, mucus in conjunction with exfoliated cells and fibrin forms a protective cap over areas of acute cellular damage, which allows cells to migrate from the gastric glands to restore epithelial continuity in a protected environment (Ito & Lacy, 1985)

4.1.2 Secretion of gastric mucin.

The mucin macromolecules generally spend several hours packed tightly together in intracellular granules prior to their release into the gastric lumen. Secretion of mucin can be achieved via three distinct mechanisms: (i) slow release by exocytosis, (ii) rapid apical expulsion or (iii) cell exfoliation (Zalewsky & Moody, 1979).

4.1.2.1 Exocytosis.

The process of exocytosis is a poorly understood sequence of events involving the migration of secretory granules towards the apical plasma membrane and possible interaction of membrane protein components prior to interaction of the two phospholipid bilayers. Fusion of the two membranes may result in a permeability change of the plasma membrane at this region allowing rapid hydration of the mucin granules causing them to swell, thus rupturing the plasma membrane causing expulsion of the now hydrated mucin (Lubbock *et al* 1981). Once the stored concentrated mucin is released into the gastric lumen it rapidly expands to form a protective gel, which is probably initiated by the loss of calcium ions present in the vesicle (Verdugo, 1984). This mechanism of secretion was demonstrated in 50-75% of resting canine foveolar and interfoveolar mucous cells examined by transmission micrographs (Zalewsky & Moody, 1979), and is probably responsible for providing a slow but sustained rate of basal secretion.

4.1.2.2 Apical expulsion.

This mechanism of secretion provides a rapid release of stored mucin into the gastric lumen and involves the fusion of a number of secretory granules to form an apical pool of mucin which then fuses *en bloc* with the apical membrane. This mechanism of mucin secretion was observed in only 10-20% of unstimulated canine interfoveolar mucous cells identified by scanning electron micrographs, suggesting that it may only be important during stimulated secretion (Zalewsky & Moody, 1979).

4.1.2.3 Cell exfoliation.

This mechanism also provides a rapid release of mucin into the gastric lumen and occurs indirectly through cell exfoliation. In unstimulated canine mucosa this type of release was observed in only 1-2% of all mucous cells (Zalewsky & Moody, 1979). However, this type of release may be an important protective measure as it is often seen following exposure of the mucosa to damaging agents such as non-steroidal anti-inflammatory drugs (Morris *et al,* 1984).

4.1.3 Methods of measuring mucus secretion.

The tendency of secreted mucins to form gels has greatly impeded studies of the regulation of mucus secretion from epithelia both *in vivo* and *in vitro.* However, techniques have been developed which provide satisfactory indices of secretion. Such procedures include direct measurement of the thickness of the mucus gel (Kerss *et al.*, 1982), assay of radiolabelled glycoprotein secretion (Heim *et al.*, 1989) and immunochemical assays using both monoclonal and polyclonal antibodies raised against mucin glycoprotein (Forstner *et al.*, 1977; Keates, 1991).

Estimates of secretory activity *in vivo* have included the very indirect quantitation of luminal sugars (Bersimbae *et al.*, 1985) and the measurement of mucus gel thickness (Bickel & Kauffman, 1981; Kerss *et al.*, 1982). *In vitro* methods involve the use of tissue explants (Seidler *et al.*, 1989b) and isolated cell preparations (Keates & Hanson, 1990b). The use of *in vitro* systems involving suspensions of cells removes systemic factors such as hormones and nervous input and allows the study of single agents in isolation. Furthermore, nutrients and oxygen are freely available if isolated cells are used. However, suspensions of cells *in vitro* do have certain disadvantages. There is a loss of cell polarity and the need to use protease enzymes and calcium chelators in the preparation of isolated cell preparations may modify cell behaviour.

4.1.4 Regulation of mucus secretion.

Despite the physiological importance of gastric mucin in protection of the underlying epithelial surface from damage by pepsin and acid, relatively little is known about the physiological regulation or intracellular control of mucus secretion in the gastric mucosa.

4.1.4.1 Acetylcholine.

The involvement of cholinergic innervation in the regulation of gastric mucus secretion was first suggested by Florey (1962), who observed a copious release of mucus into the gastric and intestinal lumina of cats and dogs following stimulation of either vagal or splanchnic nerves. More recent data has shown that topical administration of acetylcholine to the gastric mucosa of dogs produced a visible increase in the mucus gel thickness (Allen & Garner, 1980). Furthermore, the non-hydrolysable muscarinic cholinergic agonist, carbachol, increased the mucus gel thickness of rat stomach *in vivo* (McQueen, *et al*, 1984) and increased secretion of glycoprotein labelled with [^{14}C]-glucosamine by the frog gastric mucosa *in vitro* (Allen *et al.*, 1984a). Carbachol produced a small, but nevertheless significant, increase in mucus release by isolated gastric epithelial cells (Keates, 1991).

4.1.4.2 Adrenergic transmitters.

In rat intestinal slices, β-adrenergic agonists enhanced the synthetic rates of high and low molecular weight glycoproteins *in vitro* (Neutra & Forstner, 1987). Furthermore, isoprenaline has been reported to stimulate submandibular mucin secretion in the rat (McPherson & Dormer, 1984), and also increases mucin release from isolated gastric mucosal cells (Keates, 1991).

4.1.4.3 Prostaglandins.

A vast body of evidence exists for the involvement of prostaglandins in the regulation of mucus secretion. Thus, local application of the metabolically stable analogue, 16,16-dimethyl PGE_2, increased the thickness of the adherent mucus gel determined in the rat intact stomach using a pachymeter and also from direct histological measurement (Bickell & Kauffman, 1981; McQueen *et al.*, 1983). An increase in the mucus gel thickness was also observed following a 1 h topical treatment with misoprostol, a prostaglandin E series analogue, (Sellers *et al.*, 1986) Furthermore, 16,16-dimethyl PGE_2 but not $PGF_{2\alpha}$ stimulated secretion of labelled glycoprotein by cultured intact rabbit gastric mucosal preparations *in vitro* (Seidler *et al.*, 1989a).

4.1.4.4 Second messengers and gastric mucus secretion.

The intracellular signalling mechanism for cholinergic receptors in rabbit mucosal explants has been reported to be Ca^{2+} dependent and antagonised by the calmodulin antagonist W7 (Seidler & Sewing, 1989). Furthermore, glycoprotein secretion from rabbit gastric mucous cells stimulated with acetylcholine was associated with an increase in inositol phosphate production and a rise in intracellular free Ca^{2+} concentrations (Seidler & Pfeiffer, 1991) suggesting an involvement of the inositol phosphate pathway in the regulation of cholinergically stimulated mucus secretion.

A further second messenger which may be involved in the stimulation of mucus secretion is cyclic adenosine 3',5'-monophosphate (cAMP), since the cell permeable analogue of cAMP, dibutyryl-cAMP (Db-cAMP), caused a slow sustained release of radiolabelled mucin from suspensions of rat gastric mucosal cells (Keates & Hanson, 1990a). This signal transduction mechanism may be involved in mediating the secretory response to PGE_2 in rabbit gastric mucosal explants (Seidler & Sewing, 1989). The messenger system involved in mediating the response to isoprenaline is less clear, but probably involves cAMP since all known β-adrenergic receptors elevate intracellular cAMP concentrations (Bulbring & Toonita, 1987).

4.1.4.5 Other agents.

No evidence was found to support an involvement of histamine, gastrin or EGF in rat gastric mucus secretion (Keates & Hanson, 1990a) although histamine may stimulate mucin release by pig gastric cells (Heim *et al.*, 1989). Furthermore, oxygen radicals have been shown to increase mucous glycoprotein secretion from rabbit gastric mucous cells via a mechanism which does not involve either PGE_2 or cell damage (Hiraishi *et al.*, 1991).

4.1.5 Pharmacological agents used to study the involvement of nitric oxide in signal transduction processes.

One of the major aims of this section of work was to establish whether NO and its associated signal transduction pathway (see Chapter 1) was involved in the regulation of mucus secretion by rat gastric mucous epithelial cells. These intracellular pathways can be activated at various points by the addition of different pharmacological agents.

4.1.5.1 Nitric oxide donors.

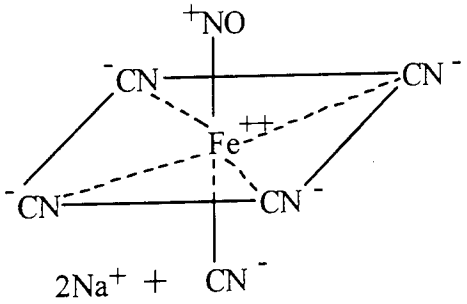
Agents that can liberate NO either spontaneously or following metabolic conversion are regarded as nitric oxide donors. Examples are isosorbide dinitrate (ISDN), sodium nitroprusside (NP) and the nitrosothiol S-nitroso-N-acetylpenicillamine (SNAP) (Fig. 4.2). Both NP and SNAP decompose spontaneously to release NO, whereas ISDN requires metabolism (Feelisch, 1991; Ignarro *et al.*, 1981). By exposing tissue or cell suspensions to aqueous solutions of the NO donors, possible NO-mediated events can be studied in the absence of a cellular source of NO.

4.1.5.2 Nitric oxide "antagonists".

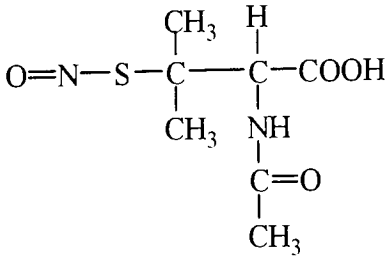
The term antagonist is usually employed to describe an agent which inhibits the action of an agonist either by inhibiting binding of the agonist to its receptor molecule or by causing a conformational change in the receptor molecule so that the agonist is no longer able to occupy the binding site. Oxyhaemoglobin and methylene blue (Fig. 4.3),

block the action of NO but it is probably incorrect to describe these agents as classical antagonists. Methylene blue inhibits the activation of the soluble form of guanylate cyclase by NO, possibly by interfering with substrate binding (Martin *et al.*, 1985). Oxyhaemoglobin acts as a scavenger of NO by chemically reacting with NO and thereby reducing its bio-availability (Martin *et al.*, 1985). Inhibition of an effect by oxyhaemoglobin therefore provides evidence for a specific effector role of NO in cellular processes.

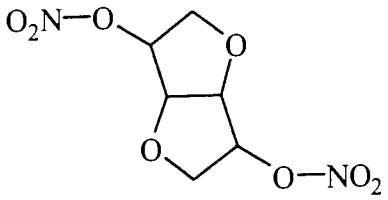
Figure 4.2 **Chemical structures of nitric oxide donors.**



Sodium nitroprusside

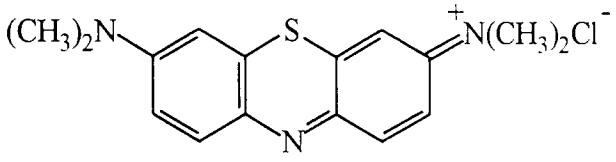


S-nitroso-N-acetylpenicillamine

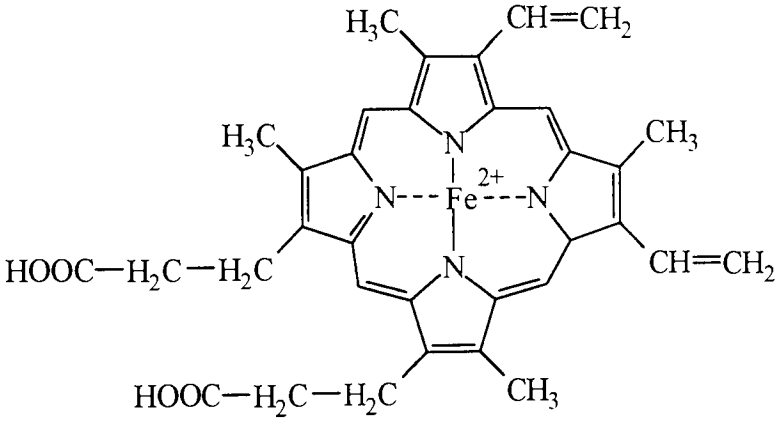


Isosorbide dinitrate

Figure 4.3 **Structures of the NO oxide "antagonists" methylene blue and the haem centre of haemoglobin.**



Methylene blue

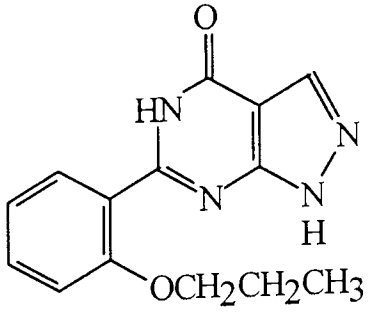


Protoporphyrin IX-ferrous iron complex of haemoglobin

4.1.5.3 cGMP phosphodiesterase inhibitors.

M&B 22948 (Fig 4.4) is a reasonably selective inhibitor of the cGMP-specific phosphodiesterase (PDE V), and will increase intracellular cGMP concentrations in tissues (Francis *et al.*, 1991). It can thus be used to either mimic or potentiate effects of NO which are mediated through increases in cGMP.

Figure 4.4 **Structure of M&B 22948.**

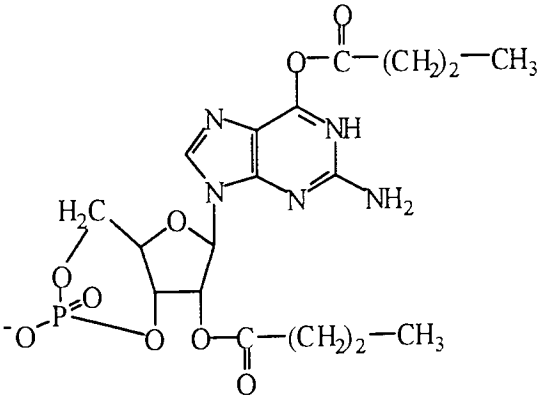


M&B 22948

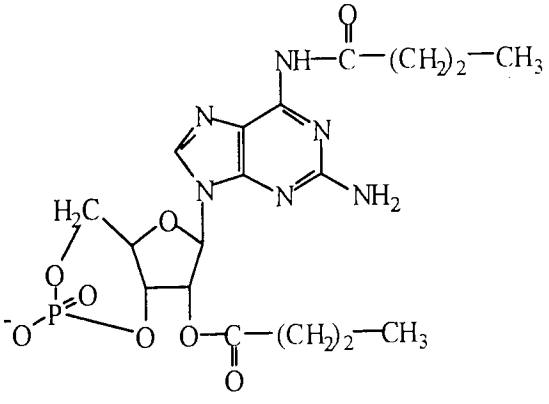
4.1.6.5 Dibutyryl cyclic nucleotide analogues.

The plasma membrane is poorly permeable to both cAMP and cGMP due to the polarity of these molecules. However, less polar, plasma membrane permeable analogues of these cyclic nucleotides (Fig 4.5) have been developed which mimic the effect of the physiological second messengers. A dibutyryl analogue of a pyrimidine base cyclic nucleotide such as dibutyryl cyclic cytidine monophosphate (Db-cCMP) can be used as a negative control to rule out non-specific effects derived from the dibutyryl moiety of these molecules.

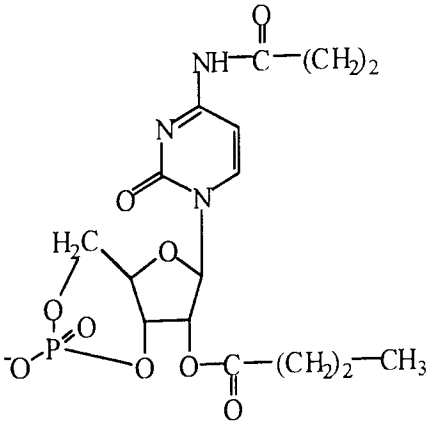
Figure 4.5 **Chemical structures of dibutyryl cyclic nucleotide analogues.**



Dibutyryl cyclic GMP



Dibutyryl cyclic AMP



Dibutyryl cyclic CMP

4.1.6 Aims of this section.

As considerable nitric oxide synthase activity had been localised to a mucous cell-rich fraction of rat gastric mucosal cell suspensions, this section aimed to investigate whether NO was involved with the regulation of mucus secretion both *in vitro* and *in vivo*. Specific aims were:

1. To establish the effect of nitric oxide donors on release of mucin from suspensions of isolated gastric mucosal cells.

2. To use oxyhaemoglobin, M&B 22948 and lipid soluble analogues of cGMP, to provide evidence for an intracellular signal transduction pathway involved with mucin release, and triggered by NO.

3. To determine whether the effects of NO donors obtained *in vitro* could be achieved by topical application of agents *in vivo* by subsequent measurement of changes in the thickness of the mucus gel.

4.2 METHODOLOGY.

4.2.1 Preparation of stock solutions.

4.2.1.1 Oxyhaemoglobin.

Oxyhaemoglobin was prepared by the oxidation of haemoglobin with sodium dithionite. Haemoglobin (154.8 mg) was gently dissolved in 0.5 ml distilled water to produce a 4.8 mM solution containing 2.4 mmoles haemoglobin. A 0.1 ml aliquot of 48 mM sodium dithionite in distilled water was added to the haemoglobin solution and the two solutions were thoroughly but gently mixed before being transferred to dialysis tubing (Sigma, Poole, U.K., cut off ≥12 kDa.) and dialysed at 4°C for 24 h against 2 x 1 litre of saline which was continually stirred with a magnetic stirrer at 60 r.p.m. The contents of the dialysis tubing were made up to 1 ml with 0.9% (w/v) sodium chloride and the oxyhaemoglobin solution was stored in 0.1 ml aliquots at -20°C until required.

4.2.1.2 Db-cGMP, Db-cAMP and Db-cCMP.

Dibutyryl cyclic nucleotide analogues were dissolved the appropriate volume of saline to produce a 0.1 M solution. Aliquots were stored frozen at -20°C and allowed to defrost for 30 min before addition to cell suspensions.

4.2.1.3 SNAP, ISDN and NP.

All the nitric oxide donors were dissolved in saline (0.9% w/v) at a concentration of 0.05 M no longer than 5 min before addition to cell suspensions.

4.2.1.4 Preparation of "exhausted" SNAP.

Stock solutions of SNAP, which had been allowed to degrade to acetyl penicillamine and oxides of nitrogen, and were therefore no longer able to generate NO, were prepared by incubating 2 ml of a 0.1 M solution in a 20 ml polypropylene scintillation vial for 48 h at 4°C with the air space above the liquid having been gassed with 95%O_2/5%CO_2 for 10 s before the vial was capped. Aliquots of 0.1 ml were then stored at -20°C until required.

4.2.1.5 M&B 22948 (Zaprinast).

Due to the poor solubility of M&B 22948 in water (<0.1%), stock solutions of 0.01 M concentration were prepared by dissolving 2.713 mg in 20 µl of 20% (v/v) triethanolamine which was made up to 1 ml volume with saline (0.9% v/v). Stock solutions were prepared no longer than 15 min before addition to cell suspensions.

4.2.2 Measurement of mucin release by crude cell suspensions.

Isolated rat fundic mucosal cells were prepared as described in section 2.1. The crude cell preparation was washed once in 20 ml of medium B' (see Table 2.1) before being resuspended in medium B' containing 8 µg/ml insulin, 10 nM hydrocortisone, 5% ($^{v}/_{v}$) foetal calf serum, 1 mM glutamine and 50 µg/ml gentamicin at a cell concentration of $1x10^{7}$cells/ml. The cell suspension was then incubated for 2 h at 37°C with continuous gassing with $95\%O_2/5\%CO_2$ and shaking at 120 cycles/min. The cells were resuspended every 30 min to reduce cell clumping and ensure adequate oxygenation. Following the 2 h incubation 0.5 mM dithiothreitol was added to the incubation medium to detach adherent mucin from the cells, and cells were incubated for a further 1 h. The cell suspension was then transferred to 10 ml plastic centrifuge tubes and centrifuged at 125 g for 7 min at 15°C. The resulting cell pellet was resuspended at a cell concentration of $1x10^{7}$cells/ml in medium B' before being transferred to 20 ml polypropylene scintillation vials containing the appropriate agent(s), to give a final volume of 1.2 ml. The air space above liquid in the vials was gassed with $95\%O_2/5\%CO_2$ before capping and incubation for 1 h at 37°C with shaking at 150 cycles/min. Two 1.0 ml samples of suspension were not added to vials but were transferred to 1.5 ml micro-centrifuge tubes and centrifuged at 10,000 g (Eppendorf Micro-centrifuge (5414), for 30 s at room temperature. A 0.9 ml aliquot of supernatant was removed from each tube and transferred to a 1.5 ml micro-centrifuge tube containing 0.1 ml of a solution consisting of 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 50 mM EDTA, 1 mM phenylmethylsulphonyl fluoride and 0.2% (w/v) sodium azide. The combined solutions were vortexed for 10 s to ensure thorough mixing and stored on

ice until the end of the experiment. These samples were used to determine the immunologically mucin present in the medium immediately after resuspension of the cells (time t=0). Following the 1 h incubation, a 1.0 ml sample was removed from each incubation vial and treated as described above. Assay of samples for mucin (4.2.3) was initiated as soon as possible after incubation and sampling had been completed.

4.2.3 Measurement of immunologically detectable mucin.

An indirect competitive solid phase enzyme-linked immunosorbent assay (ELISA) was used to detect immunoreactive rat gastric mucin. In this assay there is a preincubation step in which mucin in the sample is bound to antibody. Unbound antibody is then trapped on an ELISA plate coated with mucin and its presence detected by protein-A conjugated to alkaline phosphatase. An increase in mucin in the sample thus decreases the absorbance in the assay. The technique was developed and antiserum raised by Keates, (1991). The assay was based on a solid-phase radio-immunoassay method according to Roomi *et al.*, (1984), and was similar to that devised by Mantle & Thakore (1988) for small intestinal mucin. The procedure used is outlined in a flow diagram (Fig. 4.6). A solution containing 2% BSA in phosphate-buffered saline consisting of 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM NaH_2PO_4 was prepared and the pH adjusted to 7.4 with 1 M NaOH. A 200 ml aliquot was added to each well of a 96 well round-bottomed microtitre plate (Flow Laboratories, Rickmansworth). The plate was covered with sealing film (Flow Laboratories, Rickmansworth) and left overnight at 4°C to block non-specific binding sites. This pre-incubation plate was emptied by shaking over a sink and banging the plate on a wad of absorbent paper three times. The plate was then flooded with PBS containing 0.1% ($^{v}/_{v}$) Tween 20 from a wash bottle and allowed to stand for 3 min. This procedure was repeated twice. A 40 ml aliquot of antibody solution containing 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 5 mM EDTA, 0.1 mM phenymethylsulphonyl fluoride and 0.02% ($^{w}/_{v}$) sodium azide, with an antibody dilution of 1:1600, was added to each well of the microtitre plate except the blank wells (Table 4.1). A 40 µl volume of sample or

standard was added to the appropriate wells and the pre-incubation plate was incubated in a humid chamber at 37°C overnight.

The wells of an ELISA microtitre plate (Dynatech, USA) were coated with 400 ng of purified mucin in 50 ml phosphate-buffered saline, the plate was sealed and incubated overnight at 4°C. This plate was then washed with 0.1% (v/v) Tween 20 as described above and 200 ml of 2% BSA in phosphate-buffered saline was added to each well and the plate was sealed and left for 1 h at room temperature. The plate was washed and 50 ml of the antibody/antigen mixture from the pre-incubation plate was transferred to the ELISA plate which was sealed and left for 1h at room temperature. After washing, 50 ng of protein A-alkaline phosphatase conjugate in 50 ml of tris-buffered saline (pH 7.4) consisting of 0.14 M NaCl, 2.7 mM KCl and 20 mM tris was added to each well. The plate was sealed and left for 1 h at room temperature. The plate was then washed with tris-buffered saline containing 0.1% (v/v) Tween 20 as before.

Finally, 150 mg of disodium p-nitrophenyl phosphate in 150 ml diethanolamine buffer (composition: 1 M diethanolamine, 0.5 mM magnesium chloride, pH 9.8) was added to each well. Colour development was stopped after 1 h by the addition of 3 M sodium hydroxide (50 ml). Absorbance in each well was determined at 405 nm using an ELISA plate reader (Anthos 2001, Anthos Labtec Instruments, Austria.).

4.2.3.1 Calculation of immunologically detectable mucin present in samples.

A standard curve of absorbance at 405 nm versus concentration of mucin standard (ng mucin/40 µl) was plotted on semi-log graph paper (Fig. 4.7) following correction for absorbance of blank wells (no antibody in preincubation). The concentration of mucin present in samples was then determined by reading off from the standard curve and was expressed as µg mucin/10^7cells/h.

Figure 4.6 Flow diagram for the determination of immunologically detectable mucin.

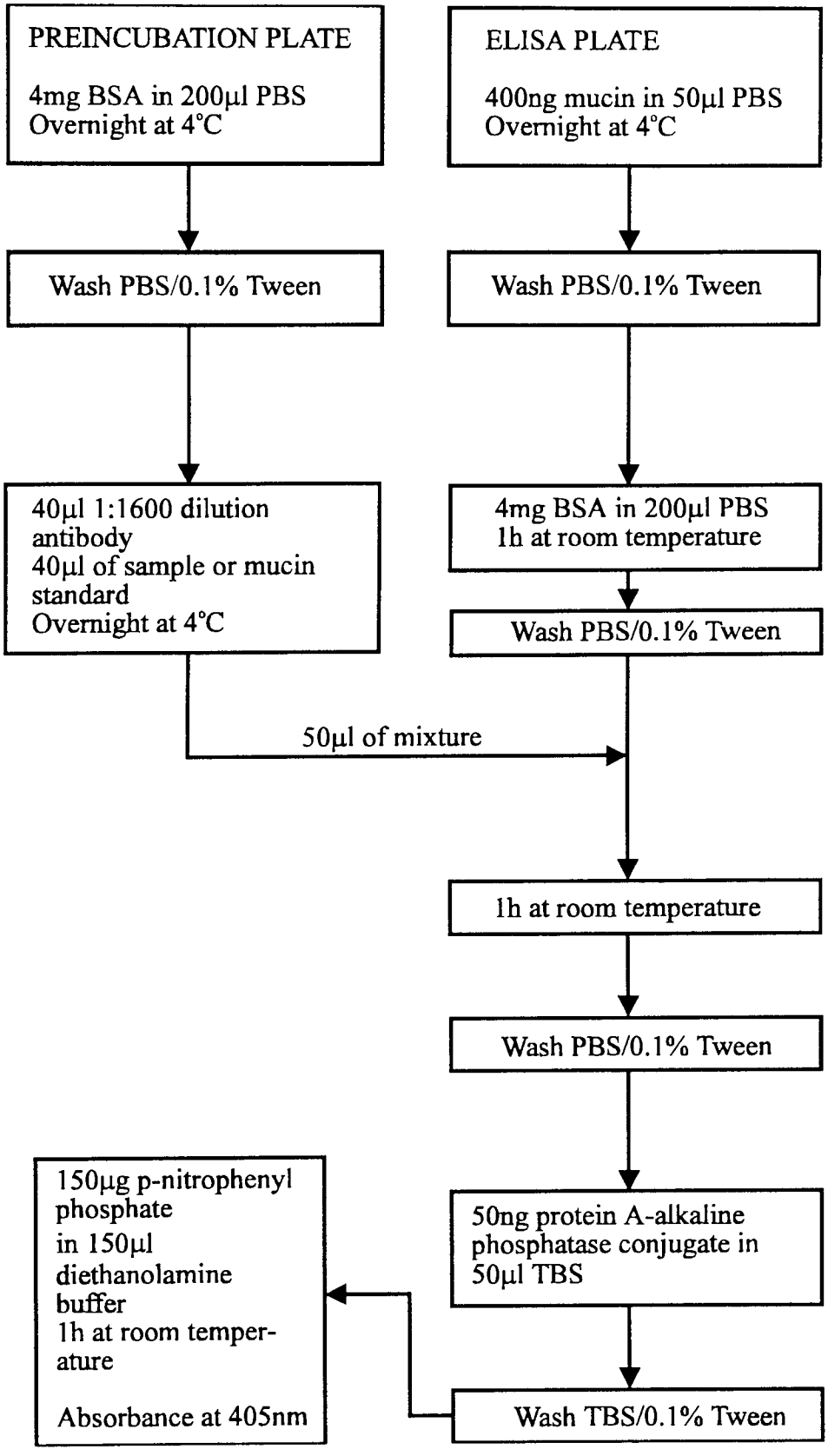
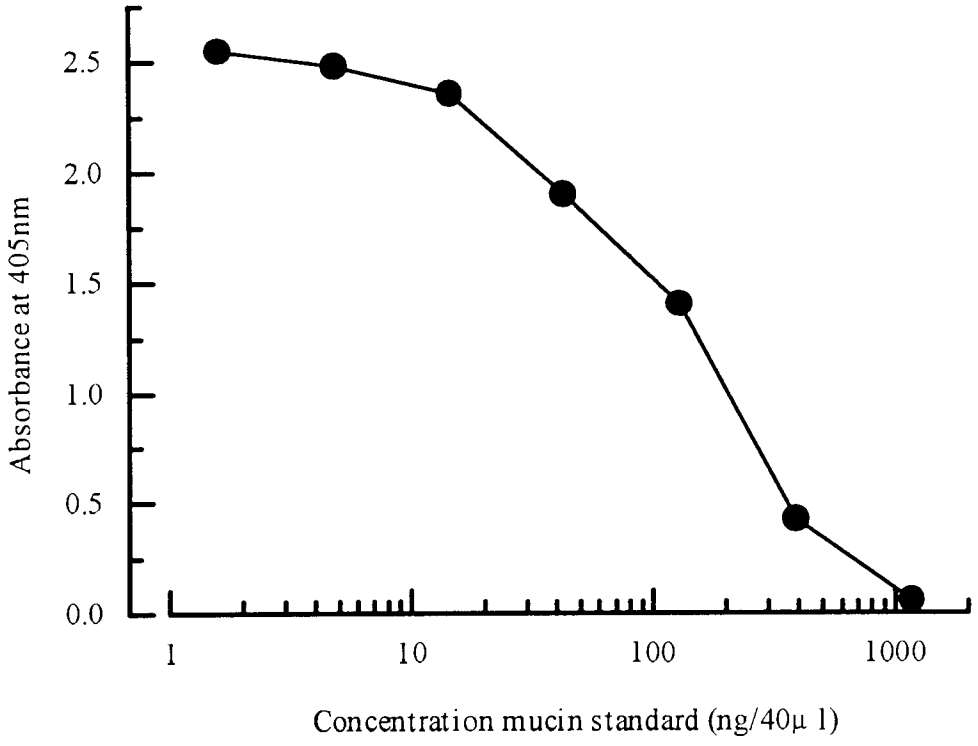


Table 4.1 **Positioning of samples on pre-incubation and ELISA plates.**
This scheme was adopted in an attempt to minimise "plate effects"

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	S7	Sa	Sa	Sa	S6	Sa	Sa	Sa	Sa	B	S7
B	Ab	Sa	Sa	Sa	Sa	S5	Sa	Sa	Sa	Sa	Ab	Sa
C	S1	Sa	Sa	Sa	Sa	S4	Sa	Sa	Sa	Sa	S1	Sa
D	S2	Sa	Sa	Sa	Sa	S3	Sa	Sa	Sa	Sa	S2	Sa
E	S3	Sa	Sa	Sa	Sa	S2	Sa	Sa	Sa	Sa	S3	Sa
F	S4	Sa	Sa	Sa	Sa	S1	Sa	Sa	Sa	Sa	S4	Sa
G	S5	Sa	Sa	Sa	Sa	Ab	Sa	Sa	Sa	Sa	S5	Sa
H	S6	Sa	Sa	Sa	Sa	B	S7	Sa	Sa	Sa	S6	Sa

Key: B = Blank (no antibody),
Ab: = Antibody without antigen (Maximum absorbance),
S1-S7 = Standards with mucin concentration of 1.6, 4.8, 14.4, 43.2, 129.6, 388.8 and 1166.4 ng/40 µl.
Sa = Samples.

Figure 4.7 **A typical standard curve for the measurement of immunologically detectable mucin.**



Results are presented from one experiment following correction for absorbance of the blank.

4.2.4 Measurement of acid phosphatase release from a crude gastric mucosal cell suspension.

Following a 1 h incubation with ISDN (0.01-2.0 mM) as described in section 4.2.2, a 0.1 ml sample of supernatant was removed and transferred to tubes containing 400 µl p-nitrophenyl phosphate (1.33 mg/ml) in 0.1 M citrate buffer (pH 4.8). The contents of the tubes were thoroughly mixed by vortexing for 5 s before incubation at 37°C for 10 min. The reaction was terminated by addition of 0.5 ml 1 M sodium hydroxide before measurement of the absorbance at 400 nm against a blank of distilled water in a dual beam spectrophotometer (Pye Unican SP30, Cambridge). Total acid phosphatase activity was determined by taking a 1 ml sample of cells at time t=0 and freeze-thawing them in liquid nitrogen three times before removing a 100 µl sample.

4.2.4 Analysis of material secreted by crude cell suspensions.

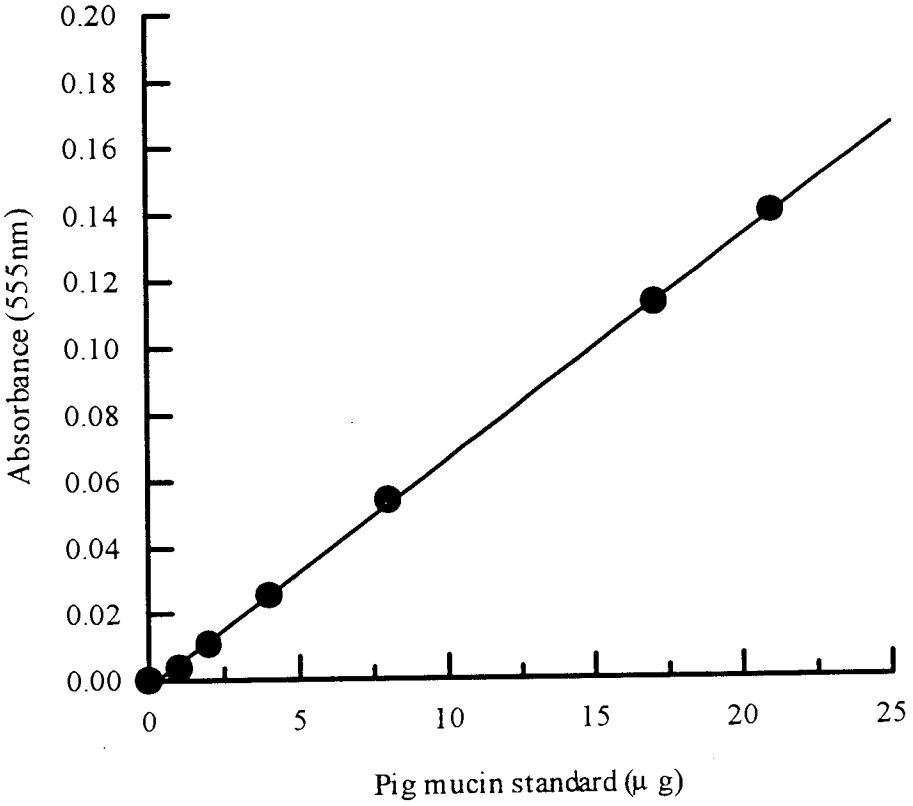
A crude gastric mucosal cell suspension was prepared as in 2.1 and pre-incubated as described in 4.2.2. The cell suspension was then transferred to a 50 ml polypropylene conical flask to which A23187 was added to give a final concentration of 10 µM. The air space was gassed with 95%O_2/5%CO_2 for 10 s before the flask was capped and incubated for 1 h at 37°C with continuous shaking at 150 cycles/min. The contents of the flask were transferred to 10 ml polypropylene centrifuge tubes and centrifuged at 125 g for 7 min at 15°C. An 8.4 ml aliquot of supernatant was removed and transferred to a 20 ml polypropylene scintillation vial containing 5.6 g $CsCl_2$. The $CsCl_2$ was allowed to dissolve before the contents were transferred to 10 ml polypropylene ultra-centrifuge tubes and centrifuged for 69 h at 150,000 g_{av} and 11°C using a 70° Ti rotor in a Beckman L8-60M ultracentrifuge. The samples were unloaded by using a peristaltic pump (Gilson Minipuls, Anachem) connected to a fraction collector (Gilson Microcol TDC 80, Anachem), to give 0.75 ml fractions. The density of each fraction was determined by measuring the mass of a 200 µl sample. The 200 µl sample was returned to the corresponding fraction tube before each fraction was transferred to dialysis tubing (6 mm diameter; cut off point >12 kDa.) and was dialysed against 2 x 2 litre of distilled

water containing 0.2%($^{w}/_{v}$) sodium azide for 24 h at 4°C. A sample of purified rat gastric mucin, in a separate tube, was treated exactly the same as the secreted material.

Following dialysis of the secreted material, a 0.5 ml aliquot was transferred to a quartz microcuvette and the absorbance at 280 nm was measured against a blank of distilled water in a dual beam spectrophotometer (Pye Unican SP30, Cambridge). Following dialysis of the secreted material, a 0.5 ml aliquot was transferred to a quartz microcuvette and the absorbance at 280 nm was measured against a blank of distilled water in a dual beam spectrophotometer (Pye Unican SP30, Cambridge). The amount of glycoprotein in the dialysed fractions of secreted material was assessed by the Periodic acid Schiff assay (Mantle & Allen, 1978). A 200 µl aliquot of dialysed sample or 0, 1, 2, 4, 8, 17 and 21 µg of pig gastric mucin standards dissolved in 200 µl of distilled water were transferred to 3 ml test tubes. The volume in each tube was made up to 1 ml with distilled water prior to addition of 100 µl of freshly prepared periodic acid solution (10% periodic acid in 10 ml (7% v/v) acetic acid) to each tube. The samples and standards were then incubated for 2 h at 37°C. After periodate oxidation, 100 µl of decolourised Schiff reagent (see section 2.5) was added to each tube and colour development was allowed to occur over 45 min at 37°C. Absorbance at 555 nm was measured for each sample and standard against distilled water using a dual beam spectrophotometer (Pye Unican SP30, Cambridge). A correction was made for absorbance of the reagent blank. The standard curve is as shown in Fig. 4.8. The absorbance values obtained with rat gastric mucin were weight to weight equivalent to those obtained with pig gastric mucin (Keates, 1991), thus justifying its use in generation of the standard curve.

Immunologically detectable mucin in the fractionated secreted material was assayed as described in section 4.2.3.

Figure 4.8 **Standard curve obtained using periodic acid Schiff colourimetric glycoprotein assay.**



Results are from a single experiment.
The equation of the regression line is: y=-0.00178+0.00684x and the correlation coefficient, r=0.9998.

4.2.5 Use of a suspension enriched with mucous cells for co-determination of mucin release and cGMP content.

Isolated rat fundic mucosal cells were separated by counterflow elutriation into five fractions, termed F_1, F_2, F_3, F_4 and F_5 as described in 2.2.3. Fraction F_3, now termed the mucous cell rich fraction, contained 61±1% (n=4) mucous cells (Fig. 3.6) as determined by periodic acid Schiff staining (3.2.3). The enriched mucous cell fraction was resuspended in medium B' at a concentration of $1x10^7$cells/ml and aliquoted into 20 ml polypropylene scintillation vials containing 0.01 mM M&B 22948 and the appropriate agents. The space above the liquid in the vial was gassed with $95\%O_2/5\%CO_2$ before the vial was capped and incubated at 37°C with continuous shaking at 150 cycles/min for 30 min. Following the incubation, two 0.5 ml samples were removed, one for the determination of mucin release, the other for measurement of cGMP. The samples for measurement of mucin release were centrifuged at 10,000 g in a bench top centrifuge at room temperature for 30 s prior to removal of 0.45 ml of supernatant which was then transferred to 1.5 ml micro-centrifuge tubes containing 50 µl of a solution consisting of 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 50 mM EDTA, 1 mM phenylmethylsulphonyl fluoride and 0.2%($^{w}/_{v}$) sodium azide. The contents were vortexed for 10 s and assayed for mucin content as described in 4.2.3. The sample removed for cGMP measurements was immediately frozen in liquid nitrogen and stored at -70°C until assayed as described in section 2.3.

4.2.6 Effects of agents on the thickness of the mucous gel layer of the rat gastric mucosa *in vivo*.

4.2.6.1 Measurement of mucous gel thickness.

The effects of various agents on the thickness of the mucous gel layer were assessed by a modification of methods established by Kerss *et al.*, (1982) and Sandzen *et al.*, (1988).

Male Wistar rats (200-250 g body weight) which had been deprived of food, but not water, for the previous 24 h were anaesthetised with sodium pentobarbitone (60 mg/kg i.p.). The abdomen was opened by a midline incision and 2 ml of isotonic

saline, containing the appropriate agent, was introduced into the gastric lumen through the wall of the forestomach by means of a syringe and a 26 gauge needle. Thirty minutes later, the stomach was removed and the animal sacrificed by rupturing the diaphragm. A small volume of saline (approximately 0.5 ml) was injected between the mucosa and the muscularis mucosae. The formation of a blister allowed the resection of the muscle layer with a sharp pair of scissors. The area of the mucosa devoid of muscle was then removed and mounted with the mucosal surface face up, on a cellulose acetate filter (Sartorious, 0.8μm pore size). The mucosa was sectioned longitudinally with two razor blades mounted either side of a glass microscope slide which consequently produced a section of the same thickness as the microscope slide (approximately 1 mm). The section was transversely mounted in a specially prepared well filled with isotonic saline (Fig. 4.9). The thickness of the mucous gel layer was determined by viewing the section under an inverted microscope (Nikon TMS F, Netherlands; Plate 4.1), and using an eyepiece graticule. Two sections from either side of the stomach were viewed and the thickness measured every 100 μm to give a total of at least 40 measurements per animal.

4.2.7 Histological assessment of the gastric mucosa after exposure to agents.

The effect of exposing the gastric mucosa to nitric oxide donors and Db-cGMP, was assessed by determining the percentage of the total length of mucosa covered with detached nucleated cells.

4.2.7.1 Preparation of sections of gastric mucosa.

Gastric mucosa devoid of muscle and mounted on cellulose acetate filters were fixed in neutral-buffered formalin overnight. Fixed samples were dehydrated by immersion in increasing concentrations of ethanol of 50, 70, 90 and 95% for 2 h at 4°C. The 95% solution of ethanol was replaced with fresh 95% ethanol and the samples were left overnight at 4°C. The tissue samples were then immersed in xylene at 4°C for 1 h after which time the solution was replaced with fresh xylene and the samples incubated for a further 1 h under the same conditions as previously. Finally, the samples were immersed in fresh xylene and incubated at room temperature for 1 h prior to immersion

Figure 4.9 **Diagram to show the method of sectioning and positioning of mucosal sections for measurement of mucus gel thickness.**

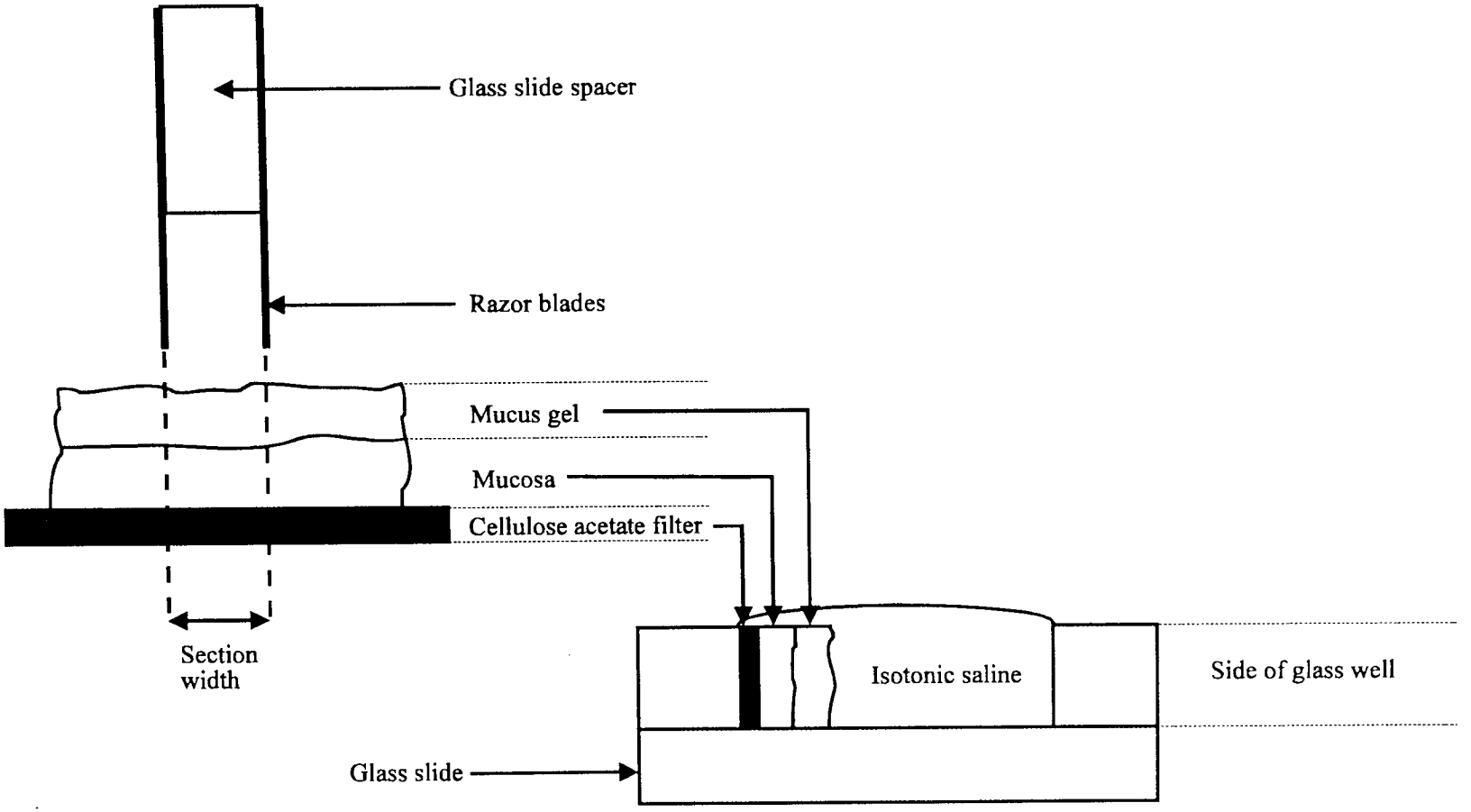


Plate 4.1 **Longitudinal section of rat gastric mucosa transversely mounted, as viewed under an inverted microscope (x50 magnification), for determination of the thickness of the mucous gel layer.**
Bar represents 100 μm.

paraffin wax at 56°C for 4 h and final embedding in paraffin when the molten wax was allowed to cool. Longitudinal sections of 5 μm thickness were cut with a microtome and floated on distilled water before being mounted on glass slides. The sections were dried in an incubator at 37°C for approximately 45 min before removal of the paraffin and rehydration by two immersions in xylene for 1 min at room temperature followed by immersion in decreasing concentrations of ice cold ethanol of 95, 90, 70 and 50% for 1 min periods. The samples were finally washed three times in ice cold phosphate buffered saline (pH 7.4) for 1 min intervals.

4.2.7.2 Staining of sections with periodic acid Schiff reagent and haematoxylin.

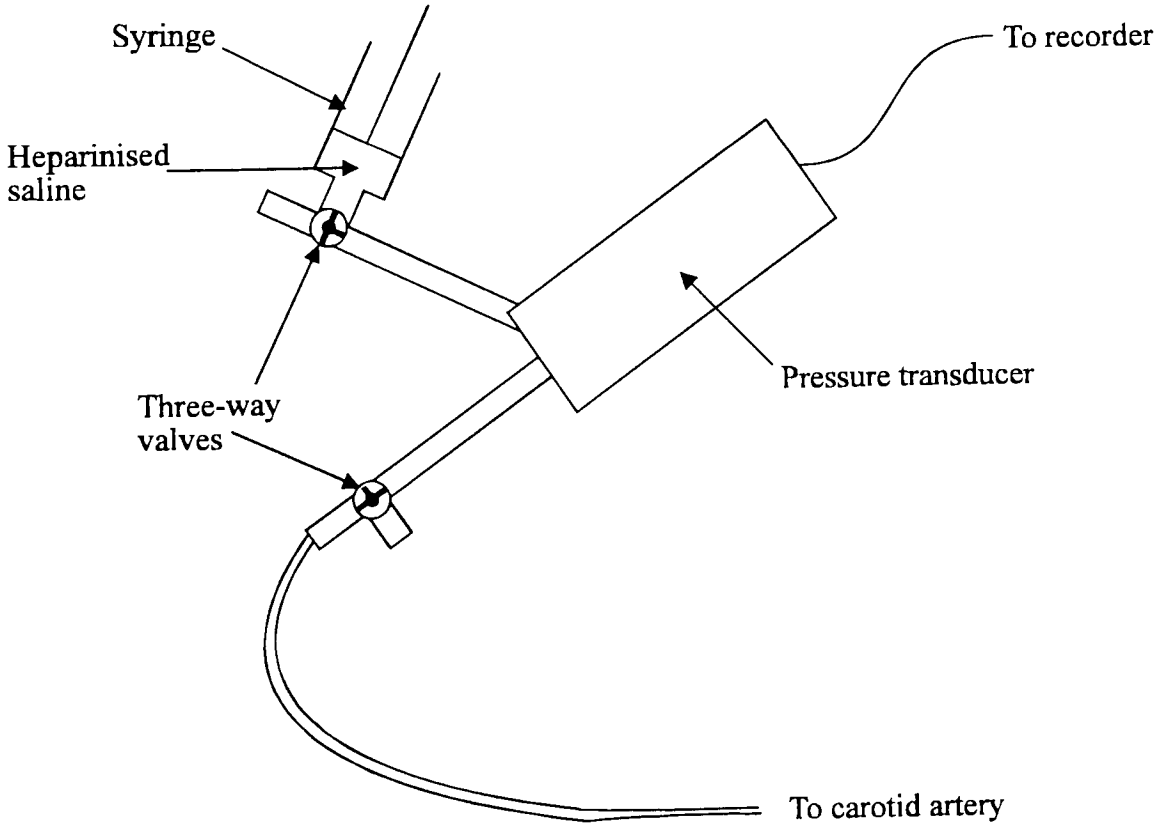
Sections were immersed in periodic acid solution (10% periodic acid solution in 10 ml (7% $^{v}/_{v}$) acetic acid) for 20 min at room temperature, washed under running tap water for 5 min before being immersed in Schiff's reagent (prepared as previously described in section 2.5) for 20 min at room temperature prior to washing under tap water for a further 10 min. Some sections were then counterstained with haematoxylin (0.1 g haematoxylin, 5 ml absolute ethanol, 2 ml 1% sodium iodate in 100 ml 5% potash alum, dissolved by boiling then cooled and filtered) by immersion for 10 min followed by washing under running tap water for 30 s.

4.2.8 Measurement of carotid arterial blood pressure.

Male Wistar rats (200-250 g body weight) were anaesthetised with sodium pentobarbitone (60 mg/kg) and the neck region was exposed to reveal the external carotid artery. Two ligatures were placed underneath the artery, the proximal ligature was tied and pulled back to expose the artery. A clamp was placed below the distal ligature, an incision made in the artery, the cannula (0.75 mm o.d. polyethylene tubing) inserted and tied and the clamp removed. The cannula was filled with saline containing 100 U/ml sodium heparin and attached to a Druck pressure transducer, the output from which was recorded on an Ectromed Multi Trace 2 chart recorder (Fig. 4.10). A measurement of mean carotid arterial blood pressure was made prior to opening the abdomen via a midline incision to expose the stomach. A 2 ml volume of agent was

introduced into the gastric lumen through the forestomach via a syringe attached to a 26 gauge needle. The abdomen was closed and the cannula cleared by introducing a small volume of heparinised saline. The recording of blood pressure was continued for 30 min to determine the mean arterial blood pressure. A 2 ml volume of saline was injected i.p. to see if this had an effect on arterial blood pressure followed by 2 ml of 1 mM ISDN (i.p.). Finally, the system was calibrated using a mercury manometer to allow the changes in height of the trace to be translated into changes in blood pressure (mmHg).

Figure 4.10 **Diagrammatic representation of the apparatus used to measure carotid arterial blood pressure.**



4.2.9 Presentation and analysis of results.

Data obtained with isolated cells are analysed by using a two-way ANOVAR and Dunnett's test (see Appendix A.6.2) for difference from basal release of mucin in the absence of added agents. This procedure, which is performed on "raw data", serves to remove the effect of variations between cell batches. Data are then presented as a percentage of basal release of mucin or as the increase in cGMP content above basal.

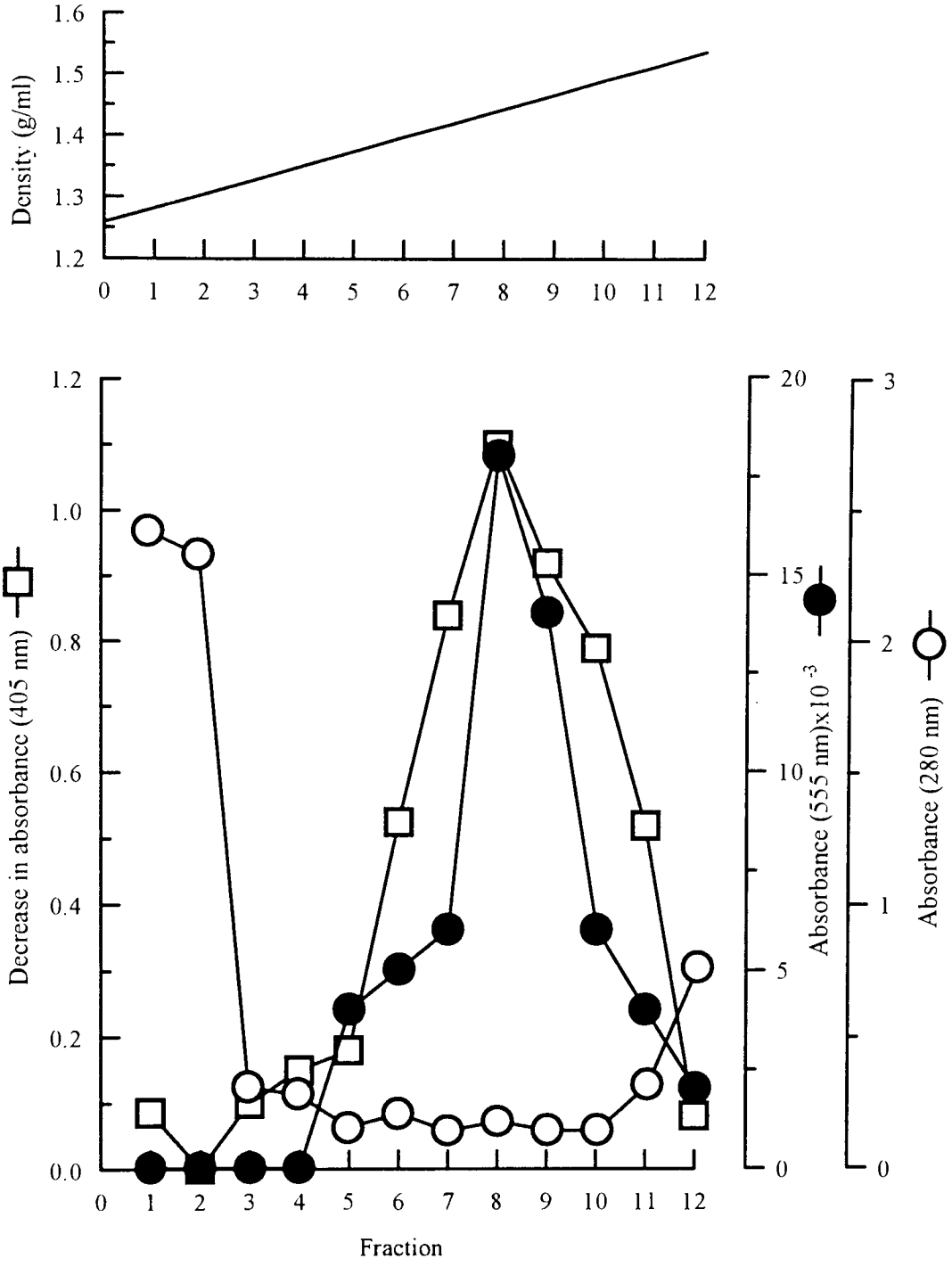
Examination of the distribution of mucus gel thickness measurements from single animals suggested that there was some, albeit slight, departure from normality. Non-parametric statistics (see Appendix A.6.3) were therefore used to analyse these data.

4.3 RESULTS AND DISCUSSION.

4.3.1 Specificity of the enzyme-linked immunosorbent assay (ELISA) for measurement of rat gastric mucin secreted from a suspension of gastric mucosal cells.

Following separation on a caesium chloride density gradient, the distribution of immunoreactive material detected by the ELISA (change in absorbance at 405 nm) did not parallel the profile for material absorbing at 280 nm (Fig. 4.11). The bulk of the material recognised by the antibody in the ELISA was not therefore protein of low density which would include the bovine serum albumin added to the incubation medium. The majority of the material detected by the ELISA was in positions on the gradient where the presence of mucin was indicated by the periodic acid Schiff assay (Fig. 4.11), suggesting that the material detected by the ELISA had a certain glycoprotein component. These findings, coupled with observations by Keates, (1991), that the ELISA did not recognise mucin in extracts of rat small intestine, crude pig gastric mucin or rat gastric mucin that had been denatured with 6 M guanidinium chloride and in which the disulphide bridges were reduced with 100 mM dithiothreitol followed by carboxymethylation of sulphydryl groups with 250 mM iodoacetamide strongly suggest that the antibody is highly specific for intact rat gastric mucin. Furthermore, serial dilutions of extracts of rat gastric mucosal cells and of material secreted into the incubation medium gave results in the ELISA that could be directly superimposed on the mucin standard curve (Keates & Hanson, 1990a). The recognition of non-mucous glycoproteins is therefore highly unlikely and the ELISA could be used to provided a quantitative index of mucus secretion from gastric mucosal cells. The term "mucin" will now be used to refer to immunologically reactive material that was detected by the ELISA.

Figure 4.11 Distribution on a caesium chloride density gradient of immunoreactive material (□), and material absorbing at 280 nm (○) from a crude gastric mucosal cell suspension following incubation for 1 h in the presence of 10 μM A23187. (●), distribution of purified mucin detected by periodic acid Schiff assay at 555 nm.



4.3.2 Effects of agents on mucin release from a crude suspension of gastric mucosal cells.

4.3.2.1 Effect of 0.01 mM-2 mM ISDN on mucin release from, and viability of, a crude suspension of gastric mucosal cells.

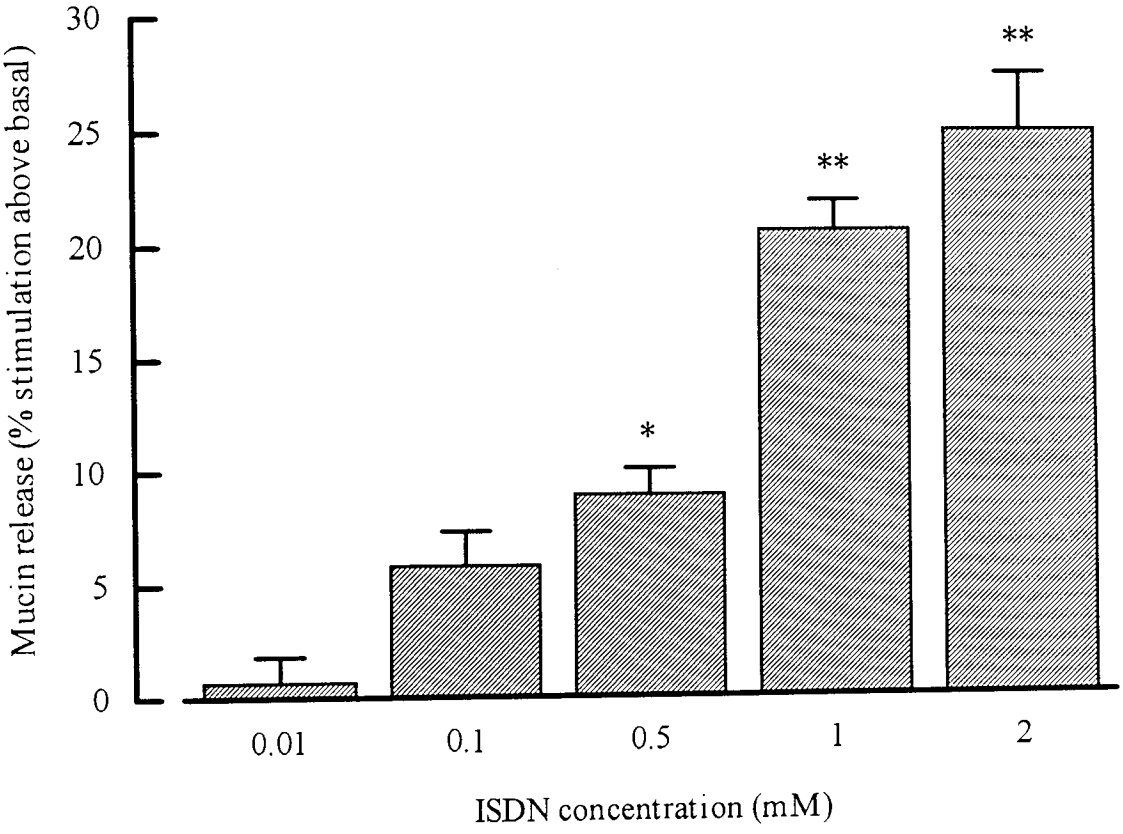
Increasing concentrations of ISDN between 0.01 and 2.0 mM caused a concentration dependent increase in the amount of mucin present in the incubation medium following a 60 min incubation period (Fig 4.12). The increase in mucin release could have been due to cell damage for although NO generators have been shown to protect against tissue damage (Boughton-Smith *et al.*, 1990), prolonged exposure to excessive levels of NO can be cytotoxic (Palmer *et al* 1992). Determination of cell viability by exclusion of trypan blue following exposure of the cells to various concentrations of ISDN (Fig. 4.13) showed no correlation between their ability to exclude trypan blue and the amount of mucin released. Similarly, no correlation could be found between the release of the lysosomal enzyme acid phosphatase and the amount of mucin in the medium following a 1 h incubation (Fig 4.14). Both exclusion of trypan blue, and release of acid phosphatase, have been shown to be reliable indices of gastric cell damage (Wallace and Whittle, 1989), and hence the stimulatory effects of ISDN on mucin release were probably not a non-specific consequence of damage to cells.

4.3.2.2 Effects of S-nitroso-N-acetylpenicillamine (SNAP), sodium nitroprusside and oxyhaemoglobin (Hb) on the release of mucin from a crude gastric mucosal cell suspension.

Two other NO donors, S-nitroso-N-acetylypenicillamine (SNAP) and sodium nitroprusside at concentrations of 0.3 and 1.0 mM respectively, caused an increase in the amount of mucin present in the incubation medium following a 60 min incubation (Fig 4.15). Furthermore, 10 μM oxyhaemoglobin (Hb), a NO scavenger (Martin *et al.*, 1985), significantly ($P<0.01$ by ANOVAR and Dunnett's test) reduced the percentage increase in mucin release caused by 0.3 mM SNAP from 17.9±1.7 to 3.5±2.4 percent above basal in the presence and absence of oxyhaemoglobin respectively (Fig 4.15). This result suggests a specific involvement of NO and not some other part of the molecule in the action of SNAP on mucus release.

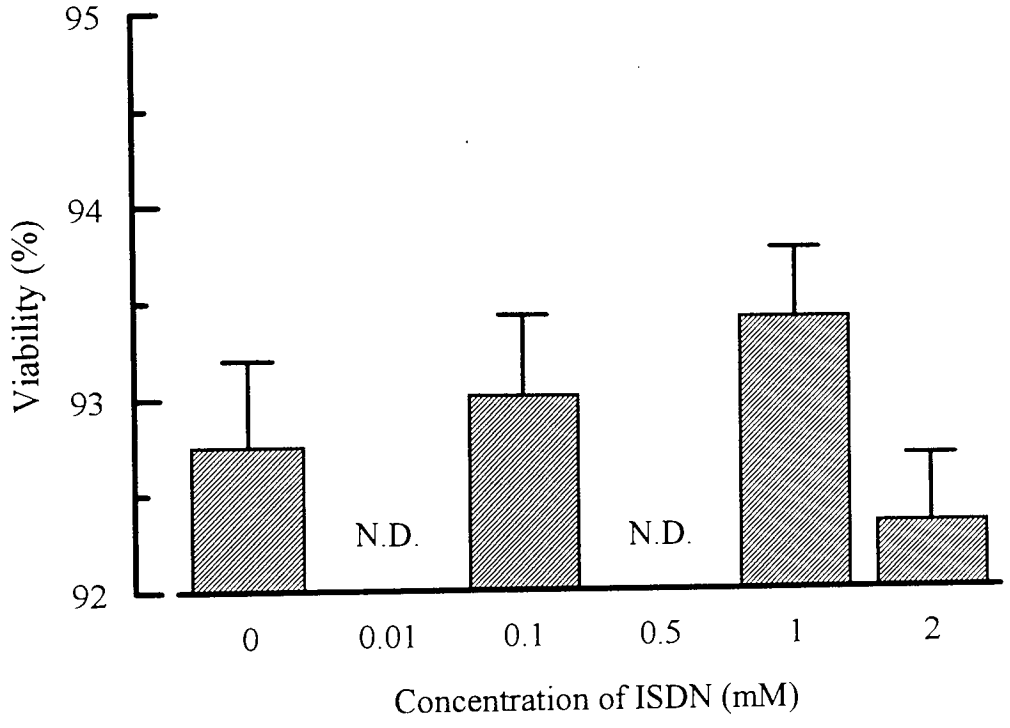
SNAP and NP spontaneously liberate NO in aqueous solution which may lead to the formation of various products including nitrites and nitrates following oxidation of the free radical (Ignarro *et al.*, 1981). The oxidised products of NO could potentially be responsible for the increase in mucin release observed following incubation with NO donors. However, "exhausted" SNAP (SNAP no longer able to spontaneously generate NO) which would contain such products, was unable to stimulate mucin release in cell batches that were responsive to "fresh" SNAP (Fig 4.22). as the stimulatory effect of SNAP, which spontaneously liberates NO, could be blocked by coincubation of the cell suspension with oxyhaemoglobin, a NO scavenger (Martin *et al* 1985), this rules out an involvement of the oxidised products and suggests a specific involvement of NO in the mediation of mucus release (Fig 4.21).

Figure 4.12 **Effects of increasing concentrations of ISDN on mucin release from a crude suspension of rat gastric mucosal cells.**



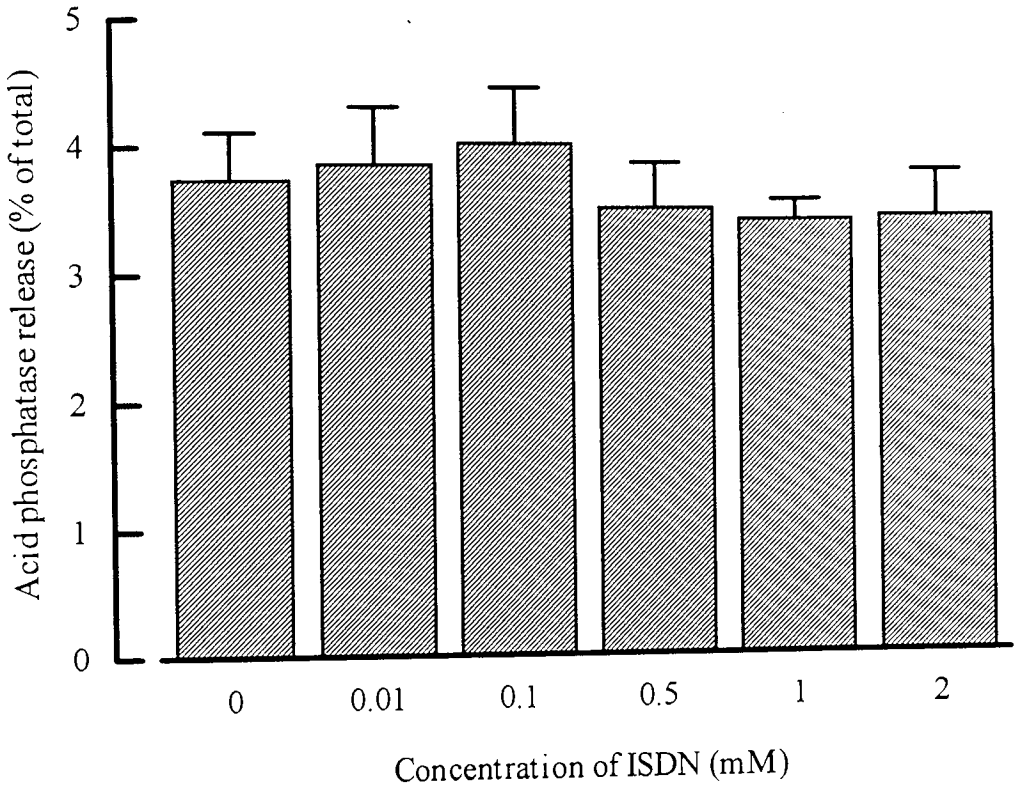
Data are presented as mean±SEM (number of cell batches n=4), where *P<0.05 and **P<0.01 by ANOVAR and Dunnett's test for difference from basal (no ISDN). Basal release over the 1 h incubation period was 3.81±0.63 μg mucin/10^7cells/h.

Figure 4.13 **Effects of increasing concentrations of ISDN on the viability of crude suspensions of gastric mucosal cells.**



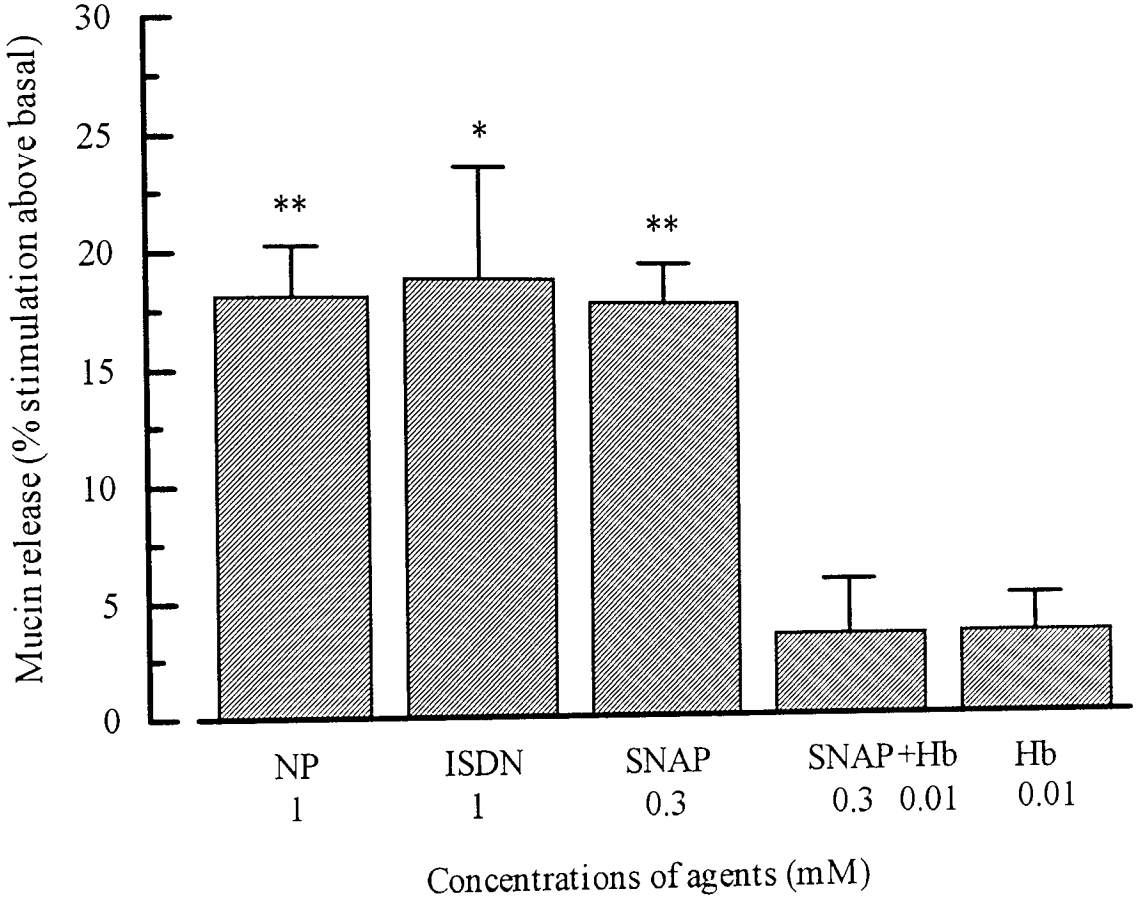
Data are presented as mean±SEM (number of cell batches n=4), with the viability expressed as the percentage of cells able to exclude the dye trypan blue. N.D. denotes that viabilities were not determined for these conditions. ANOVAR showed that the concentration of ISDN did not affect cell viability.

Figure 4.14 **Effect of increasing concentrations of ISDN on acid phosphatase release into the medium from a crude suspension of gastric mucosal cells during 1 h of incubation.**



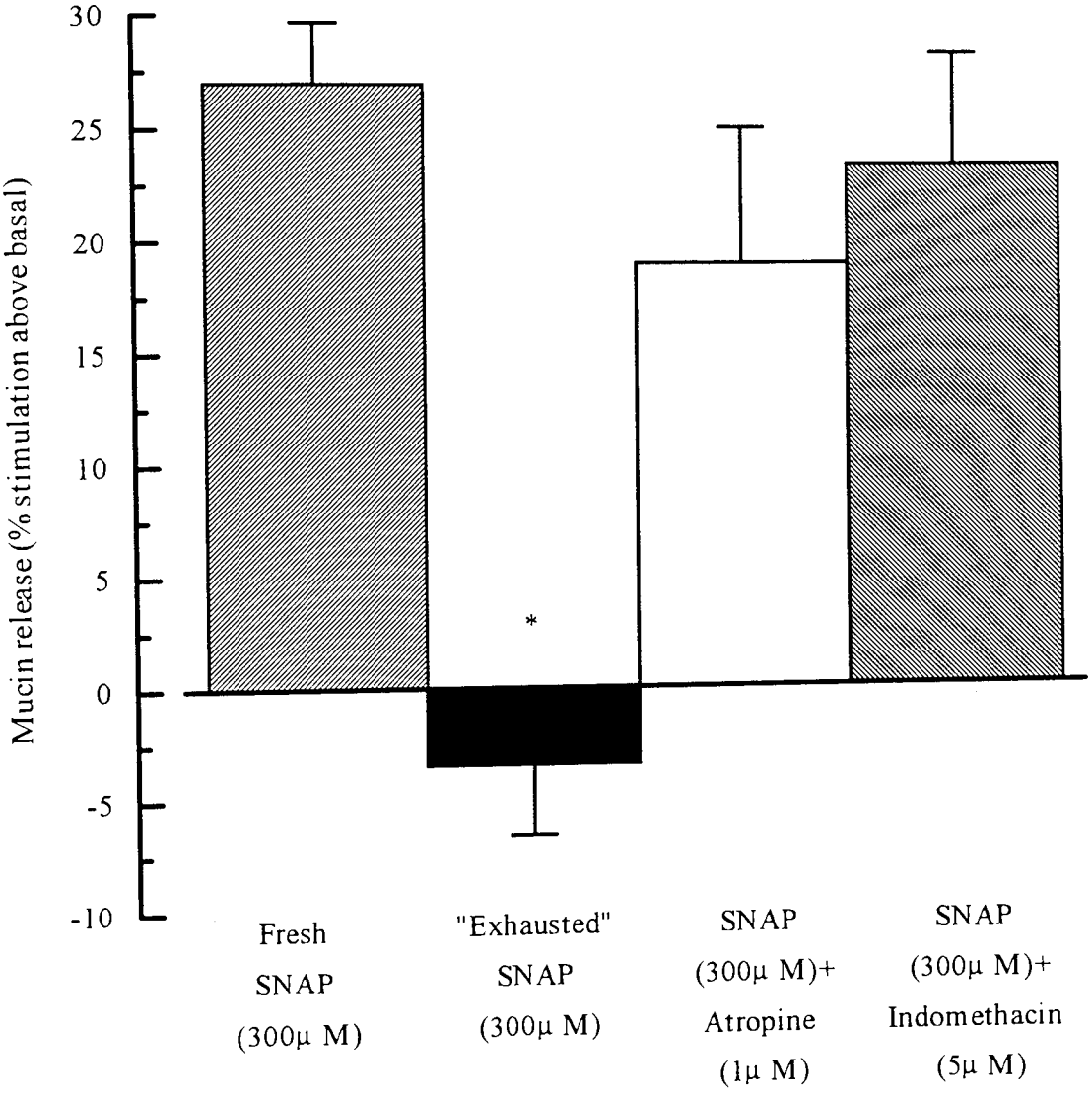
Data are presented as mean±SEM (number of cell batches n=4). Total acid phosphatase activity after freeze-thawing the cell suspension three times was 1.37±0.06 µmol/min/10^6cells. ANOVAR showed that the concentration of ISDN did not affect acid phosphatase release.

Figure 4.15 **Effects of nitric oxide donors and oxyhaemoglobin on mucin release from a crude suspension of gastric mucosal cells.**



Data are presented as mean±SEM (number of cell batches n=4), where *P<0.05 and **P<0.01 for difference from basal by ANOVAR and Dunnett's test. Basal release for nitroprusside (NP) and isosorbide dinitrate (ISDN) was 3.77±0.56 $\mu g/10^7$cells/h and for SNAP, SNAP+Hb and Hb alone was 3.33±0.25 $\mu g/10^7$cells/h.

Figure 4.16 **Effects of "fresh" and "exhausted" SNAP and of "fresh" SNAP in the presence of either atropine or indomethacin on mucin release from a crude suspension of gastric mucosal cells.**



Data are presented as mean±SEM (number of cell batches n=4), where *P<0.05 by ANOVAR and Dunnett's test for difference from "fresh" SNAP. Basal release over the 1 h incubation was 1.17±0.54 μg mucin/10^7cells.

4.3.2.3 Effects of cyclic nucleotide analogues and the phosphodiesterase inhibitor M&B 22948 (Zaprinast) on the release of mucin from a crude gastric mucosal cell suspension.

The dibutyryl analogue of guanosine 3',5'-cyclic monophosphate (Db-cGMP) caused a dose related increase in the amount of mucin present in the incubation medium following a 60 min incubation (Fig 4.17). As expected from previous work by Keates and Hanson, (1990b), the dibutyryl analogue of adenosine 3',5'-cyclic monophosphate (Db-cAMP) at a concentration of 1.0 mM, also caused an increase in

the amount of mucin present in the incubation medium (Fig. 4.17). However the dibutyryl analogue of 3',5'-cyclic cytidine monophosphate (Db-cCMP) was without effect (Fig. 4.17). These results firstly suggest that the stimulatory effects of the dibutyryl analogues of cGMP and cAMP are not due to the dibutyryl moiety as Db-cCMP had no effect on mucus release in this preparation and secondly, that both cAMP and cGMP are involved in the stimulation of mucus release from these cells.

M&B 22948 (Zaprinast), caused a significant, ($P<0.01$ by ANOVAR and Dunnett's test) 19.6±2.8% (n=6), increase in the amount of mucin present in the incubation medium following a 1 h incubation (Fig 4.17). Since M&B 22948 inhibits metabolism of guanosine 3',5'-cyclic monophosphate to guanosine 5'-monophosphate via inhibition of the cGMP-specific PDE (type V), (Francis *et al.*, 1990), the present results could be a consequence of an increase in the intracellular concentration of cGMP.

Overall, as in some other systems, such as the inhibition of platelet aggregation or smooth muscle relaxation (Moncada *et al.*, 1991b) or with the facilitation of insulin secretion (Schmidt *et al.*, 1992b), the data presented here suggest both cAMP and cGMP have comparable rather than opposing actions on the tissue response, namely mucus secretion.

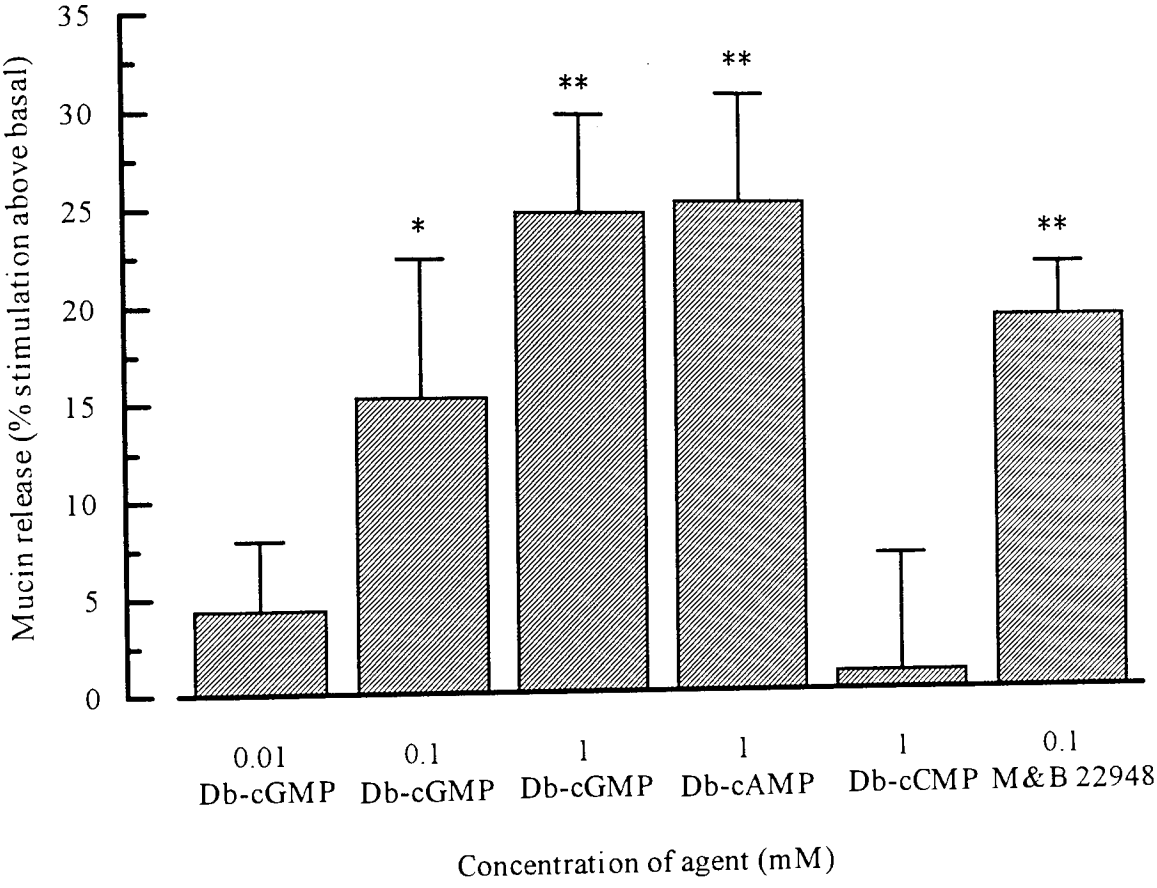
4.3.2.4 Effects of indomethacin and atropine on mucin release stimulated by SNAP from a crude suspension of gastric mucosal cells.

Coincubation with either atropine (1 μM) or indomethacin (5 μM) did not significantly affect the stimulatory effect of SNAP (300 μM) on mucus secretion from a crude gastric mucosal cell suspension (Fig 4.16).

The possibility that NO donors could mediate mucus release by stimulating the release of a secretagogue from either epithelial cells or contaminating non-epithelial cells cannot be completely excluded, although the lack of inhibition of the secretory effects of SNAP by either atropine or indomethacin argues against the involvement of two possible candidates, namely prostaglandins or acetylcholine, both potent stimulants of mucus secretion (Section 4.1.4; Neutra and Forstner, 1987). Furthermore, other potential mediators such as histamine, gastrin-17 or epidermal growth factor do not stimulate

mucus release in these cell preparations (Section 4.1.4; Keates & Hanson, 1990b). Furthermore, the effect of any potential secretagogue released from a cell into the suspension would probably be substantially limited by its dilution in the incubation medium.

Figure 4.17 **Effects of dibutyryl analogues of cyclic nucleotides and the type V phosphodiesterase inhibitor M&B 22948 on mucin release from a crude suspension of gastric mucosal cells.**



Data are presented as mean±SEM (number of cell batches n=3-6), where *P<0.05 and **P<0.01 for difference from basal by ANOVAR and Dunnett's test. Basal release after the 1 h incubation was 4.22±0.74 $\mu g/10^7$cells.

4.3.3 Effects of agents on mucin release from, and the cGMP content of, a suspension enriched with mucous cells.

4.3.3.1 Effects of ISDN (0.1-2.0 mM), SNAP (0.3 mM) and M&B 22948 on mucin release from a suspension enriched with gastric mucous cells.

Increasing concentrations of ISDN between 0.1 mM and 2.0 mM caused a concentration-dependent increase in the amount of mucin present in the incubation medium following a 30 min incubation of the cells in the presence of 0.01 mM M&B 22948 (Fig. 4.18). The PDE type V inhibitor was added to allow determination of the cGMP content of enriched mucous cell suspensions. Addition of SNAP (0.3 mM) also produced a significant increase ($P<0.05$ by ANOVAR and Dunnett's test) in the amount of mucin in the incubation medium above the basal release in the presence of 0.01 mM M&B 22948 alone (Fig. 4.18).

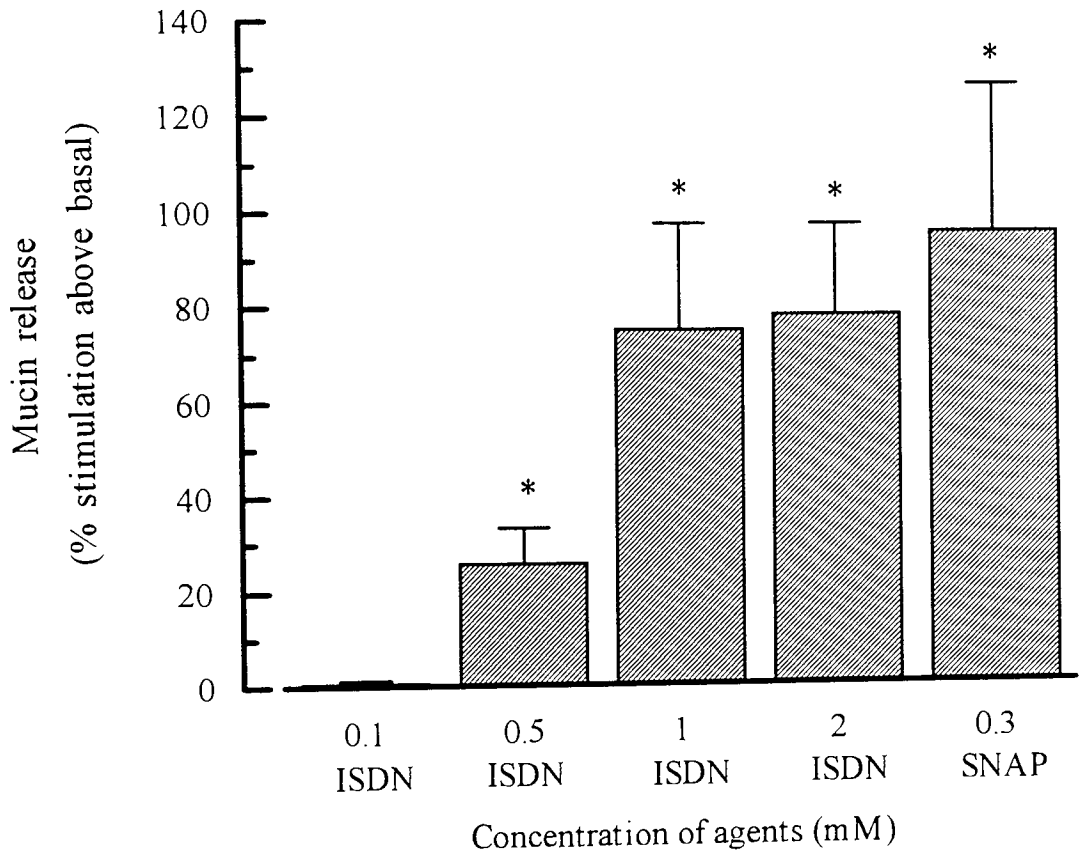
Finally, M&B 22948 caused a concentration related increase in the amount of mucin present in the incubation medium following a 30 min incubation (Fig. 4.19). These results confirm previous findings obtained from crude suspensions of cells (4.3.2).

4.3.3.2 Effects of ISDN (0.01-2.0 mM), SNAP (0.3 mM) and M&B 22948 on the cGMP content of an enriched suspension of gastric mucous cells.

ISDN caused a concentration related increase in the cGMP content of a suspension enriched with gastric mucous cells (Fig. 4.20). The nitrosothiol SNAP, also caused a significant ($P<0.01$ by ANOVAR and Dunnett's test) increase in the cGMP content of these cell suspensions (Fig. 4.20). The concentrations of agents which increased mucin release were parallel to those which resulted in an increase in the cGMP contents, strongly suggesting that the effect of NO donors might be mediated by elevation of cellular cGMP.

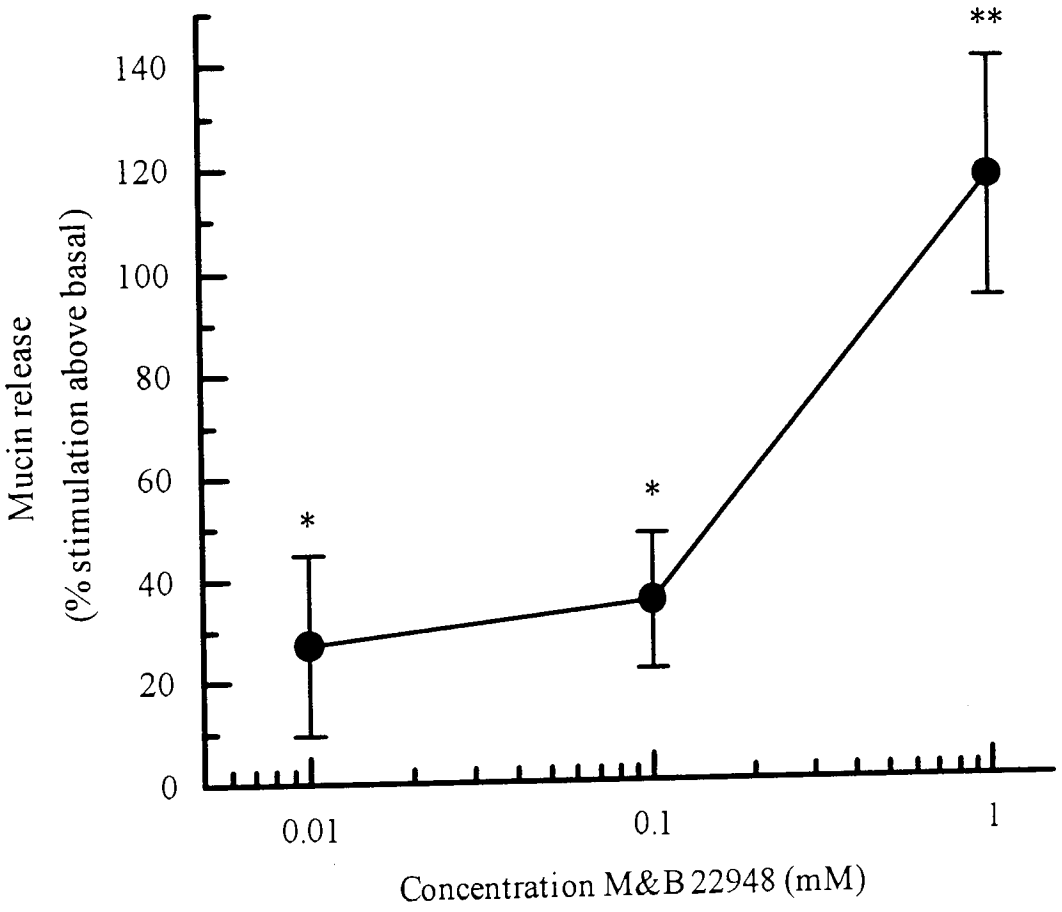
M&B 22948 caused a concentration related increase in the cGMP content of an enriched gastric mucous cell suspension between concentrations of 0.01 and 1.0 mM (Fig. 4.21). The concentrations of M&B 22948 which caused an increase in the cGMP content paralleled those which caused an increase in mucin release. This result suggests that the stimulatory effect of M&B 22948 on mucus release is mediated by elevation of cGMP.

Figure 4.18 **Effects of NO donors on the release of mucin from a cell suspension enriched with gastric mucous cells (elutriator fraction F_3) in the presence of 0.01 mM M&B 22948.**



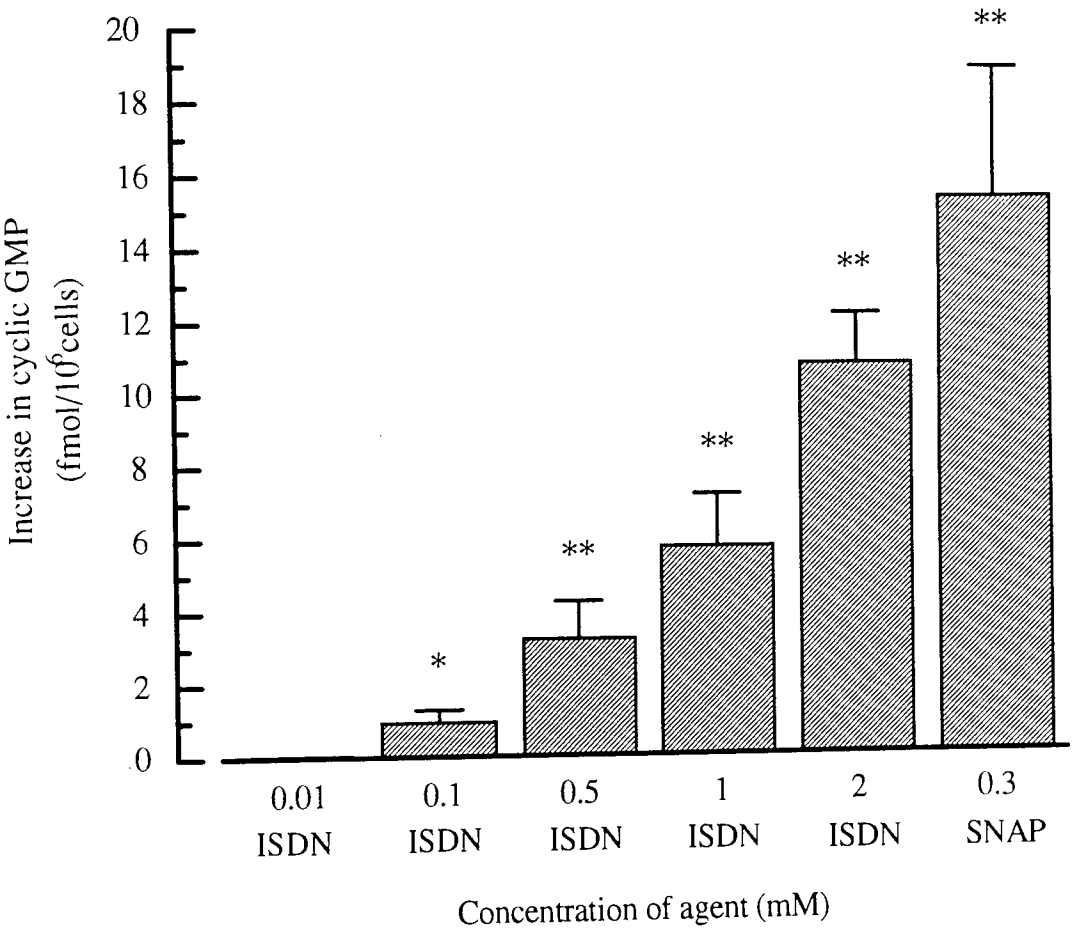
Data are presented as mean±SEM (number of cell batches n=3-4) where *P<0.05 by ANOVAR and Dunnett's test, for difference from basal release measured in the presence of 0.01 mM M&B 22948. Basal release was 13.65±5.04 $\mu g/10^7$cells/h.

Figure 4.19 **Effect of M&B 22948 on mucin release from a suspension enriched with gastric mucous cells.**



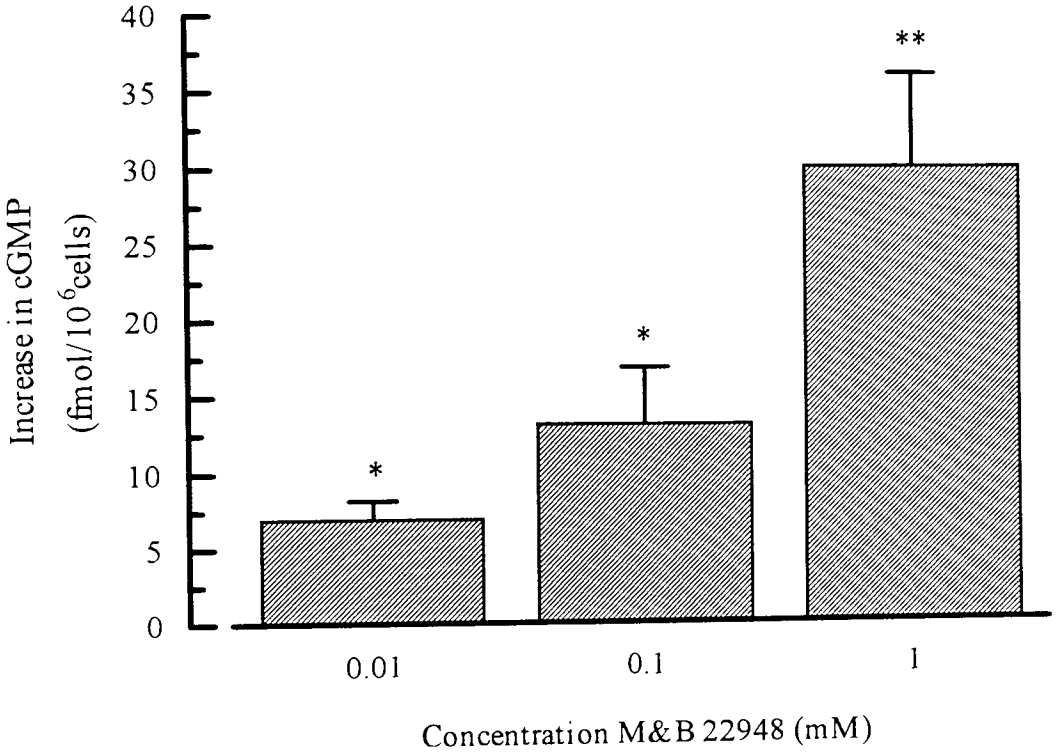
Data are presented as mean±SEM (number of cell batches n=4), where **P<0.01 and *P<0.05 by ANOVAR and Dunnett's test for difference from basal release measured in the presence of 0.001 mM M&B 22948, which was 14.04±6.16 $\mu g/10^7$cells/h.

Figure 4.20 **Effects of NO donors on the cGMP content of a suspension enriched with gastric mucous cells.**



Data are presented as mean±SEM (number of cell batches n=6), where **P<0.01 and *P<0.05 by ANOVAR and Dunnett's test for difference from the cGMP content in the presence of 0.01 mM M&B 22948 alone, which was 8.03±1.42 fmol/10^6cells.

Figure 4.21 **Effect of M&B 22948 on the cGMP content of a cell suspension enriched with gastric mucous cells.**



Data are presented as mean±SEM (number of cell batches n=5), where **P<0.01 and *P<0.05 by ANOVAR and Dunnett's test, for difference from cGMP content in the presence of 0.001 mM M&B 22948 which was 2.15±2.15 fmol/10^6cells.

4.3.4 Effects of intragastric application of NO donors and Db-cGMP on the thickness of the mucous gel of the rat corpus mucosa *in vivo*.

4.3.4.1 Effects of intragastric application of 0.1-2 mM ISDN and 300 μM SNAP on the thickness of the mucus gel layer.

ISDN caused a dose-related increase in the thickness of the layer of mucous gel that overlies the gastric mucosa (Fig. 4.22). The action of 1 mM ISDN was prevented by co-administration of 10 μM oxyhaemoglobin, which alone had no effect. Furthermore, the dimension of the mucus layer was also increased by intragastric administration of the nitrosothiol SNAP (300 μM) and the lipid permeable analogue of cGMP, Db-cGMP (1 mM) (Fig. 4.22). The ability of oxyhaemoglobin to block the effects of ISDN suggests that this agent may have undergone metabolic conversion to form NO outside the cells.

4.3.4.2 Effect of intragastric administration of ISDN (1 mM) on mean carotid arterial blood pressure.

Intragastric administration of 2 ml of 1 mM ISDN had no effect on the mean carotid arterial blood pressure which was 110±3 and 113±4 mm Hg (n=4 rats), at the beginning and end of the 30 min experimental period respectively. However, intraperitoneal injection of 2 ml of 1 mM ISDN after the 30 min experimental period did cause a significant decrease ($P<0.01$ by Student's t-test for paired data) in mean carotid arterial blood pressure with a fall of 22±3 mm Hg (n=4 rats), whereas 2 ml saline (i.p.) was without effect thereby validating the blood pressure measurement system.

These results suggest that intragastric ISDN does not increase the mucous gel thickness via alterations in the mean systemic arterial blood pressure. However, whether or not local mucosal vasodilatation by the NO generators could influence mucus release is unknown.

4.3.4.3 Effect of intragastric application of agents on mucosal histology.

Treatment with saline, ISDN (0.1, 0.5 and 1.0 mM) and Db-cGMP (1 mM) had no effect on the histological appearance of the gastric epithelium and on the % of the section length exhibiting cell exfoliation (Fig. 4.29; Plates 4.2, 4.3 and 4.4). However,

exposure of the epithelium to 2 ml of 50% ethanol, which was used as a positive control, resulted in significant damage ($P<0.01$ by ANOVAR and Dunnett's test; Fig. 4.23 and Plate 4.5). The increase in the thickness of the mucous gel layer induced by NO donors and Db-cGMP did not seem to be due to epithelial cell damage.

Plate 4.2 Transverse section of rat gastric mucosa prepared as described in section 4.2.7 (x50 magnification), following intragastric exposure to 2 ml saline for 30 min. Bar represents (200 μm).

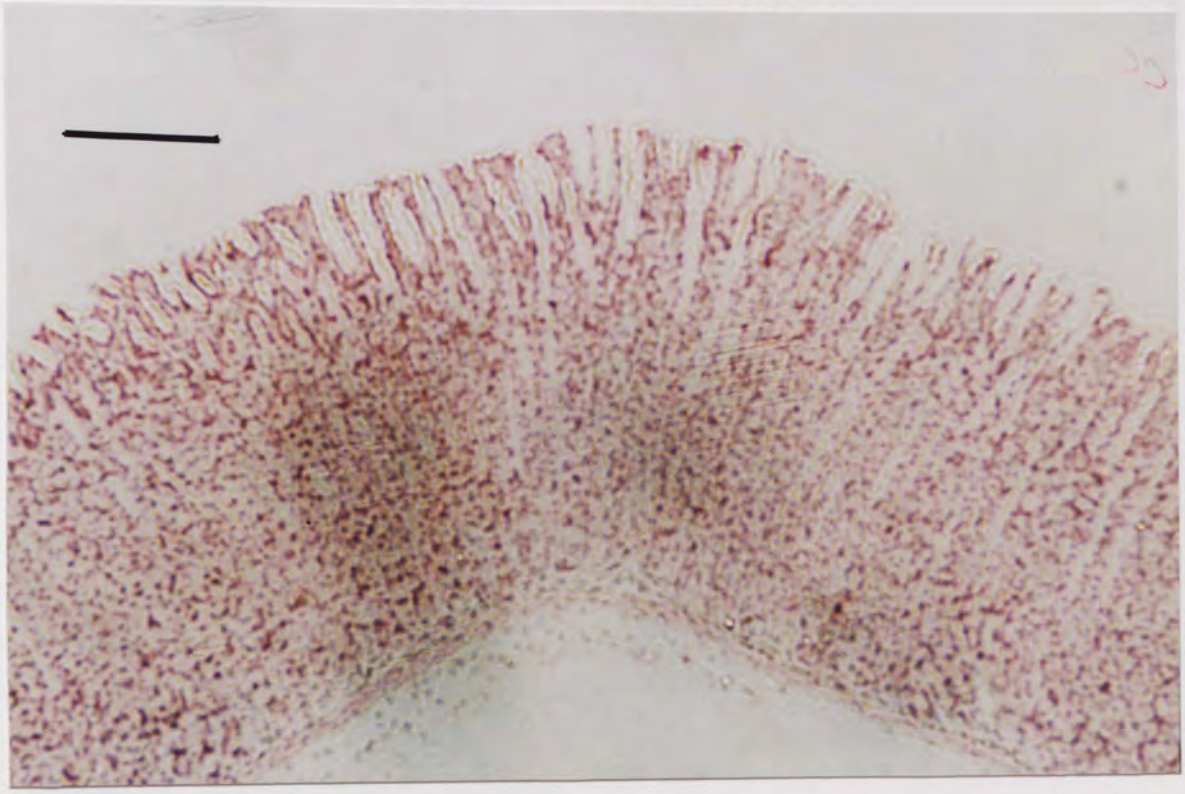


Plate 4.3 Transverse section of rat gastric mucosa prepared as described in section 4.2.7 (x50 magnification), following intragastric exposure to 2 ml of 1 mM ISDN for 30 min. Bar represents (200 μm).

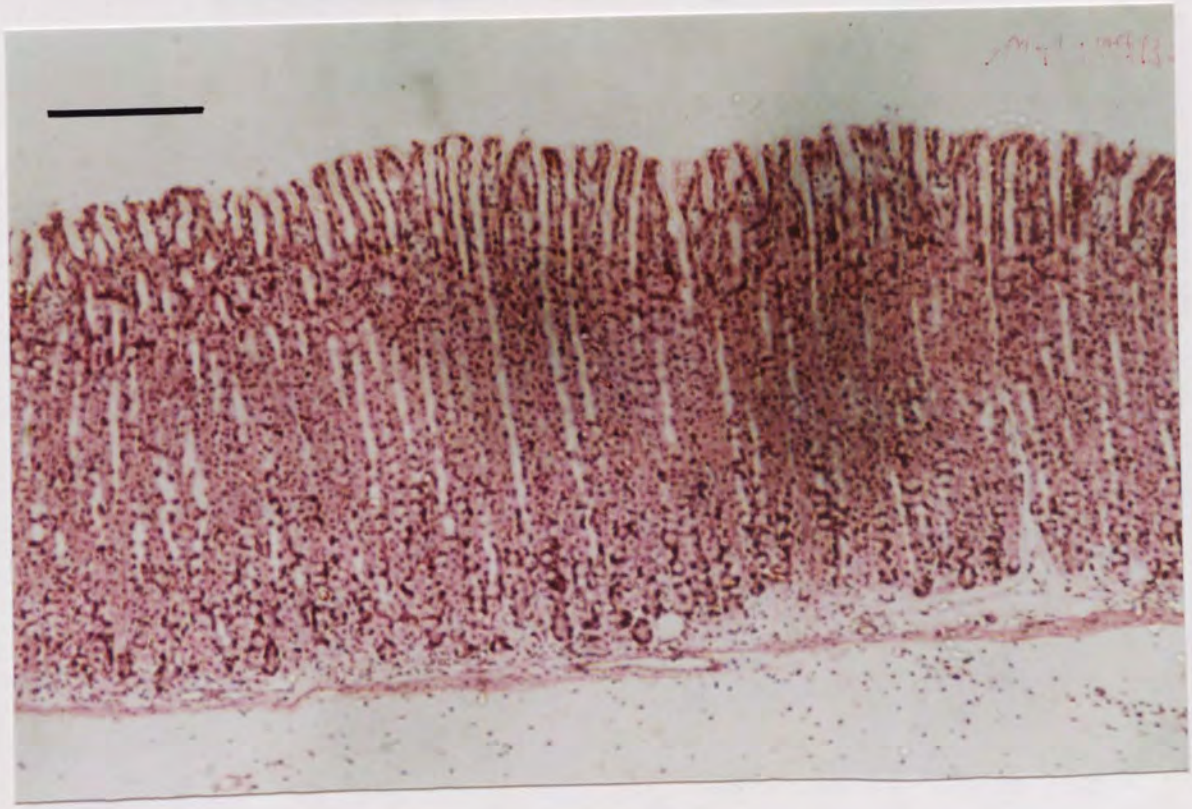


Plate 4.4 Transverse section of rat gastric mucosa prepared as described in section 4.2.7 (x50 magnification), following intragastric exposure to 2 ml of 1 mM Db-cGMP for 30 min. Bar represents (200 μm).

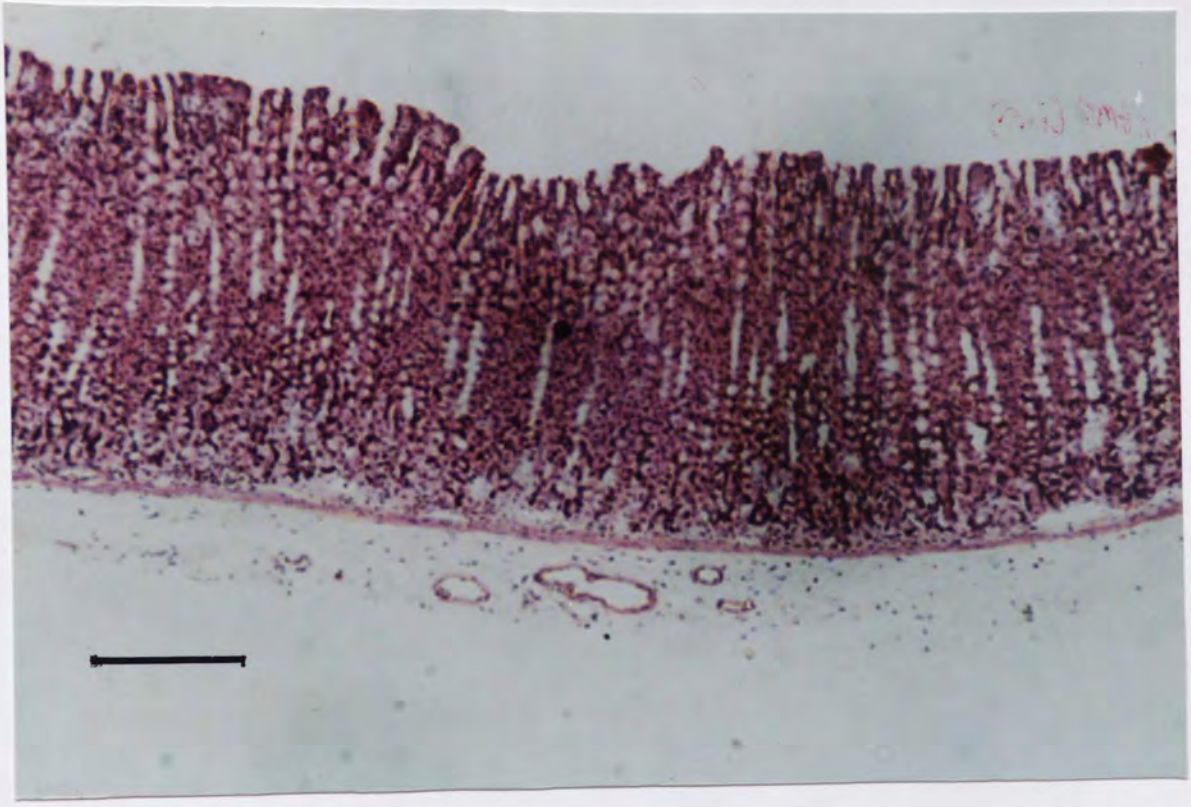


Plate 4.5 Transverse section of rat gastric mucosa prepared as described in section 4.2.7 (x50 magnification), following intragastric exposure to 2 ml of 50% ethanol for 30 min. Bar represents (200 μm).

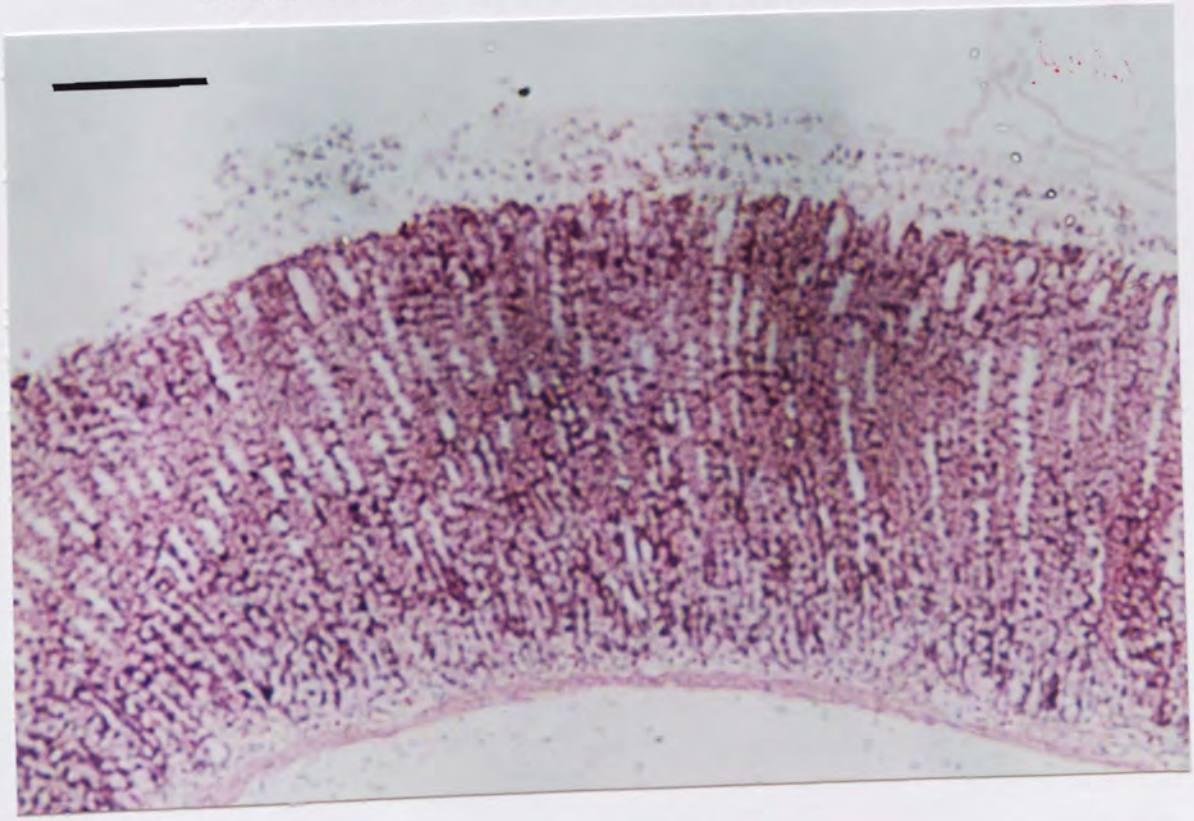
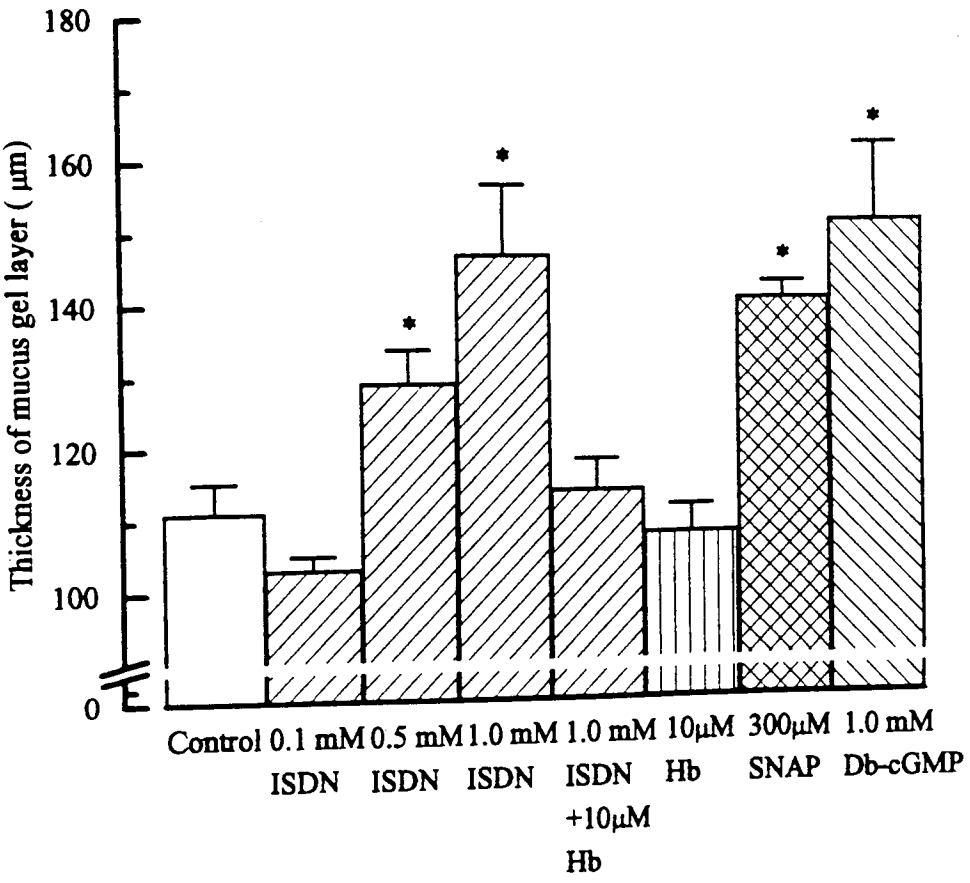
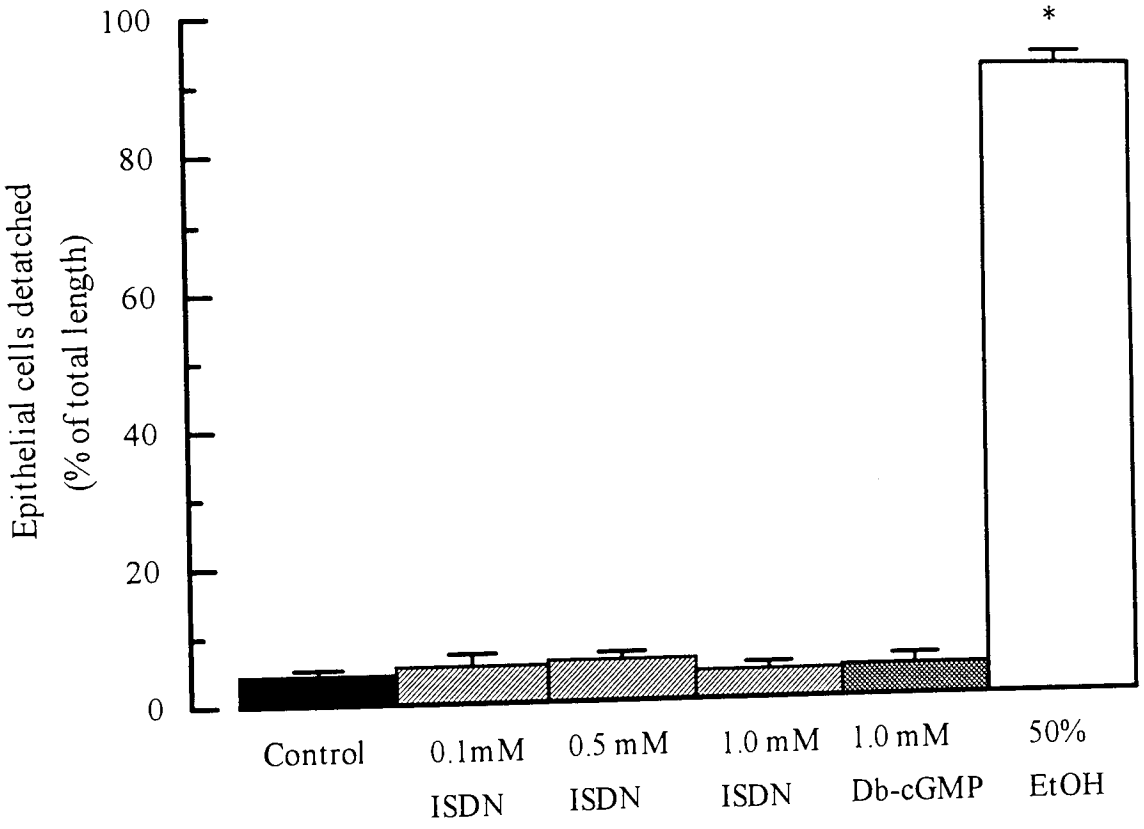


Figure 4.22 Effect of NO donors, oxyhaemoglobin (Hb) and Db-cGMP on the thickness of the gastric mucous gel layer *in vivo*.



Data are presented as mean±SEM (n=5 rats), where *P<0.01 by a Kruskal-Wallis test and a non-parametric equivalent of Dunnett's test (See Appendix A.6.3), for difference from control.

Figure 4.23 **Effect of isosorbide dinitrate (ISDN), dibutyryl-cyclic GMP (Db-cGMP) and 50% ethanol (50% EtOH) on the percentage length of longitudinal gastric mucosal sections covered with detatched nucleated cells following intragastric instillation of 2 ml of agent and an exposure time of 30 min *in vivo*.**



Data are presented as mean±SEM (n=4 different stomachs), where *P<0.01 by one-way ANOVAR and Dunnett's test for difference from control.

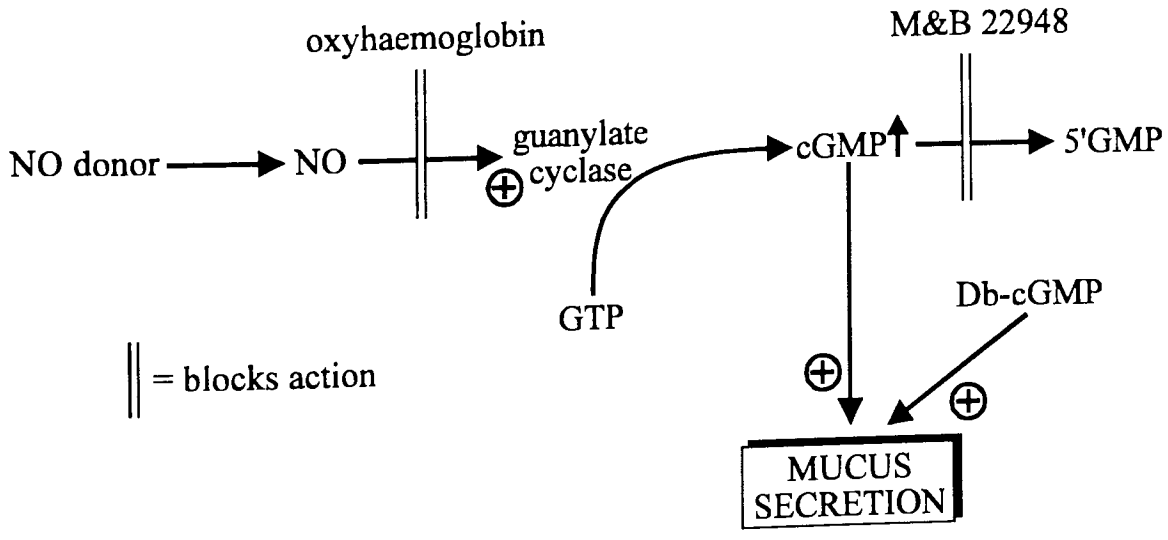
4.3.4.4 Conclusion from measurements of mucous gel thickness.

These results obtained *in vivo* suggest that NO and cGMP may play a role in the secretion of mucus from the rat gastric mucosa. The changes in the thickness of the mucus gel layer produced by intragastric administration of ISDN and SNAP *in vivo* were similar to those obtained with carbachol, an established stimulator of mucus release (McQueen *et al.*, 1984) suggesting that the effects of NO could be quantitatively important.

4.4 SUMMARY

1. The ELISA for measuring the mucin content of the incubation medium appeared a reliable and quantitative index for measurement of changes in secretory activity by gastric mucous cells.
2. Nitric oxide donors increased both mucin release from, and the cGMP content of, gastric epithelial cell suspensions at concentrations which parallelled one another without a decrease in the integrity of the cell membrane or an increase in the release of lysosomal enzymes.
3. The stimulatory effects of nitric oxide donors could be blocked by co-administration of the nitric oxide scavenger oxyhaemoglobin.
4. The cGMP-specific phosphodiesterase inhibitor, M&B 22948 increased both the cGMP content of and mucin release from, isolated gastric epithelial cells in a concentration dependent manner.
5. Intragastric administration of nitric oxide donors and the cyclic-GMP analogue Db-cGMP, caused a concentration dependent increase in the mucous gel thickness without apparent damage to the gastric mucosa.

Figure 4.24 **Summarises in diagramatic form what is suggested by the work in this chapter.**



Integration of these data with that in other chapters is presented in the general discussion (Chapter 7).

Chapter Five

INHIBITION OF SECRETORY ACTIVITY IN ISOLATED PARIETAL CELLS BY NITRIC OXIDE DONORS

5.1 INTRODUCTION.

The general morphology of the rat gastric mucosa has previously been discussed in chapter one. However, a more detailed discussion of the functional aspects of the gastric parietal cell and of the factors which influence secretory activity will be presented here.

5.1.1 Changes in Parietal Cell Morphology During Secretion.

Following exposure of the parietal cell to secretagogues the secretory canaliculi become more prominent and the microvilli elongate resulting in an increase in the surface area of the apical membrane of between 6 and 10 times that of the resting cell. This change is quantitatively matched with a decrease in the surface area of the tubulovesicles (Helander & Hirschwitz, 1972).

Two general mechanisms have been proposed to account for the changes in intracellular structure that accompany secretory activity in stimulated parietal cells. The osmotic expansion theory (Berglindh *et al.*, 1980) suggests that the tubulovesicles are collapsed canaliculi. Following stimulation, HCl accumulates inside the lumen of the acid-secreting spaces producing a hypertonic environment, which results in water movement into the canaliculi and their subsequent expansion. However, salt-induced water accumulation within tubulovesicles produced vacuolar swellings without expansion of the apical membrane surface area (Gilbert & Hersey, 1982). Hence the production of an osmotic gradient alone cannot account for both secretory canaliculi formation and expansion of the surface area of the apical membrane.

The second hypothesis (Forte *et al.*, 1977) suggested that stimulation of secretory activity was associated with migration of the cytoplasmic tubulovesicles towards the apical membrane and their subsequent fusion of the tubulovesicles with it. Immunocytochemical studies have revealed that the H^+/K^+-ATPase (or proton pump) is predominantly located in the tubulovesicles in the resting cell, but following stimulation, the H^+/K^+-ATPase also becomes detectable on the microvilli of the secretory canaliculi at the apical membrane (Smolka *et al.*, 1984). It is likely that both hypothesised mechanisms operate in combination under physiological conditions.

5.1.2 Mechanism of acid secretion.

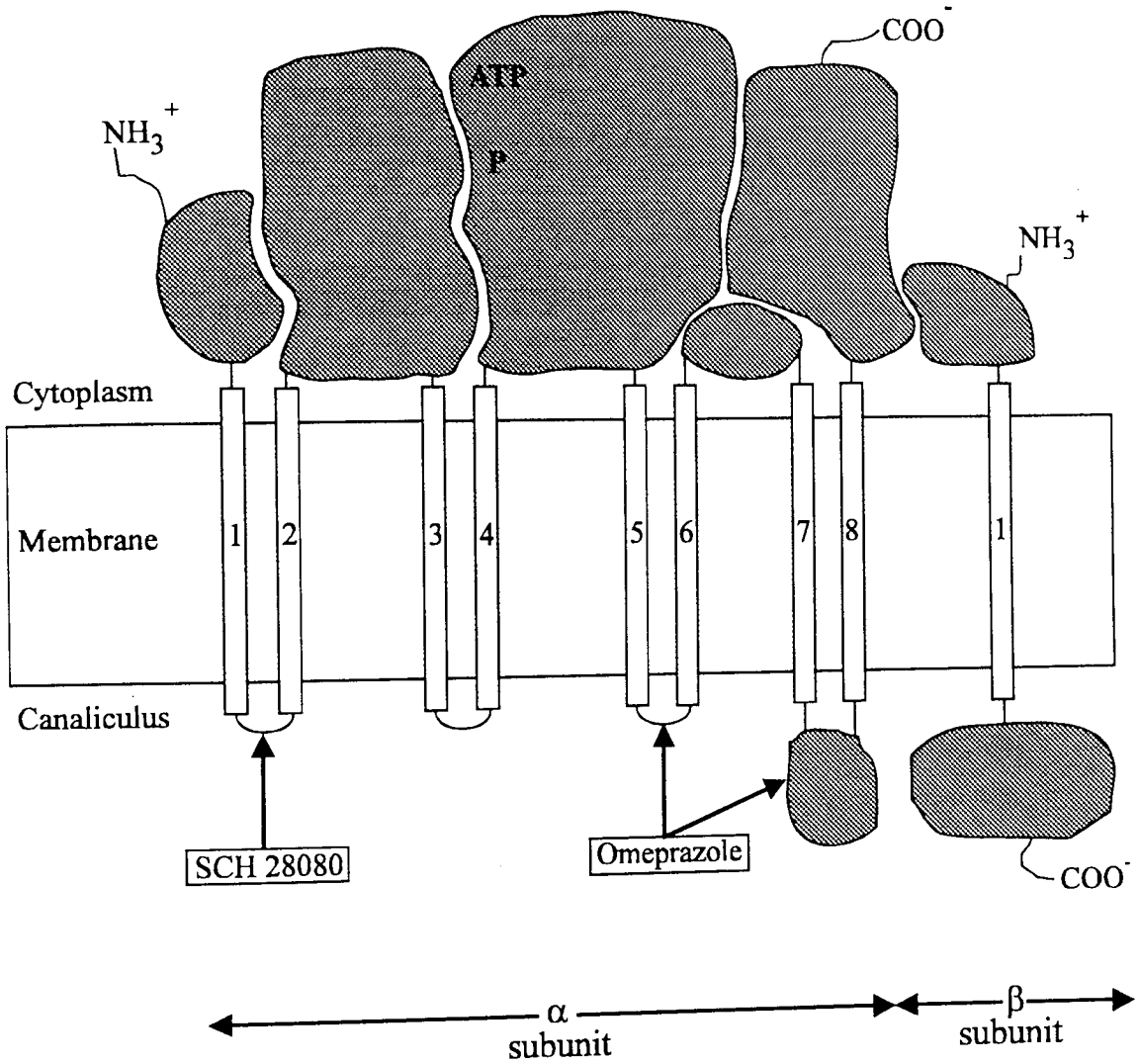
5.1.2.1 The gastric proton pump.

The protein directly responsible for the secretion of acid by the gastric parietal cell is an ATP-dependent ion pump which exchanges protons from the cytoplasm in exchange for extracellular K^+ (Lee *et al.*, 1974). The H^+/K^+ exchange pump is electroneutral (i.e. 1:1 exchange) and the enzyme became known as the gastric H^+/K^+-ATPase (Sachs *et al.*, 1976).

The H^+/K^+-ATPase is composed of a 100 kDa α-subunit and a smaller glycosylated β-subunit with a protein core of approximately 33 kDa. Molecular biological techniques have provided the primary amino acid sequences of both subunits from various species including man (Maeda *et al.*, 1989) and rat (Shull *et al.*, 1986) with the sequences of the α-subunit having a 98% homology between rat, pig and man. The α -subunit is predicted as having between 7 and 10 membrane spanning domains (Mercier *et al.*, 1991; Smolka *et al.*, 1991) with the majority of the protein being situated on the cytoplasmic side of the secretory membrane (Fig. 5.1). The β-subunit is predicted as having only one transmembrane domain with the greater part of the protein lying on the extracellular side of the membrane. The α-subunit appears to be responsible for all the activities involved in pumping and contains binding sites for ATP, the transported ions and all inhibitors known to date (Helander & Keeling, 1993). The precise function of the β-subunit is unclear although it may be involved in stabilising the α-subunit or in targeting the α-subunit to the apical membrane (Helander & Keeling, 1993).

A characteristic of the proton pump is that the α-subunit becomes transiently phosphorylated in the course of ATP hydrolysis (Waldenhaug *et al.*, 1985) classifying it as a member of the P-type (phosphorylated) ion pumps. Other members of this family include the Na^+/K^+-ATPase and the Ca^{2+}-ATPase, with the proton pump having a 63% sequence homology with the Na^+/K^+-ATPase (Helander & Keeling, 1993).

Figure 5.1 **Diagrammatic representation of the H^+/K^+-ATPase (proton pump), indicating the proposed sites of interaction for SCH 28080 and omeprazole (Helander & Keeling, 1993).**



KEY:
ATP = ATP binding site,
P = Phosphorylation site.

5.1.2.2 Associated ion fluxes.

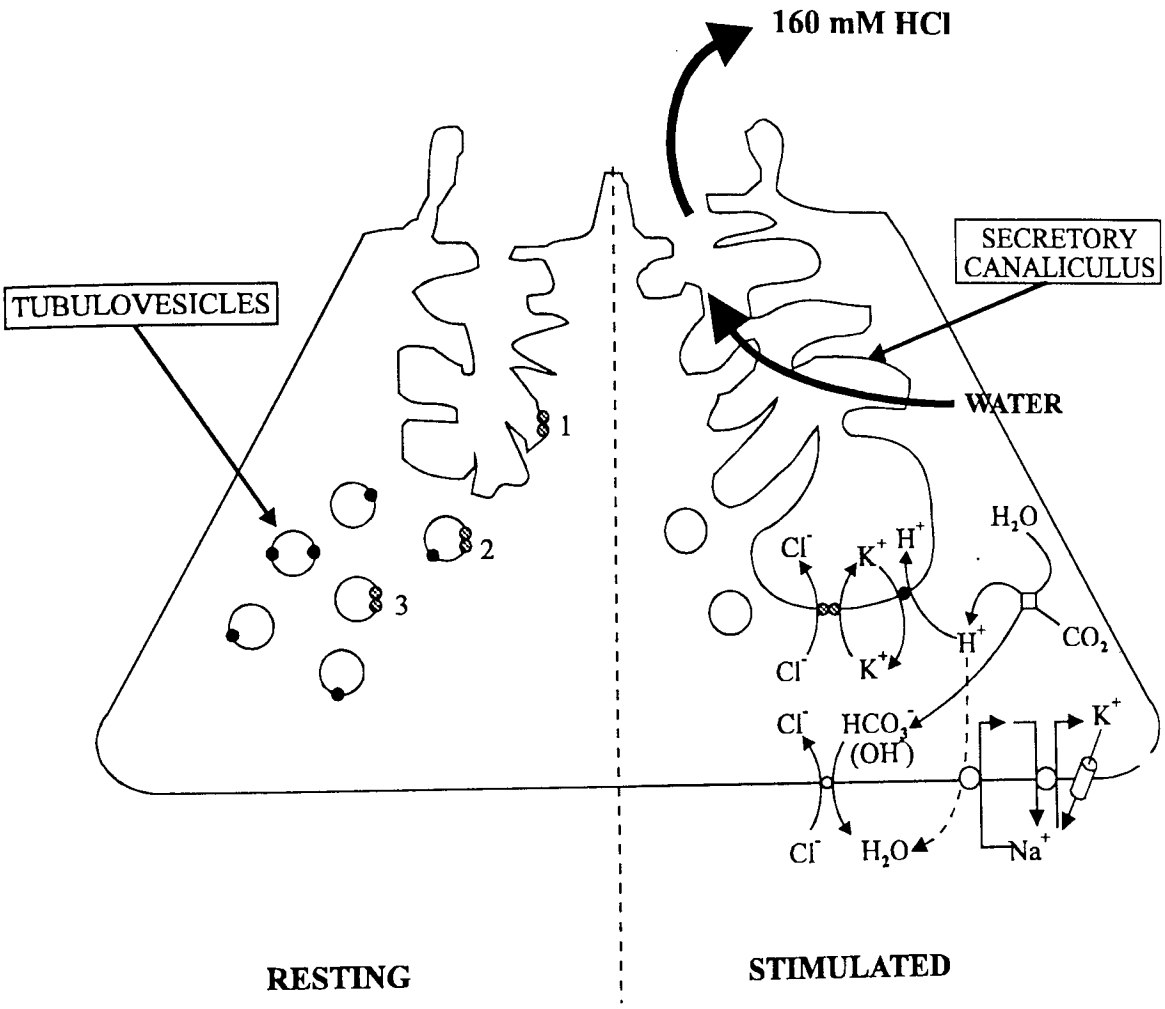
The gastric proton pump exchanges intracellular H^+ ions for extracellular K^+. The K^+ required for the exchange of H^+ enters the lumen of the secretory canaliculus via conductance pathways in the apical membrane in association with Cl^-(Wolosin & Forte, 1985). The formation of HCl within the lumen of the secretory canaliculi is achieved when the K^+ is recycled back into the cytoplasm via the H^+/K^+-ATPase. The presence of HCl within the secretory canaliculi creates an osmotic gradient and therefore a net flow of water entering the lumen (Forte & Wolosin, 1987). Berglindh *et al.*, (1980)

proposed that the swelling associated with the movement of water into the canaliculi is restricted by the basement membrane, connective tissue and muscularis mucosa and thus the HCl is forced from the cell into the lumen of the gastric gland in intact tissue.

In order to provide a ready supply of hydrogen ions to the proton pump, the parietal cell contains significant amounts of carbonic anhydrase which combines water and carbon dioxide to form carbonic acid and ultimately H^+ and HCO_3^- ions. The intracellular pH of the parietal cell during periods of secretory activity is closely regulated by a HCO_3^-/Cl^- exchanger (Fig. 5.2) which extrudes a molar equivalent of base across the basolateral membrane for every proton crossing the secretory canaliculus (Forte & Wolosin, 1987). This means that direct measurement of acid secretion by suspensions of isolated cells is not possible. When the parietal cell is not secreting acid, H^+ is produced as a product of metabolism and Na^+/H^+ exchange may be responsible for regulating intracellular pH under these conditions (Madshus, 1988).

During periods of secretory activity the Cl^- ions are provided by the HCO_3^-/Cl^- exchanger. Gastric juice has a K^+ concentration two times that of plasma K^+. Therefore, there is also a homeostatic requirement for K^+ as well as Cl^- ion uptake. During secretion a high intracellular K^+ and low Na^+ concentration is maintained by the Na^+/K^+-ATPase (Fig. 5.2). The low intracellular Na^+ concentration may provide the negative intracellular membrane potential to drive the Na^+/H^+ exchanger, which in conjunction with H^+/K^+-ATPase activity will raise intracellular concentrations of HCO_3^- ions. These elevated base levels may provide the driving force for Cl^- uptake via the Cl^-/HCO_3^- exchanger (Forte & Wolosin, 1987).

Figure 5.2 Diagrammatic representation of the associated ion fluxes involved in the secretion of acid by the gastric parietal cell.



Key to symbols:

- = K^+ pump
- ∞ = K^+/Cl^- conductances
- • = H^+/K^+-ATPase (Proton pump)
- ○ = Na^+/K^+ exchanger
- ○ = Na^+/H^+ exchanger
- □ = Carbonic anhydrase enzyme

1,2 & 3 = possible location of K^+/Cl^- conductances

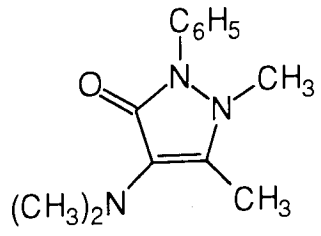
5.1.3 Methods for measuring acid secretion.

If *in vivo* systems are used then the secretory response can be measured directly by sampling the gastric juice and performing a titration to determine the concentration of hydrogen ions in the secreted fluid.

In some cases, the use of isolated cell preparations is desirable but as a result of cell disruption, polarity is lost and as mentioned above, acid secretion cannot be measured directly. Indirect procedures have to be utilised to study parietal cell function *in vitro*. Oxygen consumption has provided one method for correlating the amount of acid secretion by the gastric mucosa in various preparations and species (Bannister, 1965; Moody, 1968). This is because acid secretion which involves moving protons up a 10^6 fold gradient of concentration requires substantial activity of oxidative phosphorylation to supply the ATP power source and even in mixed cell suspensions, changes in respiratory activity are likely to reflect changes in parietal cell function (Soll, 1978). Glucose oxidation measured as the production of $^{14}CO_2$ from [^{14}C]-glucose has also been successfully used to measure secretory activity in canine parietal cells (Davidson *et al.*, 1981).

Probably the most widely used technique for measuring acid secretion in isolated parietal cell suspensions is the measurement of accumulation of the radiolabelled weak base aminopyrine (Fig. 5.3), within acidic spaces inside the parietal cell (Berglindh *et al.*, 1976, Soll, 1980a; Hatt & Hanson, 1988).

Figure 5.3 Chemical structure of aminopyrine.



pK_a=4

The pK_a of aminopyrine is about 4, which means that accumulation of the membrane impermeable protonated form of the base will occur in compartments of high acidity but not in compartments such as lysosomes or secretory granules.

The accumulation ratio (R) of the weak base is determined by the following equation:-

$$R = \frac{1 + 10(pK_a - pH_i)}{1 + 10(pK_a - pH_o)}$$

Where:-

pK_a = dissociation constant of weak base,
pH_i = pH inside the defined acidic compartment,
pH_o = extracellular pH,
R = ratio of intracellular to extracellular concentration of weak base.

It is important to point out that aminopyrine accumulation is an indirect measurement of acid secretion by parietal cells. However comparison with other indices suggests that this method does provide a quantitative and reliable index of parietal cell function *in vitro*. One problem is that aminopyrine accumulation varies quite considerably between cell batches but fortunately, relative rates of cell responsiveness are usually adequate for receptor and intracellular mechanism studies and it is usual to normalise results (Soll & Berglindh, 1987).

5.1.4 Stimulation of secretory activity.

Classical physiological studies have demonstrated essentially three types of receptors which are important in the stimulation of acid secretion by the parietal cell. These receptors are stimulated by acetylcholine, gastrin and histamine.

5.1.4.1 Acetylcholine.

Neuronal stimulation of acid secretion is achieved through parasympathetic input via vagal nerves. The nerve endings are in close proximity (100-200 nm) to the parietal cells and release acetylcholine which stimulates muscarinic M_3 receptors (Pfeiffer *et al.*, 1990) located on the basolateral membrane of the parietal cells

(Nakamura *et al.*, 1985). Activation of muscarinic receptors on the parietal cells leads to activation of phospholipase C and breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate (IP_3) (Puurunen & Schwabe, 1987) and diacylglycerol (DAG) (Pfeiffer *et al.*, 1987). IP_3 causes the mobilisation of Ca^{2+} from intracellular stores (Tsunoda *et al.*, 1988) while DAG activates protein kinase C (Park *et al.*, 1987).

Cholinergic stimulation may cause changes in intracellular protein phosphorylation by elevating intracellular Ca^{2+} concentrations, activating specific Ca^{2+}-calmodulin protein kinases or by activating protein kinase C (Brown & Chew, 1989).

5.1.4.2 Gastrin.

Gastrin is secreted by the G-cells of the gastric antrum and the upper region of the duodenum into the blood stream as two major forms, G-77 and G-34. It binds directly with gastrin receptors on the basal membrane of the parietal cell in the rat (Nakamura *et al.*, 1987). Gastrin stimulates secretory activity in dog (Soll, 1980a), rabbit (Chew & Brown, 1986) and guinea pig parietal cells (Tsunoda, 1987). In rat parietal cells it potentiates the action of histamine but does not exert a stimulatory effect by itself. Gastrin stimulated secretory activity is associated with an increase in intracellular concentrations of inositol trisphosphates (Chiba *et al.*, 1988), Ca^{2+} (Chew & Brown, 1986) and diacylglycerol (Chiba *et al.*, 1988). Therefore, the intracellular signalling mechanisms employed by gastrin appear to be the same as those utilised by carbachol, namely the activation of phosphoinositide breakdown (Hanson & Hatt, 1989).

5.1.4.3 Histamine

Histamine is stored in the rat gastric mucosa by two types of cells, namely the mast cells and enterochromaffin-like cells, although only the latter are believed to be involved in the physiological stimulation of acid secretion (Andersson *et al.*, 1990). The enterochromaffin-like cells have muscarinic type M_1 receptors which when stimulated by acetylcholine released from vagal efferent neurones results in the localised release of histamine from these cells (Helander & Keeling, 1993). Furthermore, gastrin receptors

have also been demonstrated on enterochromaffin-like cells which when activated also result in the localised release of histamine (Håkanson & Sundler, 1991).

Evidence from ligand-binding experiments for the presence of histamine receptors on parietal cells has been complicated by the occurrence of histamine uptake by parietal cells (Albinus & Sewing, 1981). However, histamine caused a concentration-dependent increase in aminopyrine accumulation, oxygen consumption and glucose oxidation in parietal cells and gastric glands (Soll & Berglindh, 1987) and the histamine H_2-receptor antagonist cimetidine competitively and completely abolished secretory activity in isolated canine parietal cells (Soll, 1980a). Thus, histamine appears to stimulate secretory activity by interacting with an H_2-receptor located on the plasma membrane. Babo *et al.*, (1992) recently demonstrated H_3-like receptors in rabbit gastric glands with the specific H_3-receptor agonist (*R*)-α-methyl-histamine reducing secretory activity and the specific H_3-receptor antagonist thioperamide enhancing secretory activity.

Histamine elevates the cAMP content of parietal cells of many species including the rat (Schepp *et al.*, 1983b). The cell permeable analogue of cAMP, Db-cAMP (Soll, 1980a) and forskolin, a direct activator of adenylate cyclase (Chew, 1983a) both stimulate secretory activity. Histamine activates adenylate cyclase in sonicates from rat parietal cells (Thompson *et al.*, 1981). Since histamine activates adenylate cyclase, in parietal cells of many species its action is potentiated if cAMP breakdown is reduced by the general cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Soll & Wollin, 1979). Furthermore, histamine has been demonstrated to activate type-I cAMP-dependent protein kinase (Chew, 1985). Histamine H_2-receptor activation also mediated increases in intracellular Ca^{2+} concentrations but it is unclear whether these events are directly related to an activation of secretory activity (Ljungström & Chew, 1991).

5.1.5 Direct inhibitors of secretory activity.

5.1.5.1 Prostaglandins.

PGE_2 inhibits histamine-stimulated secretory activity in canine and rat parietal cell suspensions (Soll, 1980a; Rosenfeld, 1986), and reduces the cAMP content of rat parietal cells stimulated with histamine (Schepp *et al.*, 1983a). The inhibitory effects of PGE_2 appears to be mediated via a PGE_2 receptor on the parietal cell (Seidler *et al.*, 1989a) and may involve a receptor-mediated inhibition of adenylate cyclase by the guanine-nucleotide dependent protein, Gi (Chen *et al.*, 1988).

5.1.5.2 Epidermal Growth Factor (EGF) and Transforming Growth Factor-α (TGFα).

EGF directly inhibits histamine-stimulated secretory activity in parietal cells and gastric glands (Chen *et al.*, 1984; Reichstein *et al.*, 1984). Furthermore, EGF inhibited the histamine-stimulated increase in cAMP content of a suspension of rat gastric mucosal cells containing over 80% parietal cells (Hatt & Hanson, 1989). These results suggest that EGF mediates its inhibitory effect on histamine-stimulated secretory activity via a decrease in the cAMP content of parietal cells. The phosphodiesterase inhibitor IBMX blocked the inhibitory effect of EGF suggesting that EGF lowers the cAMP content via stimulation of cAMP breakdown (Hanson & Hatt, 1989).

TGF-α shares approximately a 33% amino acid sequence homology with EGF (Marquardt *et al.*, 1984), and has been demonstrated to inhibit histamine-stimulated aminopyrine accumulation in suspensions of rabbit parietal cells, but unlike EGF, was without effect in cells stimulated with carbachol (Rhodes *et al.*, 1986). The inhibitory effect of TGF-α is thought to involve a specific uncoupling of histamine/cAMP signal transduction via G_i (Lewis *et al.*, 1990).

5.1.5.3 Somatostatin.

Somatostatin receptors have been identified on parietal cells from the rat and dog stomach (Reyl *et al.*, 1979; Park *et al.*, 1987). Somatostatin inhibited histamine-stimulated aminopyrine accumulation in parietal cells from guinea-pig (Batzri, 1981), rat

(Attwell & Hanson, 1988), rabbit (Chew, 1983b) and dog (Park *et al.*, 1987), with the inhibition of secretory activity being associated with a decrease in the cAMP content of the parietal cells (Park *et al.*, 1987). Preincubation of cells with pertussis toxin abolished the effect of somatostatin on both secretory activity and the cAMP content (Park *et al.*, 1987; Attwell & Hanson, 1988), suggesting that somatostatin receptors on parietal cells are linked via G_i to inhibition of adenylate cyclase.

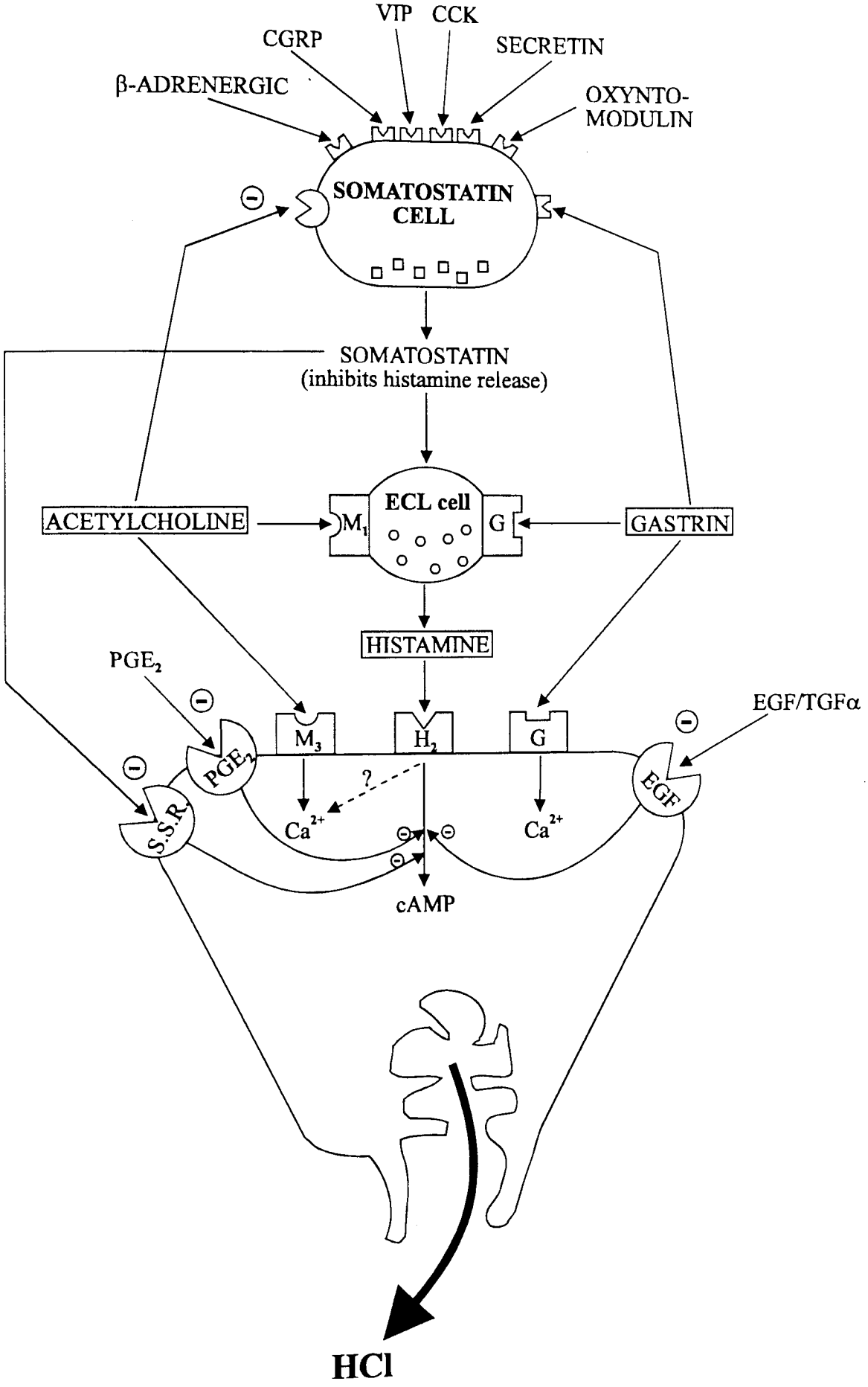
5.1.6 Indirect inhibitors of secretory activity.

Secretin, vasoactive intestinal polypeptide, cholecystokinin, calcitonin gene-related peptide, oxyntomodulin and β-adrenergic agonists may all exert indirect effects on acid secretion by promoting the release of somatostatin (Fig. 5.4).

5.1.6 Effects of nitric oxide (NO) on acid secretion.

When this work commenced, little evidence was available on whether NO was involved in the regulation of acid secretion. However, Uehara *et al.*, (1990) demonstrated that i.p. injection of lipopolysaccharide (an agent known to induce the expression of a Ca^{2+}-independent form of NOS) produced a dose-dependent (10-1000 ng/rat) inhibition of acid and pepsin secretion in pylorus-ligated male Wistar rats between 2 h and 8 h following injection. This suggests that sustained production of NO may modulate acid secretion in the rat. The aim of this section of work was to determine if NO could exert a direct effect upon histamine-stimulated secretory activity in an enriched parietal cell suspension from the rat gastric mucosa. NO was delivered by using NO donors (see Section 4.1.5).

Figure 5.4 Potential interactions between stimulators and inhibitors of acid secretion. All receptors are stimulatory unless indicated otherwise.



Key for Figure 5.4

CCK	= cholecystokinin
CGRP	= calcitonin gene related peptide
ECL	= enterochrommaffin-like cell
EGF	= epidermal growth factor
PGE_2	= prostaglandin E_2
S.S.R	= somatostatin receptor
TGFα	= transforming growth factor-α
VIP	= vasoactive intestinal polypeptide

5.2 METHODOLOGY

5.2.1 Measurement of secretory activity in intact cells.

A crude suspension of gastric mucosal cells was prepared as described in section 2.1.2 and a low density fraction containing greater than 80% parietal cells was prepared by density gradient centrifugation as described in section 2.2.1. This fraction was used to assess the effects of agents on histamine-stimulated secretory activity. To enable recovery of function (Hatt & Hanson, 1989), following separation on the density gradient and washing to remove Percoll®, the low density fraction was resuspended in medium B' (Table 2.1) containing foetal calf serum (5% v/v) and gentamicin (50 μg/ml) at a concentration of 2 x 10^6 cells/ml. The cell suspension was then incubated for 2 h at 37°C with shaking (60 cycles/min) and continuous gassing of the airspace above the cells with 95% O_2/5% CO_2 in a capped, 25 ml polycarbonate conical flask. The cells were gently dispersed every 30 min using a plastic transfer pipette to eliminate cell aggregation. Following the 2 h preincubation, cells were washed twice by centrifugation at 125g for 7 min at 15°C and then resuspended in 10 ml of medium B'. The washed pellet was finally resuspended, usually at a cell concentration of 1.0 x 10^6 cells/ml in medium B' containing 0.1 mM IBMX. The presence of IBMX increased the response of the cells to histamine and reduced the breakdown of cAMP and cGMP (Soll 1980b; Hatt & Hanson 1989). Aliquots of 1.5 ml of the cell suspension were transferred to 20 ml polypropylene incubation vials containing [^{14}C]-aminopyrine (0.1 μCi/ml, 0.9 μM) and the required secretagogues and agents. Equal volumes of vehicles were added and the combined volumes of secretagogues agents and vehicles was then made up to 100 μl with saline (0.9% w/v). The contents of the vials were gassed for 5 s with 95% O_2/5% CO_2 whilst gently swirling to ensure thorough mixing before being capped and incubated at 37°C for 30 min in a water bath with continuous shaking (120 cycles/min).

5.2.3 Determination of aminopyrine accumulation ratio.

Following the incubation period (30 min for whole cells stimulated with 0.5 mM histamine and 10 min for permeabilised cells stimulated with 2.5 mM adenosine-5'-trisphosphate, (ATP)) two 0.5 ml aliquots were removed from each incubation vial and were centrifuged at 10,000g for 30 s. The supernatant was removed by aspiration and the surface of the pellet and inside of the centrifuge tube were washed by adding 0.5 ml medium B' to each tube followed by centrifugation at 10,000g for 10 s. The supernatants were again aspirated prior to addition of 0.5 ml of 1M NaOH to each tube which was left at room temperature overnight before removal of 0.45 ml for determination of radioactive content by liquid scintillation counting. (Appendix A.3).

The aminopyrine accumulation ratio was determined for each incubation vial as described in Appendix A.4.

5.2.4 Measurement of secretory activity in permeabilised cells.

A low density fraction of gastric mucosal cells was prepared as described in 2.2.1 and was resuspended in medium B' prior to centrifugation at 125g for 7 min at 15°C. The pellet was then washed once in a sodium free medium (pH 7.4) containing 100 mM KCl, 1.5 mM $MgCl_2$, 50 mM HEPES, 1.5 mM Tris-phosphate, 10 mM succinic acid, 0.1 mM IBMX and 1 mg/ml BSA before final resuspension at a cell concentration of 2×10^6 cells/ml. A 1 ml aliquot of cells was transferred to 20 ml polypropylene incubation vials and incubated for 20 min at 37°C with shaking (60 cycles/min) in the presence or absence of either the Rp or Sp isomer of 8-bromoguanosine 3',5'-monophosphorothioate (Rp-8-Br-cGMPS, Sp-8-Br-cGMPS) or para-chlorophenylthioguanosine 3',5'-cyclic monophosphate (8-PCPT-cGMP) at a final concentration of 350 µM. Following the 20 min incubation, SNAP (final concentration 1 mM) was added where appropriate with an equivalent volume of vehicle being added to control vials and incubation was continued for a further 30 min prior to permeabilisation with digitonin (10 µg/ml) and addition of [^{14}C]-aminopyrine (0.1 µ Ci/ml) and ATP (2.5 mM). The total volume of the additions was made up to 100 µl

with the sodium free incubation medium and the cells were incubated for a further 10 min prior to determination of the aminopyrine accumulation ratio as described in section 5.2.3.

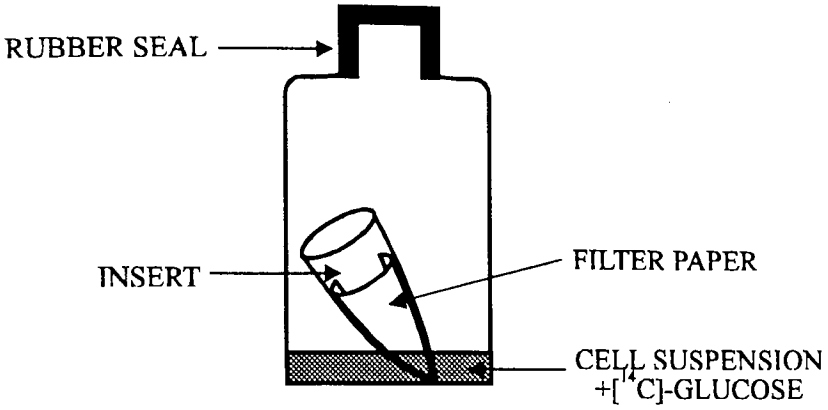
5.2.5 Measurement of glucose oxidation.

A low density cell fraction of gastric mucosal cells was prepared as described in 2.2.1 and was resuspended in medium B' at a cell concentration of 1 x 10^6 cells/ml. A 1 ml aliquot of cells was transferred to 20 ml capacity polypropylene incubation vials containing D-[U-^{14}C]-glucose (0.5 µCi/ml) and combinations of histamine (0.5 mM), SNAP (1 mM) or vehicle (final volume of additions was made up to 100 µl with saline (0.9% w/v NaCl) and all concentrations are the final concentrations following additions). Also inside the incubation vials were inserts containing a 1 cm^2 strip of filter paper (Fig. 5.5). The air space above the cell suspension was gassed for 5 s with 95%O_2/5%CO_2 prior to capping with rubber seals and incubation for 30 min at 37°C and constant shaking (60 cycles/min). Evolved $^{14}CO_2$ was collected by injecting 100 µl of 3 M $HClO_4$ through the rubber seal into the cell suspension and collecting the CO_2 released by injecting 100 µl of 0.1 M NaOH into the insert and onto the filter paper. Radiolabelled products were detected by liquid scintillation counting by transferring the filter paper strip to 20 ml polypropylene scintillation vials followed by addition of 10 ml of Optiphase HiSafe II scintillation fluid. This provided a measure of glucose oxidation via the production of $^{14}CO_2$ which was calculated as described below:

$$\text{Glucose produced (nmol / } 10^6 \text{ cells)} = \frac{\text{d.p.m. of filter paper}}{\text{SpA}} \times \frac{1 \times 10^6}{[\text{cell}]\ (\times 10^6 / \text{ml})}$$

Where:
SpA = Specific activity of cell culture medium (200 d.p.m./nmol)

Figure 5.5 System used to measure glucose oxidation.



5.2.6 Presentation of results.

Where indicated the effect of agents is expressed as the percentage inhibition of secretory activity with secretion in the presence of 0.5 mM histamine for intact cells and 2.5 mM ATP for permeabilised cells being assigned the value of 100%. Percent inhibition was calculated following correction for basal secretory activity according to the following equation:

$$\% \text{ INHIBITION} = 100 - \left(\frac{(MAX_{APR} - BASAL_{APR}) - (AGENT_{APR} - BASAL_{APR})}{(MAX_{APR} - BASAL_{APR})} \times 100\right)$$

Where:

MAX_{APR} = Aminopyrine accumulation ratio in the presence of secretagogue alone (0.5 mM histamine or 2.5 mM ATP

$BASAL_{APR}$= Aminopyrine accumulation under resting conditions

$AGENT_{APR}$= Aminopyrine accumulation in the presence of agent and secretagogue

5.3 RESULTS & DISCUSSION.

5.3.1 Effects of agents on secretory activity in whole cells.

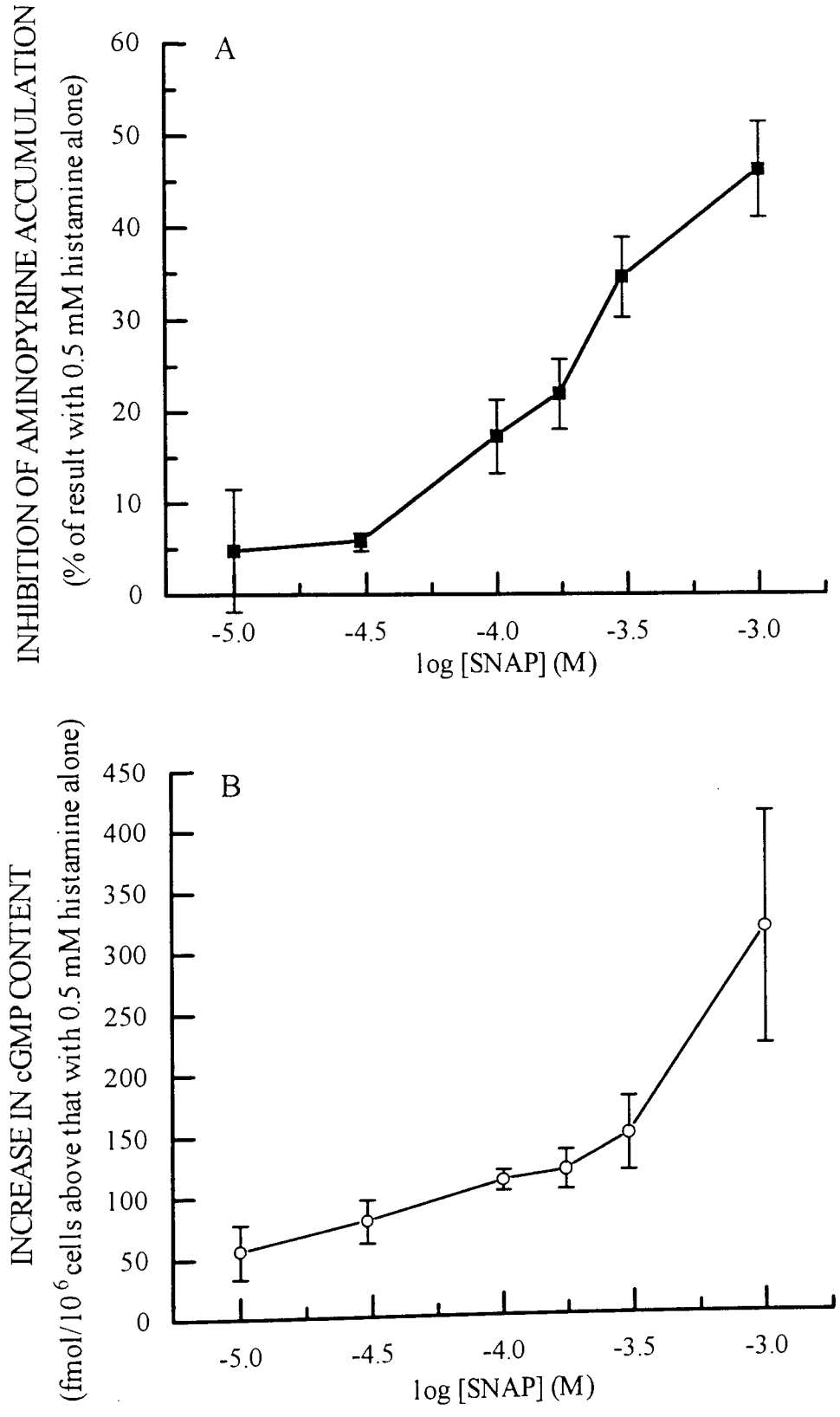
5.3.1.1 Effects of SNAP and ISDN on histamine-stimulated secretory activity.

Stimulation of a parietal cell enriched fraction of gastric mucosal cells with 0.5 mM histamine in the presence of 0.1 mM IBMX produced an increase in the aminopyrine accumulation ratio from 3.2±0.50 under basal conditions, to 41±14 under stimulated conditions (number of cell batches n=5). Coincubation with SNAP (1-1000 μM) caused a concentration-dependent inhibition of secretory activity (Fig. 5.6 A) with an IC_{50} of 247 μM (determined by the computer program FIT). ISDN (10-1000 μM) also caused a concentration-dependent inhibition of histamine-stimulated secretory activity (Fig. 5.7). These results show an inhibition by NO or NO donors of histamine-stimulated aminopyrine accumulation, but further experiments are required to determine whether the active agent is NO and whether inhibition of aminopyrine accumulation reflects a specific inhibition of the stimulatory effect of histamine.

5.3.1.2 Effects of oxyhaemoglobin and degraded SNAP on histamine-stimulated secretory activity.

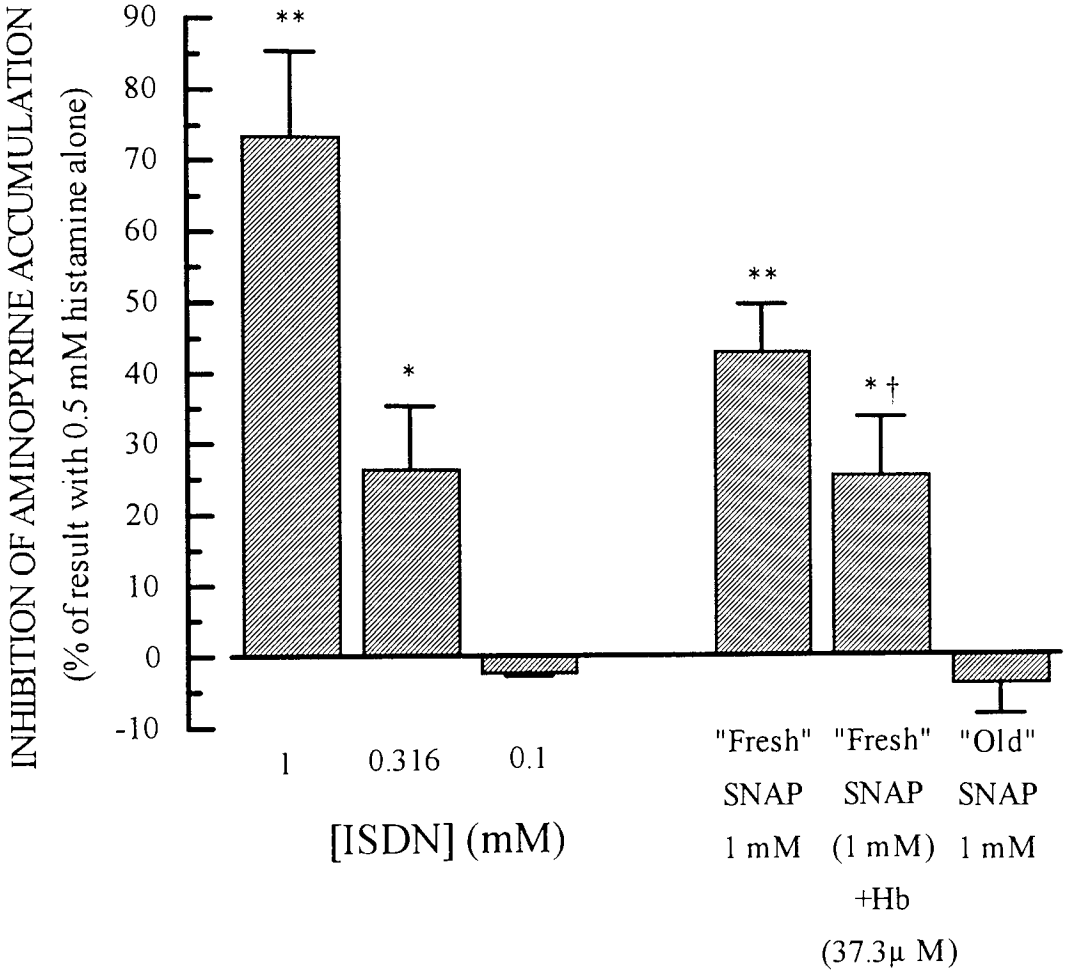
Oxyhaemoglobin (37 μM) did not affect the stimulation of aminopyrine accumulation by histamine, which was 51±11 and 49±11 (number of cell batches n=5), in the presence and absence of oxyhaemoglobin respectively, but it significantly reduced ($P<0.05$ by ANOVAR and Dunnett's test) the inhibition of secretory activity by 1 mM SNAP (Fig. 5.7). Basal secretory activity was unaffected by 1 mM SNAP with aminopyrine accumulation ratios of 3.2±0.49 and 3.3±0.45 in the absence and presence of 1 mM SNAP respectively (number of cell batches n=5). Furthermore, if the SNAP was allowed to degrade and the solution to become depleted of NO before addition to cell suspensions, histamine-stimulated secretory activity was unaffected, with aminopyrine accumulation ratios of 39±9.9 and 38±9.6 (number of cell batches n=3) in the presence and absence of degraded SNAP respectively (Fig. 5.7).

Figure 5.6 **Effect of SNAP (10-100 μM) on histamine-stimulated aminopyrine accumulation and the cGMP content of a cell suspension enriched with gastric parietal cells.**



Results are presented as mean±SEM (number of cell batches n=5 and 4 for A and B respectively. The aminopyrine accumulation ratio and cGMP content in the presence of histamine (0.5 mM) alone were 41±13, and 282±25 fmol/10^6 cells respectively.

Figure 5.7 **Effect of NO donors and oxyhaemoglobin (Hb) on histamine-stimulated aminopyrine accumulation in a cell suspension enriched with parietal cells.**

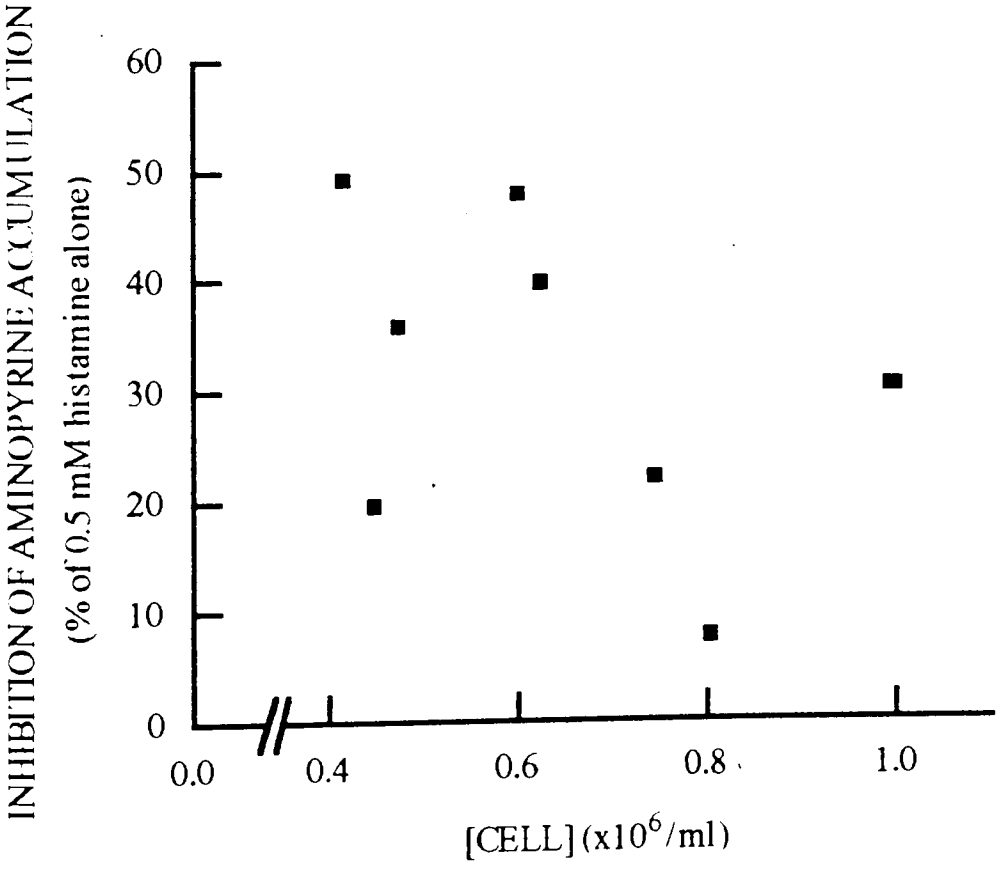


Results are presented as mean±SEM (number of cell batches n=4 and 5 for ISDN and SNAP respectively), where **P<0.01 and *P<0.05 for difference from aminopyrine accumulation in the presence of 0.5 mM histamine alone by ANOVAR and Dunnett's test, and †P<0.05 for difference from aminopyrine accumulation ratio in the presence of SNAP and histamine by ANOVAR and Dunnett's test. Aminopyrine accumulation ratios in the presence of 0.5 mM histamine alone were 52±11 and 51±11 for ISDN and SNAP experiments respectively.

Since oxyhaemoglobin, a known scavenger of NO (Martin *et al.*, 1985), reduced the inhibitory effects of SNAP and previously degraded SNAP was without effect (Fig. 5.7), these results suggest that the effects of SNAP on aminopyrine accumulation specifically involved NO in the inhibitory process. Furthermore, a non-specific effect of NO on the aminopyrine assay was rendered somewhat unlikely as only histamine stimulated and not basal values were affected.

A direct action upon the parietal cell rather than an indirect effect via release of a mediator from a contaminant cell species is suggested as inhibition of histamine-stimulated secretory activity by 0.3 mM SNAP was independent of the cell concentration of the suspension over the range $0.4x10^6$ to $1.0x10^6$ cells/ml (Fig. 5.8). If the inhibitory effects were due to release of a mediator from another cell type then one would expect a good positive correlation between the overall cell concentration and the inhibitory effect.

Figure 5.8 **Lack of effect of cell concentration on the effectiveness of 300 μM SNAP to inhibit histamine-stimulated aminopyrine accumulation in suspensions enriched with parietal cells.**



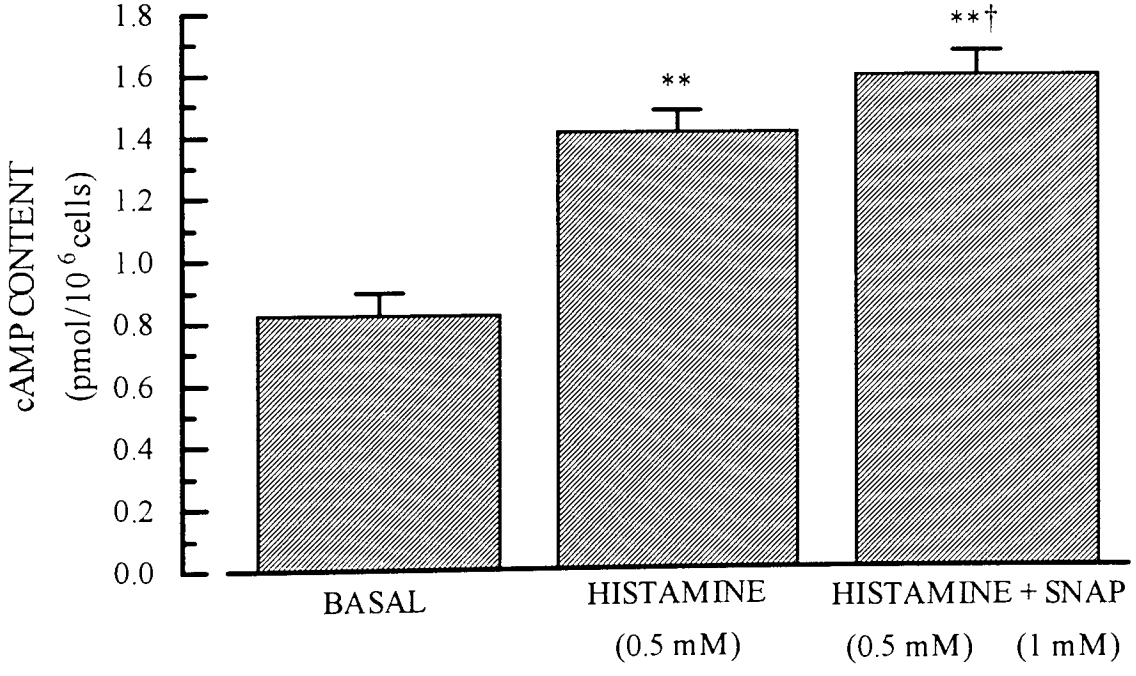
Results are presented from 9 different cell batches at different cell concentrations. The correlation coefficient r=0.3644, was not significant and so no regression line has been drawn.

5.3.1.3 Effects of SNAP on the cGMP and cAMP content of an enriched parietal cell suspension.

The inhibitory effects of SNAP were associated with a concentration-dependent increase in the cGMP content of the cell suspension (Fig. 5.6 B). This result is consistent with an involvement of NO in the inhibitory process since NO activates guanylate cyclase with a subsequent increase in intracellular cGMP content (Moncada *et al.*, 1991b).

The reduction in the secretory response to stimulation with histamine was not achieved through a reduction in the cAMP content of the parietal cell which was slightly, but significantly ($P<0.01$ by ANOVAR and Dunnett's), increased by 1 mM SNAP (Fig. 5.9).

Figure 5.9 **Effect of SNAP on the histamine-stimulated cAMP content of a cell suspension enriched with parietal cells.**



Results are presented as mean±SEM (number of cell batches n=5), where **P<0.01 for difference from basal by ANOVAR and Dunnett's test and †P<0.05 for difference from histamine alone by Student's t-test for paired data. IBMX (0.1 mM) was present in all incubations.

5.3.1.4 Effect of SNAP on cell viability and glucose oxidation.

Prolonged exposure to NO can result in cellular cytotoxicity (Curran *et al.*, 1989) and DNA damage and mutation (Nguyen *et al.*, 1992). In particular NO can inhibit enzymes such as aconitase, and components of the electron transport chain which both have Fe-S centres (Moncada *et al.*, 1991b). A reduction in ATP availability would lead to a reduction in the secretory response to histamine as ATP is required to power the H^+/K^+-ATPase. To test this possibility, conversion of [^{14}C]-glucose to $^{14}CO_2$ was measured for any reduction in the rate of glucose oxidation would suggest interference with the respiratory chain, and a decrease in ATP availability. However, addition of SNAP (1 mM) did not affect glucose oxidation by the cells which was 22.0±3.3 and 23.9±3.9 nmol/10^6cells/30 min (number of cell batches n=5) in the presence and absence of SNAP respectively (Fig. 5.9).

An increase in the permeability of the apical membrane would also result in a decrease in the aminopyrine accumulation ratio since ion leakage would occur. However, the inhibition of secretory activity by SNAP (1 mM) was not associated with a decrease in cell viability as the proportion of cells excluding trypan blue following the 30 min incubation period was unchanged with viabilities of 92±0.57, 93±0.61 and 93±0.71 for incubations under control conditions, with 0.5 mM histamine alone and with histamine plus 1 mM SNAP respectively (number of cell batches n=5, by ANOVAR).

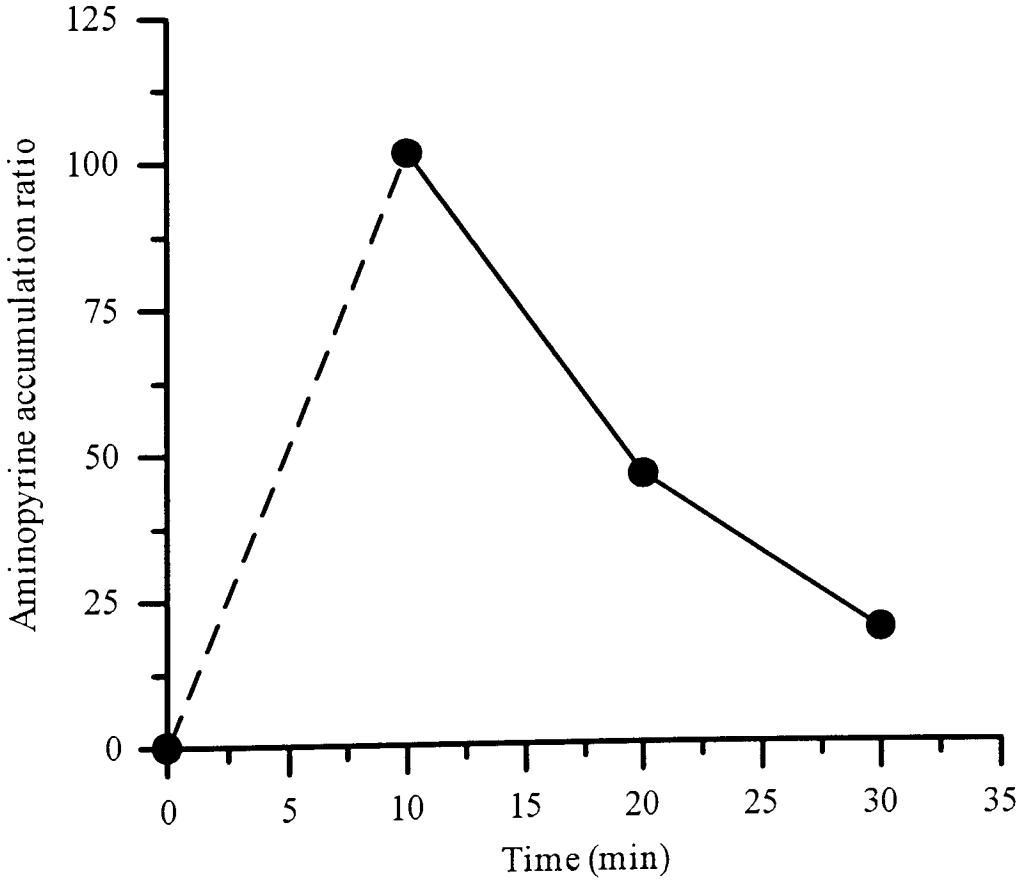
The results in sections 5.3.1.3 and 5.3.1.4 suggest that the inhibitory effects of SNAP were not due to a non-specific disruption of cellular metabolism or a disruption of cell membranes but involves a specific site. The site for the inhibitory effects of SNAP are likely to be mediated further along the secretory pathway that eventually involves the proton pump rather than the generation of cAMP. The accompanying rise in cGMP with declining aminopyrine accumulation suggests, but by no means proves, an involvement of cGMP in the inhibitory effect.

5.3.2 Effects of agents on secretory activity in permeabilised cells.

The effects of NO donors upon parietal cell secretory activity could possibly be mediated through a direct inhibitory effect upon the H^+/K^+-ATPase (proton pump). To establish whether or not this was the case, parietal cells were permeabilised with digitonin to allow a direct activation of the proton pump by ATP.

The aminopyrine accumulation ratio following stimulation of a permeabilised parietal cell suspension varied with time (Fig. 5.10) with the maximal response being seen at 10 min following addition of 2.5 mM ATP. Hence this time interval was used for all subsequent investigations involving permeabilised cells.

Figure 5.10 **Effect of time on aminopyrine accumulation ratio stimulated by 2.5 mM ATP in an enriched parietal cell suspension permeabilised with 10 μM digitonin.**



Data are presented as the results from one experiment and are means of duplicate determinations.

5.3.2.1 Effect of SNAP and ISDN.

Both SNAP and ISDN (final concentrations 1 mM) added 30 min prior to permeabilisation with digitonin (10 μg/ml), inhibited 2.5 mM ATP-stimulated secretory activity in an enriched parietal cell suspension (Fig. 5.11). However, the inhibitory effect of SNAP was only observed in the presence of 0.1 mM IBMX, whereas ISDN inhibited secretory activity if IBMX was absent from the incubation medium (Fig. 5.11). The magnitude of the secretory response obtained with ATP alone was not affected by the presence or absence of IBMX and neither was basal secretory activity. These results suggest that the inhibitory effects of SNAP are mediated via a mechanism that requires the inhibition of phosphodiesterase enzymes. This is consistent with an involvement of NO and guanylate cyclase in the inhibitory process since cGMP elevation in the presence of 1 mM SNAP was barely measurable if 0.1 mM IBMX was absent. However, since ISDN did not require IBMX to be present to produce its inhibitory effect, this may suggest that ISDN in contrast to SNAP is having a non-specific effect on secretory activity.

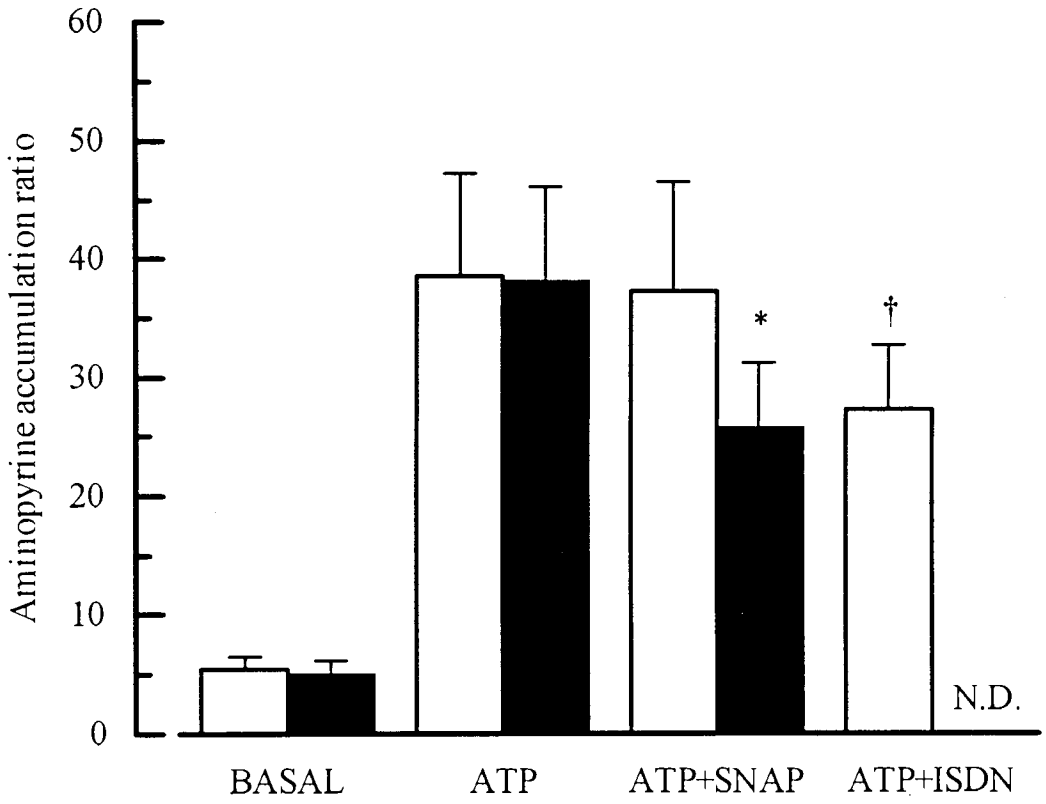
5.3.2.2 Effect of cGMP analogues.

A variety of cGMP analogues have recently become commercially available which may interact with cyclic nucleotide-dependent protein kinases (see Appendix A.5 for structures). The Rp isomer of 8-Br-cGMPS, which antagonises activation of cGMP-dependent protein kinase (Butt *et al.*, 1990), abolished the inhibitory effects of SNAP (Fig. 5.12). However, the Sp isomer, an activator of both cAMP and cGMP-dependent protein kinase (Butt *et al.*, 1990), was without such an effect (Fig. 5.12). Preincubation with 8-pCPT-cGMP, an activator of cGMP-dependent protein kinase type 1α (Sekhar *et al.*, 1992), did not however mimic the effect of SNAP on permeabilised cells. These results suggest that cGMP may mediate the inhibitory effect of SNAP on parietal cell secretory activity either stimulated by histamine or in permeabilised cells by ATP.

The overall implication of these findings is that NO, in the presence of a phosphodiesterase inhibitor to enable very large increases in cGMP, causes through interaction of cGMP with a specific site, an inhibition of acid secretion. Whether this

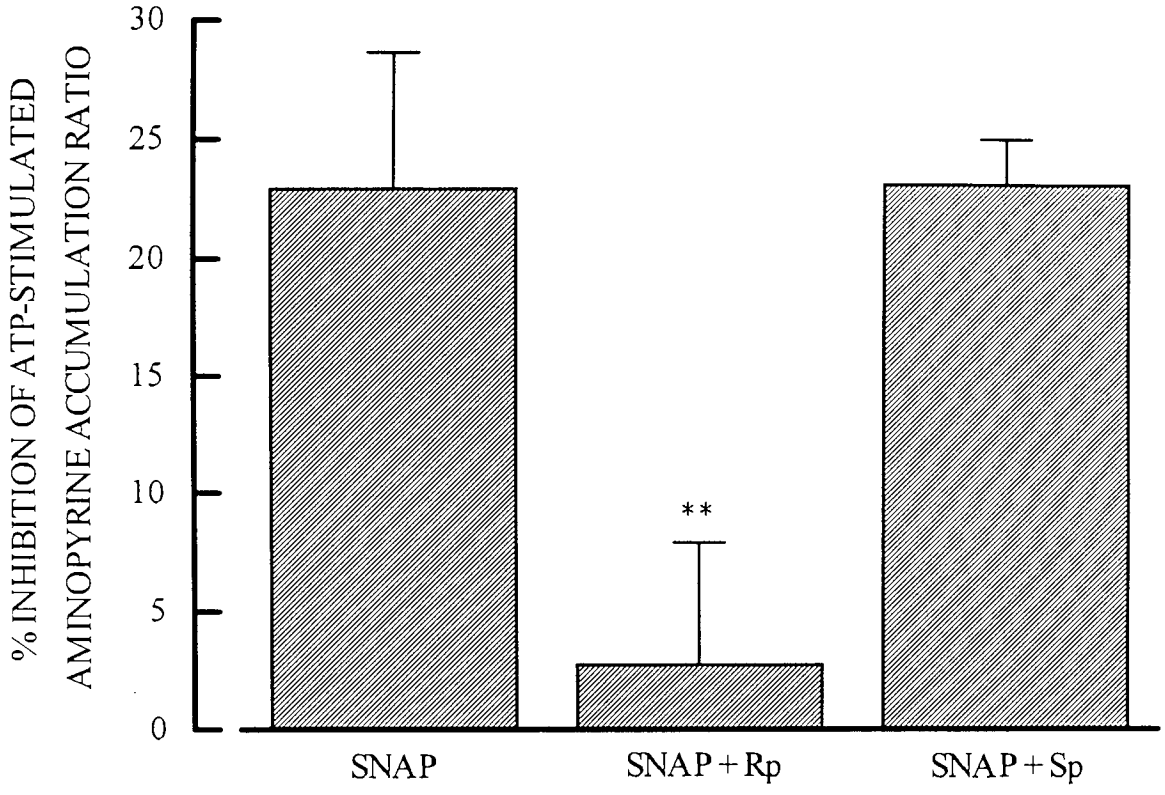
finding is more than phenomenology requires further investigation, but the apparent specificity of the effect is intriguing.

Figure 5.11 **Effect of SNAP and ISDN in the presence and absence of IBMX (0.1 mM) upon the aminopyrine accumulation ratio of permeabilised cells stimulated with 2.5 mM ATP.**



Results are presented as mean±SEM (number of cell batches n=5) where *P<0.05 for difference of aminopyrine accumulation ratios in the absence (□) and presence (■) of 0.1 mM IBMX by Student's t-test for paired data and †P<0.05 for difference from ATP alone by ANOVAR and Dunnett's test.
N.D.= not determined.

Figure 5.12 **Effect of the cGMP-dependent protein kinase regulators Rp and Sp-8-Br-cGMPS on the inhibitory effect of 1 mM SNAP on ATP-stimulated aminopyrine accumulation in permeabilised parietal cells.**



Results are presented as mean±SEM (number of cell batches n=4), where **P<0.001 by ANOVAR and Dunnett's test for comparison against percent inhibition with SNAP alone. Aminopyrine accumulation ratios were 68.2±12.1, 58.7±10.6 and 71.97±13.7 for ATP alone, ATP in the presence of 350 μM Rp-8-Br-cGMPS (Rp) and Sp-8-Br-cGMPS (Sp) respectively. The basal aminopyrine accumulation ratio was 5.8±1.4.

5.4 SUMMARY.

1. The nitric oxide donor SNAP inhibited histamine-stimulated aminopyrine accumulation, a measure of secretory activity, in preparations enriched with parietal cells and the inhibitory effect was dependent on the concentration of NO donor but was independent of cell concentration. An increase in the cGMP content of the cells occurred in response to NO donors, but SNAP had no effect on the rate of glucose oxidation or of exclusion of trypan blue by the cells.

2. Oxyhaemoglobin partially reversed the inhibitory effects of SNAP, and previously degraded SNAP was without an inhibitory effect.

3. Preincubation of whole cells with SNAP in the presence of IBMX prior to permeabilisation with digitonin inhibited ATP-stimulated secretory activity.

4. Preincubation of whole cells with the lipid soluble, metabolically stable, analogue of cGMP (Rp-8Br-cGMPS), which inhibits cGMP-dependent protein kinase activity, prior to incubation with SNAP and subsequent permeabilisation with digitonin, abolished the inhibitory effects of SNAP on ATP-stimulated secretory activity.

Chapter Six

STIMULATION OF PROSTAGLANDIN E_2 RELEASE FROM GASTRIC MUCOSAL CELLS BY BRADYKININ.

6.1 INTRODUCTION.

In Chapters 3, 4 and 5, results were presented on the production and action of a novel mediator nitric oxide. In this final chapter of results, information is given on the regulation of the production in the gastric mucosa of another more-well established type of local mediator, namely prostaglandin E_2.

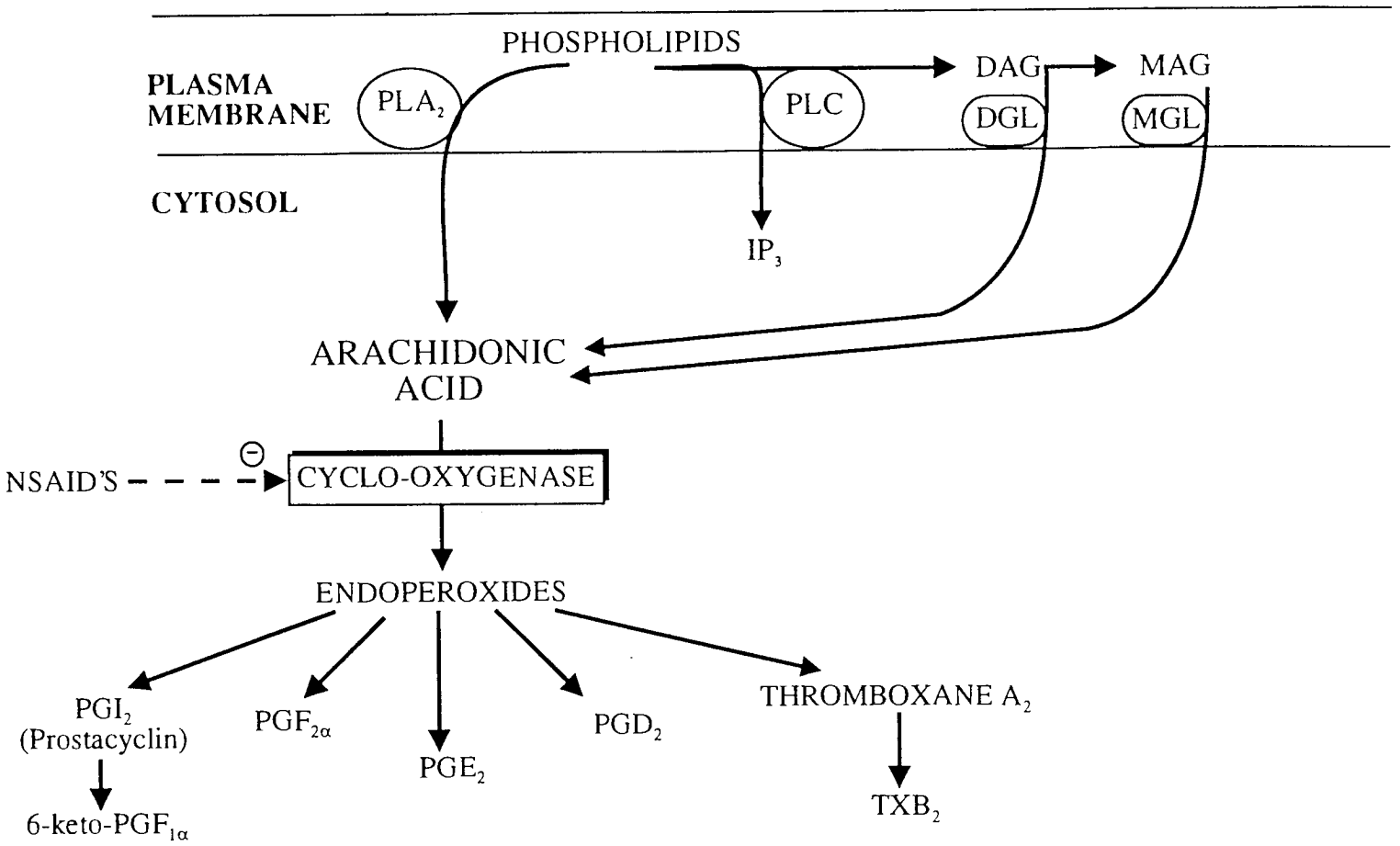
Endogenous prostaglandins seem to play an important role in modulating gastric mucosal resistance to injury (Wallace & Cohen, 1984). Inhibition of endogenous prostaglandin production by non-steroidal anti-inflammatory drugs, such as indomethacin, induces ulceration of the upper gastrointestinal tract in humans (For review see: Soll *et al.*, 1989).

Despite the apparent importance of endogenous prostaglandins, little is known of the source of prostaglandins in the gastric mucosa or of how their production may be regulated. This is mainly due to the complex structure of the tissue which consists of a collection of highly-organised and functionally-variant cell types (See Chapter 1 for a full discussion). Isolated gastric mucosal cells produce prostaglandins and thus provide a model system for studying the cellular source of prostaglandin production and its regulation.

6.1.1 Biosynthesis of prostanoids.

Phospholipase C hydrolyses phospholipids with, for example, the production of diacylglycerol and inositol 1,4,5-trisphosphate from phosphotidylinositol 4,5-bisphosphate (Prescott & Majerus, 1983). Diacylglycerol lipase and monoacylglycerol lipase may then catalyse the conversion of diacylglycerol to free arachidonic acid (Fig. 6.1) which is the precursor of prostaglandins. Activation and membrane-association of the enzyme phospholipase A_2, which in some cell types is regulated by pertussis toxin-sensitive G-proteins (Burch *et al.*, 1986; Wang *et al.*, 1988), yields lysophospholipid and free arachidonic acid from glycero-phospholipids.

Figure 6.1 Diagramatic representation of the production of lipid mediators from membrane phospholipids and arachidonic acid

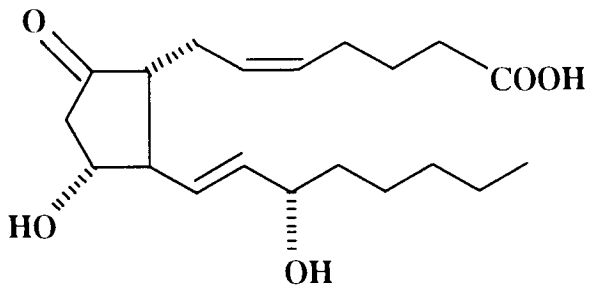


Key for Figure 6.1:

DAG	= Diacylglycerol,
DGL	= Diacylglycerol Lipase,
IP_3	= Inositol 1,4,5-trisphosphate,
MAG	= Monoacylglycerol,
MGL	= Monoacylglycerol Lipase,
NSAID'S	= Non-steroidal anti-inflammatory drugs,
PG	= Prostaglandin,
PLA_2	= Phospholipase A_2,
PLC	= Phospholipase C,

Arachidonic acid is converted into unstable intermediates termed endoperoxides via the action of the enzyme cyclooxygenase. Non-steroidal anti-inflammatory drugs (NSAID's), such as aspirin and indomethacin, inhibit cyclooxygenase and thus the formation of prostanoids (Vane, 1971). The labile endoperoxides, PGG_2 and PGH_2, can be converted to PGE_2 via the action of an endoperoxide isomerase enzyme. The formation of $PGF_{2\alpha}$ is less-well defined, although both PGE_2 and $PGF_{2\alpha}$ can arise non-enzymatically since the endoperoxides can spontaneously decompose in aqueous solution to form PGE_2, $PGF_{2\alpha}$ and PGD_2 (Moncada & Vane, 1979). PGH_2 can also be converted to PGI_2 (prostacyclin), via the action of prostacyclin synthetase (Dusting *et al.*, 1978).

Figure 6.2 **Chemical structure of PGE_2.**



6.1.2 Prostaglandin biosynthesis in the gastrointestinal tract.

Prostaglandin biosynthesis can be detected in every segment of the gastrointestinal tract. In the stomach, prostaglandins of the E, F and I types have been identified in all regions of the gastric wall of numerous species including man, especially the mucosal layer (Peskar, 1977). However, there are profound differences in the ratios of the various prostaglandins detected *in vitro* and this may reflect differences in the distribution and activity of cyclooxygenase and prostacyclin synthetase, as well as metabolising enzymes (Whittle & Vane, 1987). In addition, the levels of arachidonic acid released by phospholipase A_2 activation due to vortex mixing, can govern both the total level of prostanoids formed and the ratio of the different cyclooxygenase products (Cottee *et al.*, 1977).

The use of isolated cell preparations has allowed examination of prostaglandin formation by different cell types. Isolated cell fractions enriched with parietal cells from canine gastric mucosa produced $PGF_{2\alpha}$ as the major product followed by PGE_2 and PGI_2 (Skoglund *et al.*, 1980). However, PGE_2 can readily undergo conversion to $PGF_{2\alpha}$ by PGE-9-ketoreductase (Samuelsson *et al.*, 1975), so that the major prostanoid produced by parietal cells could in fact be PGE_2. PGE_2 output by isolated rat gastric cells was greater in a parietal cell-enriched fraction than in a cell fraction deplete of parietal cells (Ota *et al.*, 1988), suggesting that the parietal cell may be an important source of PGE_2 in the rat gastric mucosa. However, data from canine gastric mucosal cells separated by counterflow elutriation suggested that the major producers of PGE_2 were macrophages and capillary endothelial cells (Chen *et al.*, 1989).

6.1.3 Actions of prostanoids in the gastrointestinal tract.

Exogenously administered prostaglandins have a variety of gastrointestinal actions including alteration of fluid and electrolyte secretion and stimulation of gastrointestinal motility (Whittle & Vane, 1987). In the stomach, prostaglandins stimulate bicarbonate and mucus secretion (Kauffman *et al.*, 1978; Bolton *et al.*, 1978) and inhibit gastric acid secretion (Soll, 1980b). Prostanoids of the E and A series are potent vasodilators which lower systemic arterial blood pressure and could reduce gastric perfusion pressure resulting in a reduction of gastric mucosal blood flow (Main & Whittle, 1973). Prostaglandins in the gastric lumen can reduce the damaging effects (haemorrhagic lesions) of oral ethanol and are termed gastroprotective. This effect appears to be mediated primarily by the prevention of venostasis (Whittle & Vane, 1987). Furthermore, as mentioned in Chapter 1, prostanoids can interact with other mediators such as sensory neuropeptides and NO to maintain gastric mucosal integrity (Whittle *et al.*, 1990).

6.1.4 Stimulation of prostaglandin production.

6.1.4.1 Epidermal Growth Factor.

Epidermal growth factor stimulates prostaglandin production in many cell types, for example, cultured porcine thyroid cells (Kasai *et al.*, 1987) and cultured renal glomerulosa mesangial cells (Margolis *et al.*, 1988). Perfusion of EGF through the vasculature of the isolated rat stomach increased the prostaglandin content (PGE_2 and/or PGE_1) of the venous effluent (Chiba *et al.*, 1982). Furthermore, an isolated cell suspension enriched with rat gastric parietal cells (>80%) produced PGE_2 in response to stimulation with EGF with an EC_{50} of 7.5 nM (Hatt & Hanson, 1988). The signal transduction mechanism by which EGF mediates prostaglandin production is presently unclear.

6.1.4.2 Muscarinic agonists.

Carbachol stimulated both PGE_2 production and [^{14}C]arachidonic acid release in preparations of canine parietal cells with a direct requirement for extracellular Ca^{2+} (Payne & Gerber, 1987). In human parietal cells, the stimulatory effect of carbachol on PGE_2 production appeared to involve activation of both phospholipase C and protein kinase C which may synergistically interact with a calmodulin-dependent protein kinase to cause release of PGE_2 (Schepp *et al.*, 1990b).

6.1.4.3 Somatostatin.

Somatostatin, a peptide released by D-cells of the gastric mucosa has been reported to stimulate PGE_2 production by cultured rat gastric epithelial cells (Romano *et al.*, 1988).

6.1.4.4 Secretin.

Secretin stimulates PGE_2 production in injured human gastric mucosa but was without effect in normal tissue (Mine *et al.*, 1988).

6.1.4.5 Bradykinin.

Bradykinin (Fig. 6.3) is a nonapeptide, generated by the actions of kallikreins, a group of proteolytic enzymes present in most tissues and body fluids, on kininogens (Fig. 6.4). These agents are therefore local mediators released in response to tissue damage. Following the formation of bradykinin, various cleavage products can arise via the action of peptidase enzymes. For example, des-Arg^9-bradykinin is formed following removal of the N-terminal arginine group (Proud & Kaplan, 1988).

Figure 6.3 **Amino acid sequence of bradykinin.**

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Molecular Weight (Free base) = 1060.2

The kinins have diverse pharmacological properties which include their abilities to increase vascular permeability, to cause vasodilatation and pain and to contract most smooth muscle preparations. They also stimulate arachidonic acid release from phospholipids (Proud & Kaplan, 1988). Two distinct types of bradykinin receptor have been described which can be distinguished by the order of potency of various agonists and by the action of antagonists (Regoli *et al.*, 1990; Table 6.1).

An interesting characteristic of B_1 receptors is their apparent induction following various pathological insults both *in vitro* and *in vivo* (Regoli *et al.*, 1978). Hence, B_1 receptors may not be expressed under physiological conditions but may be expressed during diseased states. Bradykinin type B_2 receptors are responsible for bradykinin-stimulated PGE_2 release in Swiss 3T3 cells (Conklin *et al.*, 1988).

Bradykinin was shown to stimulate PGE_2 release predominantly in small cell fractions containing macrophages and endothelial cells, of canine isolated gastric mucosal cells separated by counterflow centrifugation (Chen *et al.*, 1989). Other workers demonstrated that bradykinin predominantly stimulated PGE_2 release from a parietal cell enriched (>80%) low density fraction of rat gastric mucosal cells, rather than a high

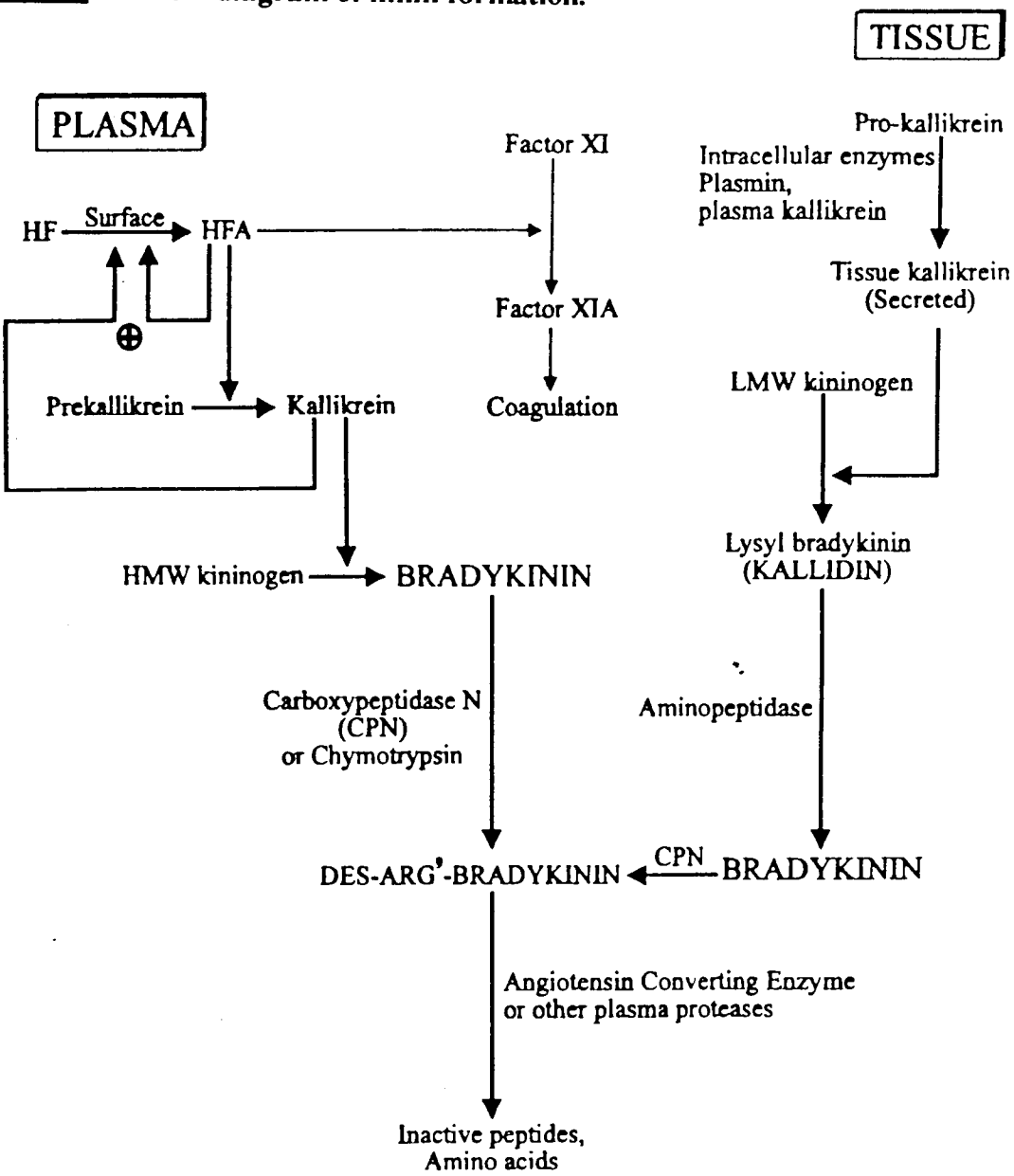
density fraction depleted of parietal cells (Hatt & Hanson, 1988). The aim of this section was to determine which type of bradykinin receptor was responsible for the stimulation of PGE_2 release in gastric cell preparations, and to establish if macrophages and or endothelial cell contaminants in the fraction enriched with parietal cells could account for PGE_2 release induced by bradykinin.

Table 6.1 **Classification of bradykinin receptors.**

RECEPTOR	ORDER OF POTENCY OF AGONISTS
B_1	des-Arg^{10}-kallidin > des-Arg^{9}-BK = Kallidin >> BK
B_2	BK = Kallidin >>des-Arg^{10}-kallidin > des-Arg^{9}-BK
	ANTAGONISTS
B_1	des-Arg^{9}-Leu^{8}-BK
B_2	D-Arg[Hyp^{3},Thi^{5,8}D-Phe^{7}]-BK

Where:
BK = Bradykinin
Thi = β-[2-thienyl]-alanine
Leu = Leucine
Phe = Phenylalanine
Hyp = Hydroxyproline

Figure 6.4 **Flow diagram of kinin formation.**



HF = Hageman Factor,
HFA = Activated Hageman Factor,
HMW = High molecular weight,
LMW = Low molecular weight.

6.2 METHODOLOGY.

6.2.1 Cell separation.

A crude gastric mucosal cell suspension was prepared as described in section 2.1.2. and was separated into two bands by density gradient centrifugation on Percoll® as described in section 2.2.1. The low density cell fraction always contained greater than 80% parietal cells and was used for the majority of experiments in this chapter after preincubation (see section 6.2.2). However, some experiments involved cells separated by counterflow centrifugation (elutriation) into fractions F_1 to F_5 as described in section 2.2.3. Cells prepared by this method were not preincubated prior to exposure to secretagogues, and were immediately resuspended in medium B' at a cell concentration of $1x10^6$cells/ml.

6.2.2 Preincubation of enriched parietal cell preparations.

Receptor mediated responses have been shown to be depressed following separation on a Percoll® density gradient (Hatt & Hanson, 1989), and to be restored by a 2 h preincubation period. Thus the high density cell suspensions were routinely preincubated for 2 h in medium B' (Table 2.1) containing foetal calf serum (5% v/v) and gentamicin (50 µg/ml) at a cell concentration of $2x10^6$ cells/ml. Two protocols for preincubation were routinely followed:-

6.2.2.1 Standard preincubation.

The cell suspension was transferred directly to the 25 ml capacity polycarbonate conical flask for the whole of the 2 h preincubation period with constant gassing of the air space with 95%O_2/5%CO_2 and shaking at 60 cycles/min. The cells were resuspended every 30 min by using a plastic transfer pipette to reduce the formation of cell clumps and to ensure adequate oxygenation.

6.2.2.2 Modified preincubation.

The cell suspension in 10 ml of medium B' (Table 2.1) was transferred to an 80 cm^3 tissue culture flask (ICN Flow) which was gassed with 95%O_2/5%CO_2 and was incubated at 37°C for 1 h. Cells remaining in suspension were then transferred from the tissue culture flask to a 25 ml capacity polycarbonate conical flask and incubated at 37°C for 1 h with constant gassing of the air space with 95%O_2/5%CO_2 and shaking at 60 cycles/min. The cells were resuspended every 30 min to reduce the formation of cell clumps and to ensure adequate oxygenation

Following the preincubation period, the cell suspensions were centrifuged at 125g for 7 min at 15°C. The supernatant was discarded and the cell pellet resuspended in the appropriate culture medium.

6.2.3 Immunological detection of macrophages and vascular endothelial cells.

Cells which had been preincubated as described in either 6.2.2.1 or 6.2.2.2 were resuspended in medium B' (Table 2.1) at a cell concentration of approximately $2x10^7$cells/ml. A 50 µl aliquot of cell suspension was transferred to 2 ml polypropylene tubes containing 50 µl of phosphate-buffered saline consisting of: NaCl (147 mM), KCl (2.7 mM), KH_2PO_4 (1.47 mM) and Na_2HPO_4 (8.1 mM), and 50 µl of a 1:50 dilution in phosphate-buffered saline of either of the monoclonal antibodies, anti-rat-macrophage (ED_2 clone), or anti-rat-endothelial (MRC-OX43 clone). All tubes were incubated at 4°C for 60 min. Following incubation, unbound antibody was removed by the addition of 1 ml medium B' to all tubes followed by thorough mixing and centrifugation at 125g for 7 min at 15°C. The supernatant was discarded and the pellet resuspended in 1 ml of fresh, "ice-cold", medium B' prior to centrifugation at 125g for 7 min at 15°C. The supernatant was again discarded and the cell pellet resuspended in 50 µl of a 1:32 dilution of fluoroscein isothiocyanate-conjugated goat-antimouse immunoglobulin G dissolved in the phosphate buffered saline. The cell suspensions were incubated at ambient temperature for 30 min prior to addition of 1 ml of "ice-cold" medium B' and centrifugation at 125g for 7 min at 15°C. The supernatant was discarded

and the pellet was washed with a further 1 ml of "ice-cold" medium B' before centrifugation at 125g for 7 min at 15°C. The supernatant was discarded prior to resuspension of the cell pellet in 10 µl of glycerol (70% v/v) containing glycine (52.4 mM), NaOH (5.25 mM), NaCl (87.3 mM), sodium azide (4.6 mM) and 1,4-diazobicyclo-(2,2,2)-octane (DABCO) (222.8 mM). A 5 µl aliquot was removed and spotted onto a microscope slide, a cover-slip was applied prior to sealing with clear nail varnish. Slides were viewed under a fluorescent microscope (Jenamed), fitted with a 35 mm single lens reflex camera (OM10, Olympus). Incident radiation was over the range 450-490 nm and fluorescence was monitored at 525 nm to allow determination of the proportion of positively-stained cells.

6.2.4 Measurement of prostaglandin release.

Cells which had either been preincubated as described in section 6.2.2.2 or separated into fractions F1 to F5 by counterflow elutriation as described in section 2.2.3 were resuspended in medium B' at a cell concentration of $1x10^6$cells/ml. Aliquots of 0.792 ml of cell suspension were transferred to 20 ml capacity polypropylene incubation vials containing either 8 µl of saline or 8µl of solutions in saline of bradykinin or des-Arg^9-bradykinin to give final concentrations after addition of cells ranging from 10^{-10} to 10^{-5}M. The air space above the cell suspension was gassed with 95%O_2/5%CO_2 for 5 s prior to capping the vials, which were then incubated in a shaking waterbath at 37°C with constant shaking at 80 cycles/min for between 10 and 30 min. Finally, two 0.6 ml aliquots of cell suspension were not incubated but were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 10,000g for 30 s. The supernatants from these samples were used for measurement of PGE_2 release upon resuspension of the cells, value t=0, and were stored briefly on ice prior to extraction of PGE_2 (6.2.4.1) and subsequent assay of the PGE_2 content by radioimmunoassay (6.2.4.2).

At the end of the incubation period, 0.6 ml of cell suspension was transferred to 1.5 ml microcentrifuge tubes, centrifuged at 10,000g for 30 s and stored briefly on ice before extraction of PGE_2 from the supernatants (6.2.4.1).

6.2.4.1 Extraction of PGE_2.

A 0.4 ml aliquot of supernatant was transferred from the microcentrifuge tube to a 2 ml capacity polypropylene tube containing 14 µl of 1 M HCl, to which 1.2 ml of ethylacetate was added immediately. The polypropylene tubes were capped and vortexed for 15 s before centrifugation at 100g for 1 min at 15°C. The upper, non-aqueous layer was transferred to a 20 ml capacity siliconised glass scintillation vial. A further 1.2 ml of ethylacetate was added to the polypropylene tubes containing the aqueous layer, which were capped vortexed and centrifuged as described above prior to removal of the non-aqueous layer which was transferred to the corresponding scintillation vial containing the previous extraction. The air space in all vials was gassed with nitrogen for 10 s prior to capping and storage at -20°C for subsequent assay of the PGE_2 content.

The percentage recovery of PGE_2 by this extraction method was determined using [^{3}H]PGE_2 to be 84.5±5.5% (number of extractions n=5). All values for PGE_2 release are corrected for loss during extraction.

6.2.4.2 Assay for PGE_2 content.

Determination of the PGE_2 content of each sample was achieved through use of a PGE_2 [^{125}I] Radioimmunoassay Kit (Dupont). Samples were removed from the freezer and allowed to reach room temperature before being evaporated to dryness under a stream of nitrogen gas. Samples were rehydrated with 1 ml of RIA assay buffer which was composed of NaCl (0.9% $^{w}/_{v}$), EDTA (0.01 M), bovine γ-globulin (0.3% $^{w}/_{v}$), Triton-X-100 (0.005% $^{v}/_{v}$), sodium azide (0.05% $^{w}/_{v}$), $NaH_2PO_4 \bullet H_2O$ (0.0255 M) and $Na_2HPO_4 \bullet 7H_2O$ (0.0245 M) at pH 6.8. All sample vials were capped and vortexed for 10 s to ensure adequate dissolution. PGE_2 standards ranging from 0.25 to 25 pg/0.1 ml were prepared by serial dilution of a 100 ng/ml PGE_2 stock solution with the aforementioned assay buffer. The [^{125}I] PGE_2 tracer (26 µCi/ml) was diluted with assay buffer (1:20 $^{v}/_{v}$) immediately before each assay. Additions to polypropylene test-tubes were made as set out in Table 6.2. All tubes were capped, vortexed for 10 s and incubated at 4°C for 16-24 h.

Table 6.2 **Radioimmunoassay of prostaglandin E_2.**

SUMMARY OF REAGENT ADDITIONS (µl)

	Tube number	Buffer	Standards	Sample	Tracer	Antibody
Total Counts	1 - 2	---	---	---	100	---
Blank	3 - 4	200	---	---	100	---
0.25 pg/0.1 ml Standard	5 - 6	---	100	---	100	100
0.5 pg/0.1 ml Standard	7 - 8	---	100	---	100	100
1.0 pg/0.1 ml Standard	9 - 10	---	100	---	100	100
2.5 pg/0.1 ml Standard	11 - 12	---	100	---	100	100
5.0 pg/0.1 ml Standard	13 - 14	---	100	---	100	100
10.0 pg/0.1 ml Standard	15 - 16	---	100	---	100	100
25.0 pg/0.1 ml Standard	17 - 18	---	100	---	100	100
Samples	19, 20 etc.	---	---	100	100	100

After incubation all tubes, except tubes 1 and 2 (total counts), were transferred to an ice bath and 1 ml of cold precipitating agent consisting of 16% w/v polyethylene glycol (PEG 6000) and 0.005% w/v sodium azide in 50 mM phosphate buffer, was mixed with the contents of each tube. The tubes were then incubated in the ice bath for 30 min before centrifugation at 1,500g for 30 min at 4°C. The supernatants were then decanted and the precipitates counted on a Compu-gamma gamma counter (LKB Instruments Ltd, Sweden) with a counting efficiency of 82%. The standard curve (cpm vs log concentration) was plotted and the concentration of PGE_2 in each sample tube calculated automatically using the curve fitting package associated with the gamma counter.

6.2.5 Presentation of results.

Due to variation in the magnitude of basal PGE_2 release between cell batches, an observation which has also been described by Chen *et al.*, (1989), results were often normalised following correction for PGE_2 present in the supernatant at the start of the incubation (t=0). Data were normalised to PGE_2 present under control conditions and were expressed as either a % of this result or as a % stimulation above this result for dose-response data.

6.3 RESULTS AND DISCUSSION.

6.3.1 Presence of macrophages and vascular endothelial cells.

When cell preparations enriched with parietal cells were prepared by density gradient centrifugation on Percoll® and preincubated according to the standard procedure (section 6.2.2.1), a mild contamination with macrophages was detected by immunofluorescent staining using an anti-rat macrophage monoclonal antibody (ED_2 clone) (Table 6.3 and Plates 6.1 & 6.2). However, following preincubation according to the modified procedure (section 6.2.2.2), the contaminating macrophages could no longer be detected. The macrophages had presumably attached to the tissue culture flask used for a 1 h incubation in the modified procedure. Vascular endothelial cells could not be detected by an anti-rat-endothelial (MRC-OX43 clone) monoclonal antibody in cell suspensions following preincubation by either protocol. Furthermore, exposure of cells, which had been incubated according to the modified preincubation procedure (see section 6.2.2.2), to zymosan (600 µg/ml), a sulphated polysaccharide that stimulates phagocytosis and subsequent production of PGE_2 in macrophages (Hsueh *et al.*, 1979), did not result in an increase in PGE_2 release above that of basal (Fig. 6.5) although the cells did respond to 1 µM bradykinin.

These results suggest that after preincubation in a culture flask the enriched parietal cell suspension was devoid of vascular endothelial cells macrophages. These cell-types were reported as being the major producers of PGE_2 in a canine gastric mucosal cell preparation (Chen *et al.*, 1989), but the above results would suggest that PGE_2 release from the preincubated cell suspension could not be attributed to these cell types. The source of PGE_2 is therefore more likely to be derived from the major cell type in these cell suspensions, namely the gastric parietal cell, but since the suspensions are only 85±2% (number of cell batches n=6) parietal cells, this cannot be stated with complete certainty.

The preincubation procedure described in section 6.2.2.1 was adopted for all subsequent experiments with the enriched parietal cell fraction.

Plate 6.1 Crude gastric mucosal cells (x125 magnification) preincubated according to the standard procedure and stained with anti-rat macrophage monoclonal antibody, viewed under visible light.
Bar represents 10 μm. F=positively stained fluorescent cell.

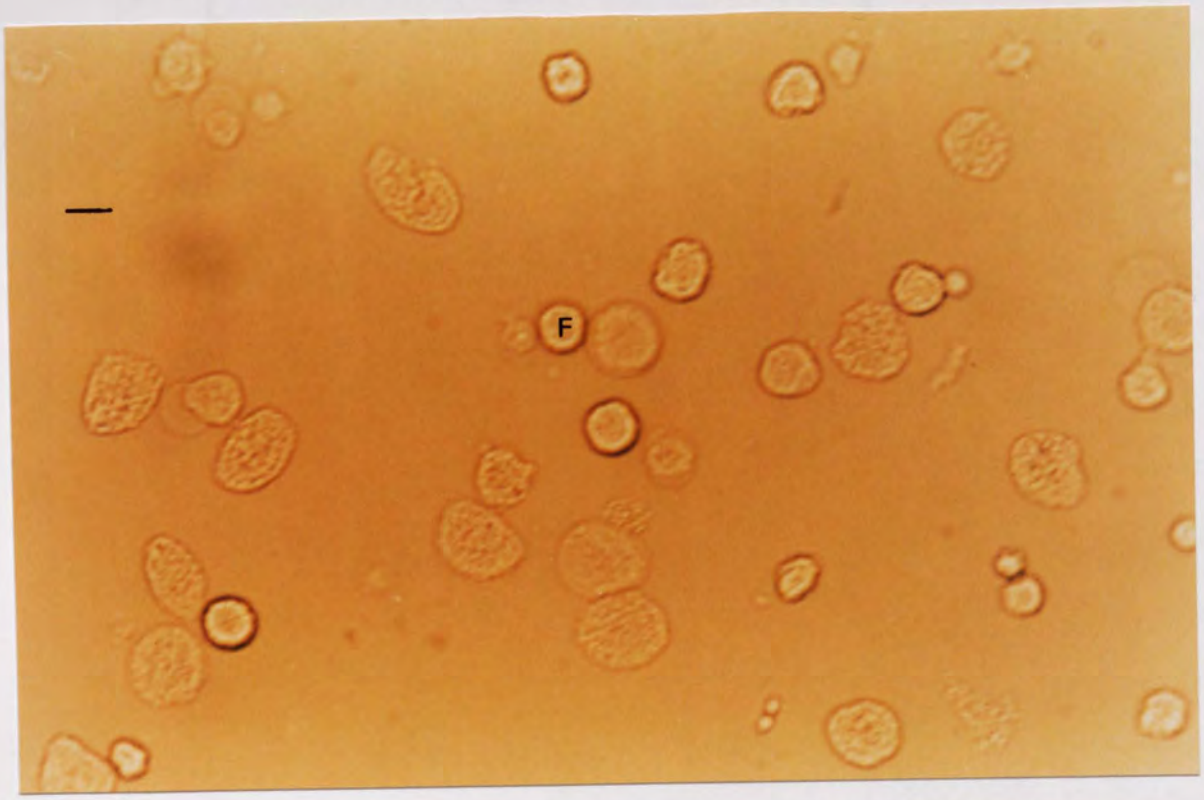
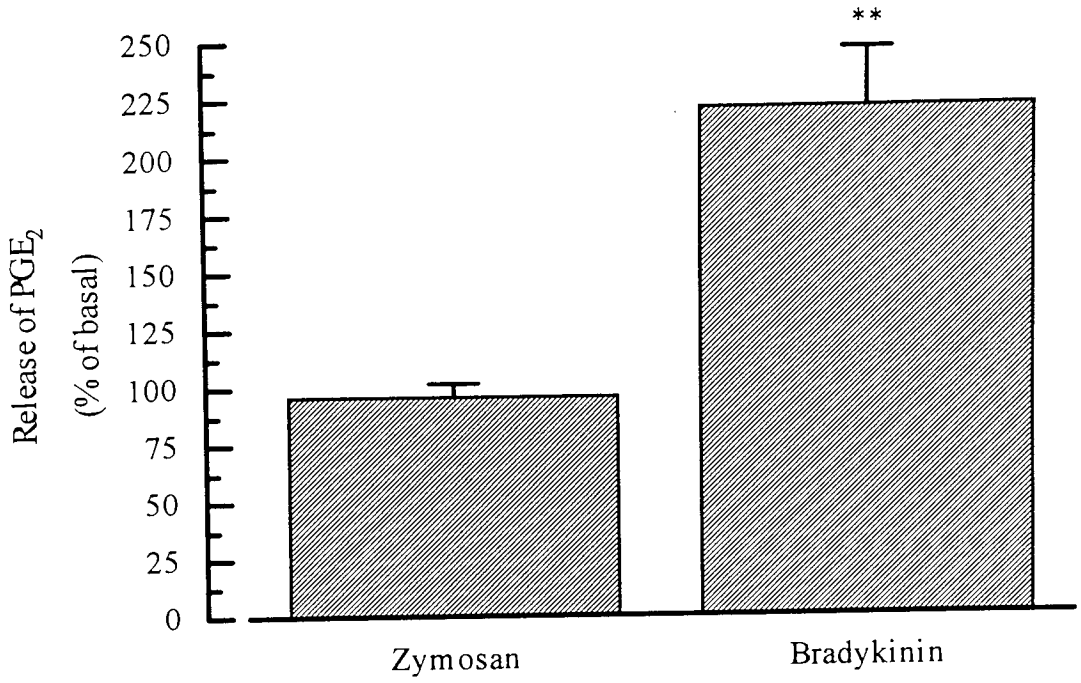


Plate 6.2 Crude gastric mucosal cells (x125 magnification) preincubated according to the standard procedure and stained with anti-rat macrophage monoclonal antibody viewed under ultra-violet light of incidence wavelength 450-490 nm.
Bar represents 10 μm, and F=positively stained fluorescent cell.

Figure 6.5 **Action of zymosan (600 μg/ml) and bradykinin (1 μM) on PGE_2 release from an enriched parietal cell preparation.**



Data are presented as mean±SEM (number of cell batches n=4), where **P<0.01 for difference from basal release by ANOVAR and Dunnett's test, and are presented as the percentage of basal release which was 117±36 pg $PGE_2/10^6$cells/30 min.

Table 6.3 **The proportion of cells in an enriched parietal cell preparation staining with anti-endothelial cell and anti-macrophage monoclonal antibodies.**

Total cells ($x10^7$)	[1]Viability (% of total)	[2]% parietal cells	[3]ED_2 positive cells (% of total)	[3]ED_2 positive cells (% of non-parietal)	[4]MRC OX-43 positive cells (% of total)
1.94±0.29	91.1±1.1	81.4±1.0	0.61±0.12*	3.57±0.86*	<0.1

Results presented as mean±SEM (number of cell batches n=4) where *P<0.05 by Students' t-test for non-paired data for difference from zero.

1 Determined as the ability to exclude trypan blue.

2 Identified by their large size and granular cytosol.

3 The ED_2 monoclonal antibody recognises most types of rat tissue macrophages including those associated with the gastric mucosa, as demonstrated by Sminia & Jeurissen, (1986).

4 The MRC OX-43 monoclonal antibody recognises all rat vascular endothelium, except that of brain capillaries (Robinson *et al.*, 1986).

6.3.2 Prostaglandin E_2 (PGE_2) release.

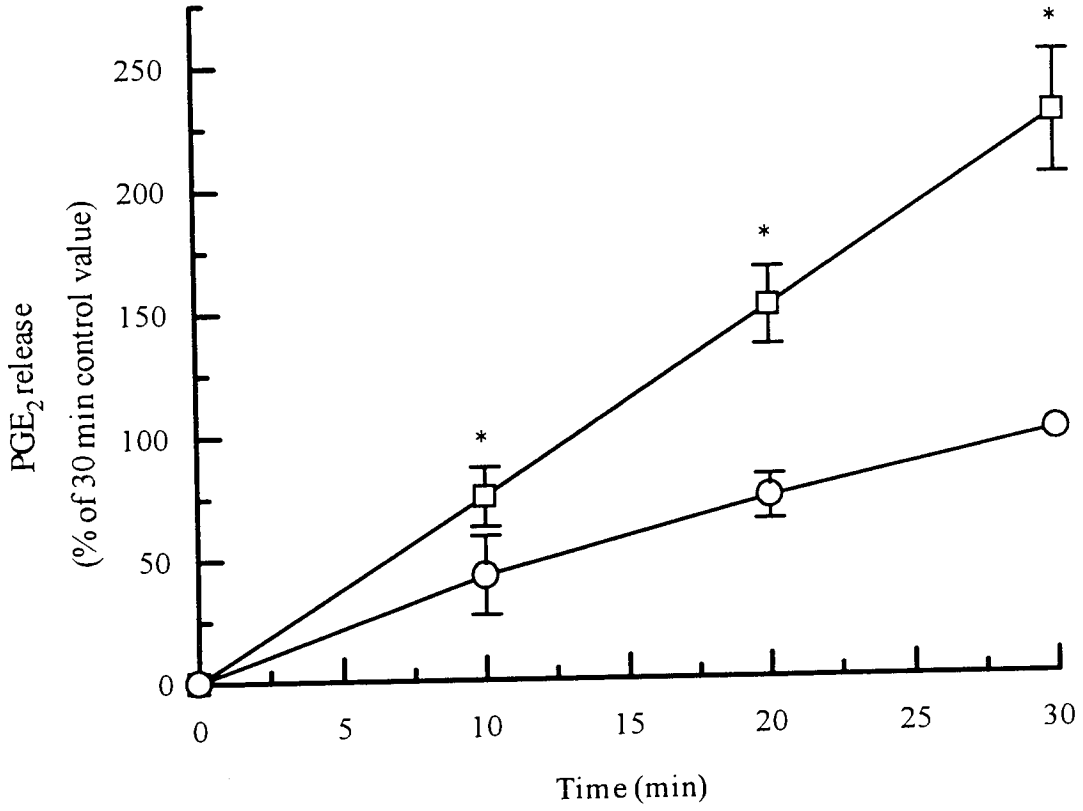
6.3.2.1 Time course of PGE_2 release.

Release of PGE_2 was essentially linearly related to time over the 30 min incubation period under both control and bradykinin (1 μM)-stimulated conditions (Fig. 6.6). The effect of bradykinin was therefore to modulate a continuous release of PGE_2 rather than to produce a sudden burst. The implication is that bradykinin produces a sustained activation of phospholipid breakdown. PGE_2 release equates reasonably well with PGE_2 production since previous work with rat gastric cells did not demonstrate intracellular accumulation of prostaglandins during incubation (Ruoff & Becker, 1982).

6.3.2.2 Bradykinin and des-Arg^9-bradykinin stimulated PGE_2 release from enriched parietal cell fractions separated by density gradient centrifugation.

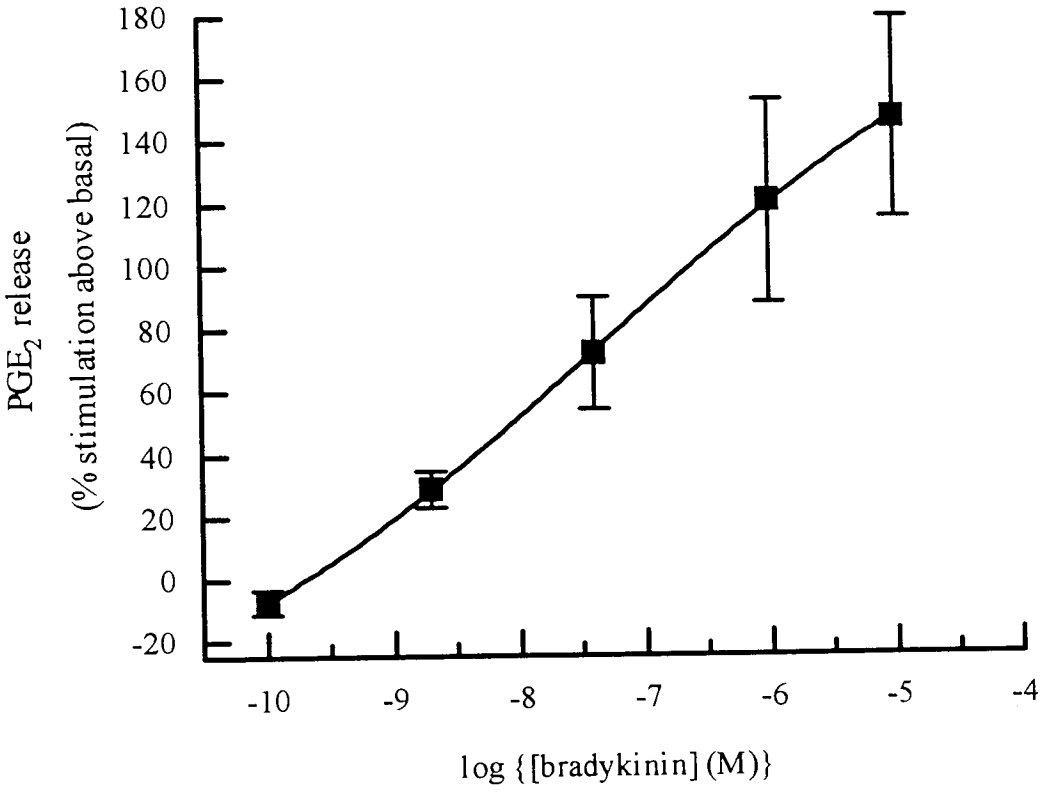
Release of PGE_2 into the incubation medium in response to both bradykinin and the selective bradykinin type B_1-receptor agonist, des-Arg^9-bradykinin, was concentration dependent (Figs 6.7 & 6.8). The ratio of the EC_{50} values for PGE_2 release stimulated by des-Arg^9-bradykinin and bradykinin was approximately 80:1. This result strongly suggests that the type of bradykinin receptor involved in mediating the response was of the B_1 type since des-Arg^9-bradykinin was much more potent in stimulating PGE_2 release. Since the likelihood of contaminating macrophages or vascular endothelial cells has been essentially eliminated, this bradykinin type B_1 receptor could be expressed by the parietal cell. The use of bradykinin antagonists to further characterise the type of receptor responsible for mediating PGE_2 release was not pursued as all "antagonists" tried, both B_1 (des-Arg^9-Leu^8-bradykinin) and B_2 (D-Arg[Hyp^3,$Thi^{5,8}$D-Phe^7]-bradykinin) showed some stimulatory effect on PGE_2 release when used alone at concentrations of 10 nM. Partial agonist activity of B_2-receptor "antagonists" has been reported previously (Regoli *et al.*, 1990).

Figure 6.6 **Time dependency of PGE_2 release from a suspension enriched with parietal cells.**



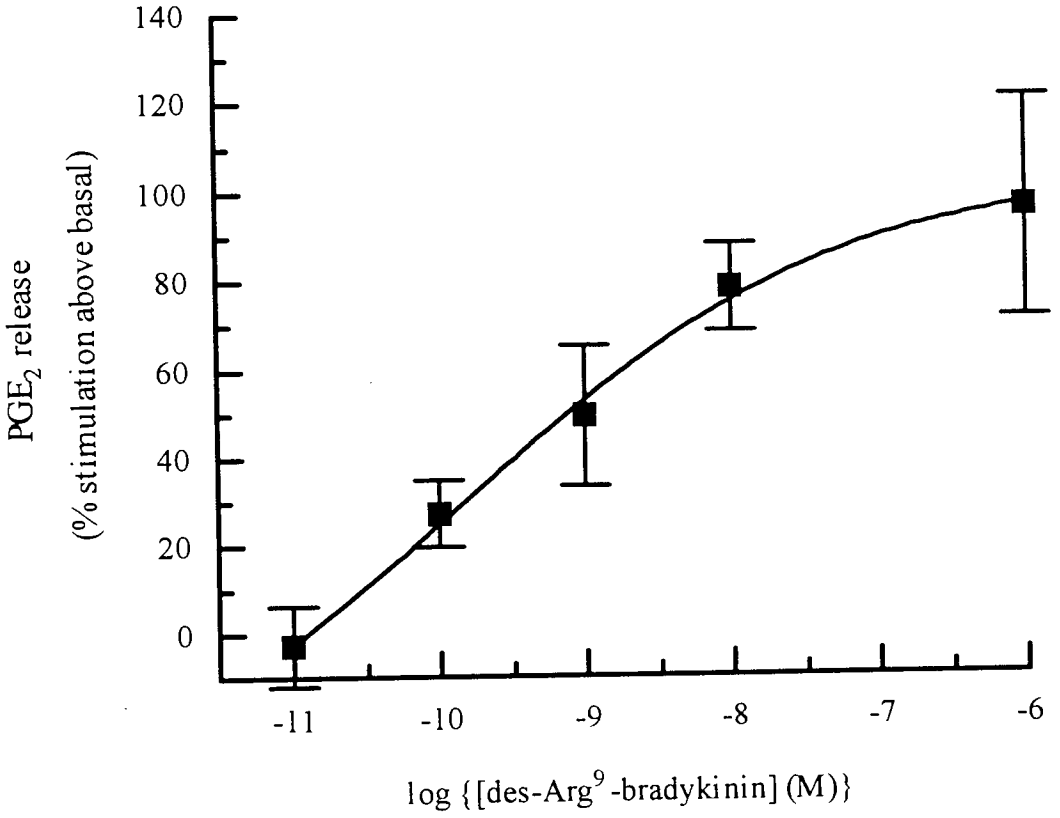
Data are presented as mean±SEM (number of cell batches n=3), and are presented as % of control release at 30 min, which was 149±64 pg $PGE_2/10^6$cells. *P<0.05 for difference in PGE_2 release in the presence (□) and absence (○) of 1 μM bradykinin by Student's t-test for paired data.

Figure 6.7 **Stimulation of PGE_2 release by bradykinin from an enriched parietal cell preparation.**



Data are presented as mean±SEM (number of cell batches n=4) and are expressed as the % release above basal, which was 329±224 pg $PGE_2/10^6$cells/30 min.
Maximum % stimulation was 161.7% with an EC_{50} of 68.9 nM (calculated by the computer program FIT).

Figure 6.8 **Stimulation of PGE_2 release by des-Arg^9-bradykinin from an enriched parietal cell preparation.**



Data are presented as mean±SEM (number of cell batches n=4) and are expressed as the % release above basal, which was 270±43 pg $PGE_2/10^6$cells/30 min.
Maximum % stimulation was 98.5% with an EC_{50} of 0.86 nM (calculated by the computer program fit).

6.3.2.3 Stimulation of PGE_2 release by des-Arg^9-bradykinin from elutriated cell fractions.

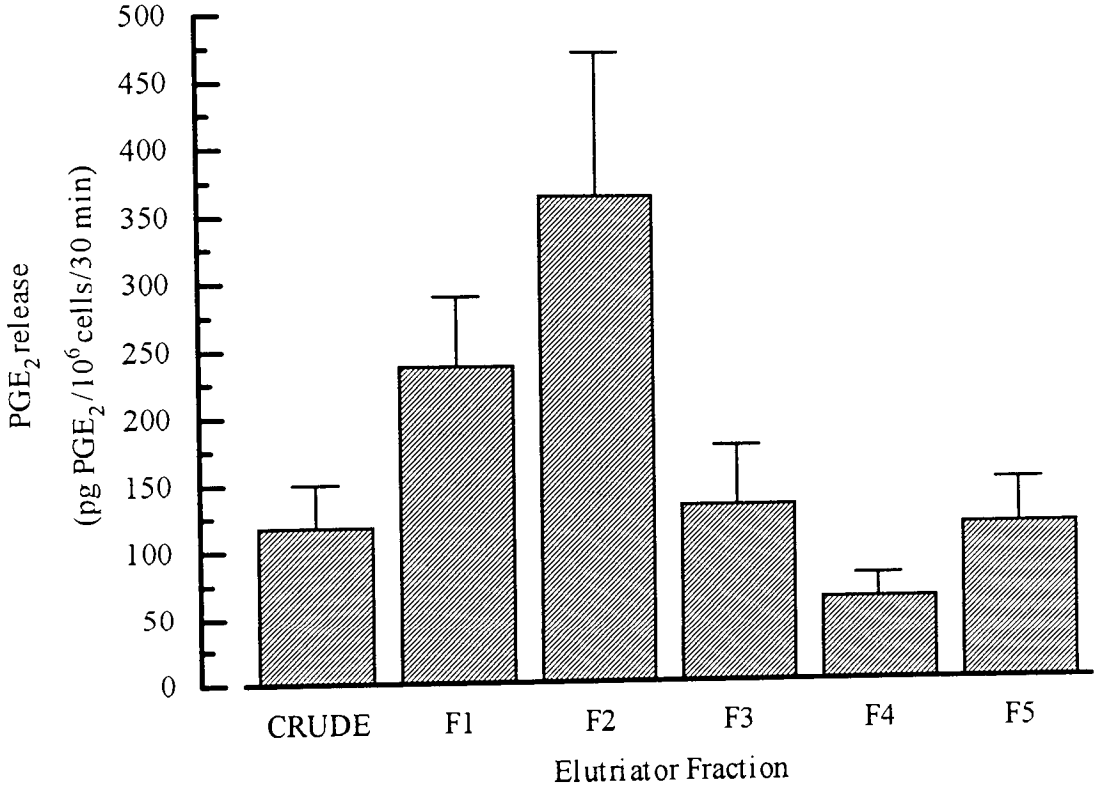
Crude suspensions of gastric mucosal cells were separated according to size by counterflow centrifugation (elutriation) into fractions termed F_1 to F_5 and stimulated with 10 nM des-Arg^9-bradykinin, a near-maximally effective concentration, (Fig. 6.8). The greatest basal release of PGE_2 was observed in fractions 1 and 2 (Fig. 6.9). The increase in release of PGE_2 following exposure of each elutriator fraction to 10 nM des-Arg^9-bradykinin was also greatest in fractions 1 and 2 (Fig. 6.10). No significant increase in PGE_2 release following stimulation with 10 nM des-Arg^9-bradykinin was observed in the cells of medium size from fractions 3 and 4, which consisted mainly of mucus and chief cells (see section 2.3.3 for full characterisation of these fractions). Elutriator fraction 5 on the other hand, which consisted of approximately 80% parietal cells, displayed a significant increase in PGE_2 release following exposure to 10 nM des-Arg^9-bradykinin.

Although these results support data from Chen *et al.*, (1989) which suggested that small cells such as macrophages and vascular endothelial cells were the major source of PGE_2 in the gastric mucosa, they also confirm suggestions by Payne & Gerber, (1987) that the parietal cell is a source of PGE_2 in the rat gastric mucosa. Nevertheless, it seems possible that the presence of small cells in low density fractions of rat gastric mucosal cells may contribute to PGE_2 release. Although in the present investigation a role for macrophages and endothelial cells is unlikely. A significant stimulation of PGE_2 release by the specific B_1 receptor agonist des-Arg9-bradykinin was found in both small cells and in the parietal cell enriched fraction 5. These results support the suggestion that a B_1 receptor linked to PGE_2 production may be present on parietal cells. No attempt was made to examine for, or remove, macrophages or endothelial cells from elutriator fractions 1 and 2. Consequently no comment can be made concerning which small gastric mucosal cells might possess B_1 receptors.

Finally, whether the involvement of a B_1 receptor, rather than the more common B_2 receptor, in regulation of gastric mucosal PGE_2 release has physiological of functional significance remains to be established. The possibility that the 'trauma' of cell

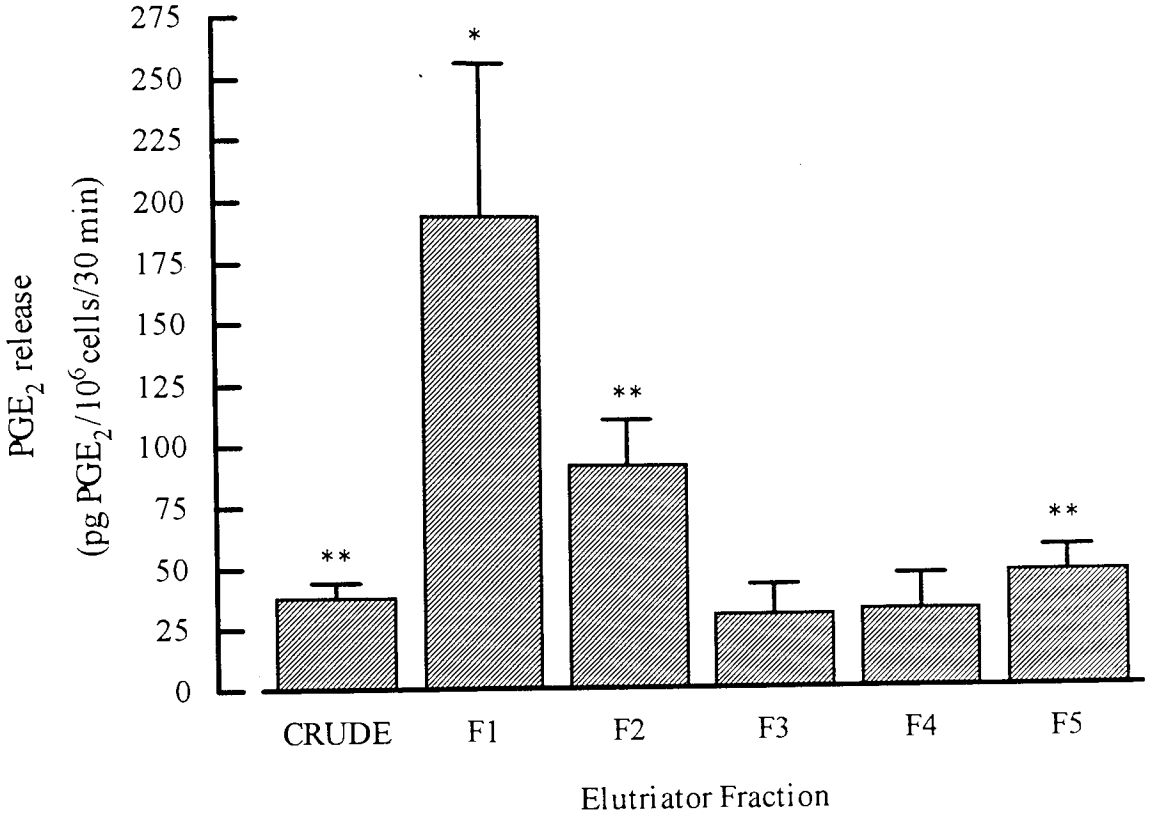
isolation causes the induction of a B_1 receptor should possibly be investigated using ligand-binding procedures.

Figure 6.9 **Distribution of basal PGE_2 release from gastric mucosal cells separated by counterflow elutriation.**



Data are presented as mean±SEM (number of cell batches n=5) and represent the amount of PGE_2 present in the incubation medium under basal conditions.

Figure 6.10 **Stimulation by 10 nM des-Arg^9-bradykinin of PGE_2 release from gastric mucosal cells separated by counterflow elutriation.**



Data are presented as mean±SEM (number of cell batches n=5), and are expressed as release above basal, with basal release of 117±33, 237±53, 364±108, 133±44, 62±18 and 117±34 pg $PGE_2/10^6$cells/30 min for crude cells and fractions F1 to F5 respectively. Where **P<0.01 and *P<0.05 for difference from basal by Student's t-test for paired data.

6.4 SUMMARY.

1. Preincubation of a cell fraction isolated from the rat gastric mucosa and enriched with parietal cells for 1 h in a tissue culture flask, successfully removed contaminating macrophages.

2. The release of PGE_2 from the preincubated parietal cell suspensions was approximately linearly related to time under both control and during stimulation with bradykinin (1 μM).

3. The low-density cell fraction which was devoid of macrophages, released PGE_2 in a concentration-dependent manner following stimulation with bradykinin and des-Arg^9-bradykinin. The higher potency of the latter agonist suggested the involvement of a B_1 receptor.

4. A significant increase in PGE_2 release was observed in fractions 1, 2 and 5 of gastric mucosal cells separated by counterflow centrifugation following stimulation with 10 nM des-Arg^9-bradykinin. The B_1 receptor may therefore be expressed on both small and large cells in the gastric mucosa.

Chapter Seven

GENERAL DISCUSSION

The major aims of this final chapter are to put the findings of this thesis into context with respect to (i) the potential physiological mechanisms involved in gastroprotection, with particular reference to the involvement of NO and cGMP, and (ii) the potential interactions between NO and PGE_2 in maintaining gastric mucosal integrity.

7.1 GASTROPROTECTIVE ROLE OF NITRIC OXIDE.

The maintenance of gastric mucosal function and integrity has been demonstrated to be critically dependent upon the status of the microcirculation, since limitations in microvascular perfusion either by direct vasoconstriction or by removal of endogenous vasodilator tone can lead to mucosal damage, erosion or ulceration (Whittle *et al.*, 1981; Whittle, 1986). Vascular endothelial cells release a labile humoral substance now known to be NO (Palmer *et al.*, 1987). Data in Chapter 3 of this thesis demonstrated that NOS-I activity paralleled the distribution of gastric mucous cells, suggesting that the mucous cell was a major producer of NO in the rat gastric mucosa. All cells of the gastric epithelium are in close proximity to the vasculature, and since NO is extremely cell permeable, it is possible that NO produced by the mucosal cells could diffuse to the vascular smooth muscle resulting in vasodilatation and an increase in microvascular perfusion. Therefore, the gastric epithelial cells as well as the vascular endothelium itself may regulate the rate of vascular perfusion via the production of NO.

The ability of NO donors to stimulate mucin release from both isolated mucosal cells *in vitro* and also to increase the thickness of the mucus gel layer *in vivo* via a cGMP-dependent mechanism was demonstrated in Chapter 4, suggesting that NO may act as an intracellular regulator of mucus release. NO may also be involved in the neural control of mucus secretion, since NO releasing NANC-neurones have been demonstrated in the rat gastric corpus mucosa (Forster & Southam, 1993). The function of myenteric NANC neurones probably involves the accommodation of food and fluid intake via relaxation of gastric smooth muscle (Desai *et al.*, 1991).

A further action of NO donors in the gastric mucosa was an ability to inhibit acid secretion (Chapter 5), since secretory activity of isolated cells stimulated with histamine

in the presence of IBMX was specifically inhibited. An inhibitory effect of NO on pentagastrin-stimulated acid secretion *in vivo* was suggested because the inhibitory effect of intravenous LPS was attenuated by L-Nitro-arginine methylester (a NOS inhibitor) (Martinez-Cuesta *et al.*, 1992). The inhibitory effects of NO donors on isolated parietal cells seemed to be associated with very large increases in the cGMP content (50-300 fmol/10^6 cells; Fig. 5.6) whereas the effects of NO donors on mucus secretion were obtained with much smaller elevations in the cGMP content (1-18 fmol/10^6 cells; Fig. 4.20). Whether or not NO is a physiological regulator of acid secretion thus remains to be established. However, NO may be an important inhibitor of acid secretion under pathological conditions such as gastritis where induction of NOS-II activity and large and sustained NO production may occur.

7.2 DETRIMENTAL EFFECTS OF NITRIC OXIDE ON THE GASTRIC MUCOSA.

The induction of a Ca^{2+}-independent NOS-II activity in gastric mucosal cells was described in Chapter 3. This activity was associated with a decrease in cell viability, an observation also made with intestinal epithelial cells (Tepperman *et al.*, 1993). The precise significance of these findings is at present unclear, although local intra-arterial infusion of high doses of SNAP (20-40 µg/kg/min) resulted in gastric mucosal injury whereas lower doses (2.5-10 µg/kg/min) protected the mucosa against the injurious effects of endothelin-1 (Lopez-Belmonte *et al.*, 1993). Hence, the unregulated release of high concentrations of NO appear to have a detrimental effect upon the gastric mucosa. In gastric disease where there is activation of the immune system, sustained production would probably inhibit several intracellular enzymes including aconitase (a citric-acid cycle enzyme) and ribonucleotide reductase (Kilbourn *et al.*, 1984; Hibbs *et al.*, 1990) which might in turn reduce bacterial colonisation. Inhibition of parietal cell function and hence acid secretion might occur through elevation of cGMP, or over the longer term, by inhibition of metabolic enzymes. Furthermore, high levels of NO would stimulate the physiological pathway of mucus release but could also damage gastric mucous cells

thereby causing the cells to lyse, release intracellular mucus granules and increase the thickness of the protective mucus gel layer. The reduction in acid secretion and enhancement of the thickness of the mucus layer could restrict the damaging effects of NO.

7.3 INTERACTION OF NITRIC OXIDE WITH OTHER EFFECTORS OF GASTRIC FUNCTION.

7.3.1 Prostanoids and sensory neuropeptides.

In Chapter 1 mention was made of the interaction between NO, sensory neuropeptides and prostanoids in the maintenance of gastric mucosal integrity. In this study the distribution of NOS-containing and PGE_2-producing cells did not overlap (Chapters 3 & 6) although the processes in the gastric mucosa regulated by PGE_2 appear to be identical to those regulated by NO such as mucosal blood flow, stimulation of mucus, and inhibition of acid, secretion. These results suggest a potential synergism between NO and PGE_2 derived from different cell types in the regulation of gastric function. Furthermore, in other tissues, interactions between NO and cyclo-oxygenase activity have been reported. For example, noradrenaline-induced PGE_2 release from the hypothalamus was reported as being mediated via the production of NO which subsequently activates cyclo-oxygenase resulting in the production of PGE_2 which in turn mediates the release of leutenising hormone-releasing hormone (Rettori *et al.*, 1992). However, in rat Kupffer cells, NO production induced by exposure to LPS and interferon-γ resulted in a decreased production of prostanoids via an inhibition of cyclo-oxygenase activity (Stadler *et al.*, 1993). These contradictory results could be due to the sustained production of NO by the Kupffer cells as a result of induced type-II NOS activity which may lead to a derangement of biochemical pathways similar to that described in macrophages (Drapier & Hibbs, 1988). The possibility of a direct interaction between NO and the regulation of cyclo-oxygenase in the rat gastric mucosa deserves further investigation.

This work demonstrated that bradykinin B_1 receptors stimulated PGE_2 release from gastric mucosal cells. Bradykinin stimulates NO production by vascular endothelial

cells (Moncada *et al.*, 1991b) and its action on NO production by gastric cells should be investigated

7.3.2 Acetylcholine.

The role of acetylcholine in the gastric mucosa is to stimulate both mucus and acid secretion (McQueen *et al.*, 1983; Ecknauer *et al.*, 1981). The signal transduction process involved in mediating cholinergic stimulation of mucus secretion involves elevation of intracellular Ca^{2+}-concentrations (Seidler & Pfeiffer, 1991). Since Ca^{2+}-dependent NOS activity has been demonstrated in gastric mucous cells (Chapter 3), it is possible that cholinergic stimulation of mucous cells not only results in probable activation of Ca^{2+}/calmodulin-dependent protein kinase activity, but may also stimulate NOS activity. It is therefore possible that cholinergically-stimulated mucus production, may at least in part, be modulated by NO via activation of NOS (Fig 7.1). Whether NO could diffuse out of mucous cells to inhibit acid secretion in parietal cells is unclear. However, it is unlikely that NO produced by cholinergic stimulation could serve as an intracellular negative feedback regulator of acid secretion because the parietal cell has negligible NOS activity (Chapter 3).

7.3.3 Interactions with other mediators.

Messengers which activate intracellular protein kinase, have the potential to regulate NO production induced by elevation of intracellular Ca^{2+}, since a phosphorylation site for cAMP-dependent protein kinase on NOS-I has been identified and phosphorylation by protein kinase C has been demonstrated to activate purified brain NOS-I (Bredt *et al.*, 1991; Nakane *et al.*, 1991). Furthermore, if the signal transduction mechanism could modulate an elevation of intracellular Ca^{2+} this agent would regulate NOS-I activity via changes in Ca^{2+}/calmodulin binding to NOS. Potential interactions therefore exist between NOS and PGE_2, β-adrenergic agonists and histamine (H_2 receptor) which elevate cAMP (Fig. 7.1).

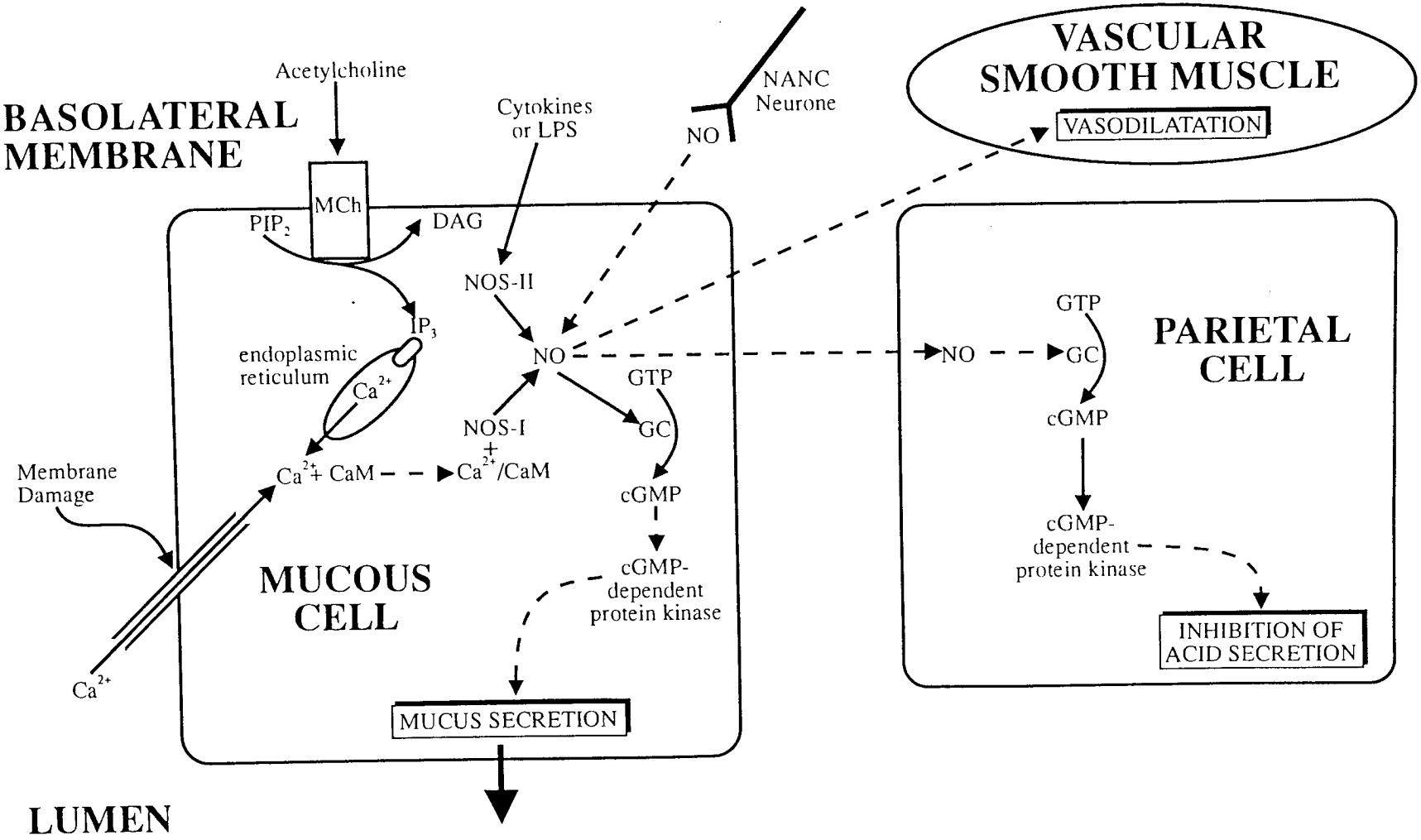
7.3.4 Interactions with antacids.

Recently, the role of NO in the mechanism of antacid induced gastroprotection has been investigated. Pre-treatment of rats with N^G-nitro-L-arginine (a NOS inhibitor) inhibited the protective effect of both Maalox® and $Al(OH)_3$ against mucosal damage by 100% ethanol, whereas indomethacin at a dose which inhibited PGE_2 production by 90% had no effect on the protective influence of these agents. The ethanol damage model is of doubtful physiological relevance but these results are of interest because they suggest that the aluminium containing antacids activate the NO system which may contribute to the gastroprotective activity of these drugs, probably via an increase in mucosal circulation (Konturek *et al.*, 1992). The findings of this thesis suggest that the epithelial cells could be the source of NO in these experiments

In conclusion, when considering the overall function of local mediators in the gastric mucosa it is apparent that for NO when produced in regulated amounts under essentially physiological conditions, a gastroprotective role is indicated since two of the major physiological mechanisms involved in maintaining gastric mucosal integrity (adequate vascular perfusion and mucus secretion to prevent access of pepsin to the mucosa) are both stimulated by the application of exogenous NO. However, during periods of sustained production following induction of NOS-II activity, NO may under these conditions be cytotoxic, possibly also causing inhibition of acid secretion and a profound mucus secretion. The other local mediator mentioned in this work, namely prostaglandin E_2 seems always to be protective in the gastric mucosa but in other tissues can be damaging.

This thesis suggests that non-specific inhibitors of NOS are unlikely to be of therapeutic importance in the treatment of gastrointestinal disease because of the protective and regulatory functions of NO produced by NOS-I. However, a specific inhibitor of NOS-II, the induced form, might be of use if gastric diseases can be identified in which this enzyme is present.

Figure 7.1 Potential roles of NO as a physiological mediator in the gastric mucosa.



Key for Figure 7.1:

— — — → = requires further investigation,
CaM = calmodulin,
DAG = diacylglycerol,
GC = guanylate cyclase,
IP_3 = inositol-1,4,5-trisphosphate,
LPS = lipopolysaccharide,
MCh = muscarinic cholinergic receptor,
NANC = non-adrenergic non-cholinergic,
NO = nitric oxide,
NOS-I = Ca^{2+}-dependent, cytosolic, nitric oxide synthase type-1,
NOS-II = Ca^{2+}-independent, cytosolic, nitric oxide synthase type-II,
PIP_2 = phosphatidylinositol-4,5-bisphosphate.

PUBLICATIONS RESULTING FROM THIS WORK.

PAPERS.

BROWN, J.F., TEPPERMAN, B.L., HANSON P.J., WHITTLE, B.J.R. & MONCADA, S. (1992). Differential distribution of nitric oxide synthase between cell fractions isolated from the rat gastric mucosa. Biochem. Biophys. Res. Commun., **184**, 680-685.

BROWN, J.F., HANSON, P.J. & WHITTLE, B.J.R. (1992). Nitric oxide generators increase mucus gel thickness in rat stomach. Eur. J. Pharmacol., **223**, 103-104.

BROWN, J.F., KEATES, A.C., HANSON, P.J. & WHITTLE, B.J.R. (1993). Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. Am. J. Physiol., **265**, G418-G422.

BROWN, J. F., HANSON, P. J. AND WHITTLE, B. J. R. (1993). Nitric oxide donors inhibit secretory activity in isolated rat gastric parietal cells. Biochem. Biophys. Res. Commun., **195**, 1354-1359

ABSTRACTS

BROWN, J.F., WHITTLE, B.J.R. & HANSON, P.J. (1992). Bradykinin stimulates PGE_2 release in cell fractions isolated from the rat gastric mucosa via a B1 receptor. Biochem. Soc. Trans. **20**, 2765.

WHITTLE, B.J.R., TEPPERMAN, B.L., BROWN, J.F. & HANSON, P.J. (1992). Distribution of nitric oxide synthase in isolated cell fractions of rat gastric mucosa. Gastroenterology, **102**, A765

BROWN, J.F. HANSON, P.J. & WHITTLE, B.J.R. (1993). Nitric oxide donors stimulate rat gastric mucus release and epithelial cyclic-GMP formation. Gastroenterology, **104**, A47.

REFERENCES

Albinus, M. and Sewing, K.F. (1981). Histamine uptake and metabolism in intact isolated parietal cells. Agents Actions, **11**, 223-227.

Allen, A., Bell, A. and McQueen, S. (1984a). Mucus and mucosal protection. In: **Mechanisms of Mucosal Protection in the Upper Gastrointestinal Tract.** (Allen Ed.), pp 195-201. Raven Press, New York.

Allen, A. and Garner, A. (1980). Gastric mucus and bicarbonate secretion and their possible role in mucosal protection. Gut, **21**, 249-262.

Allen, A., Hunter, A.C., Leonard, A.J., Pearson, J.P. and Sellers, L.A. (1989). Peptic activity and the Mucus-Bicarbonate Barrier: In **Advances in Drug Therapy of Gastrointestinal Ulceration**. (Garner A. and Whittle, B.J.R Eds.), pp 139-155, John Wiley & Sons Ltd.

Allen, A., Hutton, D.A., Pearson, J.P. and Sellers, L.A. (1984b). Mucus glycoprotein structure, gel formation and gastrointestinal mucus formation. Ciba Found. Symp., **109**, 137-156.

Andersson, K., Mattsson, H. and Larsson, H. (1990). The role of gastric histamine in acid secretion and experimentally lesions in the rat. Digestion, **46**, 1-9.

Aswad, A.W. and Greengard, P. (1981). A specific substrate for rabbit cerebellum for guanosine-3',5'-monophosphate dependent protein kinase II. Kinetic studies on its phosphorylation by guanosine-3',5'-monophosphate dependent protein kinase. J. Biol. Chem., **256**, 3487-3493.

Atwell, M.A. and Hanson, P.J. (1988). Effect of pertussis toxin on the inhibition of secretory activity by prostaglandin E_2, somatostatin, epidermal growth factor and 12-*o*-tetradecanoyl-phorbol 13-acetate in parietal cells from rat stomach. Biochim. Biophys. Acta., **971**, 282-288.

Babo, A., Moizo, L., Laigneau, J.P. and Lewin, J.M. (1992). Pharmacological characterization of histamine H3 resceptros in isolated rabbit gastric glands. Am. J. Physiol., **262**, G56-G61.

Balligand, J.L., Kelly, R.A., Marsden, P.A., Smith, T.W. amd Michel, T. (1993). Control of cardiac muscle cell function by an endogenous nitric oxide signalling system. Proc. Natl. Acad. Sci. USA., **90**, 347-351.

Bandaletova, T., Brouet, I., Bartsch, H., Sugimura, T., Hsumi, H. and Oshima, H. (1993). Immunohistochemical localization of an inducible form of nitric oxide synthase in various organs of rats treated with *Propionibacterium acnes* and lipopolysaccharide. APMIS, **101**, 330-336.

Bannister, W.H. (1965). The relationship between acid secretion and oxygen uptake by gastric mucosa of the frog. J. Physiol. (Lond.), **177**, 420-439.

Batzri, S. (1981). Direct action of somatostatin on dispersed mucosal cells from guinea-pig stomach. Biochim. Biophys. Acta., **677**, 521-524.

Batzri, S. and Dyer, J. (1981). Aminopyrine uptake by guinea-pig gastric mucosal cells, mediation by cAMP and interaction among secretagogues. Biochim. Biophys. Acta., **675**, 416-426.

Batzri, S., Harmon, J.W. and Thompson, W.F. (1982). Interaction of histamine with gastric mucosal cells: Effect of histamine antagonists on bimding and biological response. Mol. Pharmacol., **22**, 33-40.

Beauchamp, R.D., Barnard, J.A., McCutchen, C.M., Cherner, J.A. and Coffey, R.J. Jr. (1989). Localization of transforming growth factor α and its receptor in gastric mucosal cells. J. Clin. Invest., **84**, 1017-1023.

Berglindh, T., DiBona, D.R., Ito, S. and Sachs, G. (1980). Probes of parietal cell function. Am. J. Pharmacol., **238**, G165-G176.

Berglindh, T., Helander, H.F. and Obrink, K.J. (1976). Effects of secretagogues on oxygen consumption, aminopyrine accumulation and morphology in isolated gastric glands. Acta. Physiol. Scand., **97**, 401-414.

Bersimbae, R.I., Tairov, M.M. and Salganik, R.I. (1985). Biochemical mechanisms of regulation of mucus secretion by prostaglandin E_2 in the rat gastric mucosa. Eur. J. Pharmacol., **115**, 259-266.

Bickel, M. and Kauffman, J.L. (1981). Gastric gel mucus thickness: Effect of distension, 16,16-dimethyl prostaglandin E_2 and carbenoxalone. Gastroenterology, **80**, 770-775.

Blum, A.L., Shah, G.T., Wieblhaus, V.D., Brennan, F.T., Helander, F.T., Ceballos, R. and Sachs, G. (1971). Pronase method for isolation of viable cells from *Necturus* gastric mucosa. Gastroenterology, **61**, 189-200.

Bolton, P., Palmer, D. and Cohen, M.M. (1978). Stimulation of mucus and non-parietal cell secretion by the E_2 prostaglandins. Am. J. Dig. Dis., **23**, 359-364.

Boughton-Smith, N.K., Evans, S.M. and Whittle, B.J.R. (1992). Elevated nitric oxide synthase activity in inflamed colon from a rat model of colitis. Gut, **33**, 12.

Boughton-Smith, N.K., Evans, S.M., Laszio, F., Whittle, B.J.R. and Moncada, S. (1993). The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat. Br. J. Pharmacol., **109**, 1189-1195.

Boughton-Smith, N.K., Hutcheson, I.R., Deakin, A.M., Whittle, B.J.R. and Moncada, S. (1990). Protective effect of S-nitroso-N-acetylpenicillamine in endotoxin induced acute intestinal damage in the rat. Eur. J. Pharmacol. **191**, 485-488.

Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R. and Snyder, S.H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature, **351**, 714-718.

Bredt, D.S. and Snyder, S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Pro. Natl. Acad. Sci. USA., **87**, 682.

Brown, M.R. and Chew, C.S. (1989). Carbachol-induced protein phosphorylation in parietal cells: regulation by $[Ca^{2+}]_i$. Am. J. Physiol., **262**, G56-G61.

Butt, H., Boeckxstaens, G.E., Pelckmens, P.A., Jordaens, F.H., Van Maercke, Y.M. and Herman, A.G. (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature (Lond.), **345**, 346-347.

Burch, R.M., Luini, I. and Axelrod, J. (1986). Phospholipase A_2 and phospholipase C are activated by distinct types of GTP-binding proteins in response to α_1-adrenergic stimulation in FRTL5 thyroid cells. Proc. Natl. Acad. Sci. USA, **83**, 7201-7205.

Callaghan, J.M., Toh, B.H., Pettitt, J.M., Humphris, D.C. and Gleeson, P.A. (1990). Poly-*N*-acetyllactosamine-specific tomato lectin binding 60-90x10^3 M_r membrane glycoprotein of tubulovesicles. J. Cell Sci., **95**, 563-576.

Chen, M.C., Amirian, D.A. and Soll, A.H. (1984). Epidermal growth factor (EGF) binding and inhibitory effect on acid secretion in isolated canine parietal cells. Fed. Proc., **43**, 1073.

Chen, M.C., Amirian, D., Toomey, M., Sanders, M. and Soll, A.H. (1988). Prostanoid inhibition of canine parietal cells: mediation by the GTP-binding protein of adenylate cyclase. Gastroenterology, **94**, 1121-1129.

Chen, M.C., Sanders, M.J., Amirian, D.A., Thomas, L.P., Kauffman, G. and Soll, A.H. (1989). Prostaglandin E_2 production by dispersed canine fundic mucosal cells: Contribution of macrophages and endothelial cells as major sources. J. Clin. Invest., **84**, 1536-1549.

Chew, C.S. (1983a). Forskolin stimulation of acid and pepsinogen secretion in isolated gastric glands. Am. J. Physiol., **245**, G371-G380.

Chew, C.S. (1983b). Inhibitory action of somatostatin on isolated gastric glands and parietal cells. Am. J. Physiol., **245**, G221-G229.

Chew, C.S. (1985). Parietal cell protein kinases: Selective activation of type 1 cAMP-dependent protein kinase by histamine. J. Biol. Chem., **260**, 7540-7550.

Chew, C.S. and Brown, M.R. (1986). Release of Ca^{2+} and elevation of inositol trisphosphate by secretagogues in parietal and chief cells isolated from rabbit gastric mucosa. Biochim. Biophys. Acta., **888**, 116-125.

Chiba, T., Fisher, S.K., Park, J., Sequin, E.B., Agranoff, B.W. and Yamada, T. (1988). Carbamoylcholine and gastrin induce inositol lipid turnover in canine gastric parietal cells. Am. J. Physiol., **255**, G99-G105.

Chiba, T., Hirata, Y., Taminato, T., Kadowaki, S., Matsukara, S. and Fujita, T. (1982). Epidermal growth factor stimulates prostaglandin E release from isolated perfused rat stomach. Biochem. Biophys. Res. Comm., **105**, 370-374.

Cho, H.L., Xie, Q-W., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D. and Nathan, C. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. J. Exp. Med., **176**, 599-604.

Conklin, B.R., Burch, R.M., Steranka, L.R. and Axelrod, J. (1988). Distinct bradykinin receptors mediate stimulation of prostaglandin synthesis by endothelial cells and fibroblasts. J. Pharmacol. Exp. Ther., **224**, 646-649.

Cook, N.J. and Kaupp, U.B. (1986). The cGMP-dependent cation channel from from vertebrate photoreceptors: purification and function reconstitution. Photobichem. Photobiophys., **13**, 331-343.

Cornwell, T.L. and Lincoln, T.M. (1989). Regulation of intracellular Ca^{2+} by atriopeptin and 8-bromo-cyclic-GMP is mediated by cGMP-dependent protein kinase. J. Biol. Chem., **264**, 1146-1155.

Cornwell. T.L., Pryzwansky, K.B., Wyatt, T.A. and Lincoln, T.M. (1991). Regulation of sarcoplasmic reticulum phosphorylation by localised cGMP-dependent protein kinase in vascular smooth muscle cells. Mol. Pharmacol., **40**, 923-931.

Cottee, F., Flower, R.J., Moncada, S., Salmon, J.A. and Vane, J.R. (1977). Synthesis of 6-keto-$PGF_{1\alpha}$ by ram seminal vessicle microsomes. Prostaglandins, **14**, 413-422.

Davidson, W.D., Klein, K.L., Kurokawa, K. and Soll, A.H. (1981). Instantaneous and continuous measurement of ^{14}C-labelled substrate oxidation to $^{14}CO_2$ by minute tissue specimens: An ionization chamber method. Metabolism, **30**, 596-600.

De Camilli, P., Miller, P.E., Levitt, P., Walter, U. and Greengard, P. (1984). Anatomy of cerebellar purkinje cells in the rat determined by a specific immunohistochemical marker. Neuroscience, **11**, 761-817.

De Jonge, H.R. (1981). cGMP-dependent protein kinase in intestinal brush borders. Adv. Cyclic Nucleotide. Res., **14**, 315-323.

Desai, K.M., Zembowicz, A., Sessa, W.C. and Vane, J.R. (1991). Nitroxergic nerves mediate vagally induced relaxation in the isolated stomach of the guinea-pig. Proc. Natl. Acad. Sci. USA., **88**, 11490-11494.

Di Rosa, M., Radomski, M.W., Carnuccio, R. and Moncada, S. (1990). Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. Biochem. Biophys. Res. Commun., **172**, 1246-1252.

Drapier, J.C. and Hibbs, J.B. (1988). Differentiation of murine macrophages to express non-specific cytotoxicity for tumour cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. J. Immunol., **140**, 2829-2838.

Dusting, G.J., Moncada, S. and Vane, J.R. (1978). Vascular actions of arachidonic acid and its metabolites in perfused mesenteric and femoral beds of the dog. Eur. J. Pharmacol., **49**, 65-72.

Ecknauer, R., Dial, E., Thompson, W.J., Johnson, L.R. and Rosenfeld, G.C. (1981). Isolated rat gastric parietal cells: cholinergic response and pharmacology. Life Sciences, **28**, 609-621.

Elliot, K.R.F. (1979) In: **Techniques in the Life Sciences**. Vol. B 204/1, pp1-20, Elsevier/North Holland Press.

Evans, T., Carpenter, A. and Cohen, J. (1992). Purification of a distinctive form of endotoxin-induced nitric oxide synthase from rat liver. Proc. Natl. Acad. Sci. USA., **89**, 5361-5365.

Farquhar, M.G. and Palade, G.E. (1963). Junctional complexes in various epithelia. J. Cell Biol., **17**, 375-414.

Feelisch, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. J. Cardiovasc. Pharmacol. **17**, Suppl.. 3: S25-S33.

Felbel, J., Trockur, B., Ecker, T., Landgraf, W. and Hofmann, F. (1988). Regulation of cytosolic Ca^{2+} by cAMP and cGMP in freshly isolated smooth muscle cells from bovine trachea. J. Biol. Chem., **263**, 16764-16771.

Flemstrom, G. and Garner, A. (1982).Gastroduodenal HCO_3^- transport: characteristics and proposed role in acidity regulation and mucosal protection. Am. J. Physiol., **242**, G183-G193.

Florey, H.W. (1962). The secretion and function of intestinal mucus. Gastroenterology, **43**, 326-329

Forster, E.R. and Southam, E. (1993). Location of nitric oxide synthase in rat gastric corpus. Regulatory Peptides, **40**, 146.

Förstermann, U., Schmidt, H.H.H.W., Pollock, J.S., Sheng, H., Mitchell, J.A., Warner, T.D., Nakane, M. and Murad, F. (1991). Isoforms of nitric oxide synthase: characterization and purification from different cell types. Biochemical Pharmacol., **42**, (10), 1849-1857.

Forstner, G.G. & Forstner, J.F. (1985). Structure and function of gastrointestinal mucus. In: **Molecular and Cellular Biology of Digestion, (Vol. II)**, (Desnuelle, H., Sjostrom, H. & Noren, O. Eds), Elsevier, Amsterdam.

Forstner, J.F., Ofusu, F. and Forstner, G.G. (1977). Radioimmunoassay of intestinal goblet cell mucin. Anal. Biochem., **83**, 657-665.

Forte, J.G., Black, J.A., Forte, T.M., Machen, T.E. and Wolosin, J.M. (1981). Ultrastructural changes related to functional activity in gastric oxyntic cells. Am. J. Physiol., **241**, G349-G358.

Forte, T.M., Machen, T.E. and Forte, J.G. (1977). Ultrastructural changes in oxyntic cells associated with secretory function. A membrane recycling hypothesis. Gastroenterology, **73**, 941-955.

Forte, J.G and Wolosin, J.M. (1987). HCl secretion by the gastric oxyntic cell. In: **Physiology of the Gastrointestinal Tract**, (Johnson, L.R. ed.), vol 1, pp853-864. Raven Press, New York.

Furukawa, K-L., Ohshima, N., Tawada-Iwata, Y. and Shigekawa, M. (1991). cGMP stimulates Na^{+}/Ca^{2+} exchange in vascular smooth muscle cells in primary culture. J. Biol. Chem., **266**, 12337-12341.

Gibert, A.J. and Hersey, S.J. (1982). Morphometric analysis of parietal cell membrane transformations in isolated gastric glands. J. Membr. Biol., **67**, 113-124.

Granger, D.L., Taintor, R.R., Cook, J.L., and Hibbs, J.B. (1980). Injury of neoplastic cells by murine macrophages leads to inhibition of mitochondrial respiration. J. Clin. Invest., **65**, 357-370.

Graumann, W. (1965). Uber die angebbiche de Acidophile der Belegzellen. Histochemie, **5**, 437-440.

Gross, S.S., Steuhr, D.J., Aisaka, K., Jaffe, E.A., Levi, R and Griffith, O.W. (1990). Macrophage and endothelial cel nitric oxide synthesis: cell type selective inhibition by N^G-aminoarginine, N^G-nitroarginine and N^G-methylarginine. Biochem. Biophys. Res. Commun., **170**, 96-103.

Grube, D. and Forssmann, W.G. (1979). Morphology and function of the entero-endocrine cells. Horm. Metab. Res., **11**, 603-620.

Håkanson, R. and Sundler, F. (1991). Do histamine-storing cells in the gastric mucosa mediate the acid-stimulating action of gastrin? In: **Histamine and Histamine Antagonists,** (Unväs, B. ed.), pp325-346. Berlin: Springer.

Hanson, P.J. and Hatt, J.F. (1989). Intracellular signalling and regulation of gastric acid secretion. Quarterly J. Exp. Physiol., **74**, 607-634.

Harrison, S.A., Reifsnyder, D.H., Gallis, B., Cadd, G.G. and Beavo, J.A. (1986). Isolation and characterization of bovine cardiac muscle inhibited phosphodiesterase: A receptor for new cardiotonic drugs. Mol. Pharmacol., **29**, 506-514.

Hatt, J.F. (1988). Regulation of acid secretion by parietal cells isolated from the rat stomach. PhD. Thesis, University of Aston in Birmingham.

Hatt, J.F. and Hanson, P.J. (1988). Inhibition of gastric acid secretion by epidermal growth factor. Effects on cyclic AMP and on prostaglandin production in rat isolated parietal cells. Biochem. J., **255**, 789-794.

Hatt, J.F. & Hanson, P.J. (1989). Intracellular signalling and regulation of gastric acid secretion. Quarterly J. Exp. Physiol., **74**, 607-634.

Haushildt, S., Lückhoff, A., Mülsch, A., Kohler, J., Bessler, W. and Busse, R. (1990). Induction and activity of NO synthase in bone marrow derived macrophages are independent of Ca^{2+}. Biochem. J., **270**, 351-356.

Heim, H-K., Oestmann, A. and Sewing, K-F. (1989). Incorporation of N-acetyl-[^{14}C]D-glucosamine and [^{3}H]-leucine in isolated pig gastric mucosal cells. Digestion, **44**, 26-35.

Helander, H.F. and Hirschowitz, B.I. (1972). Quantitative and ultrastructural studies of microvilli and changes in the tubulovesicular compartment of mouse parietal cells in relation to gastric acid secretion. J. Cell Biol., **63**, 951-961.

Helander, H.F. and Keeling, D.J. (1993). Cell biology of gastric acid secretion. In: **Baillière's Clinical Gastroenterology,** (Tindall, B. ed.) pp 1-21, Baillière Tindal, London.

Helander, H.F. Leth, R. and Olbe, L. (1986). Stereological investigations on human gastric mucosa: I. Normal oxyntic mucosa. Anatomical Record, **216**, 373-380.

Hibbs, J.B., Taintor, R.R. and Vavrin, Z. (1987). Macrophage cytotoxicity: role for L-arginine deiminase activity and imino nitrogen oxidation to nitrite. Science, **235**, 473-476.

Hibbs, J.B., Taintor, R.R. and Vavrin, Z., Granger, D.L., Drapier, J.C., Amber, I.J. and Lancaster, J.R. (1990). Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. In: **Nitric Oxide from L-Arginine: A Bioregulatory System**, (Moncada, S. and Higgs, E.A. eds), pp 189-223, Elsevier, Amsterdam.

Hibbs, J.B., Taintor, R.R. and Vavrin, Z. and Rachilin, E.M. (1988). Nitric oxide: acytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Commun., **157**, 87-94.

Hiraishi, H., Terano, A., Ota, S., Mutoh, H., Sugimoto, T., Razandi, M. and Ivey, K.J. (1991). Oxygen metabolites stimulate mucous glycoprotein secretion from cultured rat gastric mucous cells. Am. J. Physiol., **261**, G662-G668.

Hirata, M., Kohse, K.P., Chang, C.H., Ikebe, T. and Murad, F. (1990). Mechanism of cGMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells. J. Biol. Chem., **265**, 1268-1273.

Ho, S.B., Shekels, L.L., Nietrans, G.A., Lyftogt, C.T., Kim, Y.S. and Toribara, N.W. (1993). Human gastric mucin-gene heterogeneity and alteration in gastric neoplasia. Gastroenterology, **104**, (4), 410.

Hofmann, F., Dostmann, W., Keilbach, A., Landgraf, W and Ruth, P. (1992). Stucture and physiological role of cGMP-dependent protein kinase. Biochemica. Biophysica. Acta., **1135**, 51-60.

Hofmann, F. and Flockerzi, V. (1983).Characterization of phosphorylated and native cGMP-dependent protein kinase. Eur. J. Biochem., **168**, 117-121.

Hope, B.T., Michael, G.J., Knigge, K.M. and Vincent, S.R. (1991). Neuronal NADPH diaphorase is a nitric oxide synthase. Proc. Natl. Acad. Sci. USA., **88**, 2811-2814.

Hseuh, W.C., Kuhn, W.C. and Needleman, P. (1979). Relationships of prostaglandin secretion by rabbit alveolar macrophages to phagocytosis and lysosmal enzyme release. Biochem. J., **184**, 345-354.

Hume, D.A., Perry, V.H. and Gordon, S. (1984). The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80: macrophages associated with epithelia. Anat. Rec., **210**, 503-510.

Hunt, T.E. and Hunt, E.A. (1963). Radioautographic study of proliferation in the stomach of the rat using thymidine-^{3}H and compound 48/80. Anat. Rec., **142**, 505-517.

Ignarro, L.J. (1990). Nitric oxide: a novel signal transduction mechanism for transcellular communication. Hypertension, **16**, 477-483.

Ignarro, L.J. (1991). Signal transduction mechanisms involving nitric oxide. Biochem. Pharmacol., **41**, 485-490.

Ignarro, L.J., Ballot, B. and Woods, K.S. (1984). Regulation of soluble guanylate cyclase activity by porphyrins and metalloporphyrins. J. Biol. Chem., **259**, 6201-6207.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. USA., **84**, 9265-9269.

Ignarro, L.J., Lippton, H., Edwards, J.C., Baricos, W.H., Hyman, A.L., Kadowitz, P.J. and Gruetter, C.A. (1981). Mechanisms of smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide. Evidence for the involvement of S-nitrosothiols as active ingredients. J. Pharmacol. Exp. Ther. **218**, 739-749.

Ito, S. and Lacy, E.R. (1985). Morphology of rat gastric mucosal damage defense and restitution in the presence of luminal ethanol. Gastroenterology, **88**, 250-260.

Ito, S. and Winchester, R.J. (1963). The fine structure of the gastric mucosa in the bat. J. Cell Biol., **16**, 541-578.

Kasai, K., Hiraiwa, M., Emoto, T., Akimoto, K. Takaoka, T. and Shimonda, S-I. (1987). Stimulation of PGE_2 production by phorbol esters and epidermal growth factor in porcine thyroid cells. Life Sci., **41**, 241-247.

Kauffman, G.L., Reeve, J.J. and Grossman, M.I. (1978). Gastric bicarbonate secretion: Effect of topical and intravenous 16,16-dimethyl prostaglandin E_2. Am. J. Physiol., **239**, G44-G52.

Keates, A.C. (1991) Regulation of mucus secretion by cells isolated from the rat gastric mucosa. Ph.D. Thesis, University of Aston in Birmingham.

Keates, A.C. and Hanson, P.J. (1990a). Regulation of mucus secretion by cells isolated from the rat gastric mucosa. J. Physiol. **423**, 397-409.

Keates, A.C. and Hanson, P.J. (1990b). Assay of mucus secreted from isolated stomach cells by using an enzyme-linked immunosorbent assay. Biochem. Soc. Trans. **18**, 956.

Kerss, S., Allen, A. and Garner, A. (1982). A simple method for measuring thickness of the mucous gel layer adherent to rat, frog and human gastric mucosa: Influence of feeding, prostaglandin, N-acetylcysteine and other agents. Clin. Sci. **63**, 187-195.

Kilbourn, R.G., Klostergaard, J. and Lopez-Berestein, G. (1984). Activated macrophages secrete a soluble factor that inhibits mitochondrial respiration of tumour cells. J. Immunol., **133**, 2577-2581.

Knowles, R.G., Merrett, M., Salter, M and Moncada, S. (1990). Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. Biochem. J., **270**, 833-836

Konturek, S.J., Brzozowski, T., Majka, J., Szlachcic, A., Nauert, C. and Slomiany, B. (1992). Nitric oxide in gastroprotection by aluminium-containing antacids. Eur. J. Pharmacol., **229**, 155-162.

Lee, E.R. and Leblond, C.P. (1985). Dynamic histology of the antral epithelium in the mouse stomach. IV. Ultrastructure and renewal of gland cells. Am. J. Anat., **172**, 241-259.

Lee, J., Simpson, G. and Scholes, P. (1974). An ATPase from dog gastric mucosa; Changes of outer pH in suspensions of membrane vessicles accompanying ATP hydrolysis. Biochem. Biophys. Res. Comm., **60**, 825-832.

Lewin, M., Cheret, A.M., Soumaron, A. and Girodet, J. (1974). Methode pour l'isolement at le tri des cellules de la muquese fundique de rat. Biol. Gastroenterol. (Paris), **7**, 139-144.

Lewis, J.L., Goldenring, J.R, Modlin, I.M. and Coffey, R.J. (1990). Inhibition of parietal cell H^+ secretion by transforming growth factor alpha: A possible autocrine regulatory mechanism. Surgery, **108**, 220-227.

Light, D.B., Corbin, J.D. and Stanton, B.A. (1990). Dual ion channel regulation by cyclic-GMP-dependent protein-kinase. Nature (Lond.), **344**, 336-339.

Lincoln, T.M. and Cornwell, T.L. (1993). Intracellular cyclic GMP receptor proteins. FASEB. J., **7**, 328-338.

Lincoln, T.M., Thompson, M. and Cornwell, T.L. (1988). Purification and characterization of two forms of cGMP-dependent protein kinase from bovine aorta. J. Biol. Chem., **263**, 17632-17637.

Ljungström, M. and Chew, C.S. (1991). Calcium oscillations in single cultured gastric parietal cells. Am. J. Physiol., **260**, C67-C78.

Lopez-Belmonte, J., Whittle, B.J.R. and Moncada, S. (1993). The actions of nitric oxide donors in the prevention or induction of injury in the rat gastric mucosa. Br. J. Pharmacol., **108**, 73-78.

Lubbock, R., Gupta, B.L. and Hall, T.A. (1981). Novel role of calcium in exocytosis: Mechanism of nematocyst discharge as shown by X-ray microanalysis. Proc. Natl. Acad. Sci. USA, **78**, 3624-3628.

Madshus, I.H. (1988). Regulation of intracellular pH in eukaryotic cells. Biochem. J., **250**, 1-8.

Maeda, M., Oshiman, K-I., Tamura, S. and Futai, M. (1989). Human gastric (H^+-K^+)-ATPase gene. J. Biol. Chem., **265**, 9027-9032.

Main, I.H.M. and Whittle, B.J.R. (1973). The effects of E and A prostaglandins on gastric mucosal blood flow and gastric acid secretion in the rat. Br. J. Pharmacol., **49**, 428-436.

Mantle, M. and Thakore, E. (1988). Rabbit intestinal and colonic mucins: Isolation, partial characterization, and measurement of secretion using an enzyme-linked immunoassay. Biochem. Cell Biol., **66**, 1045-1054.

Margolis, B.L., Bonventre, J.V., Kremer, S.G., Kudlow, J.E. and Skorecki, K.L. (1988). Epidermal growth factor is synergistic with phorbol esters and vasopressin in stimulating arachidoinc acid release and prostaglandin production in renal glomerular messangial cells. Bichem. J., **249**, 587-592.

Marquardt, H., Hunkapiller, M.W., Hood, L.E. and Todaro, G.J. (1984). Rat transforming growth factor type I: structure and relationship to epidermal growth factor. Science, **223**, 1079-1082.

Marsden, P.A., Schappert, K.T., Chen, H.S., Flowers, M., Sundell, C.L., Wilcox, J.N., Lamas, S. and Michel, T. (1992). Molecular cloning and characterization of human endothelial nitric oxide synthase. FEBS Lett., **307**, 287-293.

Martin, P., Lafon-Cazal, M. and Bockaert, J. (1992). A nitric oxide synthase activity selectively stimulated by NMDA receptors depends upon protein kinase c activation in mouse striatal neurons. Eur. J. Neuroscience, **4**, 425-432.

Martin, W., Villani, G.M., Jothianandan, D. and Furchgott, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. J. Pharmacol. Exp. Ther. **232**, 708-716.

Martinez-Cuesta, M.A., Barrachina, M.D., Pique, J.M., Whittle, B.J.R. and Espluges, J.V. (1992). The role of nitric oxide and platelet activating factor in the inhibition by endotoxin of pentagastrin-stimulated gastric acid secretion. Eur. J. Pharmacol., **218**, 351-354.

Mayer, B., John, M and Böhme, E. (1990). Partial purification and characterization of a Ca^{2+}/calmodulin-dependent endothelium derived relaxing factor forming enzyme from porcine cerebellum. FEBS Lett., **277**, 215-219.

McCall, T., Feelisch, M., Palmer, R.M.J. and Moncada, S. (1991). Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. Br. J. Pharmacol., **102**, 234-238.

McPherson, M.A. and Dormer, R.L. (1984). Control of secretion of mucin-type glycoproteins from human and rat submandibular acini. Biochem. Soc. Trans., **12**, 652-653.

McQueen, S., Allen, A. and Garner, A. (1984). In: **Mechanisms of Mucosal Protection in the Upper Gastrointestinal Tract**. (Allen, A. *et al.*, eds). pp 215-221. Raven Press, New York.

McQueen, S., Hutton, D., Allen, A. and Garner, A. (1983). Gastric and duodenal surface mucus gel thickness in rat: Effects of prostaglandins and damaging agents. Am. J. Physiol., **245**, G388-G393.

Mercier, F., Besancon, F., Hersey, S.J. and Sachs, G. (1991). Extracellular labelling of the H, K-ATPase: evidence for 10 membrane spanning domains. FASEB. Journal, **5**, A749.

Méry, P.F., Lohmann, S.M., Walter, U. and Fischmeister, R. (1991). Ca^{2+} current is regulated by cGMP-dependent protein kinase in mammalian cardiac myocytes. Proc. Natl. acad. Sci. USA., **88**, 1197-1201.

Mine, T., Fujisaki, J., Akimoto, K., Yoshida, S., Hasegawa, Y. and Ogata, E. (1988). Effects of secretin on content of PGE_2 and 6-keto $PGF_{1\alpha}$ in gastric mucosa. Dig. Dis. Sci., **33**, 1578-1582.

Moncada, S. and Vane, J.R. (1979). Pharmacology and endogenous role of prostaglandin endoperoxides, thromboxane A_2 and prostacyclin. Phrmacol. Rev., **30**, 293-331.

Moncada, S., Higgs, E.A., Hodson, H.F., Knowles, R.G., Lopez-Jaramillo, P, McCall, T., Palmer, R.M.J., Radomski, M.W., Rees, D. and Schulz, R. (1991a). The L-arginine: nitric oxide pathway. J. Cardiovasc. Pharmacol., **17**, (suppl. 3), S1-S9.

Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991b). Nitric oxide: Physiology, pathophysiology and pharmacology. Pharmacol. Rev., **43**, 109-142.

Moody, F.G. (1968). Oxygen consumption during thiocyanate inhibition of gastric acid secretion in dogs. Am. J. Physiol., **215**, 127-131.

Morris G.P., Harding, R.K. and Wallace, J.C. (1986). A functional model for extracellular gastric mucus in the rat. Virchows. Arch. [Cell Pathol.], **46**, 239-251.

Myers, P.R., Minor, R.L., Guerra, R., Bates, J.N. and Harrisson, D.G. (1990). Vasorelaxant properties of the endothelium derived relaxing factor more closely resembles S-nitrosocysteine than nitric oxide. Nature (Lond.), **345**, 161-163.

Nakamura M., Oda, M., Yonei, Y., Tsukada, N., Komatsu, H., Akaiwa, Y., Kaneko, K. and Tsuchiya, M. (1985). Muscarinic acetylcholine receptors in rat gastric mucosa. A radioautographic study using a potent muscarinic antagonist, ^{3}H-pirenzepine. Histochemistry, **83**, 479-487.

Nakane, M., Mitchell, J. Forstermann, U. and Murad, F. (1991). Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase. Biochem. Biphys. Res. Commun. **180**, 1396-1402.

Nakane, M., Schmidt, H.H.H.W., Pollock, J.S., Förstermann, U. and Murad, F. (1993). Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. FEBS Lett., **316**, 175-180.

Neutra, M.R. and Forstner, J.F. (1987). Gastrointestinal mucus: synthesis, secretion and function. In: **Physiology of the Gastrointestinal Tract (2nd edition)**, (Johnson. Ed.) pp 975-1009, Raven Press, New York.

Nguyen, T., Brunson, D., Crespi, C.L., Penman, B.W., Wishnok, J.S. and Tannenbaum, S.R. (1992). DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. Proc. Natl. Acad. Sci. (USA), **89**, 3030-3034.

O'sullivan, A.J. and Burgoyne, R.D. (1990). Cyclic-GMP regulates nicotine-induced secretion from cultured bovine chromaffin cells: effects of 8-bromo-cyclic GMP

Ota, S., Razanadi, M., Krause, W., Terano, A., Hiraishi, H. and Ivey, K.J. (1988). Prostaglandin E_2 output by isolated rat gastric parietal cells and non-parietal epithelial cells. Prostaglandins, **36**, (5), 589-600.

Palacios, M., Knowles, R.G., Palmer, R.M.J. and Moncada, S. (1989). Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. Biochem. Biophys. Res. Commun., **165**, 802-809.

Palmer, R.M.J., Ashton, D.S. and Moncada, S. (1988). Vascular endothelial cells synthesise nitric oxide from L-arginine. Nature (Lond.), **333**, 664-666.

Palmer, R.M.J., Bridge, L., Foxwell, N.A. and Moncada, S. (1992). The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. Br. J. Pharmacol. **105**, 11-12.

Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature (Lond.), **327**, 524-526.

Palmer, R.M.J. and Moncada, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells Biochem. Biophys. Res. Commun., **158** 348-352.

Park, J., Chiba, T. and Yamada, T. (1987). Mechanisms for direct inhibition of canine gastric parietal cells by somatostatin. J. Biol. Chem., **262**, 14190-14196.

Payne, N.A. and Gerber, J.G. (1987). Prostaglandin E_2 and [^{14}C]arachidonic acid release by carbachol in the isolated canine parietal cell. J. Pharmacol Exp. Ther., **243**, 511-516.

Pfeiffer, A., Rochlitz, H., and Noelke, B. (1990). Muscarinic receptors mediating acid secretion in isolated gastric parietal cells are of M_3 type. Gastroenterology, **98**, 218-222.

Pfeiffer, A., Sauter, G. and Rochlitz, H. (1987). Functional and biochemical interactions of dibutyryl cyclic AMP with the phosphoinositide system in isolated rat parietal cells. Biochem. Biophys. Res. Comm., **147**, 758-764.

Pique, J.M., Whittle, B.J.R. and Espluges, J.V. (1989). The vasodilator role of nitric oxide in the rat gastric microcirculation. Eur. J. Phrmacol., **174**, 293-298.

Prescott, S.M. and Majerus, P.W. (1983). Characterisation of 1,2-diacylglycerol hydrolysis in human platelets. J. Biol. Chem., **258**, 764-769.

Proud, D. and Kaplan, A.P. (1988). Kinin formation-mechanisms and role in inflammatory disorders. In: **Annual Reviews in Immunology**, (Proud, D. ed.), pp49-83. Annual Reviews Incorporated.

Puurunen, J. and Schwabe, U. (1987). Effect of gastric secretagogues on the formation of inositol phosphates in isolated gastric cells of the rat. Br. J. Pharmacol., **90**, 479-490.

Radomski, M.W., Palmer, R.M.J. and Moncada, S. (1990a). An L-arginine nitric oxide pathway present in human platelets regulates aggregation. Proc. Natl. Acad. Sci. USA., **87**, 5193-5197.

Radomski, M.W., Palmer, R.M.J. and Moncada, S. (1990b). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc. Natl. Acad. Sci. USA., **87**, 10043-10047.

Rathbone, B.J., Wyatt, J.I. and Heatley, R.V. (1986). Campylobacter pyloridis- a new factor in peptic ulcer disease? Gut, **27**, 635-641.

Rapoport, R.M. (1986). Cyclic guanosine monophosphate inhibition of contraction may be mediated through inhibition of phosphatidylinositol hydrolysis in rat aorta. Circ. Res., **58**, 407-410.

Rees, D.D., Celek, S., Palmer, R.M.J. and Moncada, S. (1990). Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone. Biochem. Biophys. Res. Commun., **173**, 541-547.

Regoli, D., Marceau, F. and Barabé, J. (1978). *De novo* formation of vascular receptors for kinins. Can. J. Physiol. Pharmacol., **56**, 674-677.

Regoli, D., Rhaleb, N-E., Dion, S. and Drapeau, G. (1990). New selective bradykinin antagonists and bradykinin B_2 receptor characterization. TIPS., **11**, 156-161.

Reichstein, B.J., Okamoto, C. and Forte, J.G. (1984). Inhibition of acid secretion by epidermal growth factor (EGF) in isolated gastric glands is secretagogue specific. Fed. Proc., **43**, 1072.

Reinhard, M., Halbrügge, M., Scheer, U., Wiegand, C., Jockusch, B.M. and Walter, U. (1992). The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts. EMBO J., **11**, 2063-2070.

Rettori, V., Gimeno, M., Lyson, K and McCann, S.M. (1992). Nitric oxide mediates norepinephrine induced prostaglandin E_2 release from the hypothalamus. Proc. Natl. Acad. Sci. USA., **89**, 11543-11546.

Reyl, F., Silvé, C. and Lewin, M.J.M. (1979). Somatostatin receptors on isolated gastric cells. In: **Hormone Receptors In Digestion and Nutrition**, (Rosselin, G., Fromageot, O. & Bonfils, S. eds), pp 391-400. Amsterdam, Elsevier/North Holland Biomedical Press.

Rhodes, J.A., Tam, J.P. and Finke, U. (1986). Transforming growth factor α inhibits secretion of gastric acid. Proc. Natl. Acad. Sci. (USA), **83**, 3844-3846.

Robinson, A.P., White, T.M. and Mason, D.W. (1986). Immunology, **57**, 231-237.

Romano, M., Razandi, M. and Ivey, K. (1988). Somatostatin stimulates prostaglandin production by rat gastric epithelial cells *in vitro*, but is not cytoprotective. Dig. Dis. Sci., **33**, 1435-1440.

Romero, J.C., Lahera, V., Salom, M. and Biondi, M. (1992). The role of the endothelial dependent relaxing factor nitric oxide on renal function. J. Am. Soc. Nephrol., **2**, 1371-1387.

Roomi, N., Laburthe, M., Fleming, N., Crowther, R. and Forstner, J. (1984) Cholera-induced mucin secretion from rat intestine. Lack of effect of cAMP, cycloheximide, VIP and colchicine. Am. J. Physiol. **247**, G140-G148.

Rosenfeld, G.C. (1986). Prostaglandin E_2 inhibition of secretagogue-stimulated [^{14}C] aminopyrine accumulation in rat parietal cells: A model for its mechanism of action. J. Pharmacol. Exp. Ther., **237**, 513-518.

Ross, I.N., Bahari, H.M.M. and Turnberg, L.A. (1981). The pH gradient across mucus adherent to rat fundic mucosa *in vivo* and the effect of potential damaging agents. J. Cell. Biol., **99**, 229.

Ruoff, J.H. and Becker, M. (1982). Histamine-sensitive adenylate cyclase in human gastric mucosa: Cellular localization and interaction by PGE_2, somatostatin and secretin. Agents and Actions, **12**, 174-175.

Sachs, G., Chang, H.H., Rabon, E., Schackmann, R., Lewin, M. and Saccomani, G. (1976). A non-electrogenic H^+ pump in plasma membranes of hog stomach. J. Biol. Chem., **251**, 7690-7698.

Sachs, G. (1987). The gastric proton pump: The H^+/K^+-ATPase. In; **Physiology of the Gastrointestinal Tract**, (Johnson, L.R. ed.) vol 1, pp 865-881, Raven Press, New York.

Salter, M., Knowles, R.G. and Moncada, S. (1991). Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. FEBS Lett., **291**, 145-149.

Salvemini, D., Masini, E., Anggard, E., Mannaioni, P.F. and Vane, J. (1990). Synthesis of a nitric oxide-like factor from L-arginine by rat serosal mast cells: stimulation of guanylate cyclase and inhibition of platelet aggregation. Biochem. Biophys. Res. Commun., **169**, 596-601.

Samuelsson, B., Granström, E., Green, K., Hamberg, M. and Hammarström, S. (1975). Prostaglandins., Annu. Rev. Biochem., **44**, 669-695..

Sandberg, M., Natarajan, V., Ronender, S., Kalderon, D., Walter, U., Lohmann, S.M. and Jahnsen, T. (1989). Molecular cloning and predicted full-length amino acid sequence of the type Iβ isozyme of cGMP-dependent protein kinase from human placenta. FEBS Lett., **255**, 321-329.

Sanders, K.M. and Ward, S.M. (1992). Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. Am. J. Physiol., **262**, (Gastrointest. Liver Physiol., 25), G379-G392.

Sanders, M.J and Soll, A.H. (1986). Characterization of receptors regulating secretory function in the fundic mucosa. Ann. Rev. Physiol., **48**, 89-101.

Sandzén, B. Blom, H. and Dahlgren, S. (1988). Gastric mucus gel layer thickness measured by direct light microscopy. An experimental study in the rat. Scand. J. Gastroenterol., **23**, 1160-1164.

Schepp, W., Heim, H.-K. and Ruoff, H.-J. (1983a). Comparisson of the effect of PGE_2 and somatostatin on histamine stimulated ^{14}C-aminopyrine uptake and cyclic AMP formation in isolated rat gastric mucosal cells. Agents and Actions, **13**, 200-206.

Schepp, W., Prinz, C., Tatge, C., Hakanson, R., Schudsdziarra, V. and Classen, M. (1990a). Galanin inhibits gastrin release from isolated rat gastric G-cells. Am. J. Physiol., **256**, G596-G602.

Schepp, W., Ruoff, H.-J. and Maslinski, S. (1983b). Aminopyrine accumulation of isolated parietal cells from the rat stomach. Effect of histamine and interactions with endogenous inhibitors. Arch. Int. Pharmacodyn. Ther., **265**, 293-308.

Schepp, W., Schneider, J., Tatge, C., Schusdziarra, V. and Classen, M. (1990b). Potential mediation of prostaglandin E_2 release from isolated human parietal cells by protein kinase C. Clin. Physiol. Biochem., **8**, 128-139.

Scherer-Singler, U., Vincent, S.R., Kimura, H. and McGeer, E.G. (1983). Demonstration of a unique population of neurones with NADPH-diaphorase histochemistry. J. Neurosci. Methods., **9**, 229-234.

Schmidt, H.H.H.W, Lohmann, S.M. and Walter, U. (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochem. Biophys. Acta., **1178**, 153-175.

Schmidt, H.H.H.W., Pollock, J.S., Nakane, M., Gorsky, L.D., Förstermann, U. and Murad, F. (1991). Purification of a soluble form of guanylyl cyclase activating factor synthase. Proc. Natl. Acad. Sci. USA., **88**, 365-369.

Schmidt, H.H.H.W., Seifert, R. and Bohme, E. (1989). Formation and release of nitric oxide from human neutrophil and HL-60 cells induced by chemotactic peptide, platelet activating factor and leukotriene B_4. FEBS Lett., **244**, 357-360.

Schmidt, H.H.H.W, Smith, R.M., Nakane, M. and Murad, F. (1992a). Ca^{2+}/calmodulin dependent nitric oxide synthase Type-I: A biopterin flavoprotein with Ca^{2+}/calmodulin-independent diaphorase and reductase activities. Biochemistry, **31**, 3243-3249.

Schmidt, H.H.H.W., Warner, T.D., Ishii, K., Sheng, H. and Murad, F. (1992b). Insulin secretion from pancreatic β cells caused by L-arginine-derived nitrogen oxides. Science, Wash. DC. **255**, 721-723.

Schulz, R., Nava, E. and Moncada, S. (1992). Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium. Br. J. Pharmacol., **105**, 575-580.

Seglen, P.O. (1976) In **Methods in Cell Biology**. (Prescott. ed) **13**, pp 29-83, Academic Press, New York.

Seidler, U., Beinborn, M. and Sewing, K-Fr. (1989a). Inhibition of acid formation in rabbit parietal cells by prostaglandins is mediated by the prostaglandin E_2 receptor. Gastroenterology, **96**, 314-320.

Seidler, U., Knafla, K., Kownatzki, R. and Sewing, K-Fr. (1989b). Effects of endogenous and exogenous prostaglandins on glycoprotein synthesis and secretion in isolated rabbit gastric mucosa. Gastroenterology, **95**, 945-951.

Seidler, U. & Sewing, K-Fr. (1989). Ca^{2+}-dependent and Ca^{2+}-independent secretogogue action on gastric mucus secretion in rabbit mucosal explants. Am. J. Physiol. **256**, (Gastrointest. Liver. Physiol. **19**), G739-G746.

Seidler, U. and Pfeiffer, A. (1991). Inositol phosphate formation and $[Ca^{2+}]_i$ in secretagogue stimulated rabbit gastric mucous cells. Am. J. Physiol. **260**, (Gastrointest. Liver Physiol., **1**), G133-G141.

Shull, G.E. and Lingrel, J.B. (1986). Molecular cloning of the rat stomach (H^+-K^+)-ATPase. J. Biol. Chem., **261**, 16788-16791.

Skoglund, M.L., Gerber, J.G., Murphy, R.C. and Alan, S.N. (1980). Prostaglandin production by intact isolated gastric parietal cells. Eur. J. Pharmacol., **66**, 145-148.

Sminia, T. and Jeurissen, S.H.M. (1986). The macrophage population of the gastrointestinal tract of the rat. Immunobiology, **172**, 72-80.

Smolka, A., Alverson, L., Fritz, R., Swiger, K. and Swiger, R. (1991). Gastric H, K-ATPase topography: amino acids 888-907 are cytosolic. Biochem. Biophys. Res. Comm., **180**, 1356-1364.

Smolka, A., Helander, H. and Sachs, G. (1984). Monoclonal antibodies against gastric H^+/K^+-ATPase. Am. J. Physiol., **245**, G589-G596.

Snary, D., Allen, A. and Pain, R.H. (1970). Structural studies on gastric mucoproteins lowering of molecular weight after reduction with 2-mercaptoethanol. Biochem. Biophys. Res. Commun., **40**, 844-851.

Sobala, G.M., Crabtree, J.E., Dixon, M.F., Schorah, C.J., Taylor, J.D., Rathborne, B.J., Heatley, R.V. and Axon, A.T.R. (1991). Acute helicobacter pylori infection: clinical features, local and systemic immune response, gastric mucosal histology and gastric juice ascorbic acid concentration. Gut, **32**, 1415-1418.

Soll, A.H. (1978). The actions of secretagogues on oxygen uptake by isolated mammalian parietal cells. J. Clin. Invest., **61**, 370-380.

Soll, A.H. (1980a) Secretagogue stimulation of [^{14}C]-aminopyrine accumulation by isolated canine parietal cells. J. Clin. Invest., **65**, 1222-1229.

Soll, A.H. (1980b). Specific inhibition by prostaglandin E_2 and I_2 of histamine-stimulated aminopyrine accumulation ratio and cyclic-adenosine monophosphate generation by isolated canine parietal cells. Gastroenterology, **83**, 216-223.

Soll, A.H., Amiriran, D.A., Park, J., Elashoff, J.D. and Yamada, T. (1985). Cholecystokinin potentially releases somatostatin from canine fundic mucosal cells in short-term culture. Am. J. Physiol., **248**, 569-573.

Soll, A.H. and Berglindh, T. (1987). Physiology of isolated gastric glands and parietal cells. In: **Physiology of the Gastrointestinal Tract**. (Johnson, L.R. ed.), vol 1, pp 883-910. Raven Press, New York.

Soll, A.H., Kurata, J. and McGuigan, J.E. (1989). Ulcers, nonsteroidal antiinflammatory drugs and related matters. Gastroenterology, **96**, 561-568.

Soll, A.H. and Wollin, A. (1979). Histamine and cyclic AMP in isolated canine parietal cells. Am. J. Physiol., **237**, E444-E450.

Spicer, S.S., Katsuyama, T, and Sannes, P.L. (1978). Ultrastructural carbohydrate cytochemistry of gastric epithelium. Histochem. J., **10**, 309-331.

Stadler, J.S., Harbrecht, B.G., Silvio, M.D., Curran, R.D., Jordan, M.L. Simmons, R.L. and Billiar, T.R. (1993). Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat Kupffer cells. J. Leukoc. Biol., **53**, 165-172.

Stamler, J.S., Singel, D.J. and Loscalzo, J. (1992). Biochemistry of nitric oxide and its redox activated forms. Science, **258**, 1898-1902.

Stark, M.E. and Szurszewski, J.H. (1992). Role of nitric oxide in gastrointestinal and hepatic function and disease. Gastroenterology, **103**, 1928-1949.

Stuehr, D.J., Cho, H.J., Kwon, N.S., Weise, M. and Nathan, C.F. (1991a). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. Proc. Natl. Acad. Sci. USA., **88**, 7773-7779.

Stuehr, D.J., Kwon, N.S., Nathan, C.F., Griffith, O.W., Feldman, P.L. and Wiseman, J. (1991b). N^{ω}-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. J. Biol. Chem., **266**, 6259-6263.

Stuehr, D.J. and Marletta, M.A. (1985). Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. Proc. Natl. Acad. Sci. USA., **82**, 7738-7742.

Takio, K., Wade, R.D., Smith, S.B., Krebbs, E.G., Walsh, K.A. and Titani, K. (1984). Guanosine cyclic 3',5'-phosphate dependent protein kinase, a chimeric protein homologous with 2 separate protein families. Biochemistry, **23**, 4207-4218.

Tepperman, B.L., Brown, J.F. and Whittle, B.J.R. (1993). Nitric oxide synthase induction and intestinal epithelial cell viability in the rat. Am. J. Physiol. **265**, G214-G218.

Thomas, L.M. and Cornwell, T.L. (1993). Intracellular cGMP receptor proteins. FASEB. J., **7**, 328-338.

Thomas, M.K., Francis, S.H. and Corbin, J.D. (1990). Characterization of a purified bovine lung cGMP-binding cGMP phosphodiesterase. J. Biol. Chem., **265**, 14964-14971.

Thompson, W.J., Chang, L.K. and Rosenfeld, G.C. (1981). Histamine regulation of adenylyl cyclase of enriched rat gastric parietal cells. Am. J. Physiol., **240**, G76-G84.

Toribara, N.W., Roberton, A.M., Ho, S.B., Kuo, W.L., Gum, E., Hicks, J.W., Gum, J.R., Byrd, J.C., Siddiki, B. and Kim, Y.S. (1993). Human gastric mucin-identification of a unique species by expression cloning. J.Biol. Chem., **268**, 5879-5885.

Tracey, W.R., Nakane, M., Pollock, J. and Förstermann, U. (1993). Nitric oxide synthases in neuronal cells, macrophages and endothelium are NADPH-diaphorases, but represent only a fraction of total cellular NADPH-diaphorase activity. Biochem. Biophys. Res. Commun., **195**, 1035-1040.

Trotman, C.N.A. and Greenwell, J.R. (1979). Computed resolution and relative specific activities of radiolabelled proteins synthesized by isolated gastric mucosal cells. Biochem. J. **178**, 159-164.

Tsunoda, Y. (1987). Ca^{2+} currents and acid secretion in the isolated parietal cell involved in response to gastrin, compound 40/80 and ethylene diamine tetraacetic acid. Biochem. Cell Biol., **65**, 144-162.

Tsunoda, Y., Yakeda, H., Asaka, M., Nakagaki, I. and Sasaki, S. (1988). Initial and sustained calcium mobilizations in the parietal cell during stimulations with gastrin, inositol trisphosphate, phorbol ester and exogenous diacylglycerol. FEBS Lett., **232**, 83-90.

Uehara, A., Okumura, T., Okumura, K., Takasugi, Y. and Namiki, M. (1990). Lipopolysaccharide-induced inhibition of gastric acid and pepsin secretion in rats. Eur. J. Pharmacol., **181**, 141-145.

Van Furth, R. (1980). Cells of the mononuclear phagocyte system. Nomenclature in terms of sites and conditions. In: **Mononuclear Phagocytes: Functional Aspects**. (Van Furth, R. ed.), pp 1-45, Martinus Nijhoff, The Hague.

Vane, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. Nature New Biol., **231**, 232-235.

Verdugo, P. (1984). Hydration kinetics of exocytosed mucins in cultured secretory cells of the rabbit trachea: A new model. In:**Mucus and Mucosa (Ciba Found. Symp., 109)**, pp 215-225, Pitman, London.

Waldenhaug, M.O., Post, R.L., Saccomani, G., Leonard, R.T. and Briskin, D.P. (1985). Structural relatedness of three ion-transport adenosine trisphosphatases around their active sites of phosphorylation. J. Biol. Chem., **260**, 3852-3859.

Wallace, J.L. (1989). Gastric resistance to acid: Is the 'mucus-bicarbonate barrier' functionally redundant. Am. J. Physiol., **256**, G31-G38.

Wallace, J.L. & Cohen, M.M. (1984). Gastric mucosal protection with chronic mild restraint; role of endogenous prostaglandins. Am. J. Physiol., **247**, (Gastrointest. Liver Physiol., **10**), G127-G132.

Wallace, J.L. and Whittle, B.J.R. (1989). The use of *in vivo* and *in vitro* markers of cellular disruption to assess damage and protection in the gastrointestinal tract. In: **New Aspects of Pathogenesis and Pharmacology,** (Szabo and Pfeiffer Eds.). pp 401-415, Roca Raton, FL: CRC Press.

Wang, J., Kester, M. and Dunn, M.J. (1988). Involvement of a pertussis toxin-sensitive G-protein-coupled phospholipase A_2 in lipopolysaccharide-stimulated prostaglandin E_2 synthesis in cultured rat messengial cells. Biochim. Biophys. Acta., **963**, 429-435.

Wernet, W., Flockerzi, V. and Hofmann, F. (1989). The cDNA's of the two isoforms of bovine cGMP-dependent protein kinase. FEBS. Lett., **251**, 191-196.

Whittle, B.J.R. (1986). The mechanisms of gastric damage by non-steroid anti-inflammatory drugs. In: **Biological Protection with Prostaglandins**, (Cohen, M. ed.), pp1-27, Florida, C.R.C. Press.

Whittle, B.J.R., Berry, S., Lopez-Belmonte, J., Boughton-Smith, N.K. and Moncada, S. (1991). Detection of the synthase enzyme that forms the endogenous vasodilator, nitric oxide in the rat gastric mucosa. Gastroenterology, **100 (2)**, A184.

Whittle, B.J.R., Kauffman, G.L. and Moncada, S. (1981). Vasoconstriction with thromboxane A_2 induces ulceration of the gastric mucosa. Nature, **292**, 472-474.

Whittle, B.J.R., Lopez-Belmonte, J. and Moncada, S. (1990). Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. Br. J. Pharmacol., **99**, 607-611.

Whittle, B.J.R. and Vane, J.R. (1987). Prostanoids as regulators of gastrointestinal function. In: **Physiology of the Gastrointestinal Tract**, (Johnson, L.R. ed.), vol 1, pp 143-179. Raven Press, New York.

Williams, G. (1977). In: **Progress in gastroenterology, (3rd ed.)** (Glass, G.B.J. ed.) pp29-51. Grune and Stratton, New York.

Wolfe, L., Francis, S.H., Landiss, L.R. and Corbin, J.D. (1987). Interconvertible cGMP-free and cGMP-bound forms of cGMP-dependent protein kinase in mammalian tissues. J. Biol. Chem., **262**, 16906-16913.

Wolosin, J.M. and Forte, J.G. (1985). K and Cl conductances in the apical membrane from secreting oxyntic cells are concurrently inhibited by divalent cations. J. Membrane Biol., **83**, 261-272.

Wyatt, T.A., Lincoln, T. and Pryzwansky, K. (1991). Vimentin is transiently co-localised with and phosphorylated by cyclic GMP dependent protein kinase in formyl-peptide-stimulated neutrophils. J. Biol. Chem. **266**, 21274-21280.

Yamada, T., Soll, A.H., Park, J. and Elashoff, J. (1984). Autonomic regulation of somatostatin release: Studies with primary cultures of canine fundic mucosal cells. Am. J. Physiol., **247**, G567-G573.

Yui, Y., Hatori, R., Kosuga, K., Eizawa, H., Hiki, K. and Kawai, C. (1991). Purification of nitric oxide synthase from rat macrophages. J. Biol. Chem., **266**, 12544-12549.

Zalewsky, C.A. and Moody, F.G. (1979). Mechanisms of mucus release in exposed canine gastric mucosa. Gastroenterology **77**, 719-729.

Zar, J.H. (1984). In: **Biostatistical Analysis**, pp 194-195, Prentice-Hall International, London.

APPENDICES

A.1. SOURCE OF REAGENTS.

A. General Chemicals.	Supplier.
Anti-rat-macrophage immunoglobulin (ED_2 clone)	Serotec
Anti-rat-endothelium immunoglobulin (MRC-OX43 clone)	Serotec
L-arginine monohydrochloride	Sigma
Bovine serum albumin (BSA), fraction V	ICN Biomedicals
Caesium Chloride	BDH
Calcium chloride	BDH
Dextran (M.Wt. 40,000 Da)	Sigma
1,4-diazobicyclo-(2,2,2)-octane (DABCO)	BDH
Dimethyldichlorosilane	BDH
Dimethylsulphoxide (DMSO)	Sigma
Di-potassium hydrogen orthophosphate	BDH
Di-sodium hydrogen orthophosphate	BDH
Di-sodium p-nitrophenyl phosphate	Sigma
Dithiothreitol (DTT)	Sigma
Ethylacetate	BDH
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Ethyleneglycol-bis-(β-aminoethylyether) N, N'-tetraacetic acid	Sigma
Fluorescein isothiocyanate (FITC)-conjugated goat- antimouse immunoglobulin G	Sigma
Foetal calf serum	ICN Biomedicals
Gentamicin sulphate	Sigma
L-glutamine	Sigma
Glycerol	BDH
D-glucose	BDH
L-glutamine	Sigma
Haemoglobin	Sigma
Hydrocortisone	Sigma
N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES)	Sigma
Insulin	Sigma
Leupeptin	Sigma
Magnesium chloride, hexahydrate	BDH
Pepstatin A	Sigma
Periodic acid	BDH
Phenylmethylsulphonylfluoride (PMSF)	Sigma
Potassium chloride	BDH
Potassium dihydrogen orthophosphate	BDH

Protein A-alkaline phosphatase conjugate	Sigma
Sodium azide	BDH
Sodium carbonate	BDH
Sodium chloride	BDH
Sodium dihydrogen orthophosphate	BDH
Sodium hydrogen carbonate	BDH
Sodium hydroxide	BDH
Sodium metabisulphite	BDH
Sodium pentobarbitone	May & Baker
Sucrose	BDH
Trichloroacetic acid (TCA)	BDH
Tris base	BDH
Trypan blue	BDH
Trypsin inhibitor, lyophillised from soyabean	Sigma
Tween-20	Sigma
L-valine	Sigma

B. Enzymes.

Pancreatin	Sigma
Pronase, 70,000 PUK/g	BDH

C. Radiochemicals and scintillation counting.

L-[U-^{14}C]Arginine monohydrochloride	NEN
Aminopyrine, dimethylamine-[^{14}C]	Amersham International
D-[U-^{14}C]Glucose	Amersham International
"Optiphase HiSafe II"	Pharmacia / LK
Prostaglandin E_2 [^{125}I] radioimmunoassay kit	NEN

D. Enzymeimmunoassay kits.

cAMP enzyneimmunoassay (EIA) system (dual range)	Amersham International
cGMP enzyneimmunoassay (EIA) system (dual range)	Amersham International

E. Secretagogues and agents.

Adenosine 1,4,5-trisphosphate, potassium salt	Sigma
Dibutyryl cyclic AMP, sodium salt	Sigma
N^G-monomethyl-L-arginine (L-NMMA)	Wellcome
Atropine	Sigma

8-Bromoguanosine-3',5'-cyclic monophosphorothioate (Rp- & Sp- isomers) (Rp- & Sp-8-Br-cGMPS)	Biolog
Bradykinin, acetate salt	Sigma
des-Arg^9-bradykinin, acetate salt (B_1 agaonist)	Sigma
des-Arg^9-Leu^8-bradykinin, acetate salt (B_1 antagonist)	Sigma
des-Arg, [Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, acetate salt (B_2atagonist)	Sigma
Calcium ionophore A23187	Sigma
Dibutyryl cyclic CMP (Db-cCMP), sodium salt	Sigma
1,4-dihydro-5-(2-propoxyphenyl)-1,2,3-triazolo[4,5-*d*]pyrimidin-7-one (M&B 22948)	May & Baker
DOWEX AG-50W8	Sigma
Digitonin	Sigma
8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP)	Biolog
Dibutyryl cyclic GMP (Db-cGMP), sodium salt	Sigma
Histamine dihydrochloride	Sigma
Indomethacin	Sigma
3-isobutyl-1-methylxanthine (IBMX)	Sigma
Isosorbide dinitrate (ISDN)	Sigma
β-Nicotinamide adenine dinucleotide phosphate (NADPH), reduced form	Sigma
Nitroprusside, sodium salt	Sigma
S-nitroso-N-aecetyl-penicillamine (SNAP)	Wellcome
Zymosan	Sigma

A.2 MEDIA PREPARATION.

A.2.1 Preparation of Eagles Minimum Essential Medium.

The cell culture medium was purchased from Sigma in powdered form and contained the following components (final concentration):- L-arginine (0.7 mM), L-cysteine (0.23 mM), L-glutamine, (2.0 mM), L-histidine (0.27 mM), L-isoleucine (0.4 mM), L-leucine, (0.4 mM), L-lysine (0.5 mM), L-methionine (0.09 mM), L-phenylalanine (0.2 mM), L-threonine (0.4 mM), L-tryptophan (0.05 mM), L-tyrosine (0.25 mM), L-valine (0.4 mM), Choline chloride (7.0 μM), folic acid (3 μM), myo-inositol (0.01 mM), niacinamide (8 μM), D-pantothenic acid Ca (2.1 μM), pyridoxal HCl (4.86 μM), $CaCl_2$ (1.8 mM), KCl (5.4 mM), $MgSO_4$ (0.8 mM), NaCl (116.4 mM), NaH_2PO_4 (1.0 mM), D-glucose (5.6 mM) and phenol red Na (0.001% w/v).

The powdered medium was dissolved in 1 litre of double distilled water with stirring at ambient temperature to which $NaHCO_3$ (25 mM) and HEPES (20 mM) were added. This medium was warmed to 37°C, gassed with 95% 0_2/5% CO_2 for 30 min and the pH adjusted to pH 7.4.

A.2.2 Preparation of Hanks Balanced Salt Solution.

Hanks' balanced salts were purchased in powdered form from BDH and consisted of (final concentrations):- KCl (1.5 mM) NaCl (96 mM), sodium citrate (27 mM), KH_2PO_4 (8 mM) and Na_2HPO_4 (5.6 mM). A 0.97% (w/v) solution was prepared by dissolving the required amount in double distilled water with stirring at ambient temperature to which $NaHCO_3$ (4.0 mM) was added. The medium was gassed with 95% 0_2/5% CO_2 for 30 min and the pH adjusted to pH 7.4.

A.3 LIQUID SCINTILLATION COUNTING.

Liquid scintillation counting was used to determine both the NOS activity of mucosal cells and also the secretory activity of parietal cells.

The [^{14}C] nuclide emits β-particles which collide with fluors present in the scintillation fluid. The collision between β-particle and the fluor result in the production of photons which are detected by photomultiplier tubes. The photomultiplier tube then converts the photons into electrical impulses which are proportinal to the number of β-particles emitted and hence the amount of [^{14}C]-L-citrulline present in the sample. Certain chemicals can act as "quenching" agents which interfere with the interaction of the β-particle with the fluor and therefore give a false low reading of the radioactive content of the sample. Quenching can be overcome by preparing a series of samples containing the suspected quenching agent along with a known amount radioactive isotope. To each tube an increasing volume of chloroform (a known quenching agent) is added and a quench curve is generated by counting the radioactivity of each tube. From this the counting efficiency can be determined which enables convertsion of counts from counts per minute to disintegrations per minute.

A.4 CALCULATION OF AMINOPYRINE ACCUMULATION RATIO.

The aminopyrine accumulation ratio was calculated according to the following procedure:

Pellet volume (µl) = dry weight (mg) x 2

Where:
dry weight (mg) = 0.2328 x cell concentration ($x10^6$/ml)

Since $1x10^6$ cells from a parietal cell enriched cell suspension prepared on a Percoll® density gradient, have been demonstrated to have a dry weight of 0.2328 mg (Hatt, 1988)

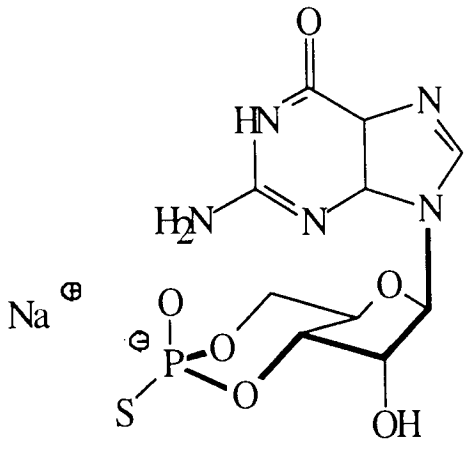
The aminopyrine accumulation rato is then calculated as follows

$$A.P.R = \frac{(\text{Pellet dpm per unit volume})}{(\text{Supernatant dpm per unit volume})}$$

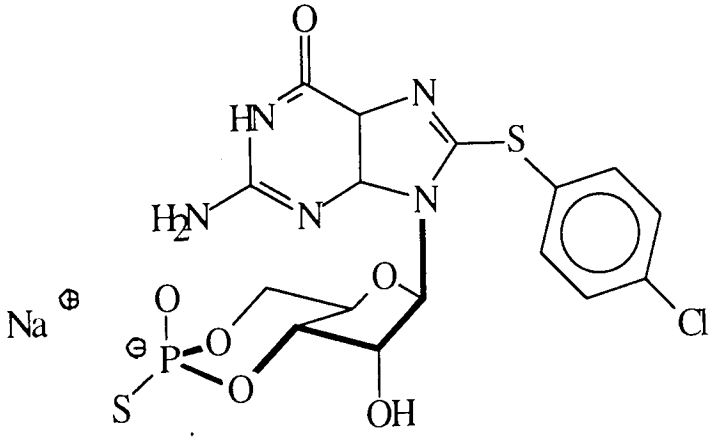
Where:
A.P.R. = aminopyrine acccumulation ratio,
dpm = disintegrations per minute,
Supernatant dpm = total dpm added to incubation medium - pellet dpm.

A.5 cGMP ANALOGUES.

A.5.1 Chemical structure of regulators of cGMP-dependent protein kinase.



Rp/Sp-guanosine-3'-5'-monophosphorothioate
(Rp/Sp-cGMPS)



Para-chlorophenylthioguanosine-3'-5'-cyclic monophosphate
(8-pCPT-cGMP)

A.6 STATISTICAL ANALYSIS.

Some examples of the statistical tests used to analyse data throughout this thesis are described below.

A.6.1 Two-way Analysis of variance (Two-way ANOVAR).

An ANOVAR of the original untransformed data in units of $\mu g/10^6$cells over a 30 min incubation period is presented below:

DATA

Experiment	Control	ISDN 2 mM	ISDN 1 mM	ISDN 0.5 mM	ISDN 0.1 mM	ISDN 0.01 mM
1	3.29	4.34	3.99	3.78	3.56	3.40
2	3.56	4.64	4.42	3.88	3.72	3.54
3	2.75	3.34	3.24	2.91	2.80	2.80
4	5.62	6.97	6.75	6.16	6.11	5.51
Mean ±SEM	3.81 ±0.63	4.82 ±0.77	4.60 ±0.76	4.18 ±0.69	4.05 ±0.72	3.81 ±0.59

TWO-WAY ANOVAR SUMMARY.

VARIANCE	d.o.f	Sum Squares	Mean squares	Variance ratio (F)
Experiments	3	34.396	11.465	
[ISDN] (treatments)	5	3.506	0.701	28.04
Error	15	0.378	0.027	

The F-ratio for treatments with 5,15 degrees of freedom had a probability of <0.01 of occurring by chance and was therefore considered significant.

6.2 Dunnett's test.

The group means are then ranked

GROUP MEANS (RANKED).

1	2	3	4	5	6
4.82	4.6	4.18	4.04	3.81	3.81
ISDN 2 mM	ISDN 1 mM	ISDN 0.5 mM	ISDN 0.1 mM	ISDN 0.01 mM	Control

Standard error for Dunnett's test is:

$$S.E. = \sqrt{\frac{2 \text{ x error of mean square}}{n}} = \sqrt{\frac{2 \text{ x } 0.027}{4}} = 0.112$$

The test statistic is given by:

$$q' = \frac{\bar{x}\text{control} - \bar{x}\text{sample}}{S.E}$$

If the resulting q' value exceeds the appropriate tabulated value then the null hypothesis is rejected and a significant difference is presumed to exist between the control and sample mean. A table of comparisons is presented in table A.6.2.1:

Table A.6.2.1 Summary of Dunnett's test.

Comparison	q	p	$q'_{0.05,v,n}$	$q'_{0.01,v,n}$	Conclusion
6 vs 1	9.018	7	2.89	3.71	P<0.01
6 vs 2	7.054	6	2.82	3.64	P<0.01
6 vs 3	3.325	5	2.73	3.55	P<0.05
6 vs 4	2.116	4	2.61	3.43	Not significant
6 vs 5	do not test				

p = number of means in the range being tested.
v = residual degrees of freedom from one-way ANOVAR.
NOTE: comparisons can only be made with the control mean i.e. multi-comparisons are not allowed. For multi-comparisons see Newman-Keuls test (A.6.4)

A.6.3 KRUSKAL-WALLIS TEST.

For comparison of data which did not follow a normal distribution, a non-parametric equivalent of ANOVAR was used to determine whether ther was an overall effect of treatments followed by a non-parametric equivalent of Dunnett's test to compare the effect of individual treatments against control data.

The measurements of mucus gel thickness for each animal exhibited a slightly skewed distribution. However, comparison of the medians and means of the mucus gel thickness for each animal by Wilcoxan signed rank test, did not show a significant difference between the two values, and mean data were used for statistical comparison

Table A.6.3.1 **Data and rank assignments for Kruskal Wallis Test.**

	Control		1 mM ISDN		0.5 mM ISDN		0.1 mM ISDN		1 mM ISDN +10 μM haemoglobin		10 μM haemoglobin		1 mM Db-cGMP		300 μM SNAP	
Expt	mean	rank	mean	rank	mean	rank	mean	rank	mean	rank	mean	rank	mean	rank	mean	rank
1	126.2	20	132.2	25	111.6	15	98.5	1	115.9	18	121.9	19	192.1	40	134.1	28
2	103.5	8	184.8	39	132.8	26	109.2	13	103.6	9	103.1	7	148.9	37	147.4	36
3	110.9	14	135.0	29	140.2	32	101.4	3	128.9	21	102.7	5	153.8	38	140.7	33
4	113.2	17	146.2	35	130.1	22	100	2	108.2	12	107.8	11	133.9	27	142.8	34
5	102.9	6	135.3	30	131.1	23	104.0	10	112.3	16	102.3	4	131.5	24	138.6	31
Σ ranks		65		158		118		29		76		46		166		162

The test statisitic H is given by:

$$H = \frac{12}{N(N+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(N+1)$$

Where: k = number of treatments, n_i = number of observations in each group, N= total number of observations in all groups,
R_i = sum of the ranks.

$$H = \frac{12}{40(41)}\left[\frac{65^2}{5}+\frac{158^2}{5}+\frac{118^2}{5}+\frac{29^2}{5}+\frac{76^2}{5}+\frac{46^2}{5}+\frac{166^2}{5}+\frac{162^2}{5}\right] - 3(41) = 31.6^*$$

The test statistic, H, is then compared to the tabulated value of χ^2 for k-1 d.f. which is 24.322, *P<0.001. Since the calculated value of H is greater than the tabulated value, a significant difference between groups is concluded.

The means are then compared for each group against the control by firstly ranking the rank sums:

RANK	1	2	3	4	5	6	7	8
Rank sum	29	46	65	76	118	158	162	166
Treatment	0.1 mM ISDN	Hb alone	Control	1 mM ISDN +Hb	0.5 mM ISDN	1.0 mM ISDN	300 µM SNAP	1 mM DbcGMP

Comparisons are now made between the control group and all treatment groups.

$$\text{S.E.} = \sqrt{\frac{n(np)(np+1)}{6}}$$

Where: n = replicates per group and p = number of sums in range tested.

The test statistic q' is given by:

$$q' = \frac{\text{Difference between rank sums}}{\text{S.E.}}$$

Table A.6.3.2 **Summary of the non-parametric equivalent of Dunnett's test.**

Comparison	p	S.E.	q'	$q'_{0.01(2)\infty p}$	Conclusion
3 vs 1	3	14.14	2.546	2.79	NS
3 vs 2	2	9.57	1.985	2.58	NS
3 vs 8	6	27.83	3.629	3.06	P<0.01
3 vs 7	5	23.27	4.168	3.00	P<0.01
3 vs 6	4	18.70	4.973	2.92	P<0.01
3 vs 5	3	14.14	3.748	2.79	P<0.01
3 vs 4	2	9.57	1.149	2.58	NS

As with Dunnett's test, the calculated q' statistic must be greater than the tabulated value in order for the difference to be significant.

A.6.4 NEWMAN-KEULS TEST.

Data from figure 3.9 fraction 3, was analysed by a Newman-Keuls test, since this allows multiple comparisons to be made between **all** groups of data, whereas Dunnett's test only allows comparisons with the control group.

This first stage of the test requires a one-way ANOVAR to be performed:

One-way ANOVAR summary.

Variation	d.o.f.	Sum Sq.	Mean Sq	Variance ratio (F)
Between groups	2	10.791	5.395	6.374*
Within groups	10	8.465	0.846	
Total	12	19.255		

The tabulated F-ratio with 2 and 10 degrees of freedom is 4.10 at a significance level of 0.05%. Thus the effect of treatments was significant, *P<0.05.

The means are then ranked:

RANK	**1**	**2**	**3**
Mean	0.072	0.093	1.955
Treatment	Control	LPS+Dexamethasone	LPS

Comparisons are now made between all groups of data:

The standard error for groups of unequal number of observations (n) is given by:

$$S.E. = \sqrt{\frac{s^2}{2}\left(\frac{1}{n_A}+\frac{1}{n_B}\right)}$$

Where: n_A = number of observations in group A and n_B = number of observations in group B,

Whereas the standard error for groups of an equal number of observations is given by:

$$S.E. = \sqrt{\frac{s^2}{n}}$$

Where: s^2 = error mean square and n = number of observations in each group.

The test statistic q' is given by:

$$q' = \frac{\bar{X}_B - \bar{X}_A}{S.E.}$$

Table A.6.4.1 Summary of Newman-Keuls test.

Comparison	Diff. of means	S.E.	q	p	$q_{0.05,10,n}$	$q_{0.01,10,n}$	Conclusion
3 vs 1	1.8834	0.436	4.320	3	3.877	5.270	P<0.05
3 vs 2	1.862	0.436	4.271	2	3.151	4.482	P<0.05
2 vs 1	0.022	0.460	0.047	2	3.151		NS

If the calculated value of q is less than or greater than the tabulated value of $q_{\alpha,v,p}$ then the difference between the two group means is significant.

A.7 ANIMALS.

Male Wistar rats were obtained from Bantin and Kingman, Hull, U.K. and were fed on Heygates diet supplied by Pilsbury, Edgbaston, Birmingham, U.K.