

BIOCHEMICAL AND PHARMACOLOGICAL APPROACHES
TO CHARACTERISATION OF OPIOID RECEPTORS

Elizabeth Peers
Department of Biochemistry
Imperial College
South Kensington
LONDON S.W.7.

Submitted in part fulfilment of the requirements for the
Degree of Doctor of Philosophy of the University of London
and the Diploma of Imperial College

1984

ABSTRACT

Following presentation of the historical and scientific background to the discovery of opioid receptors and opioid peptides, the diverse nature of opioid receptors and ligands is discussed.

NAM (14- β -(o-nitro, p-azidophenyl)-cinnamoyl-(N-cyclopropylmethyl)-normorphinone), a potential photoaffinity ligand for opioid receptors, was synthesised. It was shown that NAM and two closely-related compounds (NOM and NUM) possessed high affinity for opioid receptors in isolated tissues (c. 1000x greater than normorphine). In binding studies using rat brain membranes, the ligands' differential kinetics at opioid receptor subtypes were further investigated. They showed high-affinity, pseudoirreversible interaction with μ -receptors, a reversible interaction with δ -receptors with little demonstrable affinity for κ -receptors. NAM could not therefore be used as a photoaffinity ligand, but its use as a tool to suppress μ -receptors populations is discussed in relation to chemical affinity ligands (such as β -FNA).

The behaviour of daleck, a peptide affinity ligand, was studied in isolated tissues, and reasons for its inability to alkylate opioid receptors in these tissues are put forward.

As a development of these studies, a more hydrophilic, lower-affinity derivative was synthesised for use in affinity chromatography of opioid receptors (GANC:14- β -(^{14}C -glycyl)-amido-(N-cyclopropylmethyl)-norcodeinone). The radiolabel allowed easy estimations of incorporation into the agarose support used (Affigel-10). Free GANC showed μ -selectivity in isolated tissue and rat brain membrane preparations, and immobilised GANC was able to block μ -binding to brain membranes. The results from using the GANC affinity column are discussed with reference to the biochemical nature of solubilised opioid receptors and their relation to μ -, δ - and κ -subtypes.

It was of interest to determine whether opioid receptor subtypes coexist in a complex or reside on separate molecules. To this end, target-size analysis by radiation inactivation was carried out. The results indicated a size of around 110,000 for μ - and δ -receptors, and are discussed in relation to results from other laboratories.

Conclusions about the nature of opioid receptors and their subtypes are drawn, with reference to results from the above-mentioned approaches and to models proposed in recent and current reports.

C O N T E N T S

CONTENTS

	<u>Page</u>
Abstract	2
Contents	5
Figures	10
Tables	13
Abbreviations	14
Acknowledgements	15
 I <u>INTRODUCTION</u>	
1.1 Historical aspects of opium use	17
1.2 Elucidation of the structure of morphine	20
1.3 Development of improved analgesics	22
1.4 Opioid receptors in mammalian central nervous system	27
2.1 Isolation and identification of endogenous opioid peptides	32
2.2 Biogenesis of opioid peptides	35
2.3 Structure-activity relationships in opioid peptides and derivatives	38
3.1 Multiple opioid receptors : whole animal studies	42
3.2 Multiple opioid receptors : isolated tissues	51
3.3 Multiple opioid receptors : binding studies	59
3.4 Distribution and electrophysiology of opioid receptors	69

II GENERAL METHODS

1.	Reagents	76
2.	Brain membrane preparation	76
3.	Membrane binding assay	77
4.	Isolated tissue assays	78
5.	Solubilisation of opioid receptors in brain membranes	83
6.	Assay of solubilised receptor binding	83
7.	Protein assay	84

III APPROACHES TO AFFINITY LABELLING OF OPIOID RECEPTORS

1.	Introduction	87
2.	Methods	
1.	Synthesis of NUM, NOM & NAM	98
2.	Photolysis of NAM	112
3.	Bioassay of NUM, NOM & NAM	115
4.	Studies with daleck in MVD, GPI & MI	115
5.	Membrane binding assays with NOM	116
3.	Results	
1.	Photolysis of NAM	118
2.	Actions of NUM, NOM & NAM in MVD & GPI	118
3.	Action of daleck in MVD, GPI & MI	127
4.	Membrane binding assays with NOM	134
4.	Discussion	140

IV APPROACHES TO AFFINITY CHROMATOGRAPHY OF OPIOID RECEPTORS

1.	Introduction	146
2.	Methods	
1.	Synthesis of GAC & GANC	156
2.	Bioassay of GAC & GANC	160
3.	Membrane binding assays with GAC & GANC	160
4.	Solubilised receptor binding assays with GANC	160
5.	Incorporation of ^{14}C -GANC into Affigel-10	161
6.	Use of GANC-gel in chromatography of solubilised opioid receptors	162
3.	Results	
1.	Actions of GAC & GANC in bioassay	166
2.	Membrane binding assays with GAC & GANC	168
3.	Solubilised receptor binding assays with GANC	168
4.	Incorporation of ^{14}C -GANC into Affigel-10	172
5.	Use of GANC-gel in chromatography of solubilised opioid receptors	173
4.	Discussion	177

V RADIATION INACTIVATION OF OPIOID RECEPTORS

1.	Introduction	182
2.	Methods	
1.	Preparation of samples for irradiation	187
2.	Irradiation of freeze-dried samples	187
3.	Irradiation of frozen samples	188
4.	Resuspension of samples after irradiation	189
5.	Standard enzyme markers	191

3.	Results	
1.	Effect of freeze-drying and freezing in water alone on opioid receptor binding	193
2.	The effect of irradiation on freeze-dried samples and standard enzymes	193
3.	The effect of irradiation on frozen samples	200
4.	Discussion	207
VI	GENERAL DISCUSSION	212
VII	REFERENCES	222

LIST OF FIGURES

LIST OF FIGURES

	<u>Page</u>
1. Morphine	21
2. Correlation of analgesic potency in man with potency in GPI	23
3. Opioid agonists and antagonists	25
4. Benzomorphans	26
5. Oripavines	28
6. Opioid analgesics not derived from opium	29
7. Opioid peptides	33
8. Opioid peptide precursors and families	36
9. Enkephalin inactivation	39
10. Stabilised enkephalin analogues	40
11. Tifluadom	45
12. Allosteric coupling between μ - and δ -receptors	48
13. Receptor selectivities of dynorphin B (1-13) and fragments	56
14. Protein-lipid model of the opioid receptor	68
15. Mouse vas deferens bioassay	80
16. Protein assay calibration	85
17. Affinity ligands for opioid receptors	89
18. Reactions of nitrenes	92
19. Photoaffinity ligands for opioid receptors	93
20. Potential photoaffinity ligands for opioid receptors	96
21. Synthesis of <u>o</u> -nitrocinnamoyl chloride	106
22. Synthesis of <u>o</u> -nitrocinnamoyl chloride, <u>p</u> -azido cinnamoyl chloride	107

23.	Synthesis of protected 14- β -amino-(N-cyclopropylmethyl)-normorphinone	108
24.	Coupling and deprotection of 14- β -amino-(N-cyclopropylmethyl)-normorphinone	109
25.	Infra-red spectrum of NAM	113
26.	UV spectra of solutions of Triton-X-100	114
27.	UV spectra of NAM pre- and post-photolysis	119
28.	UV destruction of opioid binding sites in the presence and absence of 1% Triton-X-100 filter	120
29.	The effect of NOM in MVD	122
30.	Dose-response curves of NOM and normorphine in MVD	123
31.	GPI: antagonism by NAM of normorphine	125
32.	MVD: antagonism by NAM of met-enkephalin	126
33.	GPI: antagonism by naloxone of normorphine, met-enkephalin and daleck (Schild plot)	129
34.	MVD: antagonism by naloxone of normorphine, met-enkephalin and daleck (Schild plot)	130
35.	MVD: inability of daleck to inactivate opioid receptors	133
36.	Rat brain membranes: inhibition by NOM of	
	a. ^3H -DAGO binding	135
	b. ^3H -DADL binding	135
37.	Rat brain membranes: effect of 2nM NOM on specific binding of ^3H -ligands	139
38.	The principle of affinity chromatography	147
39.	Ligands synthesized for affinity chromatography of opioid receptors	154

40.	Synthesis of GAC and GANC	157
41.	Affinity chromatography of opioid receptors	164
42.	Rat brain membranes: inhibition by GANC of	
	a. ^3H -DAGO binding	169
	b. ^3H -DADL binding	170
43.	Solubilised opioid receptors: inhibition by	
	GANC of ^3H -naloxone binding	171
44.	Rat brain membranes: inhibition by GANC-gel	
	of ^3H -DAGO binding	174
45.	Elution profile of solubilised opioid receptor	
	binding collected from GANC column	176
46.	Irradiation of frozen samples at -80°C	190
47.	Rat brain membranes: effect of freeze-drying	
	on ^3H -etorphine binding	194
48.	Rat brain membranes: effect of 1mM DTT on	
	inactivation rates of μ - and δ -binding sites	
	in the freeze-dried state	197
49.	Inactivation rates of ADH & β -GAL in the	
	absence of DTT (freeze-dried)	199
50.	Inactivation rates of ADH & β -GAL in the	
	absence of DTT (frozen, series 1)	201
51.	Inactivation rates of μ - & δ -binding sites	
	in the absence of DTT (frozen, series 1)	202
52.	Inactivation rates of ADH & β -GAL in the	
	absence of DTT (frozen, series 2)	204
53.	Inactivation rates of μ - & δ -binding sites	
	in the absence of DTT (frozen, series 2)	205

TABLES

	<u>Page</u>
1. Distributions of endogenous opioid peptides	34
2. Actions of μ -, κ - and σ -agonists in the chronic spinal dog	44
3. Potencies of dynorphin A (1-17) and fragments in four bioassays	55
4. Relative receptor affinities of a variety of opioid ligands	64
5. Relative potency of normorphine, met-enkephalin and daleck in MVD, GPI & MI	128
6. Antagonism of met-enkephalin, EKC and daleck in MI by Mr2266	131
7. Rat brain membranes: effect of ethanol on ^3H -etorphine binding in the presence and absence of NOM	137
8. Antagonism of normorphine, DADL and GANC in MVD	167
9. i -values of β -GAL, ADH, μ - and σ -binding sites in freeze-dried rat brain membranes	196

ABBREVIATIONS

ADH	: alcohol dehydrogenase
β -GAL	: β -galactosidase
butyl-PBD	: 2-(4'-tert-butylphenyl)-5-(4"-biphenyl)-1,3,4- -oxadiazole
DADL	: D-Ala ² -D-Leu ⁵ -enkephalin
DAGO	: D-Ala ² -MePhe ⁴ -Gly(ol) enkephalin
DALECK	: D-Ala ² -Leu ⁵ -enkephalin chloromethylketone
DCM	: dichloromethane
DHM	: dihydromorphine
DMF	: dimethylformamide
DM2, DM5 etc	: % methanol in dichloromethane
EKC	: ethylketocyclazocine
Et ₃ N	: triethylamine
GPI	: guinea-pig ileum-myenteric plexus preparation
IR	: infra-red
LDH	: lactate dehydrogenase
MAD	: methanol 18% : acetic acid 2% : dichloromethane 80%
MI	: mouse ileum preparation
MVD	: mouse vas deference preparation
MS	: mass spectrum
N-cpm	: N-cyclopropylmethyl
NMR	: nuclear magnetic resonance
PCP	: phencyclidine
PK	: pyruvate kinase
RbVD	: rabbit vas deferens
RVD	: rat vas deferens
THF	: tetrahydrofuran
t.l.c.	: thin-layer chromatography
UV	: ultra-violet

ACTH	Adrenocorticotrophic hormone
BAM-P	Bovine adrenal medulla peptide
CLIP	Corticotropin-like intermediate lobe peptide
LPH	Lipotropin
MSH	Melanocyte-stimulating hormone
POMC	Pro-opiomelanocortin
B	Bound
SB	Specific binding
NSB	Non-specific binding
S.D.	Standard deviation
s.e.m.	Standard error of the mean

Dr. Erwin Sigel for advice.

Deepest thanks to Kay Cowen for typing the thesis and John Cavanagh for drawing many of the figures.

Finally, my gratitude to all those members of my family, friends and colleagues for continually asking me when I would finish - I have!

CHAPTER I

I N T R O D U C T I O N

1.1 Historical Aspects of Opium Use

Pain is a familiar sensation to everyone, encompassing a spectrum from an awareness to discomfort to insupportable agony, and is probably the most common single reason to seek medical aid. It saves lives, since it warns us to avoid harm, and to seek treatment when harm has been done. Its continual presence has been a driving force behind a constant search from prehistory to the present for new substances and methods to relieve pain. In Western societies, the relief of pain is considered desirable and has been most often achieved by drugs such as morphine, which acts centrally, and aspirin, which acts peripherally. The antinociceptive action of morphine is very selective, producing little impairment of consciousness, although doses larger than those required for analgesia can produce drowsiness and sleep. Other sensory modalities, such as sight, touch, smell, hearing and taste, are not affected, and indeed neither does the perception of pain as a stimulus appear to be much changed; there is, however, a subjective response which results in a greater acceptance of pain (Jaffe & Martin, 1975)

Opium is the dried resin from unripe seed capsules of the poppy Papaver somniferum and was a form of analgesic known to the ancient world; the alkaloids extracted from this poppy still form the basis of the opiate drugs used widely in clinical medicine. The Sumerians, six thousand years ago, were familiar with opium and its euphoric effects, calling the poppy 'plant of joy'. In the 3rd century B.C., Theophrastus, a pupil of

Aristotle, described the collection and use of opium in the Greek world; Arabs traded in opium with the Far East, where the Chinese first used it against dysentery, only later learning to smoke opium.

In the Middle Ages, Paracelsus the alchemist, described a suspension of opium in alcohol and water, laudanum, which gave rise to opium 'eating'. Smoking and 'eating' opium did not in general give rise to serious addiction problems because of thermal decomposition of the active ingredients in smoking, and poor absorption from the gastro-intestinal tract in 'eating'. Christopher Wren invented the hypodermic syringe when he used a bladder and quill to inject opium intravenously. This produces a sudden response, to relieve pain and produce euphoria. Constant use of opiates, however, leads to tolerance to their analgesic and euphoric effects, and physical dependence, with the consequence of unpleasant withdrawal symptoms when the drug is unavailable.

The poppy has long been a symbol of sleep and dreams, well documented in scientific and general literature. In 1680, Thomas Sydenham wrote that 'Among all the remedies which it has pleased Almighty God to give to man to relieve his sufferings, none is so universal and so efficacious as opium medicine would be a cripple without it'. Thomas de Quincey (1856) was the first to describe the dual nature of opium's effects: "Thou hast the keys of Paradise, oh just, subtle and mighty opium" and yet it is also ".... an Iliad of Woes." In the nineteenth century, opium was widely used for many complaints as well as to

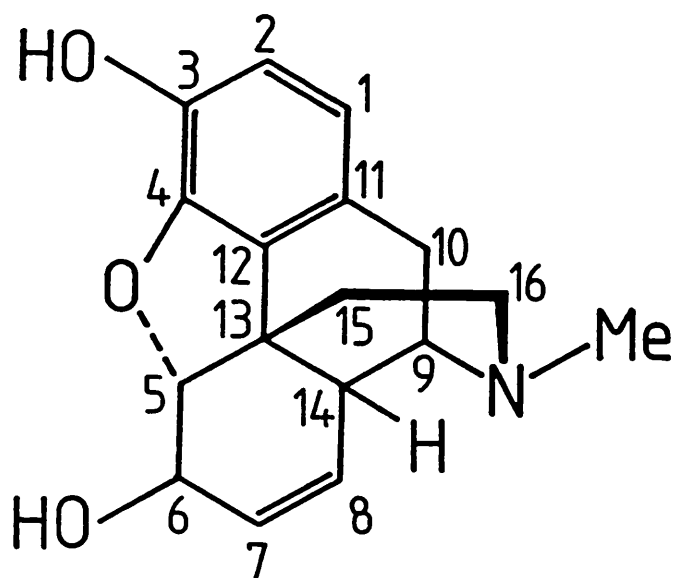
provide an escape from the drudgery of daily life; it was cheaper even than gin (Berridge & Edwards, 1982). It is well known that crying babies were silenced, often forever, with cordials and elixirs of laudanum. The wide use of opium in the city slums was also to relieve the gastro-intestinal diseases caused by overcrowded and insanitary conditions. However, when Parliament began passing Acts designed to improve the appalling living and working conditions in the cities, it also introduced the Pharmacy Act (1868) bringing the distribution of such drugs as opium more under the control of the medical profession. Indeed the improvements in sanitation, housing and working hours removed much of the need for opium use. Now, opiate abuse is considered a personal rather than a social problem, but the powerful euphoric qualities of such drugs as heroin, injected intravenously, make them open to abuse, and the consequent addictions can lead to crime.

In spite of the opiates' dangerous side effects, such as depression of respiration and, of course, physical dependence, they are still the only drugs effective against severe pain. Their use is of necessity kept under strict controls in the West and the search is still going on for safer analgesics.

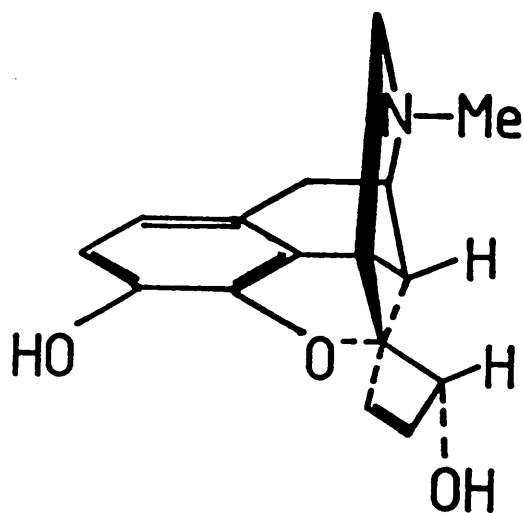
1.2. Elucidation of the Structure of Morphine

There was no success in isolation of an active principle from opium until the nineteenth century when, in 1803, Serturner isolated an alkaloid which he named morphine, after Morpheus, the Greek god of dreams. In 1832, Robiquet isolated a related alkaloid, codeine. Opium in its native state is now known to contain over twenty alkaloids, the most important being morphine (3-20%), codeine (0.3-0.4%) and thebaine (0.2-0.5%) (Gaddum 1978). Other potent alkaloids of plant origin include mescaline from the peyote cactus, and atropine from the plant deadly nightshade (Atropa belladonna). Later in the nineteenth century, much chemistry was carried out on morphine in an attempt to improve the drug by lessening its detrimental side effects and addictive qualities. In 1884, Wright synthesized morphine diacetate (heroin) (Burger, 1964). It was heralded as a non-addictive drug more potent than morphine (Dreser & Floret, 1898). Later it became clear that this is not so, and heroin is well-known as a drug of abuse because of its addictive nature. At about the same time, Dreser also introduced aspirin into clinical practice, now one of the most beneficial drugs known to man (Dreser, 1899).

In 1925, a molecular structure for morphine was proposed (Gulland & Robinson, 1925), Fig. 1, which was confirmed in 1952 when morphine was synthesized (Coates & Tschudi, 1952). It has been recognised for a long time that morphine existed as two



a. Morphine



b. D-(-)-morphine

Fig. 1

a. The morphine molecule

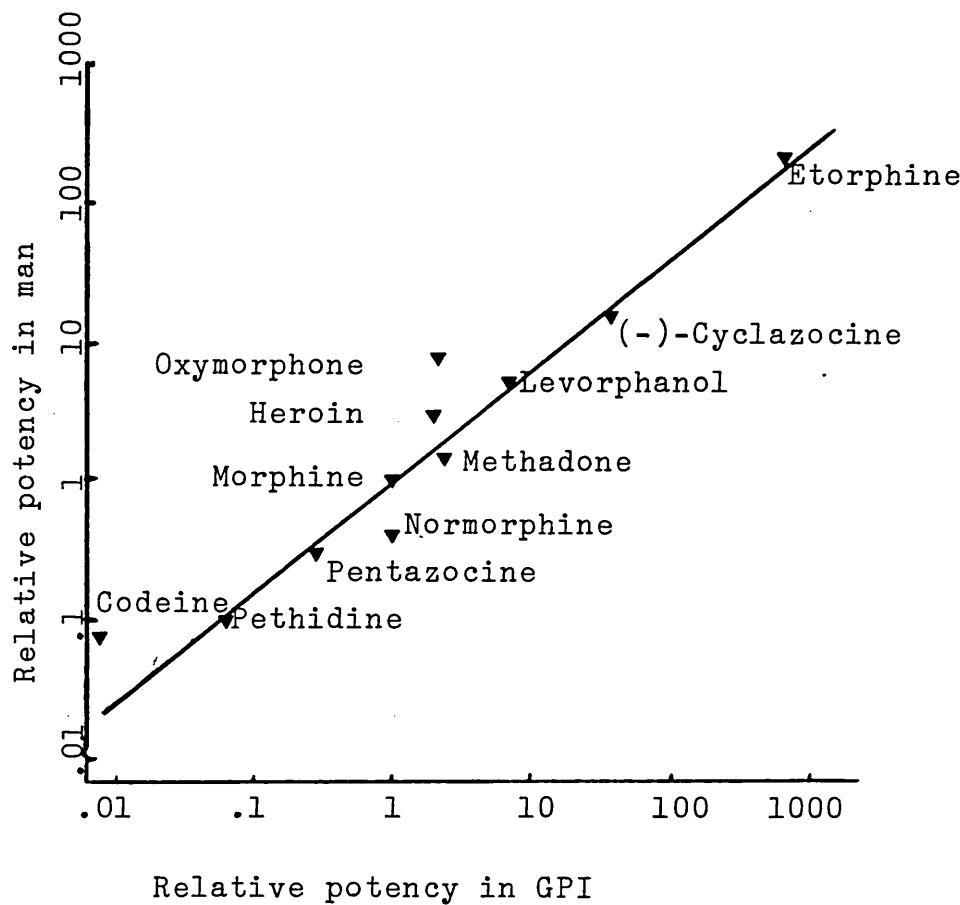
b. Stereochemistry of the morphine molecule

stereoisomers, D-(-)-morphine and L-(+)-morphine, and that only the D-(-)-isomer (Fig. 1) is active in vivo. It was proposed that this difference lay in differential uptake, distribution or metabolism, but these parameters were shown not to be significantly different, and so it was postulated that the reason was that there is stereospecific absorption of only the D-(-)-isomer to a receptor site (Beckett & Casy, 1954).

1.3 Development of Improved Analgesics

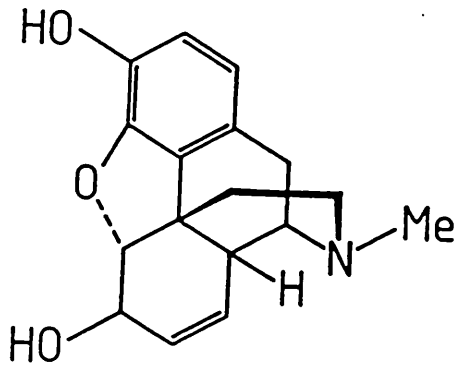
As mentioned above, much research has been directed towards synthesis of an 'ideal' analgesic, and very many compounds have been synthesized and tested, some based on morphine and others of quite different molecular structure. Not all these were able to be tested in man, and animal models such as the rat tail-flick test (D'Amour & Smith, 1941), mouse hot-plate test (Eddy & Leimbach, 1953) and mouse phenylbenzoquinone writhing test (Henderson & Forsaith, 1959) were developed to screen for analgesic agents. The use of isolated tissues, particularly the guinea-pig ileum and mouse vas deferens (Gyang & Kosterlitz, 1966; Henderson et al, 1972) has also become widespread in screening for opioid activity; the potencies of drugs in these preparations correlate well with their potencies in the animal tests and in man (Fig. 2). (For review, see Kosterlitz & Waterfield, 1975). However, morphine still remains the standard narcotic analgesic to which new agents are compared; other drugs are not superior in relieving pain, although they may have

Fig. 2 Correlation of potency in GPI with
analgesic potency in man
(from Kosterlitz & Waterfield, 1975)

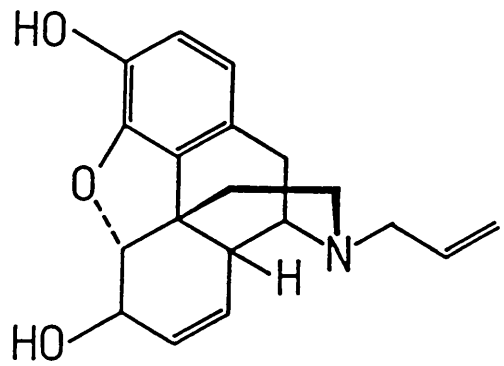


other special properties, such as duration of action, and it has not been easy to separate analgesic potency from dependence liability.

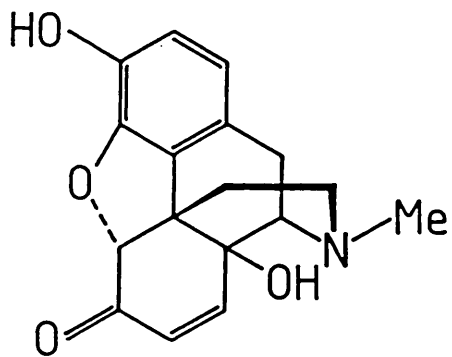
During the search for improved analgesics, antagonists of morphine's pain-relieving action were discovered, which do not affect the actions of unrelated drugs such as the barbiturates: N-allylnorcodeine (Pohl, 1915), nalorphine (Hart, 1941), naloxone (Blumberg et al, 1965) and naltrexone (Martin et al, 1973), (Fig. 3). It was striking that a small change in a molecule, from N-methyl to N-allyl, should have such dramatic consequences on the actions of the compound; the change from N-allyl to N-cyclopropylmethyl (as from naloxone to naltrexone) increases antagonist potency about three times but also introduces a small degree of agonist activity into the molecule (Lewis et al, 1971; Kosterlitz & Waterfield, 1975). In spite of nalorphine being an antagonist of morphine's action, it was shown that nalorphine could itself produce analgesia in man (Lasagna & Beecher, 1954). However, its dysphoric effects mean it is not used as an analgesic, although it suggested that partial agonists might be non-addictive analgesics (See I.3.1). Naloxone was different in that it was shown to be a pure antagonist (Blumberg et al, 1966, Jasinski et al, 1967; Kosterlitz & Watt, 1968); naloxone has been described as 'the most useful single tool in the study of endogenous opioids' (Terenius, quoted in Garfield, 1983). Other partial agonists are of the benzomorphan class of drugs, for example pentazocine and cyclazocine (Fig. 4). Although these are potent analgesics



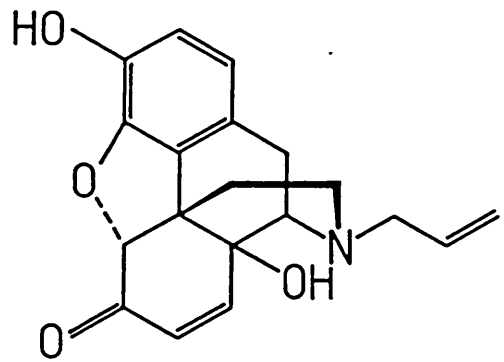
a. Morphine



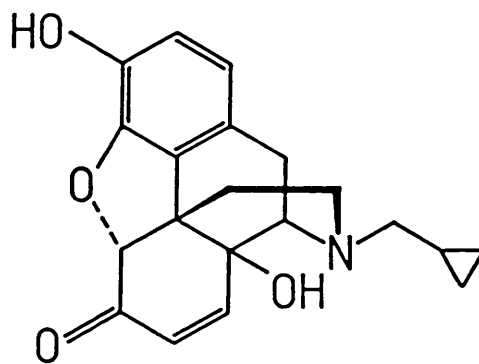
b. Nalorphine



c. Oxymorphone

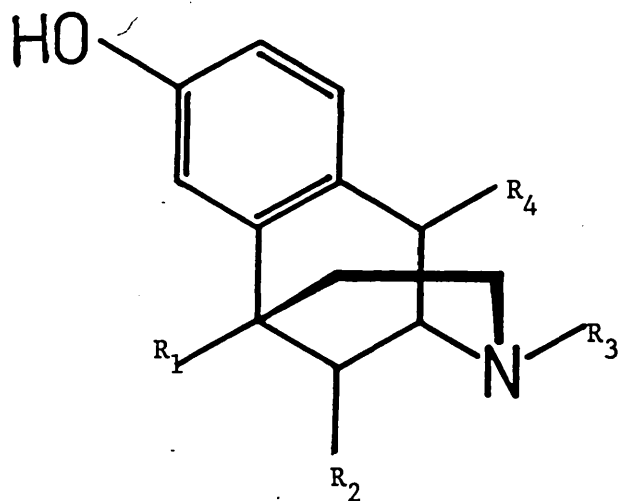


d. Naloxone



e. Naltrexone

Fig. 3 Relationship of opioid agonists and antagonists

Fig. 4Benzomorphans

	R_1	R_2	R_3	R_4
a. Cyclazocine	Me	Me	N-cpm	H
b. EKC	Et	Me	N-cpm	=O
c. Phenazocine	Me	Me	$CH_2CH_2\phi$	H
d. Bremazocine	Et	DiMe	$CH_2C(OH)CH_2CH_2$	H
e. SKF-100,47	H	Me	CH_2CHCH_2	H

their actions differ from those of opiate alkaloids such as morphine, particularly in their abstinence syndromes (For review see Martin, 1979 and § I.3).

Thebaine (Fig. 5a) from the opium poppy is the precursor of the oripavine group of compounds, of which many have been synthesized and tested (For review see Lewis et al, 1971). The parent oripavine (Fig. 5b) is forty times, and etorphine (Fig. 5c) a thousand times, as potent as morphine (Blane et al, 1967). Etorphine is used in low doses as an immobilizing agent for wild and domestic animals (Harthoorn & Bligh, 1965).

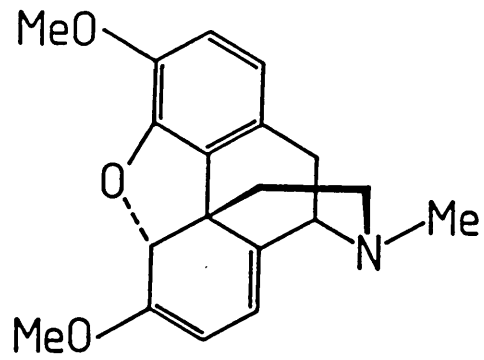
Diprenorphine (Fig. 5d) is an antagonist more potent than naloxone in vivo, but it shows some agonist activity in the isolated guinea-pig ileum (Kosterlitz & Watt, 1968).

Buprenorphine (Fig. 5e) is a potent analgesic which is a partial agonist at morphine receptors; this, along with its slow onset and offset of action, mean its dependence liability is low, and so it is safer to use than pure agonists such as levorphanol or pethidine (Lewis & Rance, 1978; Rance, 1979; Martin, 1979).

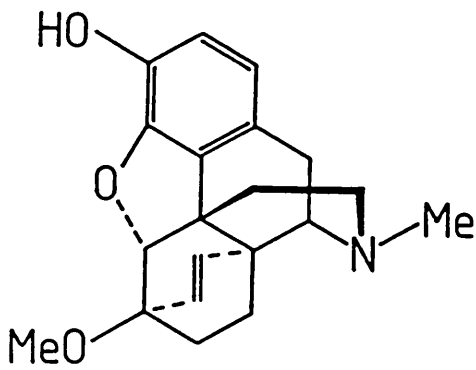
This drug also demonstrates that it has been possible to separate high analgesic potency from high addiction potential.

1.4 Opioid Receptors in Mammalian Central Nervous System

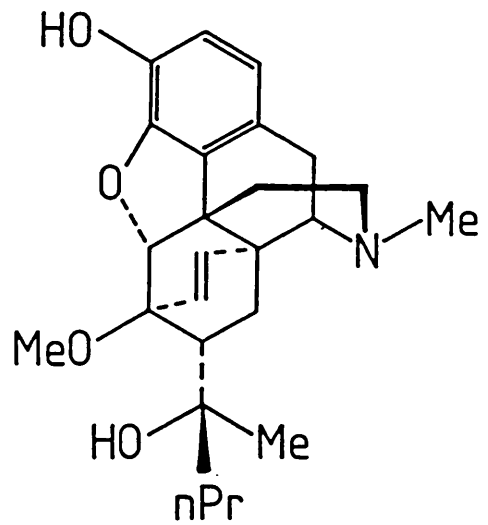
The concept of receptors was postulated about eighty years ago, in the forms of a 'receptive substance' present in muscle for a chemical stimulus released from the nerve, at the neuromuscular junction (Langley, 1907), and of 'chemoreceptors' in micro-



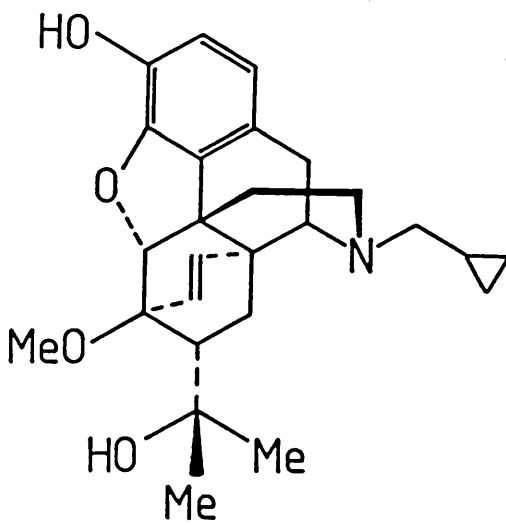
a. Thebaine



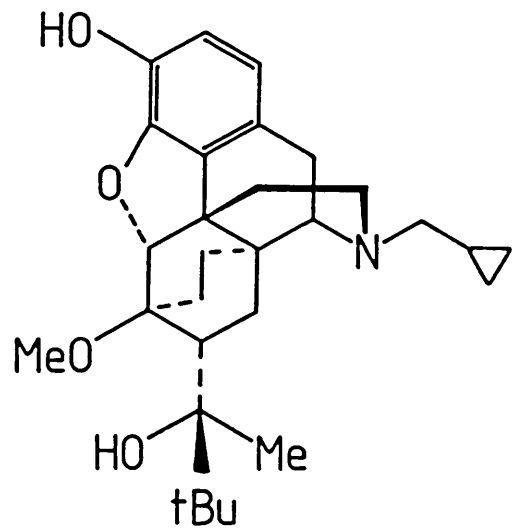
b. Parent oripavine



c. Etorphine



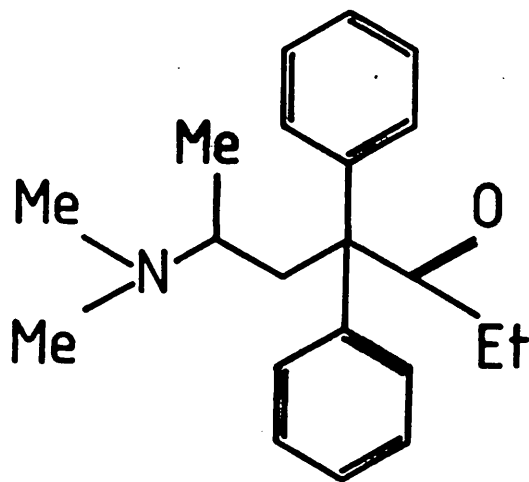
d. Diprenorphine



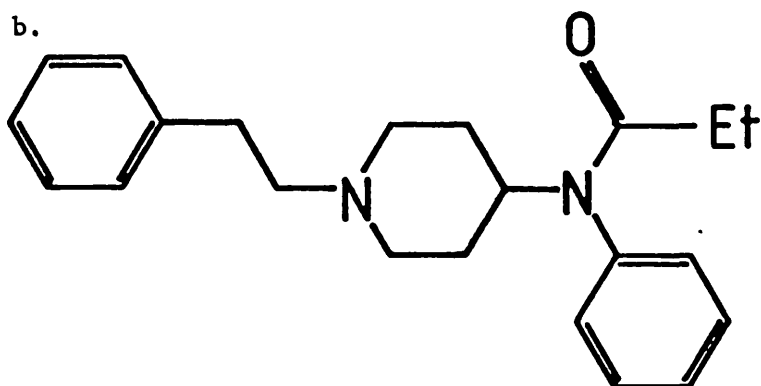
e. Buprenorphine

Fig. 5 Thebaine and related oripavines

a.



b.



c.

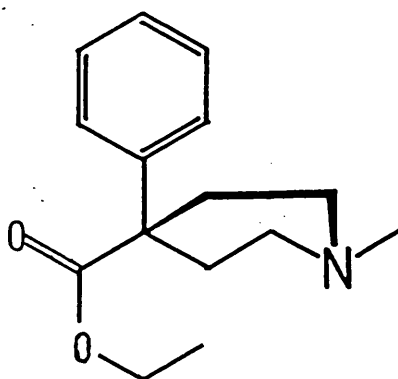


Fig. 6 Opioid analgesics not derived from opium

a. Methadone

b. Fentanyl

c. Pethidine

organisms not present in man (Ehrlich, 1913); this was the principle of selective toxicity whereby Ehrlich likened drugs such as Salvarsan to poisoned arrows whose target existed only in the microorganisms which caused disease. The precise structural qualities for analgesia, the existence of specific opioid antagonists and the very high potency of certain opiates, led to the proposal of specific opioid receptors present in the mammalian central nervous system long before the demonstration of stereospecific opioid binding to mouse brain membranes (Goldstein et al, 1971). However, only 2% of total binding was stereospecific because the concentration of ^{14}C - and ^3H -levorphanol used was so high ($1.95\mu\text{M}$), but the basic requirements to demonstrate the presence of stereospecific opioid binding in the CNS had been established; with the development of this technique i.e. use of higher specific activity ligands and removal of non-specifically bound radioligand came the demonstration of saturable, high-affinity binding of opioids to brain membranes of mouse, rat, guinea-pig and man (Pert & Snyder, 1973; Simon et al, 1973; Terenius, 1973). The dissociation constant for the ligands used were in the nanomolar range and saturation of stereospecific binding was demonstrated. However stereospecific binding per se is not enough evidence that binding-sites are pharmacological receptors; further evidence comes from the close correlation between affinity in brain homogenates and potency in the guinea-pig ileum (Kosterlitz and Waterfield, 1975) and in man. Apparent exceptions, etorphine and codeine can be explained by differences in lipid solubility and metabolism. Thus,

etorphine is a thousand times more potent in vivo than morphine and with an affinity fifty times higher; codeine is a tenth as active as morphine in vivo, but its affinity to the binding-sites is a hundred times lower. Drugs in other classes affecting the CNS, for example neuroleptics, antidepressants, barbiturates and benzodiazepines (with the exception of tifuladom) (Römer et al, 1982) do not interact with the opioid binding-sites in brain membranes at pharmacologically relevant concentrations (Pert & Snyder, 1973).

Metal ions and guanine nucleotides have been shown to affect opioid binding; as little as 0.5mM sodium decreased agonist binding but increased antagonist binding (Snyder & Childers, 1979), but it was not certain that this was because of a change in affinity (Simon et al, 1975a) or in number (Pert & Snyder, 1974) of binding-sites. The sodium effect was specific; lithium was the only other cation to demonstrate a similar effect. Physiological concentrations of sodium would mean the receptor would be in an 'antagonist conformation', and this correlates with the low level of activity of the endogenous opioid system. Divalent metal ions also affect opioid binding in vitro; magnesium calcium and manganese have been reported to increase agonist binding, an effect enhanced by the presence of sodium (Pasternak et al, 1975). The physiological relevance of these studies is however questionable because of the concentrations of ions used.

2.1 Isolation and Identification of Endogenous Opioid Peptides

It is now accepted that in most cases neurons communicate by chemical neurotransmitters at synapses, and by 1963 the identified neurotransmitters included the monoamines such as noradrenaline and dopamine, acetylcholine and possibly γ -aminobutyric acid (McLennan, 1963). There were some drugs known to act by enhancing or blocking these endogenous neurotransmitters, for example atropine at muscarinic acetylcholine receptors and isoprenaline at β -adrenergic receptors, and it was suggested that morphine might mimic an ongoing biochemical process (Martin, 1965), although the observation that there was an analgesic substance present in pituitary glands did not attract attention (Murray & Miller, 1960). Further evidence for an endogenous analgesic substance was the observation that electrical stimulation of particular brain areas, notably the peri-aqueductal grey matter, could produce analgesia (Reynolds, 1969) which is very like that produced by morphine, and is reversed by naloxone (Akil et al, 1972; 1976).

Following the demonstration of opioid receptors present in the CNS in 1973, increased effort was made in the search for an endogenous ligand for these receptors; it seemed unlikely that they should exist with the sole purpose of occasional combination with alkaloids from the opium poppy. Shortly afterwards, substances which inhibited binding of

Fig. 7 Structures of opioid peptides

a. Tyr-Gly-Gly-Phe-Met

Met⁵ enkephalin

b. Tyr-Gly-Gly-Phe-Leu

Leu⁵ enkephalin

c. Tyr-Gly-Gly-Phe-Met-Thr-Ser-Gly-Lys-Ser-Gln-Thr-Pro-Leu-Val-
Thr-Leu-Phe-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln

Porcine β -endorphin (1-27)

TABLE 1 Distributions, concentrations and molecules associated
with, endogenous opioid peptide families

	POMC	Proenkephalin A	Proenkephalin B
Type of cell	Endocrine; discrete neurons	Short neurons (diffuse) Few endocrine cells	Widespread neurons
Highest concentration	Adenohypophysis	Striatum, hypo- thalamus, adrenal medulla, gut	Neurohypophysis, hypothalamus, gut
Associated biomolecules	ACTH, MSH (co-synthesis)	catecholamines (co-storage)	ADH (co-storage)
Action	Hormonal	neurotransmitter neurohormonal	hormonal neurotransmitter
Opioid receptor selectivity	μ, δ	μ, δ	$\kappa(\delta)$

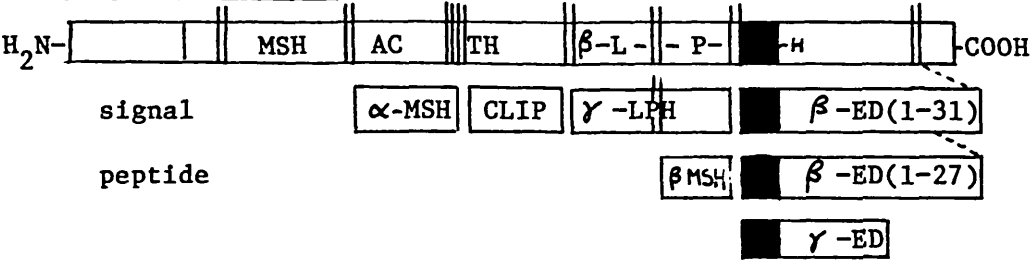
³H-dihydromorphine to opioid receptors were detected (Terenius & Wahlstrom, 1974, Hughes et al, 1975) and later identified (Hughes et al, 1975). The compound isolated and identified by the latter group consisted of two pentapeptides, leu-enkephalin and met-enkephalin (Fig. 7), and it was shown that they are potent naloxone-reversible agonists in the isolated guinea-pig ileum and mouse vas deferens. Many other opioid peptides have been identified (Table 1).

2.2 Biogenesis of Opioid Peptides

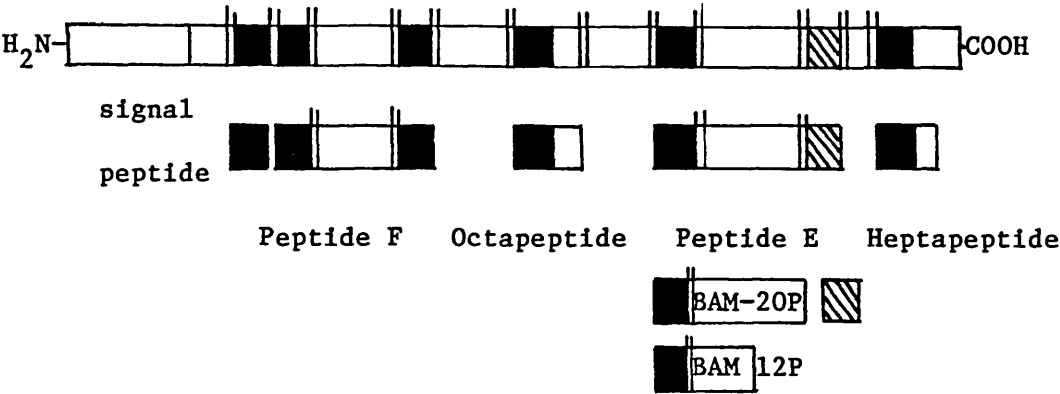
The opioid peptides can now be defined in terms of three families according to the gene product from which they originate (Fig. 8). Pro-opio-melanocortin is the precursor for β -endorphin and for adrenocorticotrophic hormone; pro-enkephalin (A) is the precursor for met- and leu-enkephalin; prodynorphin (proenkephalin B) is the precursor for dynorphins and neo-endorphins. The primary structures of these precursors was elucidated by recombinant DNA techniques (Nakanishi et al, 1979; Comb et al; 1982, Kakidani et al, 1982). The three precursors are similar in size (about 260 amino-acids); they contain repeated sequences, for example, there are three MSH sequences in pro-opiomelanocortin, six copies of met-enkephalin in proenkephalin, and three copies of leu-enkephalin in prodynorphin; they each have a hydrophobic 'signal' peptide necessary for transport across endoplasmic reticulum membranes; the biologically active domains are adjacent to pairs of basic

Fig. 8 Processing of opioid peptides

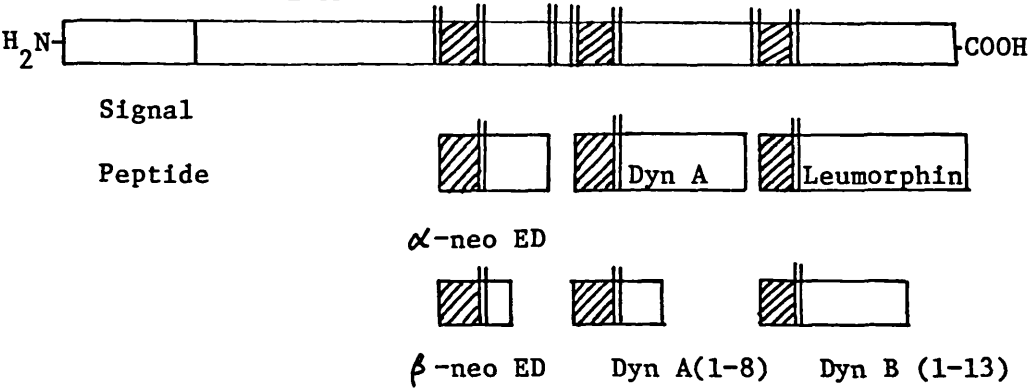
a. Pro-opiomelanocortin (265 amino acids)



b. Prepro-enkephalin A (263 aminoacids)



c. Prepro-enkephalin B (256 aminoacids)



||| Basic amino-acid pair

■ met-enkephalin

▨ leu-enkephalin

ED endorphin

Dyn dynorphin

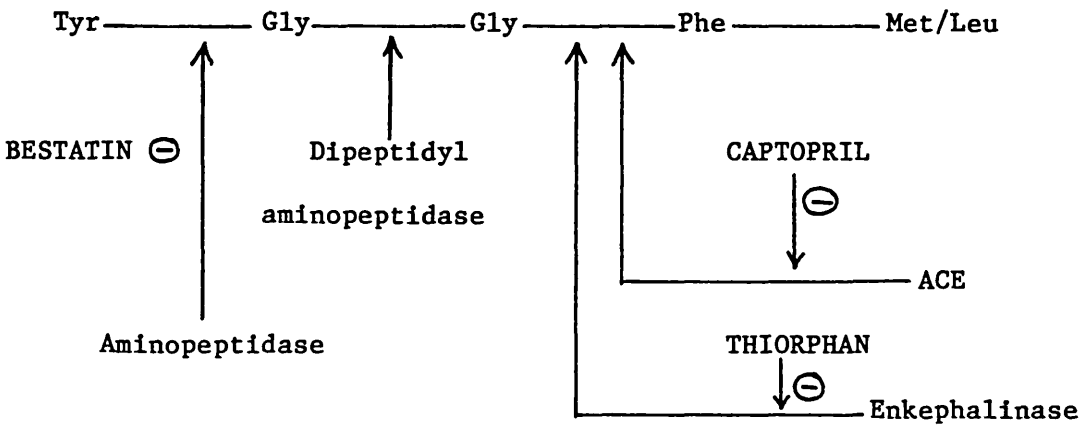
amino acids which are processing signals for a trypsin-like enzyme. However, it does not appear necessary to have a basic amino-acid pair as a signal as a single basic amino-acid appears to function as a similar processing signal (Weber et al, 1982); neither does it appear that such a basic doublet is an obligatory signal since it has been shown that the enkephalin sequences are not all processed down to the pentapeptides, as in β -endorphin from POMC, i.e. there is differential processing of the precursors depending on the region of the brain (Seizinger et al, 1983; Cuello, 1983). The physiological relevance of such peptides as met-enkephalin-Arg⁶-Phe⁷ (heptapeptide) and peptide E still remains to be elucidated. The distributions of the three families is different (Table I), and the opioid peptides together constitute a diffuse system of communication at levels from short-acting putative neurotransmitters such as the pentapeptide enkephalins and dynorphin A (1-8), which are known to be co-stored and co-released with catecholamines in the adrenal medulla (Viveros et al, 1979) to longer-acting putative hormones such as dynorphin (1-17) and β -endorphin which is co-synthesized with other well established hormones such as ACTH and MSH. In general, the opioid peptides are said to have a 'modulator' role, for example, they contribute to regulation release of growth hormone, prolactin, luteinizing hormone and oxytocin, all found in the hypothalamic-pituitary axis (Grossmann and Rees, 1983) and have been implicated in memory processes and disorders such as schizophrenia, although definitive evidence is lacking in these areas (Koob & Bloom, 1983).

2.3 Structure-Activity Relationships in Opioid Peptides and Derivatives

Although the opioid peptides are potent agonists in isolated tissue preparations (Kosterlitz et al, 1975), when first injected (intravenously) into animals and man they were not found to be analgesic. This is because they do not cross the blood-brain barrier satisfactorily, and are metabolised quickly in the blood (Hambrook et al, 1976). Injected directly into the brain, β -endorphin and the enkephalins (Koob & Bloom, 1983), but not dynorphins (Holtt et al, 1982) can produce analgesia. Several enzymes are involved in the inactivation of enkephalins in vivo and in vitro (Fig. 9), and inhibitors of these enzymes have been reported to block metabolism of enkephalins up to 75% (Baume et al, 1983) and to potentiate enkephalin action in bioassays (McKnight et al, 1983). Thiorphan has been shown to increase brain levels of met-enkephalin in vivo, accompanying an analgesic effect (Hughes, 1983). The metabolism of dynorphins and β -endorphin is not yet well-understood, but β -endorphin (1-31) undergoes degradation to give β -endorphin (1-26) and (1-27), at least in rat brain, and these peptides may also undergo N-acetylation, although this is dependent on brain region.

In an attempt to produce enkephalin analogues which are stable in vivo, many synthetic peptides have been studied. It was relatively easy to produce stable enkephalins by means of such substitutions as D-amino-acids for the L-forms, substitutions

Fig. 9 Enkephalin metabolism



ACE Angiotensin converting enzyme

⊖ = inhibition

Fig. 10 Examples of stabilised enkephalin analogues

Tyr-Gly-Gly-Phe-Met/Leu (all L-amino acids)

Tyr-D-Ala-Gly-Phe-D-Leu	DADL	(Baxter <u>et al</u> , 1977)
Tyr-D-Ala-Gly-MePhe-Met(0)ol	FK-33,284	(Romer <u>et al</u> , 1977)
Tyr-D-Met-Gly-Phe-Pro-NH ₂		(Bajusz <u>et al</u> , 1977)
Tyr-D-Ala-Gly-MePhe-Gly(01)	DAGO	(Handa <u>et al</u> , 1981)
Tyr-D-Ser-Gly-Phe-ProNH ₂		(Shaw <u>et al</u> , 1978)
Tyr-D-Ser-Gly-Phe-Leu-Thr	DSLET	(David <u>et al</u> , 1982)

of different amino acids in the sequence, amidating the C-terminal, and extending the enkephalin sequence (For review see Morley, 1983 a) (Fig. 10). These procedures increased metabolic stability, but also changed the properties of enkephalins (see I.3.1). It has not been easy to make a peptide antagonist at opioid receptors; the N-allyl-tyrosine derivatives are of low affinity and are partial agonists in bioassays (Pert et al, 1977; Hahn et al, 1977). Further modification has produced the pure antagonist ICI-154,129, but this is again of low potency (Shaw et al, 1982). The structure-activity relationships in opioid peptides have been intensively studied (Morley, 1980; 1983a) and the basic unit of binding to opioid receptors is Tyr-Gly-Gly-Phe, common to all endogenous opioids; properties of particular peptides depend on subsequent amino-acids (Smyth, 1980; Chavkin & Goldstein, 1981), a conclusion confirmed by the use of a monoclonal antibody to this sequence (Meo et al, 1983).

In spite of exhaustive studies to deduce the conformation of opioid peptides at the receptor (Jones et al, 1976; Sprites et al, 1978), the flexibility of small peptides means it is difficult to assign any particular three-dimensional conformation to them, and it is likely that the conformation at the receptor is constrained during the binding process (Wessels et al, 1973).

As indicated above, stabilized enkephalins are potent analgesics, but they are not non-addictive, and there is good

correlation between analgesic activity and dependence liability (Wei, 1981). This is not surprising in view of their action at the same receptors as morphine to produce analgesia (Gacel et al, 1981) and see § I.3.1.

3.1 Multiple Opioid Receptors : Whole Animal Studies

As mentioned above, when endogenous ligands for opioid receptors were discovered, there was considerable investigation into their receptor profiles, and evidence soon accumulated for the existence of opioid receptor subtypes. That acetylcholine had two quite distinct sets of actions on nicotine and muscarinic receptors had been known for many years (Dale, 1914), and other systems with receptor subtypes include the adrenergic β_1 , β_2 and α_1 , α_2 receptors, histamine H_1 and H_2 receptors and 5-hydroxytryptamine types 1 and 2 receptors.

As mentioned in § I.1.3, while nalorphine was apparently an antagonist of the pain-relieving properties of morphine, it also produced analgesia itself in higher doses. This behaviour led to studies on the clinical efficacy of different morphine-nalorphine mixtures to control pain in cancer patients, using a double-blind crossover design (Houde & Wallenstein, 1956). The result showed a biphasic dose-response relationship; using a fixed dose of morphine combined with a varying dose of nalorphine, low doses of nalorphine showed antagonism of morphine's analgesic action. At higher doses, nalorphine began to exert an analgesic effect of its own.

This concept was also based on further observations of the different effects in man of morphine compared to nalorphine. Both were analgesics, but with different accompanying effects, for example, nalorphine was sedative and sometimes hallucinogenic, but morphine was not. The abstinence syndromes in dependent subjects were also quite distinct.

Further evidence in support of multiple opioid receptors came from the observation that some benzomorphans, powerful analgesics in themselves, could not substitute for morphine in morphine-dependent monkeys (Villarreal & Seevers, 1972); neither did they show morphine antagonist activity, unlike nalorphine (Albertson, 1974). Also, using rats dependent on either fentanyl (morphine-like) or ethylketocyclazocine (benzomorphan), rats dependent on fentanyl did not generalise to ethylketocyclazocine and vice versa (Shearman & Herz, 1982a). Further studies, using the chronic spinal dog preparation, suggested three receptors through which a variety of opioid drugs can act, and be at least partially reversed by naloxone (Gilbert & Martin, 1976; Martin *et al.*, 1976); these were called μ -, κ - and σ -receptors, and the effects they mediate in the chronic spinal dog are indicated in table 2. It is not surprising that morphine can suppress the cyclazocine abstinence syndrome, probably through its own pharmacological actions, although this datum alone does not preclude action on the cyclazocine receptor. There is now evidence that μ - and κ -receptors have different actions producing analgesia; μ -receptor agonists are active in tail-flick and hot-plate tests

TABLE 2 Actions of μ -, k and σ -agonists in the chronic spinal dog (from Martin et al, 1976)

	μ (morphine)	k (ketocyclazocine)	σ (SKF-10,047)
Heart rate	↓	-	↑
Respiratory rate	↓	-	↑
Pupil constriction	↑	↑	↓
Temperature	↓	-	↑
Skin twitch reflex	↓	↓	-
Suppress morphine abstinence	yes	no	no
Suppress cyclazocine abstinence	yes	yes	no
Overall behavioural effect	-	sedation	delirium

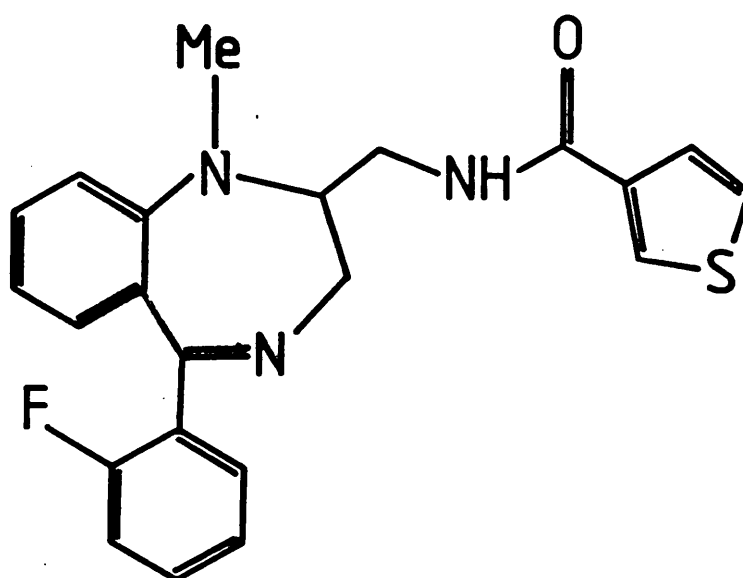


Fig. 11

Tifluadom

while κ -agonists, believed active in writhing and paw pressure tests, show little activity in tail-flick and hot-plate tests (Tyers, 1982; Hayes *et al*, 1983). It appears that κ -agonists, such as ethylketocyclazocine and dynorphin, act to produce analgesia at a spinal rather than a supraspinal level, and that μ -agonists, such as morphine, act more at the supraspinal level (Wood *et al*, 1981; Piercey *et al*, 1982a, b). It has also been proposed that the μ -sites which mediate analgesia supraspinally are a subset of these sites, the μ_1 -receptors, while those μ -sites which mediate analgesia in the spinal cord are μ_2 -receptors. μ_1 -receptors are those which are irreversibly, and μ_2 -receptors reversibly, blocked by the antagonist naloxazone/naloxonazine (Ling & Pasternak, 1982). The σ -receptor identified by Martin has a less well-established role as an opioid receptor, for several reasons: σ -agonists such as cyclazocine and SKF-10,047 have similar psychotomimetic effects to phencyclidine (PCP) (Brady, 1982; Henderson, 1982) and not to morphine (Shearman & Herz, 1982c). Further, the classical opioid antagonists naloxone and naltrexone both fail to antagonise σ -effects fully (Holtzman, 1982; Shannon, 1982). Also naltrexone fails to antagonise the effects of SKF-10,047 in the chronic spinal dog (Vaupel, 1983). The σ -receptor may be, therefore, a PCP-receptor, although the physiological role of such a receptor has yet to be defined. It has also been difficult to assign a physiological role for the δ -receptor so easily identified in bioassays and binding assays (Sections I.3.2 & 3). This has been partly due to the lack of stable selective agonists for the δ -receptor. In early experiments,

intracerebroventricular injection of the natural enkephalins did not produce analgesia; however, local injections into certain nuclei in the medulla, or periaqueductal grey matter, did. (For review, see Koob & Bloom, 1983). The advent of enkephalin analogues stable to deactivation which were also potent analgesics appeared to confirm the conclusion that the natural enkephalins produced little analgesia because of plasma and brain enzymatic inactivation; it soon became clear that the structural alterations which produced stable analogues also changed the receptor profile from δ to μ , and so the analgesia seen was due to action on μ -rather than δ -receptors. Even δ -selective peptides such as Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET) produce analgesia by acting through μ -receptors (Gacel *et al*, 1981; Chaillet *et al*, 1983). The stabilised enkephalins also produce dependence which shows cross-tolerance to μ -ligands such as fentanyl (Shearman & Herz, 1982a; Wei, 1981).

In spite of the lack of evidence for a direct involvement in analgesia, δ -receptors do appear to modulate analgesia produced by morphine or μ -selective enkephalins: sub-analgesic doses of δ -selective peptides have been shown to enhance morphine analgesia, while μ -selective peptides have no such effect (Vaught & Takemori, 1979; Lee *et al*, 1980; Barrett & Vaught, 1982). It is postulated that the μ -receptor mediates analgesia and the δ -receptor modulates either the μ -receptor itself, or the μ -receptor-effector mechanism, through an allosteric interaction (Fig. 12). There is further evidence for a model of this kind from binding studies (§ I.3.3). However,

Fig. 12 Allosteric interaction between μ - and δ -receptors (from Rothman & Westfall, 1983).

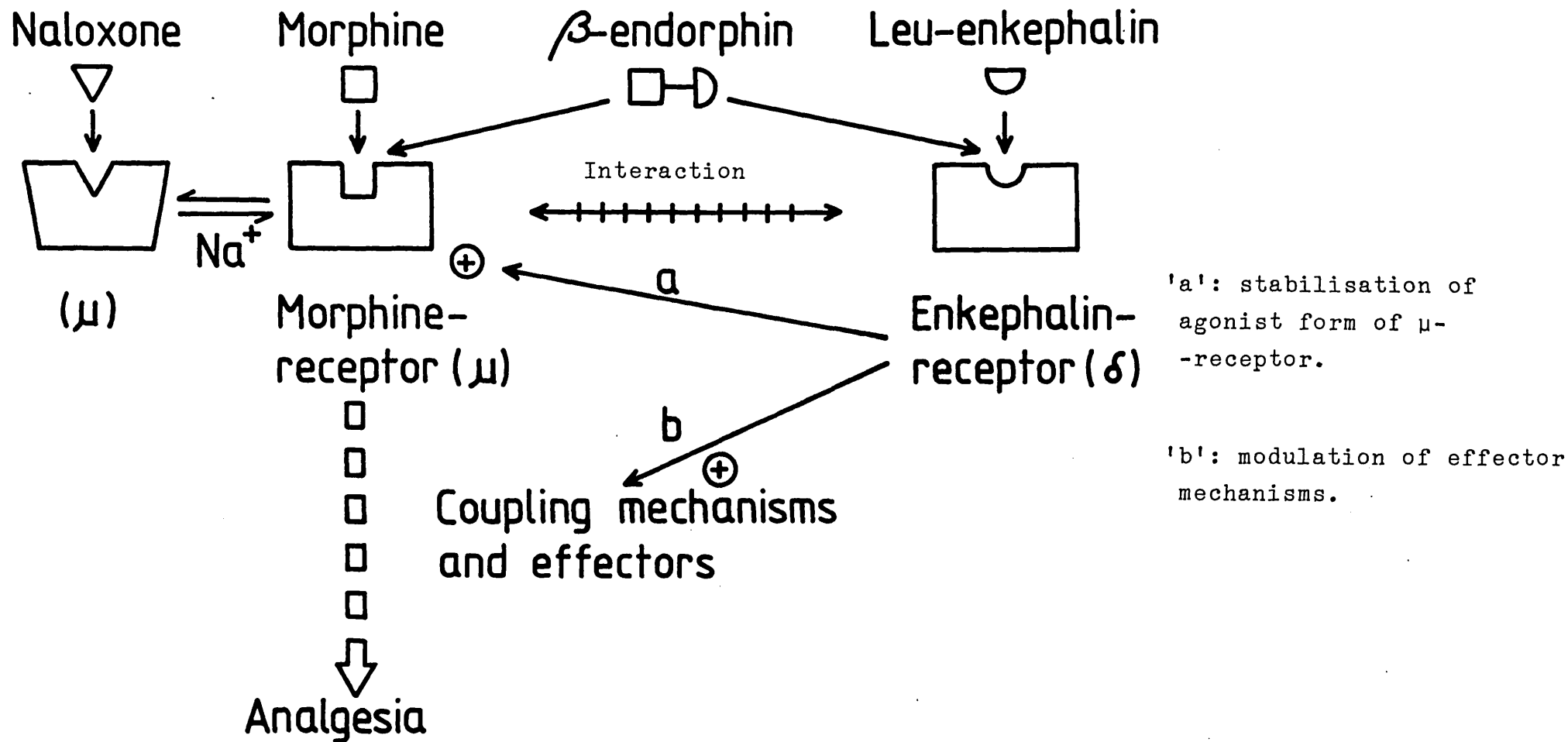


Fig. 12 Allosteric interaction between μ - and δ -receptors (from Rothman & Westfall, 1983)

this is again complicated by the lack of stable δ -selective ligands, both DADL and DSLET showing a degree of cross-reactivity with μ -receptors (Paterson et al, 1983); the δ -antagonist, ICI-154,129 has been shown to block putative δ -mediated effects such as the slowing by etorphine of the rat striatally-evoked head-turn, at doses which leave morphine analgesia unaffected (Gormley et al, 1982). The compound shows no analgesic action of its own unlike the weak partial agonist N-allylenkephalins (Pert et al, 1977). The conclusion that both enkephalins and morphine produce analgesia through a common (μ) receptor has also been supported by studies in vivo using β -funaltrexamine^(β -FNA) (Ward et al, 1982), whereby blockade of high-affinity morphine, β -endorphin and D-Ala²-Met-enkephalinamide binding sites correlates with blockade of analgesia produced by β -FNA. Naloxonazine has also been used to show that morphine produces analgesia and respiratory depression through separate systems (Ling et al, 1983).

The problems of studying opioid receptor subtypes in vivo are compounded by the complexity of the CNS; for example, in the dog, ethylketocyclazocine produces effects (E_{EKC}) at at least three levels: supramedullary (analgesic), medullary (hyperalgesic) and spinal (analgesic), so that

$$E_{\text{EKC}} = E_{\text{SPINAL}} - E_{\text{MEDULLARY}} + E_{\text{SUPRAMEDELLARY}}$$

(Martin, 1984; Wu et al, 1983)

Because opioid antagonists do not affect pain thresholds except in certain experimental conditions, for example when there is already a level of pain, and because inhibitors of enkephalin breakdown, such as thiorphan, are not good analgesics (Greenberg & O'Keefe, 1982), the endogenous opioid system is not thought to be tonically active (Watkins & Mayer, 1982). In spite of this low level of activity, endogenous opioids have been postulated to be involved in many physiological processes, and it is true their distribution is wide. Examples include a role in the cardiovascular, respiratory and digestive systems (Maas, 1982; Pfeiffer et al, 1983); effects on food intake (Sanger & McCartley, 1982) and on memory (Izquierdoui et al, 1980). Opioid antagonists have been used to treat shock, one of their main beneficial effects being reversal of shock-induced hypotension (Faden et al, 1981).

Experimental data from studies in vivo have supported the existence of multiple opioid receptor subtypes, in spite of the difficulties of overlapping ligand specificities and CNS complexity, at least three opioid receptor subtypes have been identified: the μ -receptor, which mediates classical-narcotic-induced analgesia, probably euphoric effects and dependence on opioids, the κ -receptor, which mediates analgesia different to μ -receptor mediated analgesia, the δ -receptor which modulates the μ -receptor and may mediate behavioural and mood effects of opioids, and finally there is the σ -receptor with which some opioids are able to interact, but appears in fact to be a PCP-receptor, not an opioid receptor.

3.2 Multiple Opioid Receptors in Isolated Tissues

Certain isolated tissues have been used as in vitro assay systems for opioid ligands; one advantage they have over radioligand binding studies is that they can unequivocally distinguish agonists from antagonists. The first tissue in which opioids were shown to produce a consistent effect was the isolated guinea-pig ileum (GPI) in which they produce a dose-dependent inhibition of electrically-evoked contractions by decreasing acetylcholine output from cholinergic nerve terminals (Paton, 1957; Schaumann, 1957; Kosterlitz & Watt, 1968). Another preparation widely used for assay of opioid activity is the mouse vas deferens (MVD); in this tissue, opioids inhibit the neuronal release of noradrenaline, which depresses electrically-evoked contractions (Henderson et al, 1972). The action of endogenous enkephalins in these tissues, and its reversal by the opioid antagonist naloxone, was part of the primary evidence for enkephalins being endogenous opioid ligands (Hughes et al, 1975). It was immediately recognised that the enkephalins were about ten times more potent in the MVD than in GPI; also the K_e value of naloxone against the enkephalins in the MVD was ten to twenty-fold the K_e against morphine; K_e is equal to the antagonist concentration which requires a doubling of agonist concentration to produce the same pharmacological response as that obtained in the absence of antagonist (Kosterlitz and Watt, 1968). In the GPI, however, K_e values against morphine and enkephalins are similar (Lord et al, 1976). Further studies, including binding assays, led this

group to propose the existence of μ -receptors, through which morphine acts, and δ -receptors, through which the enkephalins act, the GPI containing μ -receptors and the MVD μ - and δ -receptors. The enkephalins have a higher affinity for δ - than μ -receptors, and therefore appear more potent in the MVD than GPI, where they have to interact with μ -receptors in the absence of δ -receptors (Lord et al, 1977). The use of a δ -antagonist has confirmed these conclusions (Shaw et al, 1982). Studies in MVD on the differential effects of temperature on potencies of opiate alkaloids and enkephalins also indicate the presence of at least two types of opioid receptor in this tissue (Miller & Shaw, 1983).

The actions of stabilised enkephalin analogues in bioassays showed that nearly all structural modifications of enkephalins produced μ -selective ligands, as shown by naloxone K_e values and relative potencies in both tissues, and correlating with their analgesic effects in whole animals (Kosterlitz et al, 1980; Fournie-Zaluski et al, 1981).

It also became clear that the benzomorphans classified as k-agonists by Martin, such as ethylketocyclazocine, which do not substitute for morphine in morphine-dependent monkeys, yet are potent analgesics, showed differential activities in the two assays; they were more potent in the GPI than MVD and had naloxone K_e values three to seven times higher than that of morphine. However, the benzomorphans which do substitute for morphine in dependent monkeys, such as phenazocine, showed no

differences between the MVD and GPI (Hutchinson et al, 1975).

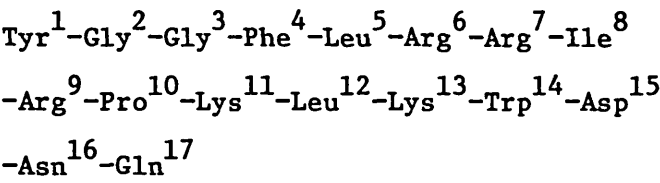
It was not immediately apparent whether the k-agonist benzomorphans were acting on different receptors to morphine, and again the elucidation of receptor populations present in the tissues was made more difficult by the proposed k-ligands' lack of selectivity, especially apparent in binding assays (e.g. Johnson & Pasternak, 1983).

However, use of two further isolated tissues helped clarify the situation, these being the rat and rabbit vasa deferentia (Lemaire et al, 1978; Oka et al, 1980). In the rat vas deferens (RVD), β -endorphin and enkephalins are pure agonists, morphine shows partial agonist/antagonist action and the k-benzomorphans such as EKC and MR 2034 are pure antagonists (Gillan et al, 1981; Ishii et al, 1981; Henderson et al, 1982). Some studies suggested multiple receptors in the RVD (Huidobro-toro et al, 1980; Jacquet et al, 1980) or proposed a novel opioid ϵ -receptor (Schulz et al, 1981a); however, a more satisfactory explanation is that the RVD contains one receptor, and that this is the μ -receptor, coupled to its effector system in such a way that only μ pure agonists can produce a response (Smith & Rance, 1983). This also implies the existence of very few or no spare receptors, and that k-ligands act as antagonists at μ -receptors, even though they do not precipitate withdrawal in morphine-dependent dogs (Gilbert & Martin, 1976). The low potency of natural enkephalins compared to β -endorphin is explained by their lower affinity for μ -receptors, and possibly by their lower metabolic stability, although this has not been studied with reference to the RVD.

The rabbit vas deferens (RBVD) also contains a single type of opioid receptor, the k-receptor, and only the benzomorphans and k-ligands such as tifluadom and U50-488H are able to inhibit the electrically-evoked contractions in a naloxone-reversible manner; μ -, δ - and σ -ligands are inactive as agonists (Oka et al, 1980).

The four bioassay systems (MVD, GPI, RVD, RbVD) can together provide information about the receptor profile of any opioid ligand, an example of this being the elucidation of the opioid receptor specificities of dynorphins A and B and fragments of them. Dynorphin A (1-13) was shown not to act at μ -receptors in the GPI on the basis of naloxone pA_2 values (negative logarithm of K_e) (Vaught, 1981), and cross-tolerance studies (Chavkin & Goldstein, 1981b). It was proposed to be acting at k-receptors because of the differential potencies of three opioid antagonists, naloxone, WIN-44,441 and MR 2266, against dynorphin A (1-13) and enkephalins in the GPI (Yoshimura et al, 1982). It was not until full results of the actions of dynorphin A (1-13) in all four assays were known that it was clear that its action was that of a selective k-agonist (Oka et al, 1982). Studies on the full dynorphin A (1-17) sequence, and fragments of it, showed that dynorphin A (1-17) is k-selective, and that losing successive amino-acids from the C-terminal, down to dynorphin A (1-8), retains k-activity; from then on, a δ -selectivity emerges, going down to dynorphin (1-5) which is leu-enkephalin (Corbett et al, 1982), Table 3. The most k-selective peptides were shown to be dynorphin A (1-17) and dynorphin A (1-9) (Corbett et al, 1983a). Similar studies

TABLE 3 Potencies of Dynorphin A (1-17) and its fragments in
four bioassays (from Corbett et al, 1982)



	GPI(μ + <u>K</u>)	MVD(μ + δ + <u>k</u>)	RVD(μ)	RbVD(<u>k</u>) (IC ₅₀ ,nM)
Dyn 1-5	29	1.7	550	10,000
1-6	103	26	720	10,000
1-7	107	11	790	3,000
1-8	5	9	10,000	12
1-9	2.4	6	10,000	6
1-13	0.3	0.33	10,000	2.4
1-17	0.3	0.91	10,000	3

Fig. 13 Relative receptor selectivities of Dynorphin B (1-13)
and fragments

Tyr¹-Gly²-Gly³-Phe⁴-Leu⁵-Arg⁶-Arg⁷-Gln⁸-Phe⁹-
 Lys¹⁰-Val¹¹-Val¹²-Thr¹³

1-5	$\delta \gg \mu \simeq \underline{k}$	most δ -selective
1-6, 1-7	$\delta > \mu \gg \underline{k}$	
1-8	$\underline{k} > \mu = \delta$	
1-9	$\underline{k} \gg \mu = \delta$	most $\underline{k}$ -selective
1-13	$\underline{k} > \mu > \delta$	

From Corbett et al, 1983b

on dynorphin B (1-13) and fragments have recently shown a similar pattern of behaviour, i.e. the larger peptides are k-selective, and the smaller δ -selective (Fig. 13). The four bioassays have also been used to show the k-selectivity of tifluadom (Romer et al, 1982) and U-50,488H (Gillan et al, 1983).

In addition to the μ - and k-receptors in the GPI, it has been suggested that there are also σ -receptors present (Su et al, 1981; Itzhak et al, 1981), because SKF 10-047 produced a naloxone reversible depression of twitch, but with a K_e value between that for morphine (μ) and EKC(k). However, SKF-10,047 may be acting through both these receptors, evidence to support this coming from binding studies (Paterson et al, 1983).

Studies on cross-tolerance and cross-protection against inactivating ligands in isolated tissues have also been used to demonstrate the receptor specificities of peptide and non-peptide opioids. In cross-tolerance studies, tolerance may be induced in vivo prior to removal of the vasa, or in vitro, these procedures providing similar results (Schulz et al, 1980a). Vasa made tolerant to μ -agonists were up to 1000-fold less sensitive to other μ -agonists without showing tolerance to δ -agonists; vasa made tolerant to DADL, a δ -agonist in this tissue, were up to 800-fold less sensitive to δ -agonists, with an unchanged sensitivity to μ -agonists (Schulz et al, 1980b). Parallel experiments in the GPI similarly revealed that tolerant μ -receptors can apparently coexist with k-receptors of unchanged sensitivity (Schulz et al, 1981b).

These data were used to demonstrate that dynorphin A (1-13) acts on κ -receptors in the GPI (Chavkin & Goldstein, 1981b).

Tolerance studies in the MVD suggested there may exist subtypes of the μ -receptor, on the basis that various μ -agonists did not show complete cross-tolerance (Schulz & Wuster, 1981); this might however be explained by the action of some of these ' μ -agonists' on δ - or κ -receptors in μ -tolerant vasa.

Cross-protection studies in isolated tissues using affinity labelling reagents such as β -funaltrexamine (β -FNA) (Takemori *et al*, 1981a), β -chlornaltrexamine (β -CNA) (Portoghese *et al*, 1978) and naloxazone/naloxonazine (Pasternak & Hahn, 1980), are also consistent with the existence of multiple receptors in the MVD and GPI. It has been shown that β -CNA is a non-selective opioid receptor-directed alkylating agent whose irreversible effects are those of an antagonist at opioid receptors (Caruso *et al*, 1979); β -FNA, on the other hand, is a μ -selective antagonist alkylating agent contaminated with a reversible agonist action, seen prior to the onset of its irreversible antagonist action. This agonist action may be due to action on κ -receptors (Ward *et al*, 1982b). The amount of irreversible blockade produced is dependent on both the concentration and the length of time of incubation of β -FNA: i.e. β -FNA is a non-equilibrium antagonist. β -FNA's selective inactivation of μ -receptors in MVD has been shown to produce tissues in which it is proposed that morphine produces its effects by acting on κ -receptors and not δ -receptors (Ward *et al*, 1982c), a result in conflict with the relative affinity of morphine for

δ - and κ -binding-sites in binding assays (Paterson et al, 1983). An explanation might be that morphine is an antagonist at δ -receptors in MVD. The non-selective compound β -CNA has been used to demonstrate the presence of a greater number of 'spare' receptors in GPI compared to MVD (Cox & Chavkin, 1983); also it has been used in cross-protection studies whereby β -CNA is incubated with the tissue in the presence of high concentrations of reversible ligands, which can protect certain subtypes of opioid receptors against inactivation. Results of such studies have been used to show the existence of a third opioid receptor in the MVD, apart from μ -or δ -receptors, presumably the κ -receptor (Ward et al, 1982c).

Clearly, then, isolated tissue studies have provided much data which has been used to support the existence of three opioid receptor subtypes in peripheral tissues. The actions of opioids on these three proposed subtypes have been distinguished particularly by the use of pharmacological antagonists displaying selectivity for one subtype.

3.3 Multiple Receptors : Binding Studies

Since the demonstration of stereospecific opioid binding to brain membranes ten years ago (Simon et al, 1973; Pert & Snyder, 1973; Terenius, 1973), there has been much further study of the CNS opioid binding sites, in an attempt to elucidate how opioids produce their effects. It soon became apparent that the binding

sites seen did represent physiological opioid receptors, as outlined in §I.1.4. Parallel with the observation that the natural enkephalins acted on δ -receptors and opiate alkaloids on μ -receptors in MVD, was the demonstration that the enkephalins displaced completely the specific binding of ^3H -leu-enkephalin, while morphine could only displace a proportion of it (Lord et al, 1976). Later, in further displacement binding studies, it was shown that opiate alkaloids could displace the specific binding of ^3H -naloxone better than could the enkephalins. The rank order of potencies of enkephalins and opiates in GPI, MVD, ^3H -naloxone displacement and ^3H -leu-enkephalin displacement suggested a close relationship between the MVD δ -receptors and ^3H -leu-enkephalin binding sites, and between GPI μ -receptors and ^3H -naloxone binding sites (Lord et al, 1977). It was suggested that both μ - and δ -receptors are present in brain membranes, but studies on the binding capacities of ^3H -dihydromorphine (a μ -ligand), ^3H -DADL (a δ -ligand) and ^3H -etorphine (universal ligand), suggested that there were sites to which ^3H -etorphine binds which were neither μ - nor δ -sites. These observations were complicated by the lack of ligand specificity, especially in saturation binding studies. The alterations in enkephalin structure which bring about stability to enzymatic degradation but also change receptor binding characteristics also made interpretation of binding studies difficult; some analogues were extremely selective for μ -receptors, for example DAGO (Handa et al, 1981),

while others bound equally well to both μ - and δ -receptors, for example D-Ala²-met-enkephalinamide (Gillan et al, 1980). This mirrored the results seen in bioassay and in vivo studies, (§§I.3.1 and 2). The use of iodinated analogues has the advantage that the higher specific activity obtainable with ¹²⁵Iodine means lower concentrations of radioactive ligand can be used, therefore the specificity of receptor binding can be increased compared to ³H-ligands. ¹²⁵I-DADL has been used as a δ -receptor probe (Chang & Cuatrecasas, 1979), but it has been shown to be much less potent than natural enkephalins in MVD and GPI bioassays (Miller et al, 1978). This is probably due to the large increase in molecular mass accompanying iodination, and maybe to the increase in hydrophobicity, thus changing the nature of enkephalin molecules substantially. ³H-derivatives are now the most commonly used radioligands for opioid receptor binding studies, because the substitution of tritium for hydrogen has little effect on molecular mass or hydrophobicity. However, this has not always been possible, as for example with the κ -probe U50-488H, where the ³H-derivative shows extensive radiolysis (Lahti et al, personal communication).

Initial studies on opioid receptors in brain membranes suggested a sodium effect whereby agonists and antagonists were reported to be differentially affected by sodium ions; it was uncertain whether this was because of a change in the number (Pert & Snyder, 1975) or affinity of sites (Simon et al, 1975).

Assuming a two-state model, it has been calculated that about 10% of sites are in an agonist conformation at the synapses in physiological sodium concentrations (Cheney et al, 1982). This might explain the apparent low level of endogenous

opioid activity. The 'sodium ratio' has been postulated as a method to screen for new opioid agonists and antagonists, and there have been classifications of opioid ligands based on their affinities for opioid receptors in the presence and absence of sodium (e.g. Snyder et al, 1979). However, much of this early work needs now to be reinterpreted in the light of the existence of multiple opioid receptors in mammalian CNS.

There are however differential effects of other compounds on μ - and δ -receptors. Short-chain alcohols selectively inhibit δ -receptor binding more than μ -receptor binding (Hiller et al, 1981). The differential effects of guanine nucleotides on binding have also suggested a subclassification scheme into types 1 (more affected) and 2 (less affected) opioid receptors (Pert & Taylor, 1980); however, it seems that the type 1 is a μ -receptor, and type 2, a δ -receptor (Chang et al, 1981a; Simon, 1981).

Cross-protection studies have again provided evidence for the existence of opioid receptor subtypes. Ligands used have been phenoxybenzamine and N-ethylmaleimide. It was observed that phenoxybenzamine reduced the number, but not the affinity of, mouse brain opioid receptors (Spiehl et al, 1978); it was shown that μ -ligands protected ^3H -dihydromorphine binding, and δ -ligands protected ^3H -DADL binding (Robson & Kosterlitz, 1979). Similar results were reported for inactivation of opioid receptors by N-ethylmaleimide (Smith & Simon, 1980). These data suggest the existence of independent μ - and δ -binding sites in brain membranes. Similar experiments using

EKC as protecting ligand suggested there was protection of a κ -receptor (Kosterlitz *et al*, 1981); this was not however so clear because EKC and other proposed κ -agonists such as bremazocine bind readily to μ - and δ -receptors with only slightly lower affinities (Paterson *et al*, 1983; Pfeiffer & Herz, 1981; Zukin & Zukin, 1981) (Table IV).

In rat particularly, it was suggested that κ -ligands produced their effects *in vivo* through action at both μ - and δ -receptors (Chang *et al*, 1980). However, with the development of improved assay methods to measure κ -receptors, such as suppression of μ - and δ -binding by unlabelled ligands relatively specific for these receptors (such as DADL and DAGO), it became clear that there are κ -receptors in brain membranes (Kosterlitz *et al*, 1981; Chang *et al*, 1981b). The prototype σ -ligand, SKF-10,047, has affinity for μ -, δ - and κ -receptors in brain membranes, but also binds to a site to which the 'universal' opiate, etorphine, does not bind (Su, 1981). This site may be the ^3H -PCP binding-site (Henderson, 1982); the properties of ^3H -PCP binding-sites correlate with *in vivo* tests of PCP and its analogues (Vignon *et al*, 1982).

Binding studies using naloxazone, the irreversible opiate antagonist, suggested the presence in brain of high-affinity and low-affinity binding-sites (Pasternak & Snyder, 1975), but further examination led to the conclusion that the high-affinity sites are μ -receptors, and the low-affinity sites, δ -receptors (Zhang & Pasternak, 1980). Studies on inhibition of binding by

TABLE 4 Relative receptor affinities of a variety of opioid ligands according to Paterson et al (1983) and Chang et al (1981), in parentheses.

		μ -site	δ -site	κ -site
Morphine	μ	1 (1)	0.02 (0.01)	0.01 (0.01)
DAGO	"	1 (-)	0.01 (-)	0.01 (-)
Naloxone	"	1 (1)	0.07 (0.07)	0.11 (0.06)
Met-enkephalin	δ	0.1 (0.5)	1 (1)	0.01 (0.01)
Leu-enkephalin	"	0.06 (0.16)	1 (1)	0.01 (0.01)
DADL	"	0.1 (0.4)	1 (1)	0.01 (0.01)
U-50, 488H	κ	0.01 (-)	0.01 (-)	1 (-)
Dynorphin A				
(1-17)	"	0.15 (-)	0.05 (-)	1 (-)
β -endorphin	$\mu + \delta$	1 (-)	0.9 (-)	0.02 (-)
EKC	$\mu + \delta + \kappa$	0.5 (1)	0.1 (0.3)	1 (0.06)
SKF-10,047	$\mu + \delta + \kappa$	1 (1)	0.25 (0.6)	0.7 (0.05)
Etorphine	$\mu + \delta + \kappa$	0.2 (-)	0.4 (-)	1 (-)
Diprenorphine	$\mu + \delta + \kappa$	1 (1)	0.6 (1)	0.4 (0.4)
Bremazocine	$\mu + \delta + \kappa$	0.3 (-)	(0.2) (-)	1 (-)

the alkylating antagonists β -CNA and β -FNA have been difficult to interpret, because it does not appear possible to remove unreacted ligand from brain membranes under conditions in which control receptor binding remains unchanged. In MVD, more than twenty washes are necessary to remove unreacted ligand (Ward et al, 1982b). Ligands derived from fentanyl (fentanyl isothiocyanate, FIT) and oripavine (fumarimido oripavine, FAO), have been shown in binding assays to be selective irreversible ligands for δ -receptors (Rice et al, 1983).

The evidence discussed above points to the existence of three subtypes of opioid receptors in brain membranes, μ -, δ - and κ -receptors; this has been confirmed by detailed computerised studies of the binding of various opioid ligands (Pfeiffer & Herz, 1982). However, the proportions of μ : δ : κ -receptors estimated by saturation analysis vary between species: in guinea-pig brain, the proportions are 20% : 40% : 40%, but in rat brain 46% : 42% : 12% (Paterson et al, 1983). The low proportion of κ -sites in rat brain may have contributed to the earlier difficulties in identifying them. There is support for the three opioid receptor subtypes also from endogenous opioid peptide specificities (Table 4). Dynorphins A and B, and fragments of them appear to be endogenous κ -ligands (Corbett et al, 1982; 1983a, b), while the natural enkephalins may be δ -ligands (Lord et al, 1977). There is not such an obvious μ -ligand, although β -endorphin binds with high affinity to the μ - as well as the δ -receptor; however the octapeptide metorphamide may be an endogenous μ -ligand (Weber et al, 1983). At present, the most selective ligands for

binding studies on brain opioid receptors are ^3H -DAGO (μ) (Handa et al, 1981; Kosterlitz & Paterson, 1981) and ^3H -DSLET (δ) (David et al, 1982). The most κ -selective ligands are U-50,488H (Gillan et al, 1983) and, oddly, a benzodiazepine, tifluadom (Romer et al, 1982), but these have not been successfully radiolabelled, and the most κ -selective radioligand appears to be ^3H -dynorphin (Young et al, 1983).

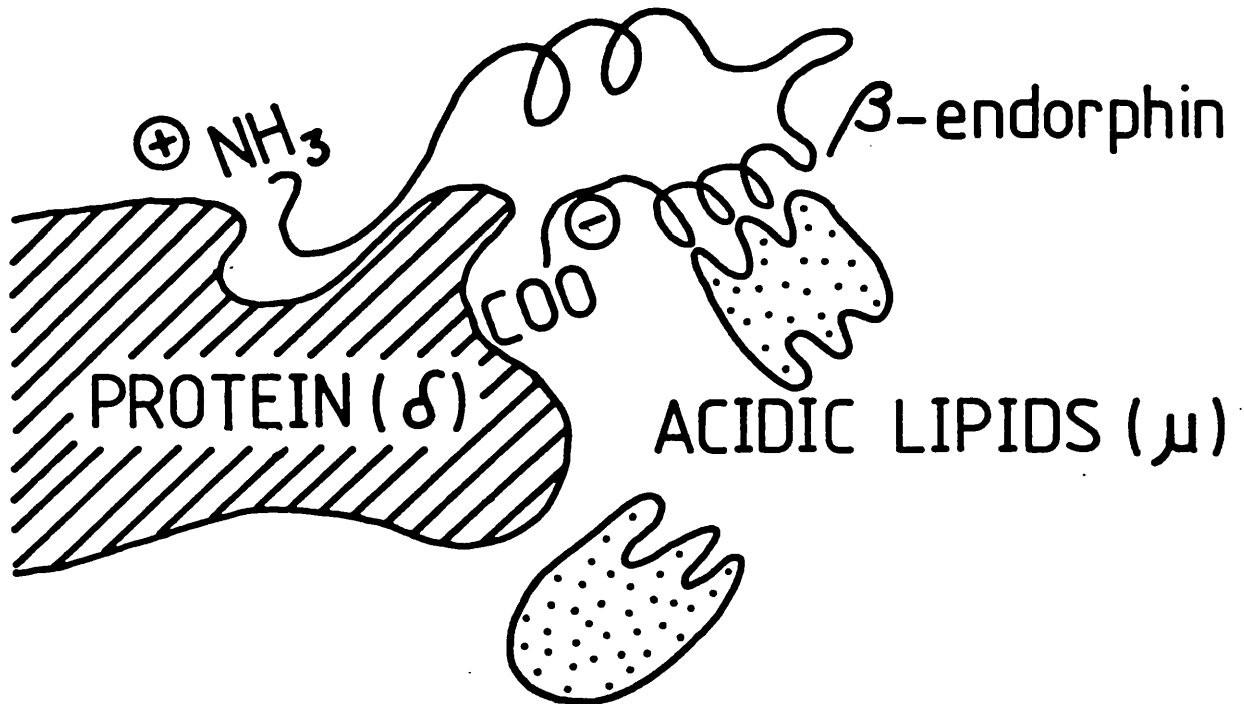
As an alternative to using brain membranes to study opioid receptor binding in vitro, it has been demonstrated that there are opioid receptors on the membranes of cultured cell lines (Klee & Nirenberg, 1974) and that these are δ -receptors (Chang et al, 1978). Studies on these cells using fluorescent enkephalin derivatives have shown that these ligands bind slowly to the receptors of neuroblastoma cells, and that there are clusters of receptors in the cell membranes which are not internalised (Hazum et al, 1979a). Sulphydryl reagents have been shown to disrupt cluster formation, but not to affect binding, implying that a sulphydryl-disulphide exchange reaction is involved in clustering (Hazum et al, 1979b; 1980). In neuroblastoma and neuroblastoma X glioma cells, opioid agonists, but not antagonists, inhibit prostaglandin E-stimulated cyclic AMP formation (Traber et al, 1975), an effect also reported in rat brain membranes (Collier & Roy, 1974). The irreversible alkylating antagonist, β -CNA, has been shown to block the opioid inhibition of cyclic AMP formation, presumably by inactivating the δ -receptors; it has also been employed to demonstrate the presence of large numbers of 'spare receptors' (Stephenson, 1956) for this effect (Fantozzi et al, 1981).

Studies of tolerance and dependence using cell lines have produced differing results: it has been suggested by Chang et al that chronic exposure to opioids leads to a decrease in the number of receptors, but that these are not internalised (Chang et al, 1982); however, Law et al suggested that internalisation is the reason for the decrease in receptor numbers, because this decrease is temperature-dependent and can be blocked by inhibitors of metabolism (Law et al, 1983a). In contrast to this, there is no detectable difference in the number of receptors in brain membranes of naive and morphine-dependent mice (Klee & Streaty, 1974; Dum et al, 1977); this may be related to a large number of spare receptors (Law et al, 1983b). It therefore seems more likely that tolerance and dependence are mediated through changes in receptor-effector coupling rather than in receptor numbers or affinity, a conclusion supported by studies on isolated GPI using β -CNA (Porreca & Burks, 1983).

The steps which follow the binding of an opioid agonist to a receptor, and lead to the diverse effects opioids bring about, are poorly characterised at present, in spite of considerable knowledge of the receptor-binding properties of many opioid ligands. As mentioned above, there is much evidence to suggest that at least one opioid receptor subtype affects adenylate cyclase activity, as do many other hormone and neurotransmitter receptors (e.g. those for adrenaline, glucagon and dopamine). GTP may also affect binding of ligands to μ -receptors, as mentioned above, as well as the coupling of ligand- δ -receptor

Fig. 14 The protein-lipid model of the opioid receptor

(from Lee & Smith, 1980).



Alkaloids bind to acidic lipids, ' μ ' site, and enkephalins to protein, ' δ ' site. β -Endorphin binds to both sites.

complex to adenylate cyclase (Cooper et al, 1982). It has also been postulated that opioid agonists may produce their effects by increasing cyclic GMP levels (Gwynn & Costa, 1982); other possible effector mechanisms include calcium mobilisation (Berridge, 1980) and/or the stimulation of phosphoinositol metabolism (Michell et al, 1981). (See also I.3.4).

3.4 Distribution and electrophysiology of opioid receptors

Opioid receptors are widespread in neural tissue but they are not uniform in their distributions as studied by autoradiography using ligands labelled to high specific activity (Pert et al, 1976, Goodman et al, 1980) and by binding assays of particular brain regions (Chang & Cuatrecasas, 1979; Cox et al, 1980). They are associated mainly with the neuroendocrine, limbic and sensory systems in the CNS. In the spinal cord, there are many small interneurons in the dorsal horn (particularly the substantia gelatinosa), known to modulate the sensory transmission of such modalities as pain (Kerr, 1975). Morphine injected into the epidural spinal spaces of humans has been shown to be potent in producing analgesia (Behar et al, 1979). Dorsal rhizotomies in monkey spinal cord show loss of opioid binding in dorsal horn (LaMotte et al, 1976). The exact type of opioid receptor involved in spinal cord-mediated antinociception is not completely clear; it is known that primary afferent fibres contain μ - and δ -receptors (Fields et al, 1980) and it has also been suggested that spinal analgesia is mediated by κ -receptors (Piercey et al, 1982b). Presynaptic

localisation of receptors suggests an effect on neurotransmitter release, and studies have produced results consistent with such a role for enkephalin reception in the spinal cord (Jessell & Iversen, 1977) and brain synaptosomes (Brennan et al, 1980).

At a subcellular level, it is clear that opioid receptors are found in both synaptic and microsomal membrane fractions (Glaser et al, 1980), and it has been suggested that the microsomal fraction opioid receptors are precursors of the receptors that later appear in the synaptic membrane fraction (Roth et al, 1981). There appears not to be an intracellular binding-site for tritiated opioid ligands (Killian et al, 1981)

Using low concentrations of tritiated and iodinated ligands relatively specific for μ -, δ - and κ -sites, it has been shown by autoradiography that the distributions of these sites are different (Goodman et al, 1980; Duggan et al, 1981; Foote & Maurer, 1982; Slater & Patel, 1983). However, Quirion et al have postulated the existence in rat striatal slices of receptors which appear to change their binding characteristics according to conditions of temperature and metal ions. Thus, at 4°C in the presence of 100mM NaCl, it is proposed that ^3H -EKC binding is solely to κ -sites, but at 25°C, it binds to μ -sites as well (Quirion et al, 1983). How these sites might behave in vivo, at 37°C, is a question still to be addressed, but it is clear there may be differential effects on μ - and κ -binding, suggesting different binding-sites. The distribution of the PCP/ σ -receptor is quite different to those of μ -, δ - and κ -receptors, again suggesting its separate identity (Quirion et al, 1981).

There are high densities of opioid receptors within the limbic system, in particular the basal ganglia and striatum; because of the involvement of these areas in motor and behavioural control, these opioid receptors have been studied in relation to such disorders as Parkinson's Disease and schizophrenia (Snyder, 1979; Mackay, 1981). The striatum receives dopamine, acetylcholine and GABA terminals, and lesions of the dopamine input from the substantia nigra have been shown to result in a loss of opioid binding-sites in the striatum. These binding-sites may represent presynaptic opioid receptors, but it appears that most opioid receptors in the striatum are post-synaptically located on striatal neurones (Murrin et al, 1980). There have been conflicting reports about the role of striatal presynaptic opioid receptors : some have reported an inhibition of dopamine release (Loh et al, 1976), while others have seen an increase in dopamine transmission (Wood & Richard, 1982). There is also evidence for the presence of μ - and δ -receptor subtypes within the striatum, the μ -receptors being clustered together, and the δ -receptors being diffusely spread (Atweh & Kuhar, 1983).

Within the neuroendocrine system, there are opioid receptors in the hypothalamus, indicating a role in regulation of hormone release from the pituitary, and indeed opioids have been shown to decrease release of luteinising hormone and thyroid-stimulating hormone, and to increase release of growth hormone and prolactin, although there are different effects in male and female animals (Herz, 1983). As mentioned previously,

opioid peptides are co-stored with such well-established hormones as vasopressin (dynorphin) and ACTH (β -endorphin) (I.2.2.). It has also been shown that in many of the brain regions where there are opioid receptors, there are also enkephalin- or endorphin containing neurons, which suggests that those endogenous opioids may act on these receptors (Wamsley, 1983).

Electrophysiological studies have examined the effects of exogenous opioids on neurons throughout the CNS (Duggan, 1983; Henderson, 1983) and periphery (North & Egan, 1983). Within the brain, neurons in the hippocampus, locus coeruleus and cortex have been particularly examined. In the case of the hippocampus, study has been made easier by the presence of large pyramidal cells suitable for single-cell recording. The actions of opioids on primary afferent fibre terminals in the spinal cord have also been examined with regard to the possible role of these fibres in conveying the sensation of pain. Peripherally, neurons in the MVD and GPI myenteric plexus have also been studied, because of the known presence of opioid receptors in these tissues. In almost all tissues studied, individual neurons were inhibited in response to opioids. For example, single locus coeruleus neurons were hyperpolarised by normorphine and enkephalins, but not dynorphin; the use of selective antagonists now available showed that the receptors involved are μ -opioid receptors (North & Williams, 1983a). Similarly, single neurons in the substantia gelatinosa were hyperpolarised by exogenous enkephalin (Yoshimura & North, 1983). Not all recorded responses to opioids have been

inhibitory; it has been shown in hippocampus (Duggan, 1983) that opioids produce an excitation of the pyramidal cells, and in cerebral cortex there is a similar naloxone-reversible effect (Stone, 1983). However, it is likely that these excitatory effects are due to disinhibition, i.e. inhibition of inhibitory interneurons (Duggan, 1983).

So far, evidence has not pointed to the existence of multiple subtypes of opioid receptor on one cell, although there has been one report of the co-existence of μ - and δ -receptors on a single neuron in GPI myenteric plexus (Egan & North, 1981). However, the evidence is somewhat equivocal, and there is no pharmacological evidence for the coexistence of these two subtypes in GPI myenteric plexus where only the μ - and κ -subtypes are identifiable. δ -Receptors have however been identified in binding-studies on homogenates of GPI myenteric plexus (Leslie *et al*, 1980), suggesting μ - and δ -receptors more likely exist on different populations of neurons in this tissue.

The basis of this inhibitory action on neuron firing might lie in the hyperpolarisation of cell membranes seen, which can inhibit the initiation and/or propagation of action potentials, and also lead to a decreased release of neurotransmitter. Examination of the mechanism of this hyperpolarisation in single CNS and peripheral neurons has led to the conclusion that opioid agonists specifically increase potassium conductance by opening membrane potassium channels, thus producing the membrane

hyperpolarisation seen which constitutes their inhibitory action on cells (North & Williams, 1983b).

As well as μ - and δ -agonists having this effect of increasing potassium conductance, it has been reported that κ -agonists such as dynorphin may decrease calcium entry into dorsal root ganglion cells, thus producing a decrease of calcium-dependent action potential duration (Werz & Macdonald, 1983).

However, although responses to exogenous opioids are relatively clear and consistent, identical synaptic potentials and susceptibility to pharmacological antagonists must be shown for endogenous opioid peptides before it can be accepted that they are indeed the neurotransmitters at the synapses studied.

CHAPTER II

GENERAL METHODS

II GENERAL METHODS

1. Reagents

All reagents and solvents used for synthesis of ligands for photoaffinity and affinity chromatographic purposes were of analytical grade, except boron tribromide (standard laboratory reagent grade), see § III.3.2. Reagents for buffer solutions used in the preparation of brain membranes for opioid binding assays, and in the assays themselves were of analytical grade, as were those used in solubilisation of binding activity from membranes, and in bioassays. Water was always distilled or deionised.

2. Brain membrane preparation

Buffer A : 10mM Tes-KOH pH 7.4, 1mM sodium edetate, 1mM benzamidine hydrochloride, 1mM dithiothreitol, 0.01% bacitracin, 0.002% soybean trypsin inhibitor, 0.1mM phenylmethylsulphonyl fluoride.

Buffer B : buffer A with the addition of 0.3M sucrose.

5 male Sprague-Dawley rats (150-200g) were stunned by a blow to the head and killed by cervical dislocation. The brains, minus cerebella, were excised into 40ml ice-cold buffer B for rinsing to remove blood, then, in 40ml fresh buffer, were chopped coarsely with scissors prior to homogenisation using a Polytron tissue homogeniser : 2 x 15 seconds at speed 3 followed by

1 x 15 seconds at speed 6. The resulting suspension was centrifuged at 2000 x g for 10 minutes. The supernatant was retained on ice and the pellet rehomogenised at speed 6 for 15 seconds in 40ml ice-cold buffer B, and centrifuged as before. The supernatants were combined and centrifuged at 45,000 x g for 30 minutes. The resulting supernatants were discarded and the pellets resuspended in 40ml ice-cold buffer A, and the suspension stirred on ice for 30 minutes before centrifugation as before. The pellets were resuspended in 10mM Tes-KOH pH 7.4, recentrifuged and finally resuspended in 15-20ml 10mM Tes-KOH pH 7.4, divided into 1ml aliquots in plastic 1ml centrifuge tubes. These aliquots were frozen in liquid nitrogen and stored at -20°C until use.

3. Membrane binding assay

The binding assay used tritiated ligands (Amersham) : for μ -sites, $^3\text{H-D-Ala}^2\text{-MePhe}^4\text{-Gly(ol)}$ enkephalin ($^3\text{H-DAGO}$) or $^3\text{H-dihydromorphine}$ ($^3\text{H-DHM}$); for δ -sites, $^3\text{H-D-Ala}^2\text{-D-Leu}^5\text{-enkephalin}$ ($^3\text{H-DADL}$) in the presence of a 25-fold excess of DAGO or DHM (unlabelled); for κ -sites, $^3\text{H-etorphine}$ ($^3\text{H-Etn}$) in the presence of 25-fold excesses of unlabelled DAGO or DHM and DADL. Non-specific binding was measured in the presence of a 250-fold excess of unlabelled ligand.

The buffer used for binding assays was 10mM Tes-KOH pH 7.4 with 100mM KCl and 2mM MgCl_2 .

Briefly, tritiated and unlabelled ligands, buffer and membranes were mixed together in a volume of 1ml at a concentration of 100-150µg/ml protein. The resulting suspension was incubated at 30°C for 40 minutes in a shaking water bath, to allow binding of ligands to reach equilibrium. The assay was always performed in triplicate. 900µl of the incubated suspension was then rapidly filtered through Whatman GF-C glass fibre filters under reduced pressure, and the membranes retained on the filters were washed twice with 5ml ice-cold 10mM Tris-HCl pH 7.4. The filters were dried and the radioactivity retained counted in 4ml scintillant by a Kontron-Intertechnique scintillation counter at an efficiency of 35-37%. The scintillant used was 10% Soluene, 0.7% 2,5 diphenyloxazole and 0.06% 1,4-bis-[2-(4-methyl-5-phenyloxazole)] benzene in toluene. Samples were usually counted twice for 4 minutes.

4. Isolated tissue assays

1. Mouse vas deferens ($\mu + \delta + \kappa$ receptors)

Male mice (MF1/OLA, over 30g) were stunned by a blow to the head and killed by cervical dislocation. The vasa deferentia were removed and placed in modified, oxygenated krebs solution (magnesium-free). This was 111mM sodium chloride, 11mM glucose, 25mM sodium hydrogen carbonate, 2.7mM calcium chloride, 4.7mM potassium chloride, 1.2mM potassium dihydrogenphosphate, pH 7.4.

The blood vessel running the length of the vas was stripped off along with connective tissue remaining. Vasa were mounted in 50ml organ baths as shown in Fig. 15. The organ bath was positioned in a larger water bath whose temperature was maintained at 30°C. A glass sinter provided a steady supply of 95% O₂ : 5% CO₂ bubbles. Two platinum electrodes connected to a Grass S88 dual channel stimulator provided stimulation at 0.1 Hz for 3msec of 80-120V. This gave slightly submaximal twitches. Using this apparatus, stimulation at 100V gave a current of 290mA.

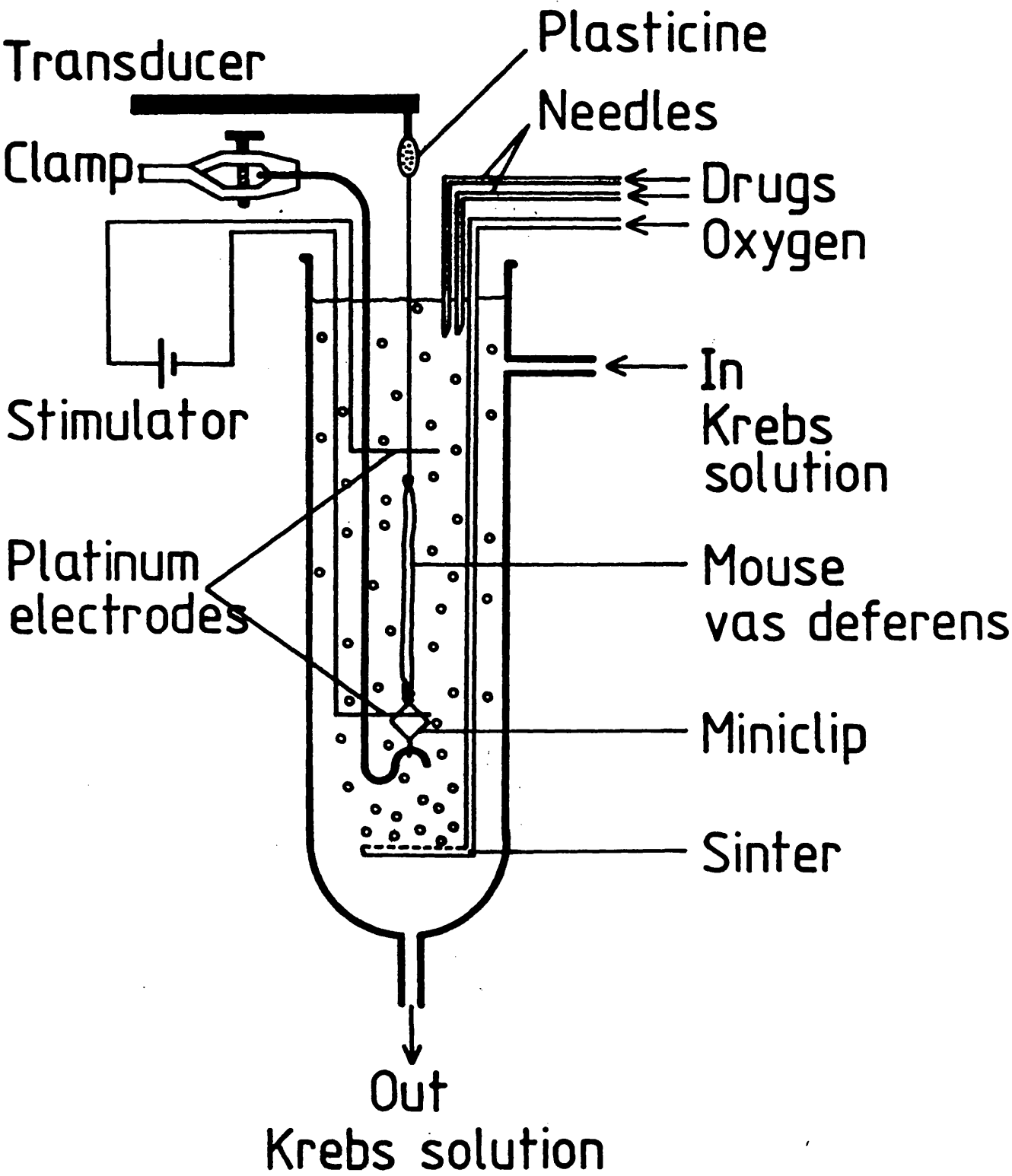
Twitches were recorded isotonicallly through a Statham Goldcell UC-2 transducer on a standard Smiths Servoscribe flat-bed recorder. Twitches were calibrated, and those vasa giving less than 100mg twitches after about 30 minutes equilibration in the bath were discarded.

2. Guinea-pig ileum (μ + κ receptors)

Guinea-pigs (Duncan-Hartley, 300-400g) were killed by a blow to the head. The abdomen was opened and the ileum removed and placed in warm oxygenated modified Krebs solution (as above, with the addition of 1.95mM magnesium sulphate). Undigested food was flushed from the ileum and 2-3cm sections were cut from it; sections with Peyer's Patches lymphoid tissue were discarded. The sections were then everted onto a glass rod and the surface mucosa stripped off gently with a Kleenex tissue. The sections were reverted and set up in 50ml organ baths for

Fig. 15 Mouse vas deferens bioassay

Method described in Ch. II.4.1



transmural stimulation via platinum electrodes (as above, but requiring a lower voltage, 15-40V, to give just submaximal twitches). For stimulation at 40V, the measured current was 100inA.

3. Mouse ileum (δ + k receptors)

2cm sections of mouse ileum were set up as the GPI, with the difference that the mucosa were not stripped. Stimulation parameters were different because of the strong spontaneous contractions. Their rate was monitored, and stimulation consisting of twin 1 msec pulses 0.1 msec apart were applied at the same frequency, at a supramaximal voltage (40-80V). It had been shown that this tissue responded only to opioid δ - and k-agonists, apparently having no μ -receptors (C.F.C. Smith, unpublished observations).

The three tissues above were then used to determine the receptor profiles of various ligands. The standard agonists used were normorphine (μ), met⁵-enkephalin and D-Ala²-D-Leu⁵-enkephalin (δ) and ethylketocydazocine (k).

Before any twitch measurements were made, all tissues were allowed to equilibrate in the bath for about half an hour with washes at 5 minute intervals.

PET Commodore bench-top computers were used in the construction of dose-response curves. The flat-bed chart recorders were interfaced with the computers in such a way that the computer

was able to monitor and record twitch heights and so was able to deliver doses of standard drugs automatically when steady states had been reached. Generally, a dose would be delivered when the twitch heights were within 3% of each other for 4 or 5 twitches; doses were in the ratio 1 : 3 : 9 : 27 to give a cumulative dose-response curve. Once the twitch heights were depressed more than 50% and had 'plateaued', the bath would automatically empty and refill. The next dose-response curve would not be commenced unless the steady state twitch height was at least 95% of the previous dose-response curve's twitch height.

After two dose-response curves to any one agonist had been constructed which had ED_{40}^* values differing less than 25%, the computer sounded an alarm requesting delivery of a dose of test agonist. The test drug dose-response curve would then be constructed manually, and an ED_{40} value obtained if possible. This applied to agonists and partial agonists.

For antagonists, a different computer programme was used which did not demand doses to produce a dose-response curve, but requested the antagonist to be infused in Krebs solution. Meanwhile, the computer constructed dose-response curves to standard agonists continually until ED_{40} values differed less than 25% to one agonist; an increased dose of test antagonist could then be infused in the Krebs solution.

* (ED_{40} is 40% depression of twitch height, not 40% of the maximum effect)

5. Solubilisation of brain membrane-bound opioid
 receptors

Rat brain membranes were prepared as described above. Membranes were resuspended into a 10-fold volume buffer C, containing 10mM Tes-KOH pH 7.4, 50mM MgSO_4 , 1mM EGTA, 0.1mM phenylmethylsulphonylfluoride, 0.02% bacitracin, 0.002% soybean trypsin inhibitor, 1mM benzamidine hydrochloride, and centrifuged at 45,000 x g for 30 minutes. The pellet was resuspended into the same buffer to give a protein concentration of about 10mg/ml. Digitonin solution was added, to a final concentration of 1%. The mixture was shaken for 1 hour at 10° - 15°C prior to centrifugation at 100,000 x g for 1 hour. The yellowish supernatant was stored at -20°C in 500ul aliquots. Total protein solubilised was about 40-50%.

6. Assay of soluble receptor binding

(C. Demoliou-Mason, personal communication)

The same tritiated ligands were used as in the membrane assay. The buffer used was slightly different, being 50mM KCl, 50mM NaCl, 10mM MgCl_2 , 1mM EGTA, 0.1mM phenylmethylsulphonylfluoride, 0.02% bacitracin, 0.002% soybean trypsin inhibitor, 1mM benzamidine hydrochloride, 10mM Tes-KOH, pH 7.4. The assay volume was 500µl, and the incubation started after the addition of 50µl solubilised receptor (to give an assay concentration of 0.1% digitonin). The incubation was at 30°C for 30 minutes. Tubes were then removed onto ice and allowed to stand for 10 minutes before addition of 50µl 0.6% gammaglobulin solution and

200µl 36% polyethylene glycol solution, to precipitate soluble protein. The samples were vortexed and incubated on ice for 30 minutes before 2 x 300µl aliquots were filtered through Whatman GF-C glass fibre filters, washed twice with 10% PEG, 0.01% digitonin, 5mM Tes-KOH pH 7.4. The filters were dried and the radioactivity retained counted as above.

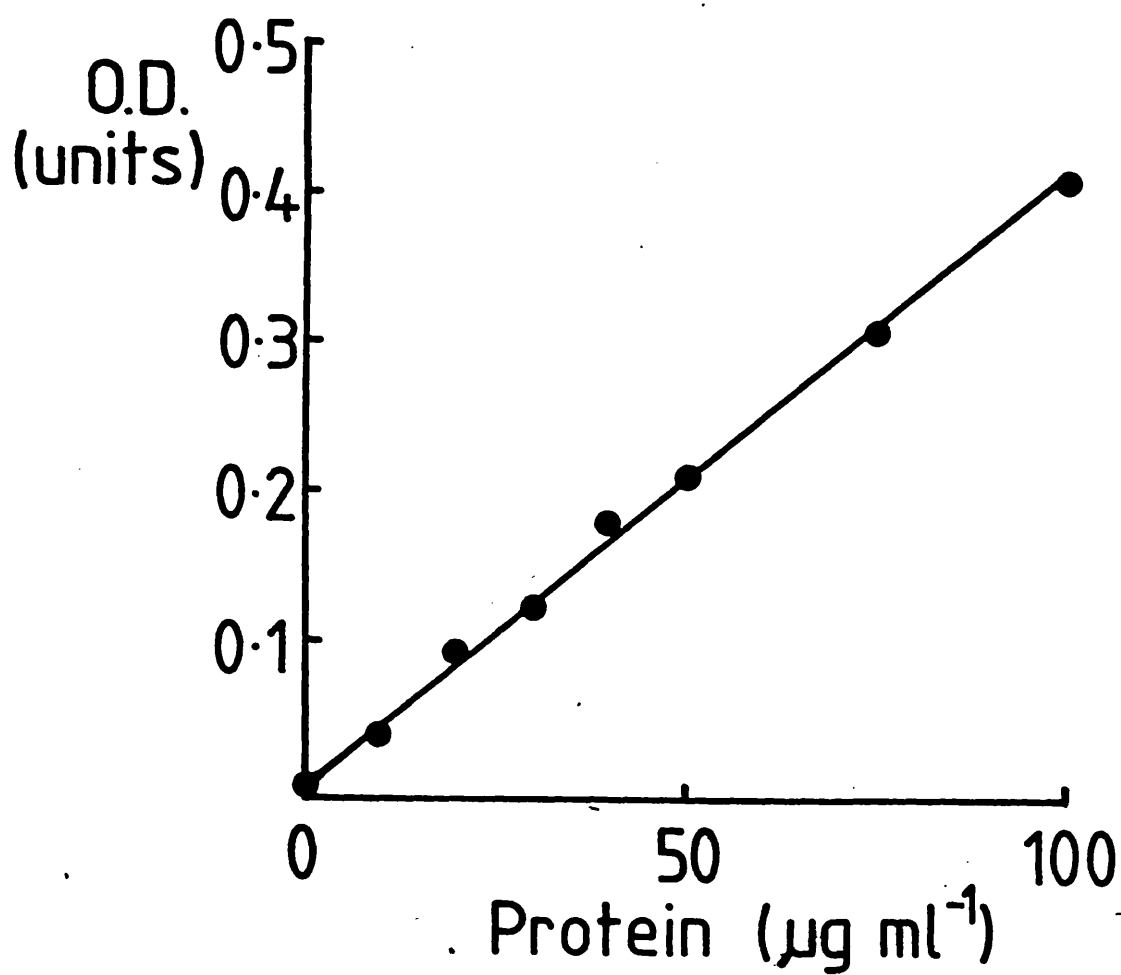
7. Protein Assay

The protein assay used was based on a modification of the procedure devised by Lowry (Markwell et al, 1978). Briefly, a solubilising mixture was used to break down proteins into amino acids. This mixture was 2% sodium carbonate, 0.4% sodium hydroxide and 1% sodium dodecyl sulphate. This solution was also used to make up standard protein (bovine serum albumin) concentrations from 0 to 100 µg/ml. Membrane suspensions were diluted 1 : 50, 1 : 100, 1 : 200 and 1 : 400.

Reagent A : 2% sodium carbonate, 0.4% sodium hydroxide, 0.16% sodium tartrate and 1% sodium dodecyl sulphate.

Reagent B : 4% copper sulphate.

A and B were mixed in the ratio 100 : 1 to give reagent C. 800 µl 'C' was added to 200µl standard or test protein solution and allowed to stand for about 20 minutes at room temperature. 60µl Folin-Ciocalteu reagent was then added, and the mixture vortexed and incubated at room temperature for 45 minutes. The optical absorbance at 750nm was read on a Cecil spectrophotometer. This procedure was a reproducible and reliable method to measure protein (Fig.16).

Fig. 16Calibration curve for protein assay(modified from Markwell et al, 1978)

CHAPTER III

APPROACHES TO AFFINITY LABELLING OF OPIOID RECEPTORS

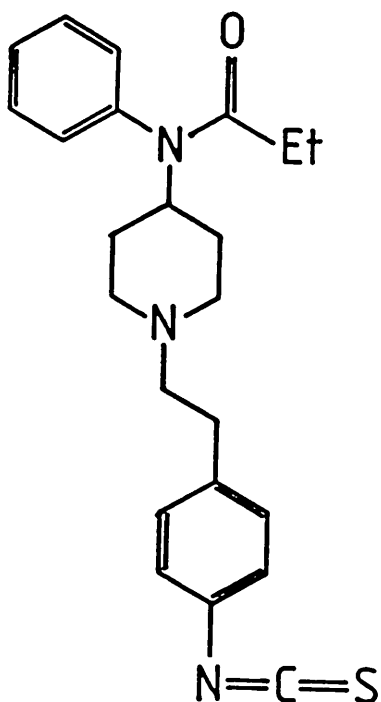
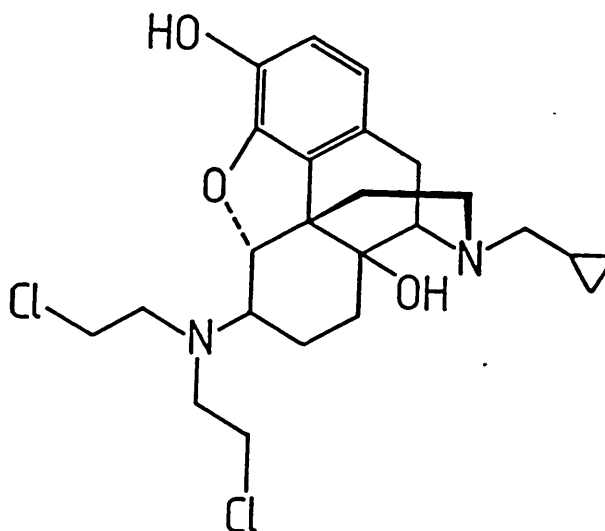
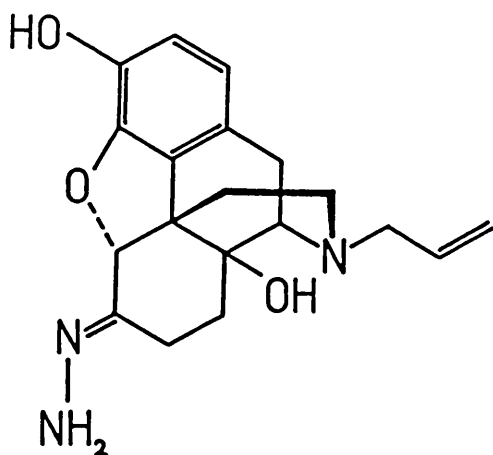
III.1 AFFINITY LIGANDS FOR OPIOID RECEPTORS

Introduction

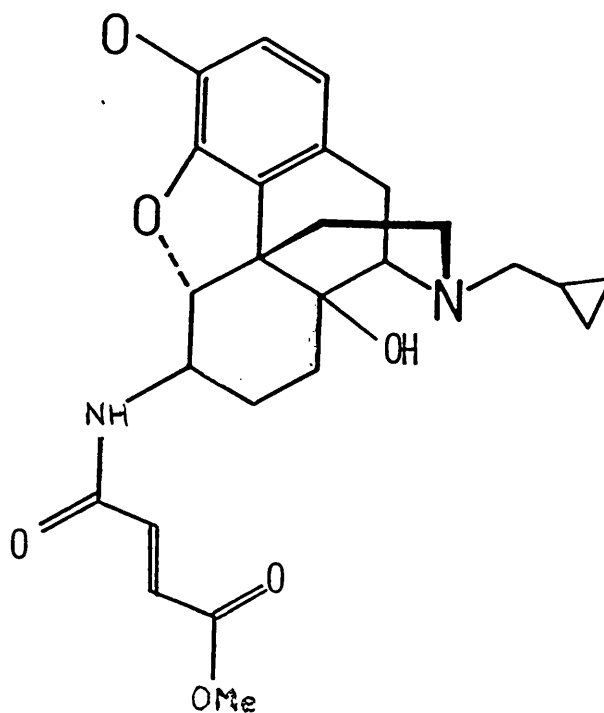
As indicated in chapter I, the study of hormone and neurotransmitter receptors requires their characterisation, including isolation, and determination of physical and chemical properties such as molecular weight, number of subunits etc. Some properties can be established by examination of the receptor in situ, where such treatments as trypsinisation or heat may reveal the involvement of protein, and the use of lectins may reveal the presence of sugar moieties, in the receptor studied. Most hormone and neurotransmitter receptors are intrinsic membrane proteins, that is they are embedded in the plasma membranes of cells; treatment with detergents is often necessary to remove them from the membrane, other methods such as treatment with high salt or sonication often proving inadequate. In the case of opioid receptors, sonication has been reported to solubilise the receptor (Cho et al, 1981), although it is possible that this procedure merely produced very small receptor-containing membrane fragments. For other receptors, solubilisation has proved possible and the solubilised receptor may retain many of the properties it possessed in the membrane. Examples include the nicotinic acetylcholine receptor (Changeux et al, 1971) and the GABA-benzodiazepine receptor (Stephenson & Olsen, 1982). It has proved more difficult to solubilise opioid receptors because they appear to be highly sensitive even to low concentrations of detergents.

Other approaches to characterising receptors have included affinity labelling, i.e. the covalent attachment of a radioactive ligand to its receptor, followed by solubilisation of the complex, and examination of the properties of the radiolabelled species. Clearly, in this case, full activity of solubilised receptor is not required because the ligand is covalently attached. The non-specific binding must be kept to a minimum to enable identification of specifically labelled receptor rather than non-specifically labelled molecules. The specificity of labelling can be tested according to certain criteria, such as prevention of labelling in the presence of known specific ligands. The affinity label used must have a high affinity for its receptor, but not so high as to make the removal of non-covalently bound ligand difficult (Knowles, 1972). Affinity labelling of opioid receptors has been described using various alkaloid and enkephalin derivatives (Fig. 17.) Fentanyl isothiocyanate is one such ligand, which apparently selectively labels the δ -receptor binding-site and suggests this may be a subunit of 58,000 daltons (Klee et al, 1982). However, affinity labelling has disadvantages; if the ligand to be used is not reactive enough, or is too reactive, leading to high non-specific labelling. The technique of photoaffinity labelling was designed to overcome such difficulties. Briefly, a derivative of a biologically active molecule is synthesized which contains a chemical entity stable in the dark, but which on irradiation with ultraviolet light is transformed to a highly reactive species. This species can then link the molecule to the binding site. This method has

Fig. 17

Affinity ligands for opioid receptorsa. Fentanyl isothiocyanate
(FIT)b. β -Chlornaltrexamine
(β -CNA)c. D-Ala²-Leu⁵-enkephalinchloromethylketone (DALECK)

d. Naloxazone/naloxonazine

e. β -funaltrexamine
(β -FNA)

theoretical advantages over conventional affinity labelling:-

- a. the ligand-binding-site interaction can be studied in the dark, in the absence of activation;
- b. the ligand is not activated until it is bound to the binding site;
- c. the photogenerated species is highly reactive.

Points (b) and (c) imply an increased specificity of covalent labelling.

The concept of photoaffinity labelling was first proposed by Westheimer using ^{14}C -p-nitrophenyldiazoacetate to label the enzyme chymotrypsin. The ligand and enzyme were allowed to equilibrate together in the dark, and the complex then photolysed. After this, the radiolabelled aminoacids were to be identified following hydrolysis (Singh et al, 1962). The experiment failed, but the principles of photoaffinity labelling had been established. Later, the technique was used to label the binding-site of antibodies, using a nitrene derived from a phenylazide precursor (Fleet et al, 1969). Aryl azides fulfil the criteria for successful photoaffinity labels:-

- (a) the precursor (arylazide) is relatively chemically inert;
- (b) the reactive species (nitrene) does not rearrange to a less reactive one (this does not apply to aliphatic azides);
- (c) the reactive species has a short half-life (phenylnitrene $< 10^{-4}$ sec.) to minimise non-specific labelling caused by diffusion away from the binding-site;

(d) generation of the reactive species does not lead to dissociation from the binding-site provided the azide group is not placed at a part of the original ligand critical for binding activity.

Some of the reactions nitrenes undergo are shown in Fig. 18. It is an advantage that they can insert into the ubiquitous carbon-hydrogen bonds.

Examples of hormone receptors successfully labelled using aryl azides include ACTH receptors (Ramachandran et al, 1980), insulin receptors (Jacobs et al, 1979) and glucagon receptors (Demoliou, 1980). A neurotransmitter receptor so labelled is the GABA-benzodiazepine receptor in brain membranes (Chang & Barnard, 1982).

In the case of opioid receptors, the first report of attempted photoaffinity labelling used the derivative of levorphanol shown in Fig. 19a, but the experiment was unsuccessful (Winter & Goldstein, 1972). This was largely because true stereospecific opioid receptor binding activity had not yet been demonstrated. This ligand also appeared to give very high non-specific labelling since incorporation of radio-activity was not significantly differently affected by the presence of levorphanol or dextrorphan. In part this may have been because of the need to use a high concentration of ligand ($7.7\mu\text{M}$) in order to incorporate enough radioactivity to count. This concentration was probably of the order of a thousand

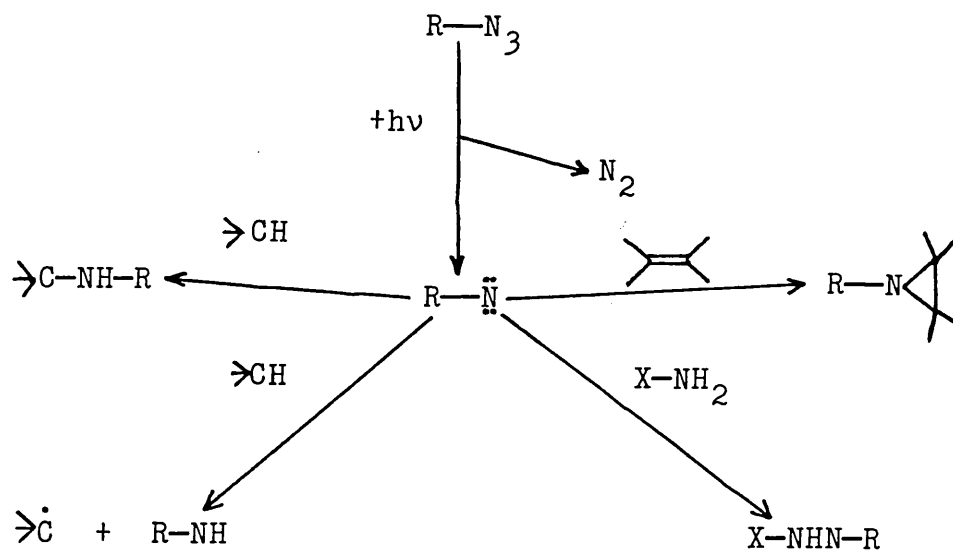
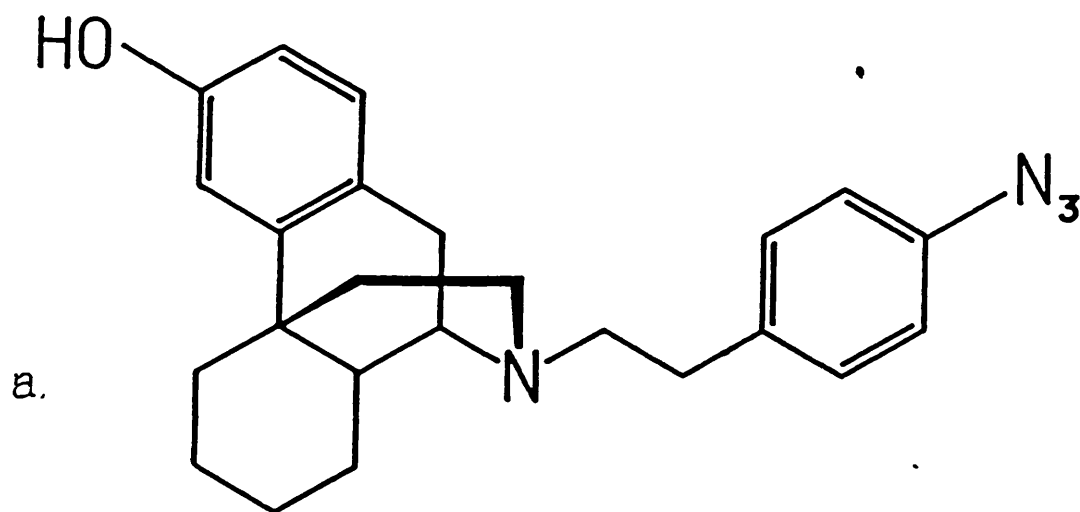
Fig. 18Reactions of nitrenes

Fig. 19 Photoaffinity ligands for opioid receptors



- a. $^3\text{H-N-}\beta\text{-(p-azidophenyl)ethylnorlevorphanol}$ (Winter & Goldstein, 1972)
- b. $\text{D-Ala}^2\text{-Met}^5\text{-(o-nitro, p-azidophenyl)-Tyr}^6\text{-enkephalin}$ (Lee et al, 1979)
- c. $\text{D-Ala}^2\text{-Phe}^4\text{-(p-azidophenyl)-n-Propylamide}$ (Smolarsky & Koshland, 1980)
- d. $\text{D-Ala}^2\text{-Leu}^5\text{-(o-nitro, p-azidophenyl)-N}^\epsilon\text{-Lys-enkephalin}$
(Hazum et al, 1979c)
- e. $\text{D-Ala}^2\text{-Leu}^5\text{-(o-nitro, p-azidophenyl)-diglycylamide-enkephalin}$
(Zioudrou et al, 1982)
- f. $\text{D-Ala}^2\text{-(p-azido-Phe}^4\text{)-Gly(ol)-enkephalin}$
(Garbay-Jaureguiberry et al, 1983)

times the K_d of the ligand. In an attempt to reduce non-specific labelling by decreasing lipophilicity, a quaternary analogue of the ligand was synthesized, but this was no more successful (Schulz & Goldstein, 1975).

Later, azide derivatives of enkephalins were used as photoaffinity probes (Fig 19b-f); these offer the advantage over alkaloids of being less hydrophobic, but their more flexible conformation means it can be more difficult to achieve specific receptor labelling. So far, it has been difficult to demonstrate a significant difference between opioid receptors irreversibly blocked by photoaffinity labelling, and those inactivated by ultraviolet light damage (e.g. Smolarsky & Koshland, 1980). There has not yet been synthesized an enkephalin derivative specific for the δ -subtype of opioid receptors, although there has been a report of a μ -selective enkephalin photoaffinity ligand based on DAGO (Garbay-Jaureguiberry *et al*, 1983; Fig 19g).

It was known that arylazide derivatives of $14\text{-}\beta$ -aminomorphinone had high μ -receptor binding activity and could inhibit electrically evoked contractions of MVD and GPI; such compounds appeared to be potential photoaffinity ligands for opioid receptors (Rance *et al*, 1981). However, since more of these ligands needed to be synthesized for further study, because of decomposition, it was decided to modify the molecule described by Rance *et al*, and to continue using the basic $14\text{-}\beta$ -aminomorphinone structure rather than use an enkephalin analogue

because more was known about the structure-activity relationships in this series than about enkephalins (data on file, Reckitt & Colman).

The ligand synthesized is shown in Fig.20a, and the modifications were:

a. An o-nitro group was introduced into the arylazide ring, for two reasons, firstly to increase the wavelength of the absorption maximum of the compound (Knowles, 1972), and therefore presumably decrease photodestruction of opioid receptors, and secondly to increase the reactivity of the resulting nitrene (Chowdry & Westheimer, 1979). The latter means there is less likelihood of diffusion of the nitrene away from its binding site and hence, theoretically, an increase in specific compared to non-specific labelling.

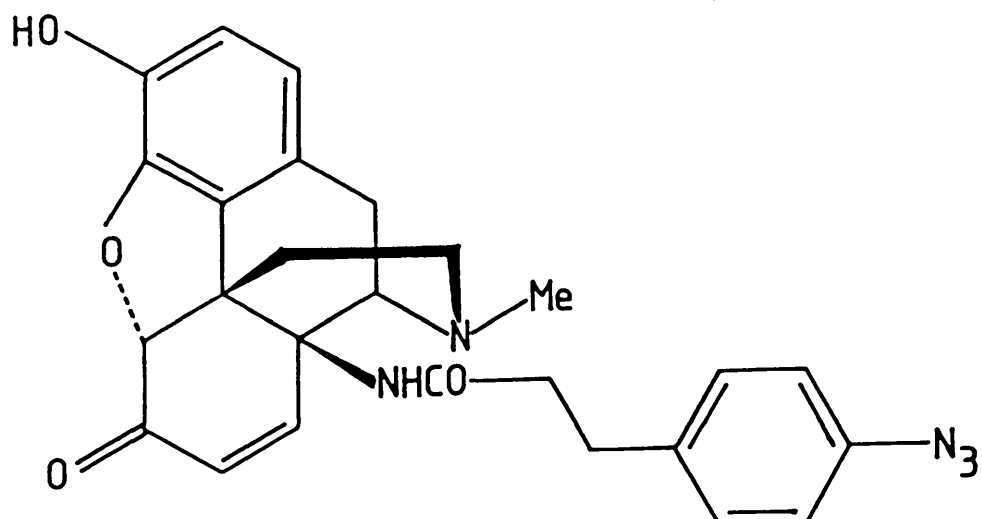
b. Alteration of the N-methyl group to an N-cyclo-propylmethyl group. This has the effect of changing the profile of the molecule from agonist to antagonist, and increasing its affinity, all other parts of the molecule remaining the same (Kosterlitz & Waterfield, 1975). An antagonist photoaffinity label has the advantage in isolated tissues of leaving the tissue intact to respond to agonists acting on other receptors in the system.

c. A double bond was incorporated in the 14- β - side chain so that radiolabelling with tritium gas could be carried out, should studies with unlabelled compound prove promising.

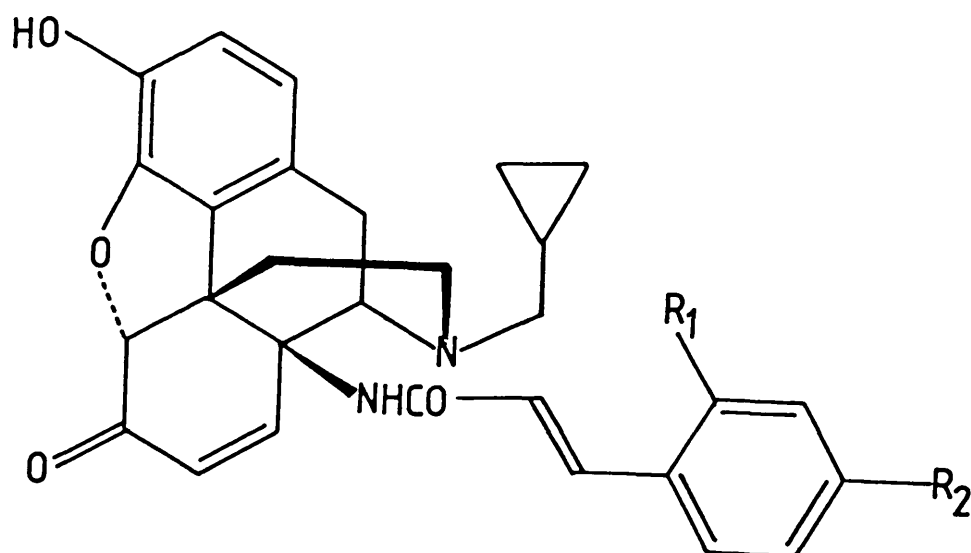
Fig. 20

Potential photoaffinity ligands for opioid receptors

a.



b.



	R ₁	R ₂
<u>NUM</u>	H	H
<u>NOM</u>	NO ₂	H
<u>NAM</u>	NO ₂	N ₃

- a. 14-β-(p-azido)-dihydrocinnamoylamidomorphinone (Rance et al, 1981).
- b. 14-β-(o-nitro, p-azido)-cinnamoylamido-(N-cyclopropylmethyl)-nor-morphinone, NAM, and the related compounds NUM and NOM.

The compound described was synthesized [14- β -amido (o-nitro, p-azido) cinnamoyl, N-cyclopropylmethylnormorphinone, 'NAM'] (Fig. 20b), along with two related compounds lacking respectively, the p-azido and both the p-azido and o-nitro moieties [14- β -amido (o-nitro cinnamoyl), N-cyclopropylmethyl-normorphinone 'NOM' and 14- β -amidocinnamoyl N-cyclopropylmethyl-normorphinone 'NUM']]. These last two were synthesized to show that the introduction of an o-nitro group into the 14-cinnamoyl sidechain did not change the receptor profile or affinity of the compound. The activity of the compounds synthesized was studied first in isolated tissues before brain membranes, so that their activity on physiologically relevant opioid receptors could be demonstrated before photoaffinity labelling was attempted.

NAM and NOM were both novel compounds, and while NUM had been synthesized before, there was not much information about its action in isolated tissues or in brain membranes.

The behaviour of daleck, a potential chemical affinity ligand for opioid receptors (Fig. 17) was also studied in isolated tissues. Daleck had been shown to be an agonist in isolated tissues (Venn & Barnard, 1981); its receptor selectivity had not however been established and neither had the question of its ability to activate irreversibly opioid receptors in isolated tissues been investigated.

III.2 METHODS

1. Synthesis of NUM, NOM and NAM

All reactions were followed by t.l.c. on silica or alumina plates run in suitable solvent systems; the IR and NMR spectra of products were taken at each stage, and sometimes also the mass spectrum, elemental analysis and melting points were determined.

All reagents and solvents used were of analytical grade.

Often, reactions were carried out up to four times; yields quoted are the average values obtained. Small-scale 'pilot' reactions were carried out before full-scale reactions were attempted, to try to ensure optimal conditions for the full-scale reaction.

All reactions involving an azide derivative were carried out in foil-covered flasks, to prevent photolysis.

The scheme for NAM, NOM and NUM syntheses is shown in Figs. 21-24. In short, the protected morphinone was synthesized, and the three cinnamoyl derivatives were coupled to the morphinone in separate reactions to give the three products NAM, NOM and NUM.

1.1 Synthesis of o-nitro cinnamoyl chloride (Fig. 21)

o-Nitrocinnamic acid (386mg) was suspended in dichloromethane (10ml) in a round-bottomed flask with a drying tube and stirred at room temperature. Oxalyl chloride (10ml) was added, followed by DMF (3 drops) as catalyst. Effervescence evolved CO and HCl gases. The mixture was stirred for 30 mins to give a pale yellow solution of o-nitrocinnamoyl chloride. Excess oxalyl chloride and dichloromethane were removed under reduced pressure by rotary evaporator. The product was used immediately in the coupling reaction with protected morphinone, and consequently was not analysed.

2. Synthesis of o-nitro, p-azido cinnamoyl chloride (Fig. 22)

2.1 Nitration of p-bromoaniline

p-Bromoaniline (20g) was dissolved in CH_2SO_4 (90ml) to give a dark brown solution. A nitration mixture of CH_2SO_4 (15ml) and CHNO_3 (15ml) was added dropwise while stirring, at 0°C . The temperature was not allowed to rise above 15°C , and, after the nitration mixture was added, maintained at 5°C . Reaction, followed by t.l.c. was judged complete after 1 hour. The mixture was poured into water (1 litre) and basified using 0.88 ammonia solution, bringing down a precipitate of product, which was filtered off. The product was dissolved in DCM, dried over $\text{Na}_2\text{SO}_4(\text{s})$ and evaporated

under vacuum to give a brown solid (19g).

This solid was adsorbed onto alumina beads and extracted with hexane in a Soxhelt extractor to give 11.7g reddish solid.

This solid was adsorbed onto extra-fine silica beads and eluted from a 50cm x 4cm silica column with MAD. Yield of pure product, bright yellow needle-like crystals (m.p. 134-137°C) was 32% (7.9g).

2.2 Substitution of bromine by methylcinnamate

o-Nitro, p-bromoaniline (21.5g), methyl acrylate (7.4ml), Et₃N (29ml), palladium acetate (190mg) and triphenyl-phosphine (430mg) were combined in a glass tube which was sealed and incubated inside a steel jacket at 100°C for 24 hours.

The tube was allowed to cool, and broken open. The red oily liquid was poured into water (700ml) and extracted with DCM to give a viscous red liquid (c. 30g). T.l.c. showed this consisted of about 5 products, including the desired one. It was adsorbed onto silica gel (70-230 mesh) and the products eluted from a 25cm x 4cm silica column using DCM. Combined fractions containing desired product gave 2-nitro, 4-amino methylcinnamate, orange crystals (3.7g) in a yield of 16%. Other products isolated, some in greater yield than the desired one, included one which showed that 2 mole equivalents of ester had substituted onto the phenyl ring, thus reducing availability of ester to other molecules.

The ester was hydrolysed to the free acid by stirring overnight at room temperature in a mixture of KOH pellets (10g) in methanol (110ml) and water (10ml). The mixture was then poured into water (400ml), acidified with 2M HCl to give an orange precipitate. This was filtered off, and dried in a vacuum oven, to give orange crystals in a yield of 64% (2.4g)(2-nitro, 4-amino cinnamic acid).

2.3 Substitution of 4-amino by azido in 2-nitro, 4-amino cinnamic acid

2-Nitro, 4-amino cinnamic acid (2.2g) was added to CHCl_3 (80ml) to give an orange suspension to which was added water (50ml) to dissolve the acid. The mixture was cooled to 0°C . Sodium nitrite (625mg) was dissolved in distilled water (4ml) and added dropwise, keeping the temperature below 0°C , and stirred for 1 hour to give a diazonium salt. The reaction flask was covered in foil, and sodium azide (595mg), dissolved in water (3ml) was added dropwise, producing effervescence and a flocculent precipitate, the product, which was filtered off and dried in a vacuum oven to give an orange-brown solid in 18% yield (430mg). The low yield was proposed to be due to decomposition of the diazonium salt to phenols, prior to addition of sodium azide.

2.4 Preparation of o-nitro, p-azido cinnamoyl chloride

o-Nitro, p-azido cinnamic acid (420mg) was dissolved in DCM (10 ml). Oxalyl chloride (10ml) was added, with DMF (3 drops) as catalyst. After 30 mins, the reaction mixture was evaporated under reduced pressure; re-dissolved in DCM and again evaporated. The resulting acid chloride was used immediately in coupling to the protected morphinone (4.5).

3. Preparation of protected 14- β -amino (N-cyclopropylmethyl) normorphinone (Fig. 23)

3.1 Preparation of 14- β -formylamido (N-cyclopropylmethyl) norcodeinone

14- β -amino-(N-cpM) norcodeinone dimethyl ketal (37g) was placed in a round-bottomed flask in a heating mantle with a water condenser above; 98% formic acid (45ml) was added and the reaction maintained at 60°C for 2 hours. The colour of the solution changed from pale yellow to brown on formation of product, as determined by t.l.c. It was then poured into water (300ml), basified with 2M NaOH to give a chalky cream precipitate which was filtered off, washed, and dried in a vacuum oven to give a 95% yield of 14- β -formyl-amido-(N-cpM) norcodeinone (33g).

3.2 Dealkylation of 14- β -formylamido (N-cyclopropylmethyl)
norcodeinone to corresponding morphinone

This reaction was carried out on small quantities, so that it was easier to control the reaction temperature.

The codeinone (e.g. 7.8g) was dissolved in DCM (200ml) to give a pale yellow solution. After cooling to -70°C , boron tribromide (20ml) was added dropwise, keeping the temperature at -70°C . The boron tribromide successfully used was brownish in colour, i.e. it contained HBr as an impurity. However, it seems that HBr may act as a catalyst for the dealkylation reaction (A.S. Haynes and E.M. Peers, unpublished observations).

The reaction temperature was raised to -20°C for 2 hours, after which time, t.l.c. showed disappearance of starting material, and the mixture was recooled to -70°C . Methanol (200ml) was added very slowly to hydrolyse excess boron tribromide, evolving profuse fumes of HBr gas. The reaction mixture was poured into 2M NaOH (300ml) to give an orange solution acidified to give a yellow solution, and back-neutralised to pH7, producing a cloudy apricot-coloured suspension, from which the products were extracted with 20% methanol in dichloromethane. The organic layer was dried over $\text{Na}_2\text{SO}_4(\text{s})$ and evaporated under reduced pressure to give impure product (7.5g). This was adsorbed onto silica gel (70-23- mesh) and pure produce eluted from a 10cm x 4cm column using 5% methanol in dichloromethane. Fractions containing desired morphinone were pooled and evaporated to dryness giving a yellowish solid in 80% yield (6.9g).

3.3 Preparation of the hydrochloride salt of 14- β -amino
 (N-cyclopropylmethyl) normorphinone

Morphinone from 3.2. (18.5g) was placed in a flask with a mixture of methanol (250ml) and 2M HCl (250ml), and stirred at 20°C overnight. cHCl was added (15ml) and the temperature raised to 79°C for 2 hours. T.l.c. showed disappearance of starting material, and there were yellow crystals precipitated, of the hydrochloride salt. The crystals were filtered off and dried in a vacuum oven to give a yield of 76% (15g).

3.4 Protection of 3-hydroxy group in 14- β -amino
 (N-cyclopropylmethyl) normorphinone salt by silylation

A modification of the method of Corey and Venkatswarlu (1972) was used. The dihydrochloride salt (2.6g) was converted to free base by dissolving it in water, neutralisation with NaHCO₃(s), extracting the precipitate with DM5, drying over NaHCO₃(s) and evaporating the organic solution to dryness under reduced pressure, to give free base (2g).

Imidazole (1.9g, 4.5 equivalents) and tButyldimethylsilyl chloride (2.25g, 2.5 equivalents) were dissolved in DMF (8ml). The morphinone was dissolved in DMF (8ml) to give a yellow solution, and added to the reaction mixture. After 20 mins, t.l.c. showed disappearance of starting material, and the reaction mixture was poured into water (300ml) to give a milky white suspension, from which silylated product was extracted

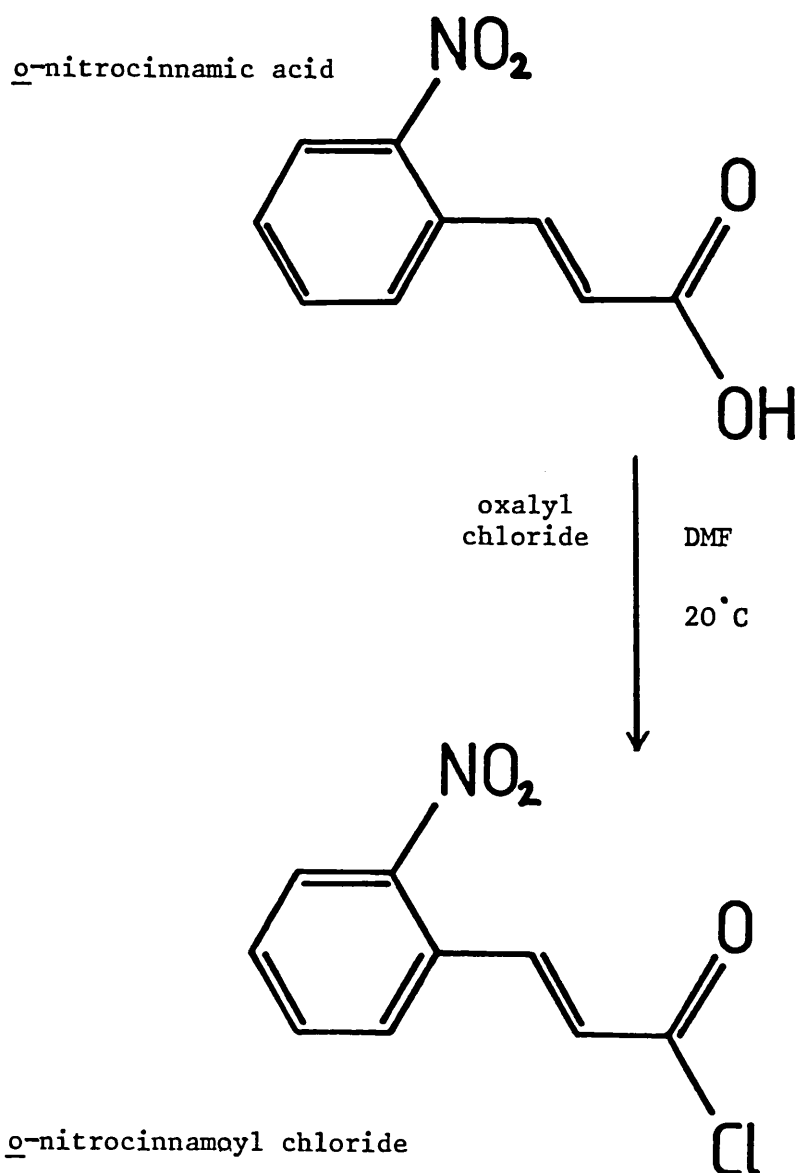
with ether, and evaporated to dryness on a high-vacuum rotary evaporator, to remove ether and DMF; yield was 80% (2.3g).

The protected morphinone could now be coupled to

- (a) cinnamoyl chloride to give NUM;
- (b) o-nitrocinnamoyl chloride, to give NOM, and
- (c) o-nitro, p-azido cinnamoyl chloride, to give NAM.

4.1 Coupling of protected morphinone to cinnamoyl chloride (analogous to Fig. 24)

Protected morphinone (700mg) was dissolved in DCM (100ml), cooled to 0°C, and 2ml Et₃N added. Cinnamoyl chloride (1ml, 1.5 mole equivalent) was added and the solution darkened from pale yellow to brown. The temperature was allowed to rise to 20°C, and the reaction judged complete after 30 mins, by t.l.c. The organic solution was washed with saturated NaHCO₃ solution, to remove unreacted acid, dried over Na₂SO₄(s) and evaporated under reduced pressure, to give a viscous orange liquid. This was adsorbed onto silica (70-230 mesh) and the desired product eluted from a 15cm x 2cm silica column with DCM. Pure product was a yellow glassy solid in a yield of 69% (630mg).

Fig.21Synthesis of o-nitrocinnamoyl chloride

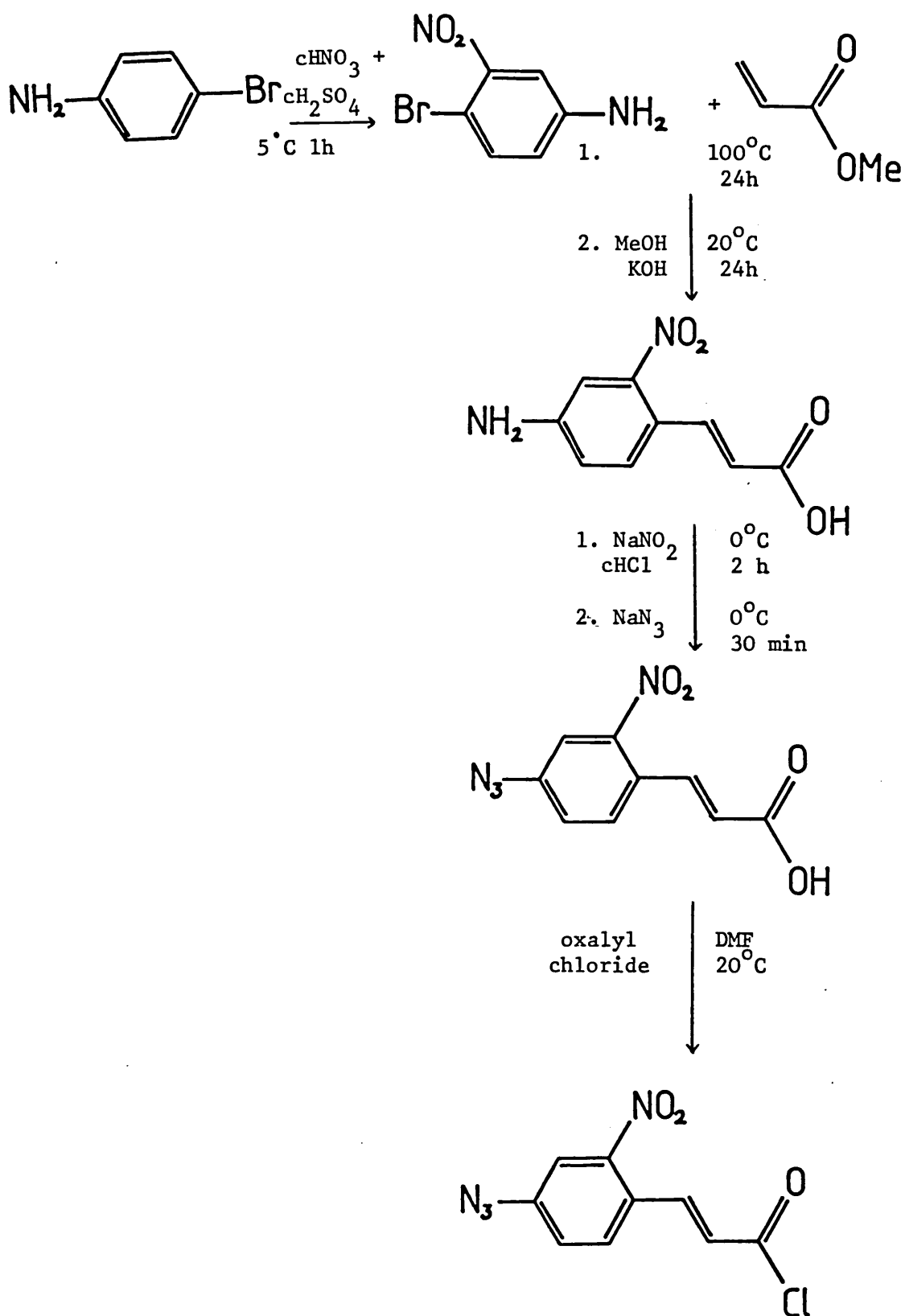


Fig. 22

Synthesis of o-nitro,p-azidocinnamoyl chloride

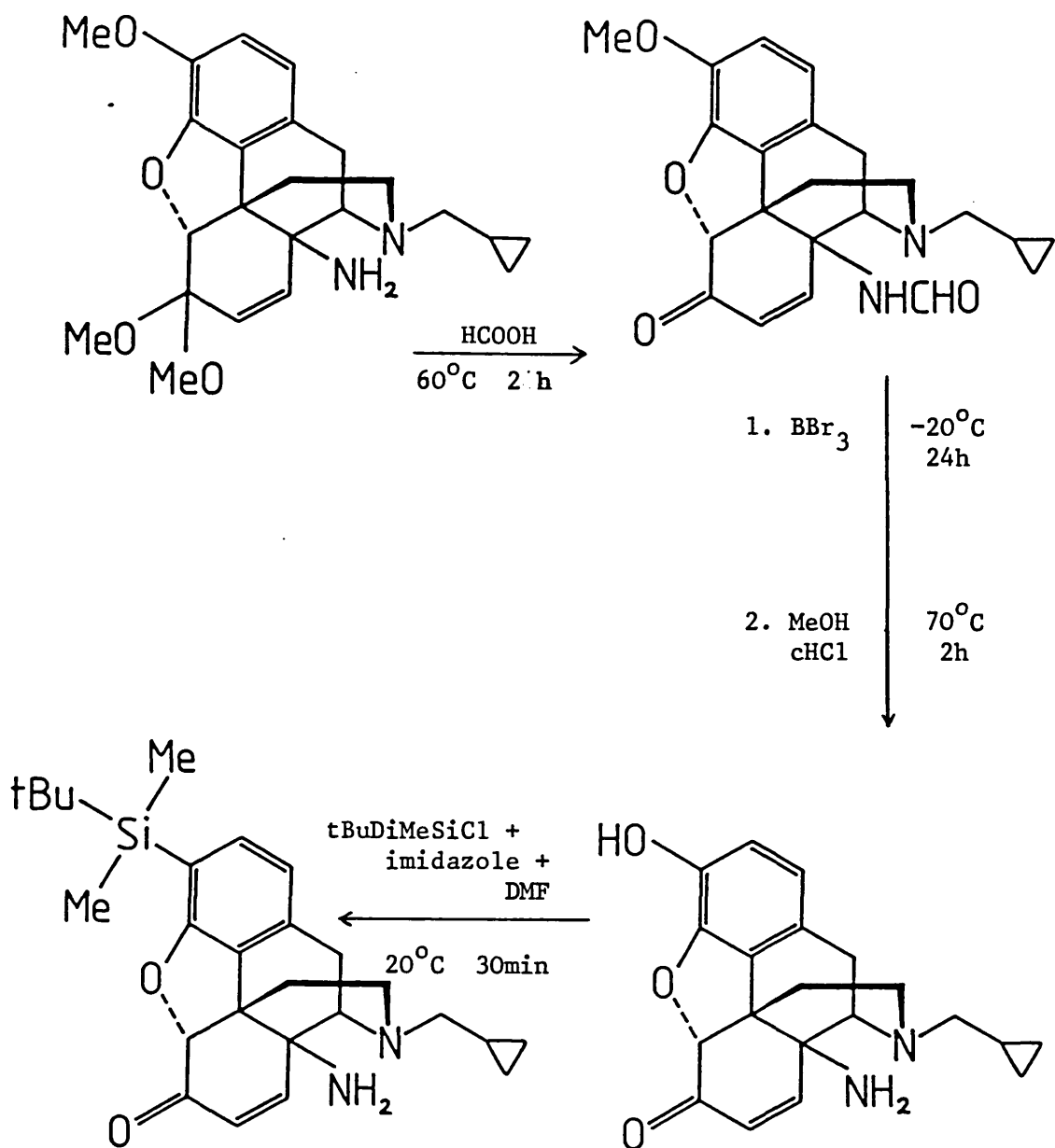


Fig. 23

Synthesis of protected 14-β-amino-(N-cyclopropylmethyl)
normorphinone

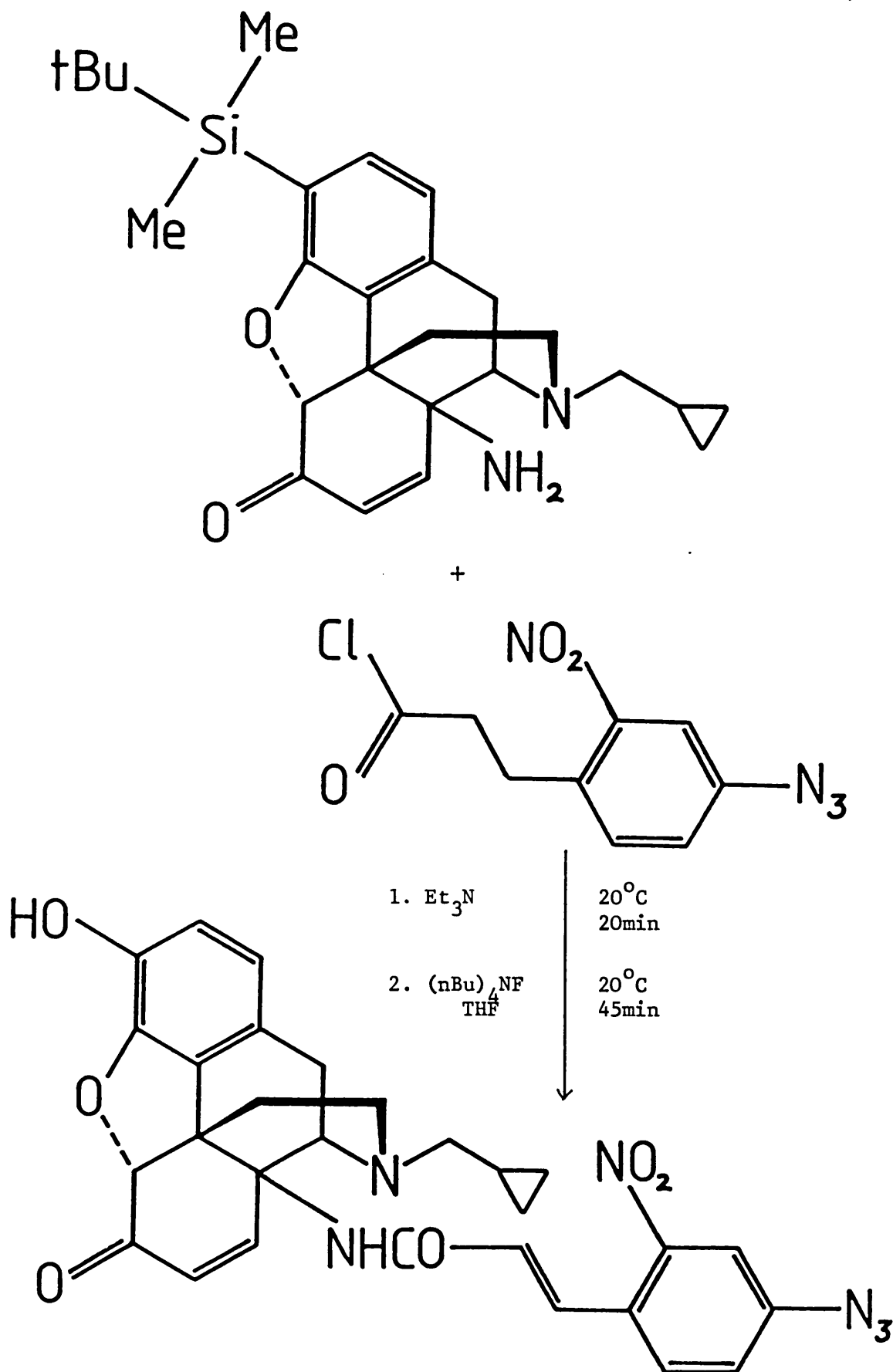


Fig. 24

Coupling and deprotection of 14-β-amino-(N-cyclopropylmethyl)-normorphinone

4.2 Deprotection of coupled morphinone

The product from 4.1 (600mg) was dissolved in tetrahydrofuran (10ml) at 0°C.. 1M tetra-N-butyl ammonium fluoride (3ml, in THF) was added, and the temperature allowed to rise to 20°C. T.l.c. showed disappearance of starting material after 30 mins. Water (50ml) was added to precipitate product, which was extracted with DM5, dried over Na₂SO₄(S) and evaporated under reduced pressure to give impure product and silanols. Impure product was adsorbed onto silica (70-230 mesh) and pure product eluted from a 10cm x 2.5cm silica column with DM2. Fractions containing pure product were combined and evaporated under reduced pressure to give a yellow crystalline solid. The yellow crystals were washed with ether, filtered and dried in a vacuum oven to give the desired product, 14- β -cinnamoyl-amido (N-cyclopropylmethyl) normorphinone (NUM), in a yield of 75% (362mg). NMR, IR and MS all showed this to be the product, but elemental analysis revealed the presence of a half-mole equivalent of water. Since the compound was synthesized for use in pharmacological and binding assays when it would be dissolved in water, it was decided that it would not be worth attempting further purification.

4.3 Coupling of protected morphinone to o-nitrocinnamoylchloride (analogous to Fig. 24)

Method as in 4.1, using 600mg protected morphinone. Yield of protected coupled morphinone (intense yellow glassy solid) was 96% (800mg).

4.4 Deprotection of coupled morphinone

Method as in 4.2, including purification of the product by column chromatography, to give 14- β -(o-nitrocinnamoyl) amido (N-cyclopropylmethyl) normorphinone (NOM), and intense yellow crystalline solid, in a yield of 50% (325mg). NMR, IR and MS confirmed the structure of the product, but as in 4.2, elemental analysis revealed the presence of a half-mole equivalent of water, again not removed.

4.5 Coupling of protected morphinone to o-nitro, p-azido cinnamoyl chloride (Fig. 24)

Method as in 4.1 and 4.3, using foil-covered glassware to protect the azide from photolysis, and using 799mg protected morphinone.

Yield of protected coupled morphinone (orange glassy solid) was 75% (760mg).

4.6 Deprotection of coupled morphinone (Fig. 24)

Method as in 4.2 and 4.4 up to the stage of obtaining crude product. In 4.2 and 4.4, the crude product was adsorbed onto silica and pure product eluted from a silica column. However here, because of the difficulties of column chromatography for a light-sensitive product, it was decided simply to wash the product with petrol (30°C-40°C boiling fraction), filter

and dry it, to give an orange solid. NMR showed the presence of silanols, which column chromatography would normally have removed. The product still showed the presence of silanols after intensive washing with ether at 4°C. A short (5cm x 2cm) silica (70-230 mesh) column was therefore set up, the product adsorbed onto silica, and eluted from the column with DMZ. Fractions containing pure product were pooled and evaporated down to give a yield of 45% (274 mg) 14- β - (o-nitro, p-azido cinnamoyl) amido (N-cyclopropylmethyl) normorphinone (NAM). Again, NMR, IR and MS confirmed the structure of pure produce and elemental analysis showed the presence of a half-mole equivalent of water, which was not removed.

The IR spectrum showing a characteristic azide band at 2100cm^{-1} is shown in Fig. 25.

2. Photolysis of NAM

The UV spectra of NAM were examined before and after various time intervals of UV irradiation with a 50watt t.l.c. hand lamp (Camag Universal UV lamp TL-900) set at 254nm. Photolysis was also attempted with the lamp set at 366nm. Because light of higher energy, i.e. lower wavelength, was thought to be the more destructive element in UV irradiation of opioid receptors, a filter was devised to remove light of lower wavelengths. Solutions of several compounds at various concentrations were examined for their ability to filter out light of lower wavelengths. The most satisfactory was a 1% solution of the

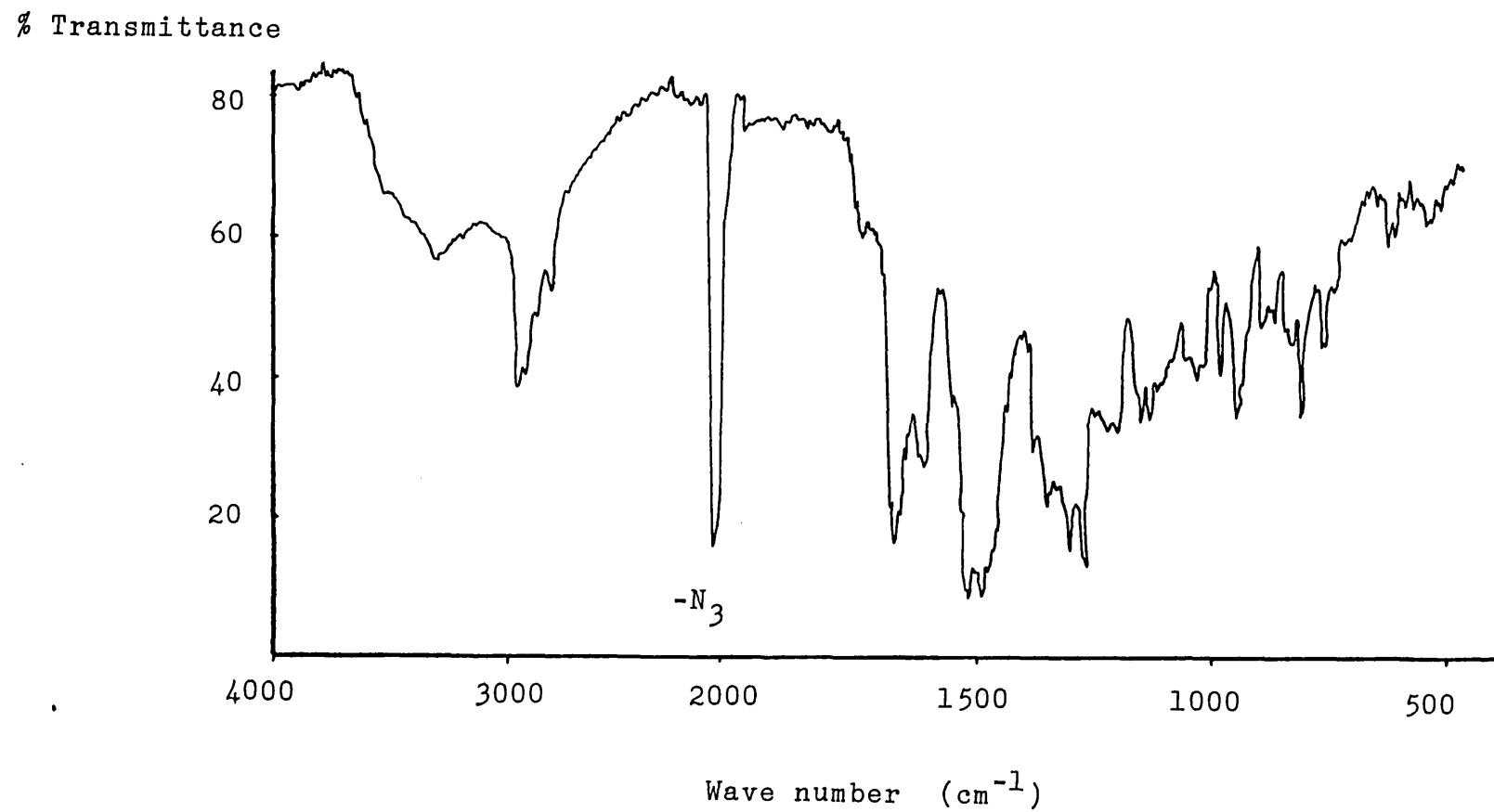
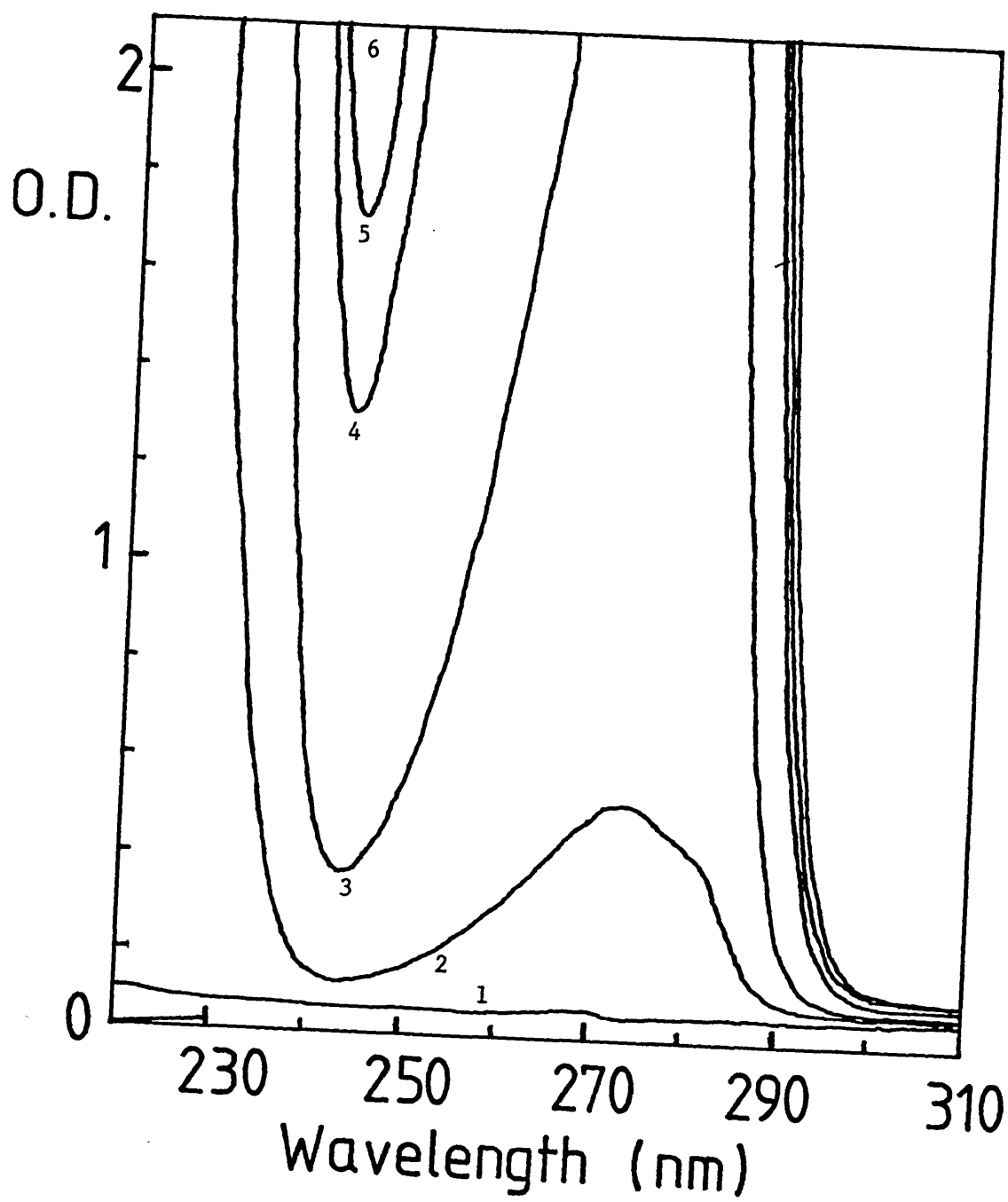


Fig. 25

Infrared spectrum of NAM

Fig. 26 UV spectra of solutions of Triton-X-100



1	Baseline	4	0.5%
2	0.01%	5	0.75%
3	0.1%	6	1%

ionic detergent Triton-X-100 in water. It showed a sharp block to transmission of light of wavelength below 295nm (Fig 26). A 2cm layer of this solution was interposed between the UV source and brain membranes in an attempt to reduce photodestruction of opioid binding sites.

3. Bioassay of NUM, NOM and NAM

NUM, NOM and NAM were suspended in distilled water with a few drops of 2M HCl, at a concentration of 1mg/ml. The resulting suspensions were sonicated until they became transparent solutions.

The types of experiment described in II.5 were carried out with the qualification that only one dose of ligand was applied to any single tissue (See III.3). The isolated tissues used were MVD, GPI and MI. All studies with NAM were carried out in the dark.

4. Studies with daleck in MVD, GPI and MI

Receptor selectivity

The relative potencies (nM) of normorphine, met-enkephalin and daleck were estimated in each tissue, to give an estimate of the receptor selectivity of daleck. These studies were extended by examining the shifts in dose-response curves of the 3 agonists to various concentrations of naloxone in MVD and GPI and to Mr 2266 in MI.

Attempted irreversible activation of opioid receptors
in MVD

The conditions under which brain membrane opioid receptors were irreversibly blocked by daleck were reproduced as far as possible for MVD. These were to incubate membranes in 25mM sodium borate pH 8.1 at 30°C for 1 hour. Clearly it was not possible to use this buffer system for isolated tissues, so ordinary (magnesium-free) Krebs solution was modified by the addition of sodium hydroxide solution to bring the pH up to 8.0. It was not possible to attain pH 8.1 without the precipitation of calcium salts. To maintain this pH it was necessary to stop the O₂/CO₂ supply to the organ bath and therefore electrical stimulation was also stopped. The length of time of incubation was increased to 2 hours at 30°C and incubation medium (with or without daleck, 200nM = 100 x ED₄₀) was changed every 30 minutes. These conditions were applied to MVD preparations in the absence of daleck to ensure that they alone had no lasting effect on twitch height, tissue viability or responses to met-enkephalin and normorphine.

5. Membrane binding assays with NOM

NOM was chosen for binding studies initially, because it lacked the reactive azide moiety of NAM, and the kinetic behaviour of all three ligands in bioassay appeared very similar. Initial experiments examined the ability of NOM to displace the specific binding of 1nM ³H-DAGO and of 1nM ³H-DADL and IC₅₀ values

were calculated for the particular conditions used. Concentrations of NOM from 10^{-12} M to 10^{-5} M were used, NOM being added to the assay tubes before the membranes and the assay then carried out as usual.

In later experiments, membranes were incubated with 2nM or 10nM NOM for 30 minutes at 30°C; the membranes were then diluted 10-fold with buffer and centrifuged at 45,000 x g. The pellet was resuspended in the same buffer volume incubated for 20 minutes and again centrifuged. This washing procedure was carried out 3 times, and the membranes were then assayed for remaining binding activity. Experiments were also undertaken using 1% ethanol or dimethylformamide in buffer, in an attempt to increase the wash-out of NOM from membranes.

III.3. RESULTS

1. Photolysis experiments

The UV spectra of NAM pre-and post-photolysis, in the presence of the Triton-X-100 filter, are shown in Fig. 27. There is an absorbance maximum between 270 and 300nm. NAM was not effectively photolysed with the lamp set at 366nm. The half-time for photolysis of NAM under the conditions studied was 1.7 minutes, in the absence of membrane fragments. In the presence of the Triton-X-100 filter there was less photodestruction of opiod binding activity in membranes than in its absence (Fig. 28). However, there was still significant photodestruction of binding activity even in the presence of the filter.

2.1 Actions of NOM and NUM in MVD and GPI

NOM and NUM behaved in very similar manner in all studies, and NOM only will be referred to, although the results obtained apply equally to NUM.

The first indication of the problems later experienced in working with these compounds came with the difficulty of dissolving them in aqueous solution prior to any bioassay or binding assay experiments. This showed that the compounds were hydrophobic. When doses of NOM were applied to MVD or GPI, there was no immediate response, but a gradual decline in twitch

Fig. 27

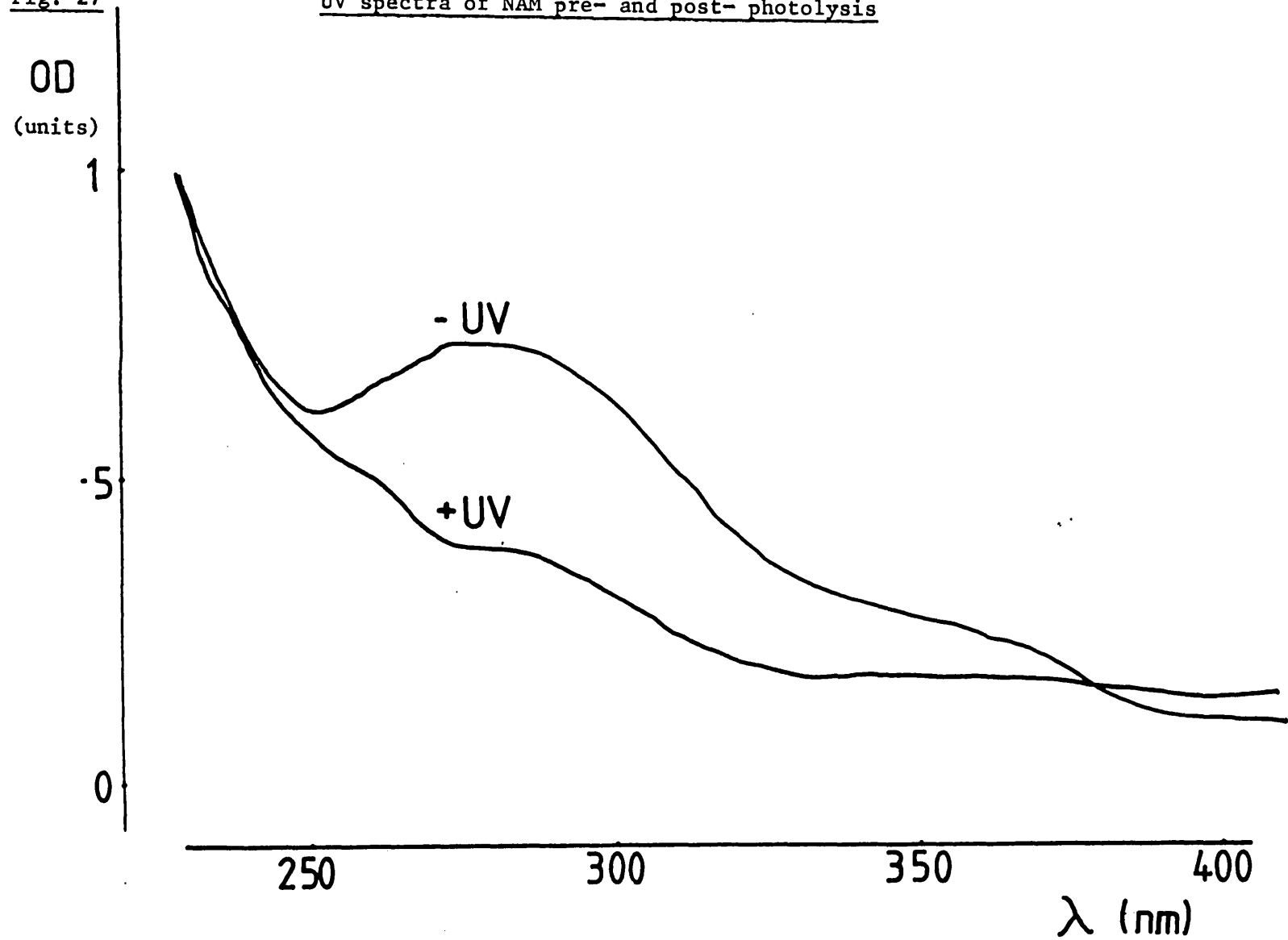
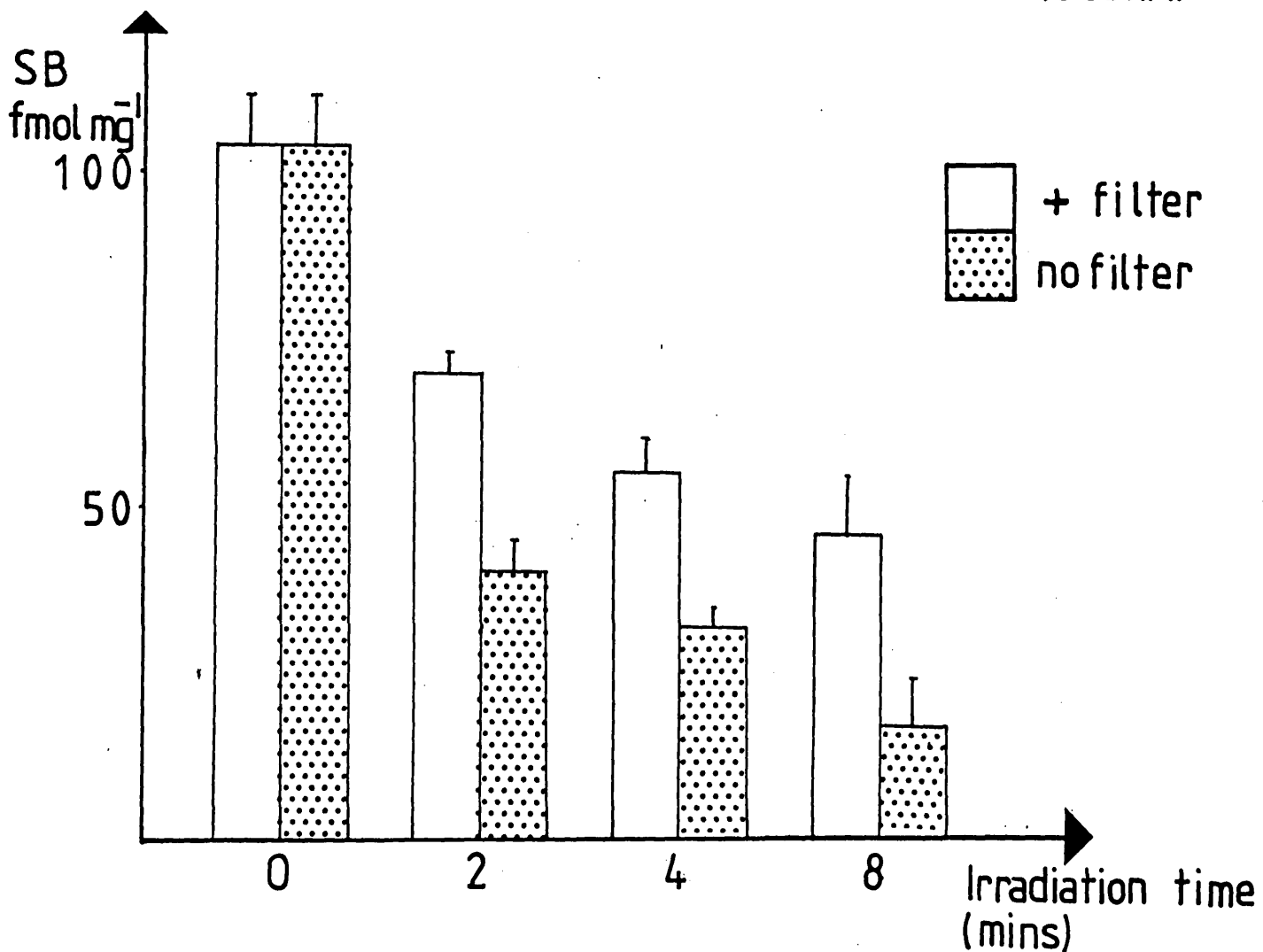


Fig. 27 UV spectra of NAM pre- and post-photolysis

Fig. 28

UV irradiation of rat brain membranes with & without
2cm 1% Triton-X-100 filter: loss of ^3H -DAGO specific binding
(0.85nM)



Each histogram represents the mean of 5 observations + or - s.e.m.

Fig. 28 UV destruction of opioid binding sites in the
presence and absence of 1% Triton-X-100 filter

height which eventually plateaued leaving between 40% and 80% twitch height (Fig. 29). Repeated washes (at 10 minute intervals for 4 hours) were not able to reverse this depression, neither could naloxone at any concentration used. Because of the slow onset of effect and extremely slow offset which gave apparent irreversibility, only one dose of NOM could be applied to any one preparation, and only estimates of % depression of twitch could be made for each dose. However, it was clear that NOM was of high potency, possessing an ED_{40} value about 1000 times lower than that of normorphine (Fig. 30). The dose-response curve of NOM was less steep than that of normorphine showing NOM was a partial agonist, in both preparations. NOM was unable to depress twitch height by more than about 60%.

It was important to show that NOM produced its effects by action on opioid receptors. Since it was not possible to reverse the depression of twitch by naloxone administered after NOM, an attempt was made to block the onset of NOM's effect by adding high concentrations of naloxone to the bath before adding NOM. The dose of NOM chosen (4nM) was known to produce a certain amount of depression of twitch after a certain time interval. 800nM naloxone shifted the normorphine dose-response curves in GPI and MVD about 300-fold. The normorphine responses seen in the presence of this concentration of naloxone were abolished on increase of naloxone concentration to 8 μ M. 800nM naloxone did appear to delay the onset of NOM's action, but once it had started, the twitch depression proceeded inexorably.

Fig. 29 The effect of NOM in isolated MVD preparation

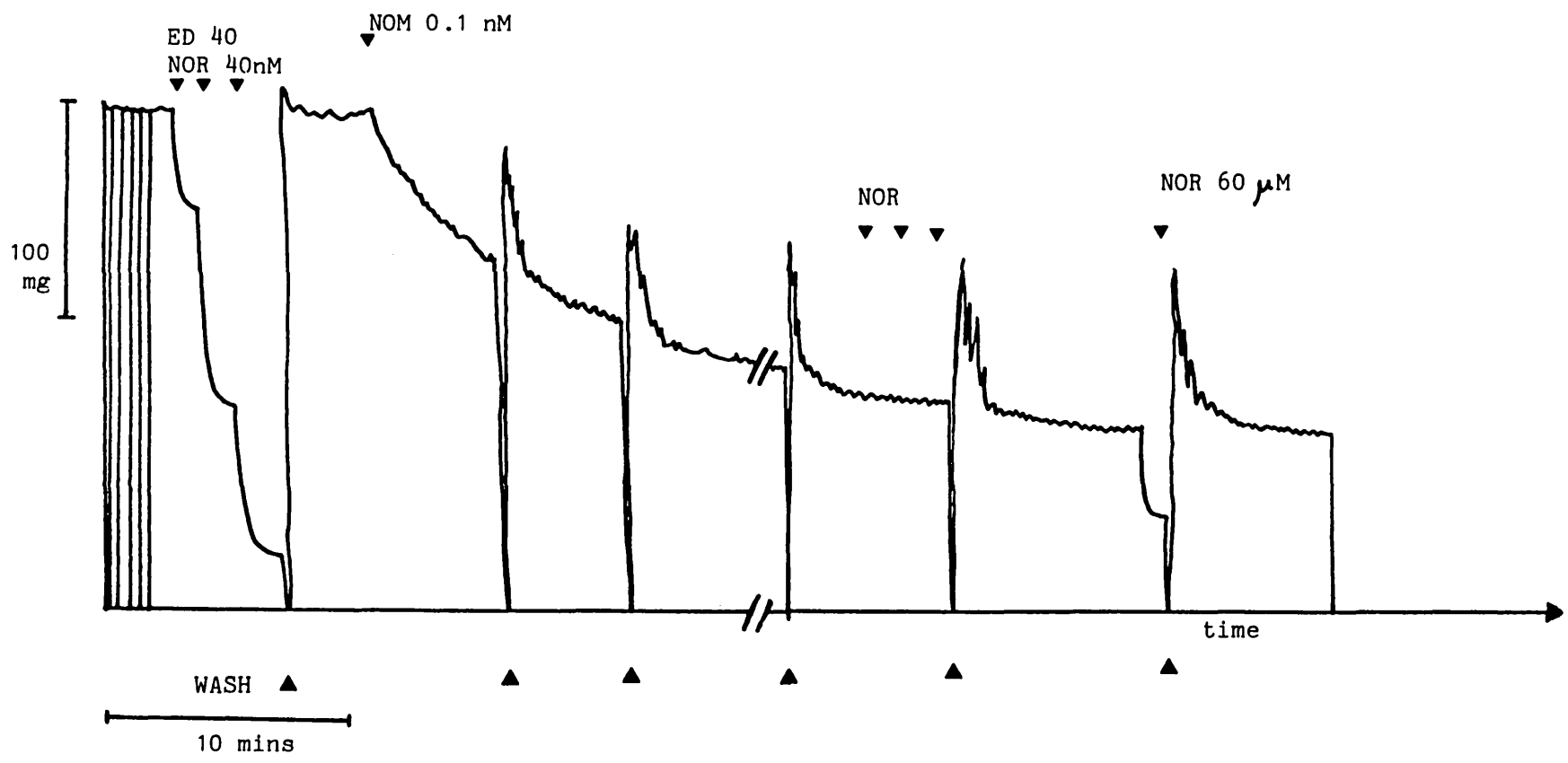
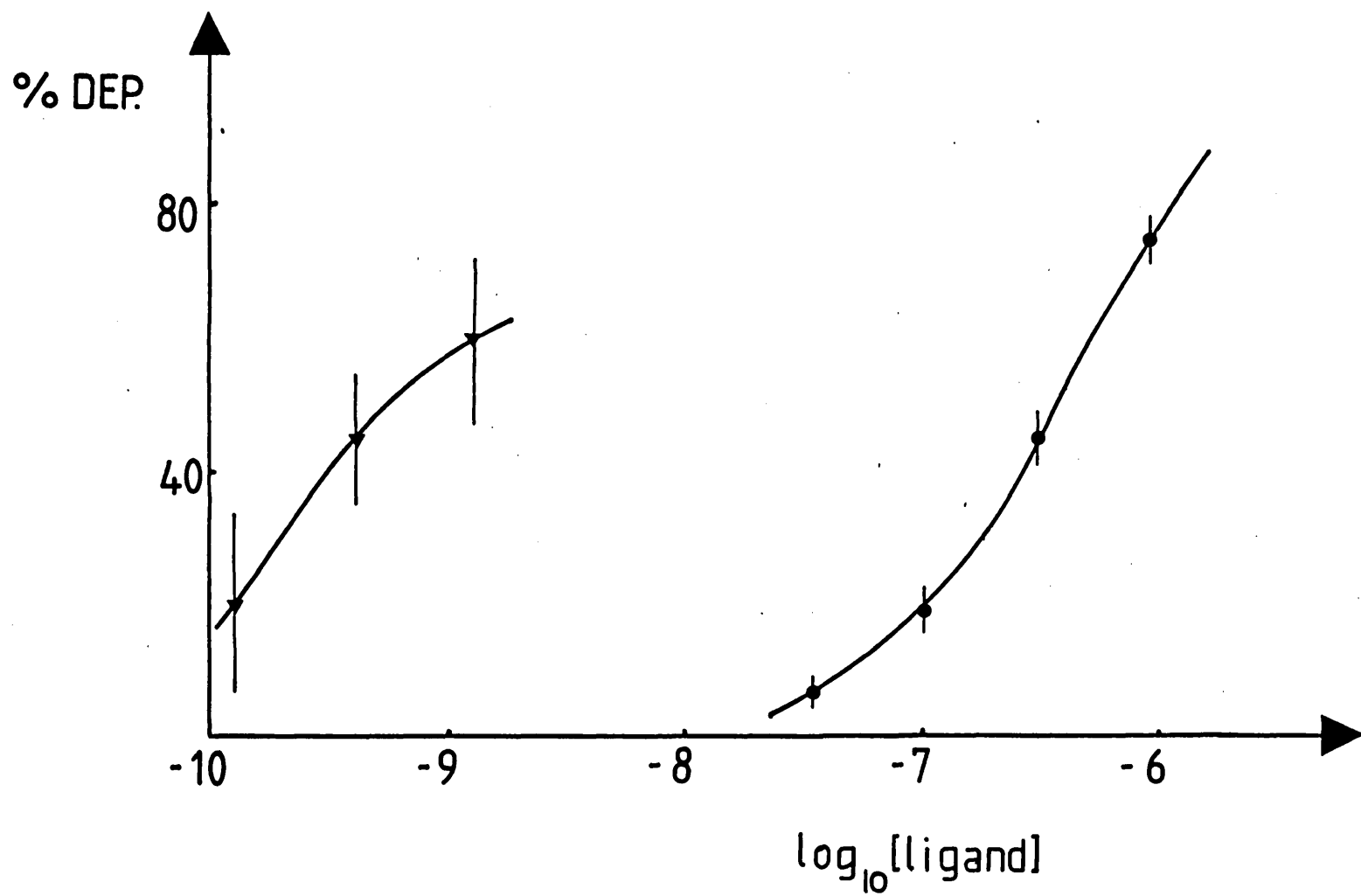


Fig. 30

MVD : Dose-response curves to normorphine and NOM

For normorphine, each point represents the mean of 52 observations, and for NOM the mean of 15 observations, both + or - S.D.



2.2 Actions of NAM in MVD and GPI

In contrast to NOM and NUM, NAM was a pure antagonist, producing no depression of twitch at doses up to $1\text{ }\mu\text{M}$. However, like NOM and NUM, it was of high potency and its effects were of slow onset. Antagonism of normorphine responses in MVD and GPI were similar but antagonism of met-enkephalin responses in the two tissues differed in nature. In GPI, NAM's antagonism of met-enkephalin responses was very similar to its action against normorphine responses: 0.54 nM NAM perfused in Krebs solution

*
produced a pseudocovalent non-competitive antagonism of normorphine and met-enkephalin responses which increased with time, until after 4 hours, no response could be obtained to either agonist (Figs. 31 , 32). Antagonism of met-enkephalin in MVD was competitive in nature, with no decrease in maximum effect after 4 hours (Figs. 31 , 32). After this time, responses of MVD to clonidine were unaffected. Prephotolysed NAM infused into either tissue under the same conditions into MVD produced only about a doubling of ED_{40} values after 4 hours

*
i.e. the behaviour of NAM was as if it interacted irreversibly with μ -receptors but without a covalent chemical reaction.

When etorphine ('universal' ligand, acting on μ -, δ - and κ -receptors, Paterson et al, 1983) was used as the agonist in MVD, infusion of 1.8 nM NAM produced a shift in ED_{40} value for etorphine from 0.4 nM at zero time to 400 nM after 2 hours NAM infusion. At this time, infusion of NAM was stopped. 1 hour later, the ED_{40} dropped to about 150 nM , showing a component of reversibility in NAM's antagonism of opioid-receptor mediated depression of twitch in MVD.

Fig. 31

Method as described
in the text (Ch. III.2
& Ch. II).

One typical result of 5 separate experiments.

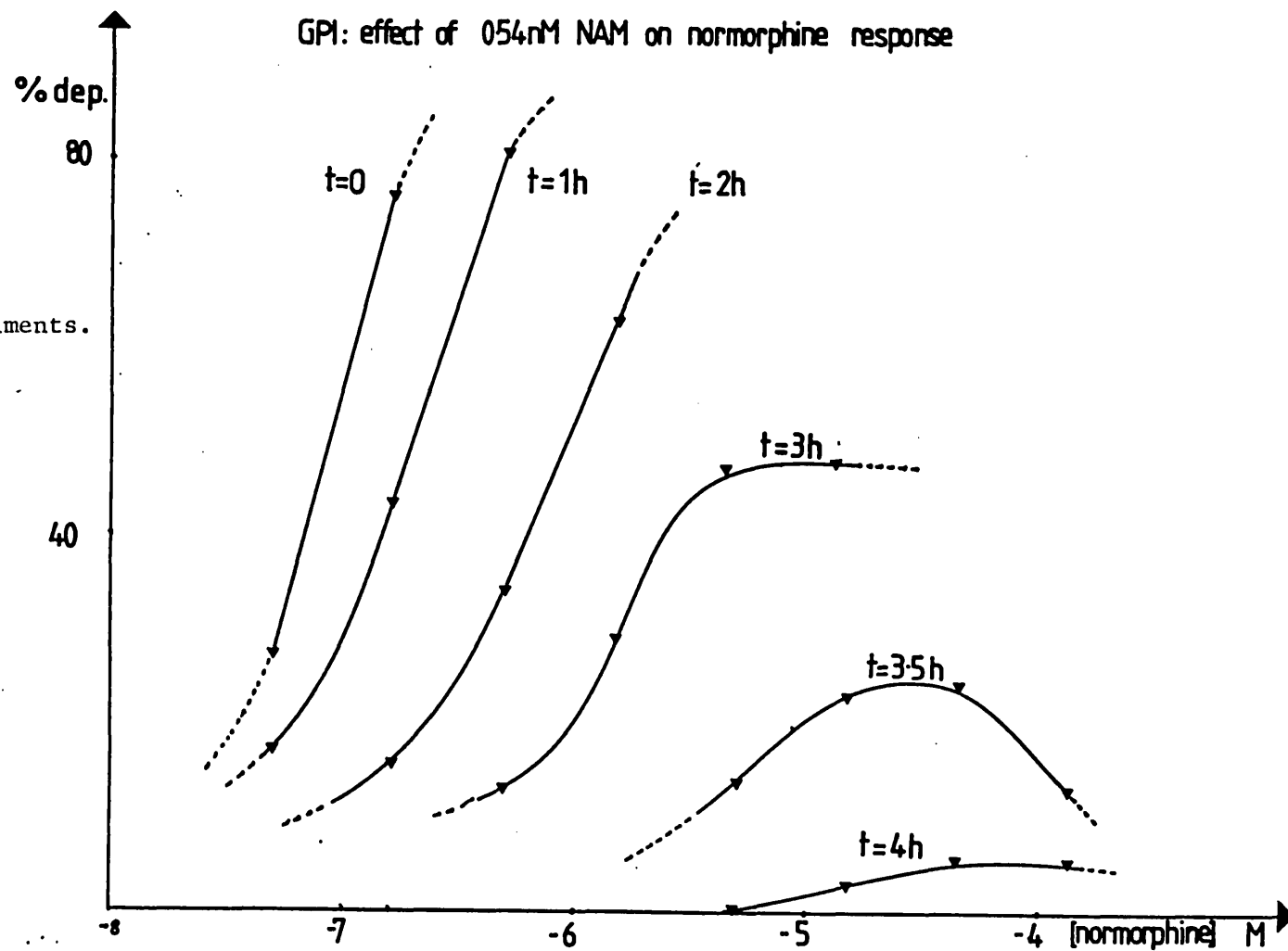
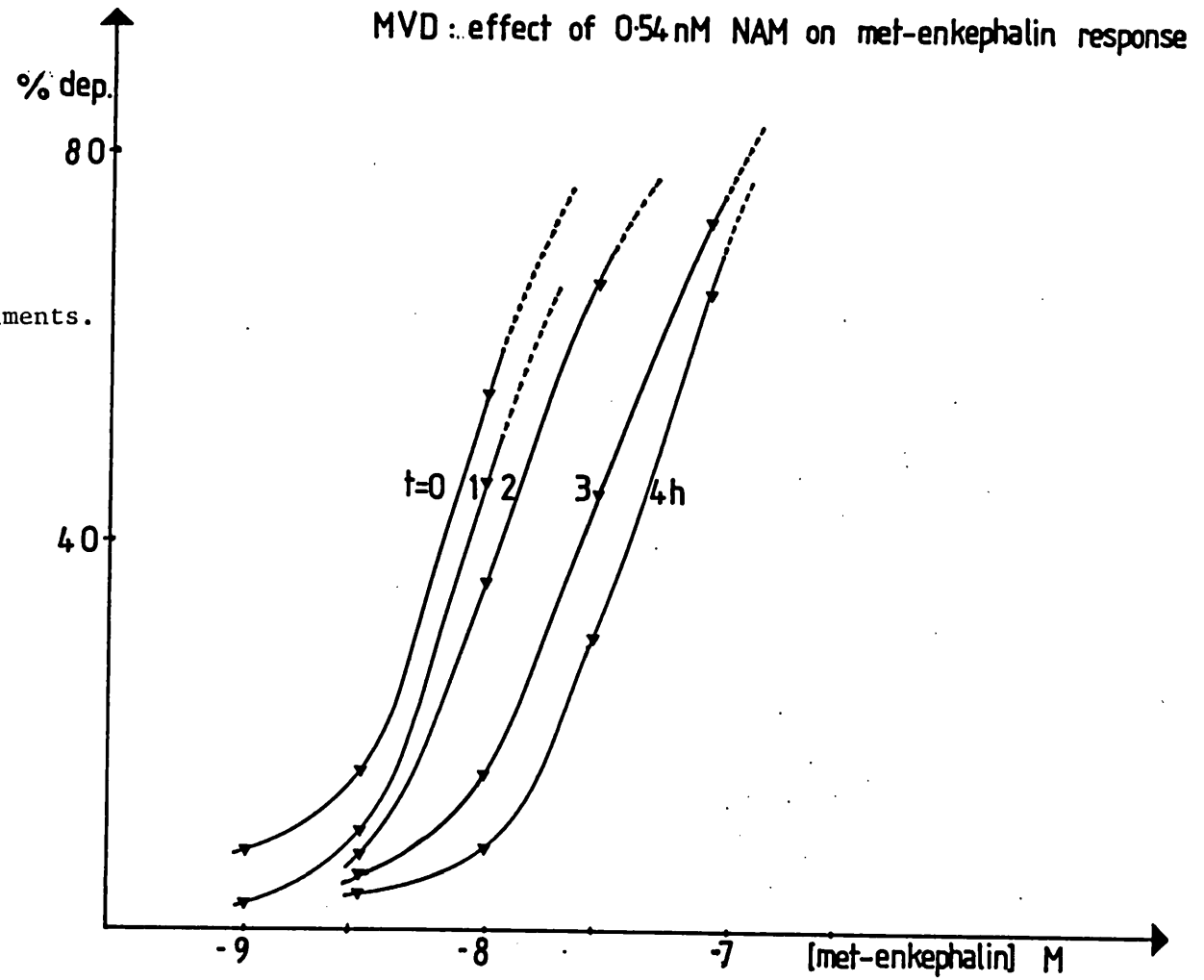


Fig. 32

Method as described
in the text (Ch. III.2
& Ch. II).

One typical result of 5 separate experiments.



3.1 Receptor selectivity of daleck in MVD, GPI and MI

In MVD, dose response curves obtained with daleck were compared to those of normorphine and met-enkephalin. Daleck was a pure agonist and could produce up to 90-95% depression of twitch. The ED_{40} values indicated that daleck was about 40 times more potent than normorphine in GPI and half as potent as met-enkephalin in MI (Table 5).

To enquire further into daleck's receptor selectivity, naloxone was used in various concentrations to antagonise the responses of MVD and GPI to normorphine, met-enkephalin and daleck. In GPI, 8.3nM, 28nM and 83nM naloxone were used to show that the shifts in ED_{40} to normorphine met-enkephalin and daleck were similar (Schild plot, Fig. 33). It is clear that all three compounds are acting on the same receptor because the slopes and intercepts of the three lines are so similar. However, in MVD, the results were not similar. Again, three concentrations of naloxone (8.3nM, 28nM and 83nM) were used to examine the shift in ED_{40} values of the three agonists. The data are shown in Fig. 34 as a Schild plot. Met-enkephalin was deduced to be acting on a different receptor to normorphine and this receptor has a lower affinity for naloxone than that of normorphine. Daleck is different, in that it appears to act through the same receptor as normorphine at lower concentrations of naloxone i.e. the μ -receptor, but at higher concentrations, naloxone appears to have relatively less effect on shifting the ED_{40} value of daleck. This shows that daleck can also act through another

TABLE 5 Relative potencies of daleck, normorphine and met-
enkephalin in 3 bioassays

Relative to met-enkephalin = 1

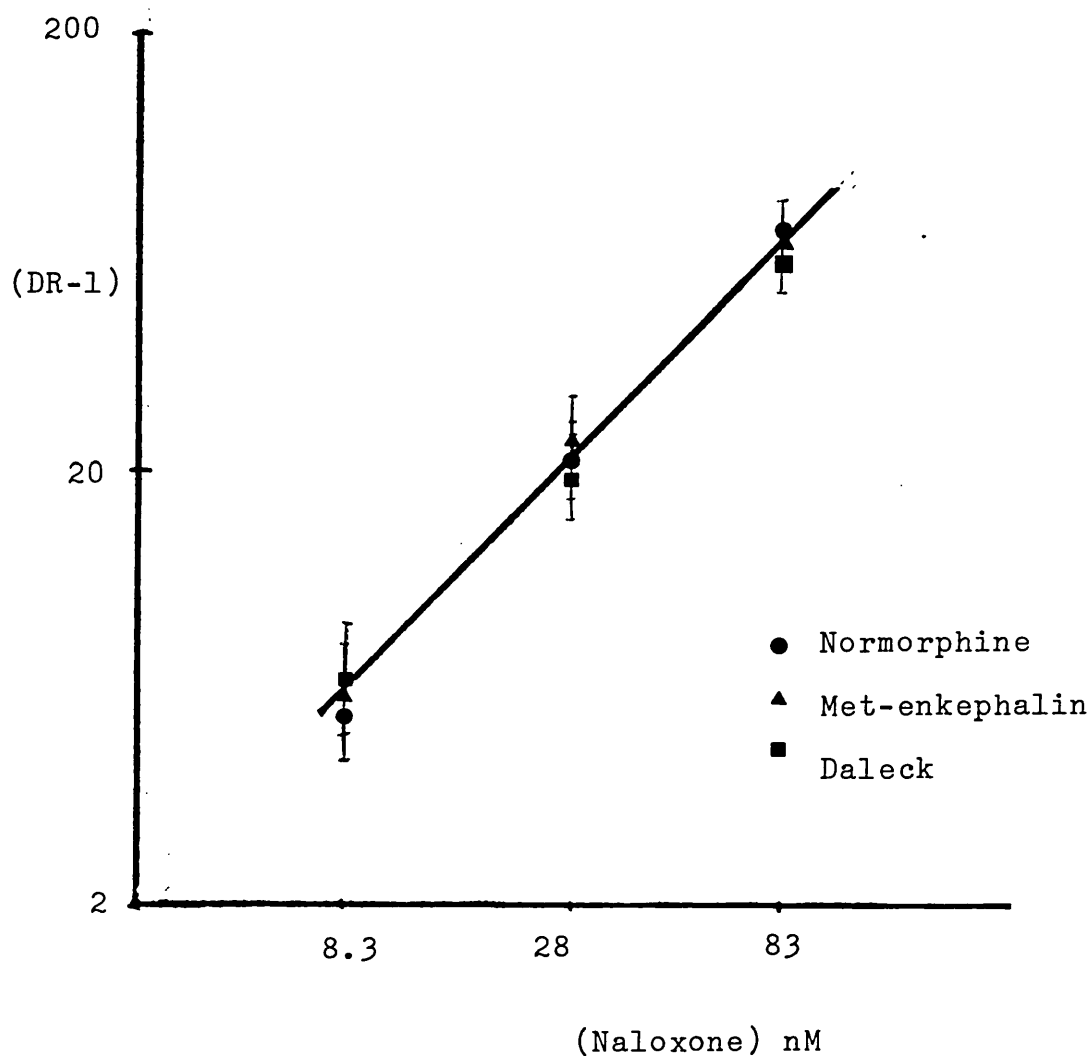
	Tissue		
Drug	MVD ($\mu + \delta + \underline{k}$)	GPI ($\mu + \underline{k}$)	MI ($\delta + \underline{k}$)
Normorphine	0.1 (230nM)	1 (180nM)	-
Met-enkephalin	1 (20nM)	1 (190nM)	1 (20nM)
Daleck	10 (2nM)	38 (5nM)	0.5 (40nM)

n = 50

n = 10

n = 9

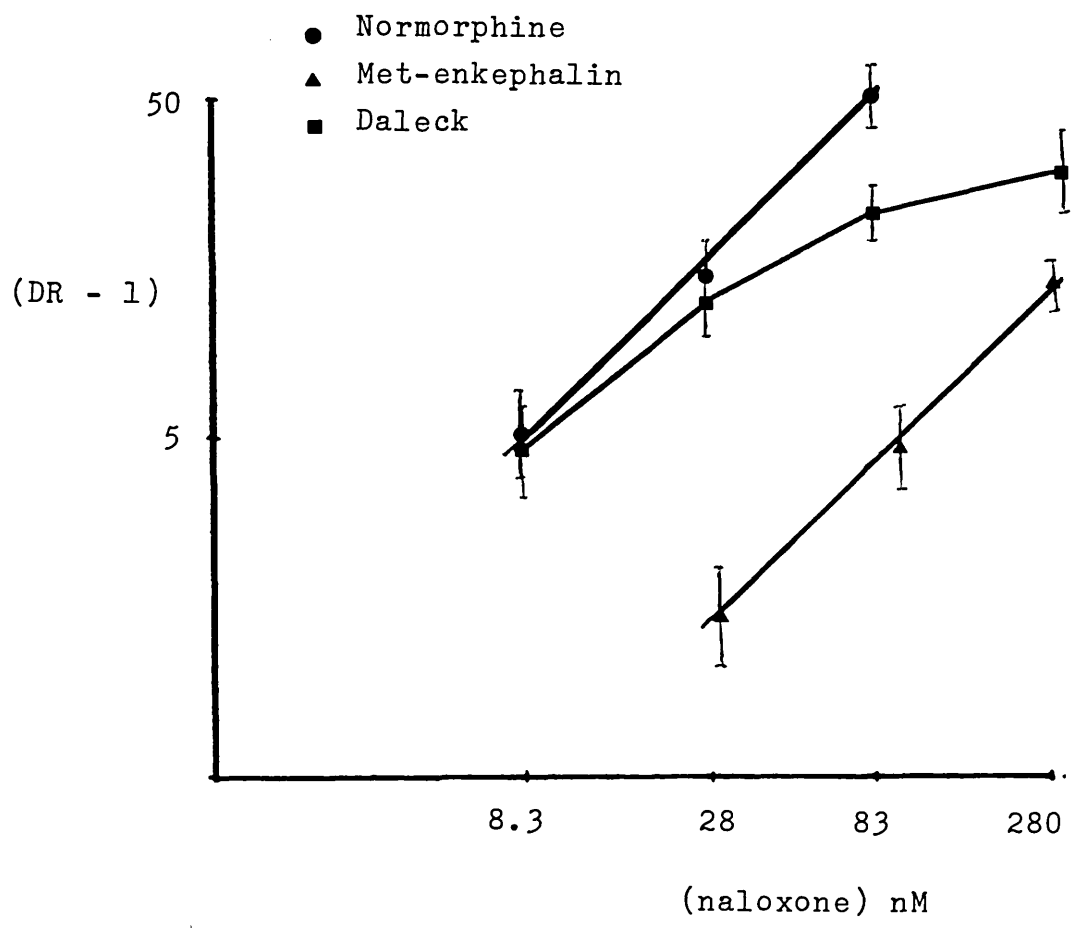
Fig. 33 GPI : antagonism by naloxone of normorphine,
met-enkephalin and daleck (Schild plot)



Each point represents the mean observation from 3 points in
each of 3 preparations, + or - s.e.m.

DR = dose ratio

Fig. 34 MVD : antagonism by naloxone of normorphine,
met-enkephalin and daleck (Schild plot)



Each point represents the mean observation from 3 points
in each of 3 tissues, + or - s.e.m.

TABLE 6 ED40 values of met-enkephalin, ethylketocyclazocine and
daleck in MI in presence and absence of 9nM Mr2266 (nM)

	Control	Mr 2266	Shift	
Met-enkephalin	20	40	2.0	n = 6
EKC	2	45	23	n = 6
Daleck	40	105	2.1	n = 6

receptor apart from the μ -receptor which may be the δ -opioid receptor.

Clarification of this point came with studies on the antagonism of met-enkephalin, ethylketocyclazocine and daleck responses in MI, by 9nM Mr 2266. The ED_{40} value of ethylketocyclazocine increased about 23-fold in the presence of 9nM Mr 2266, while those of daleck and met-enkephalin shifted about 2-fold. These data suggest that daleck acts as an agonist on δ -receptors, although with a lower potency than met-enkephalin (Table 6).

3.2 Attempted irreversible activation by daleck of opioid receptors in MVD

Incubation of MVD at pH8, in the absence of oxygen and electrical stimulation for 2 hours, showed that these conditions alone had no lasting effect on twitch height or sensitivity to agonists, once normal magnesium-free Krebs solution was restored and stimulation restarted (4 preparations).

When 200nM daleck was included in the incubation medium (8 preparations), and the preparations washed thoroughly with normal magnesium-free Krebs (5 times) to ensure removal of non-covalently bound daleck. Electrical stimulation was then restarted and dose-response curves to normorphine, met-enkephalin and daleck compared to those obtained before incubation.

Four preparations showed no change in (A typical result is shown in fig. 35) twitch height or sensitivities to the three agonists, and one

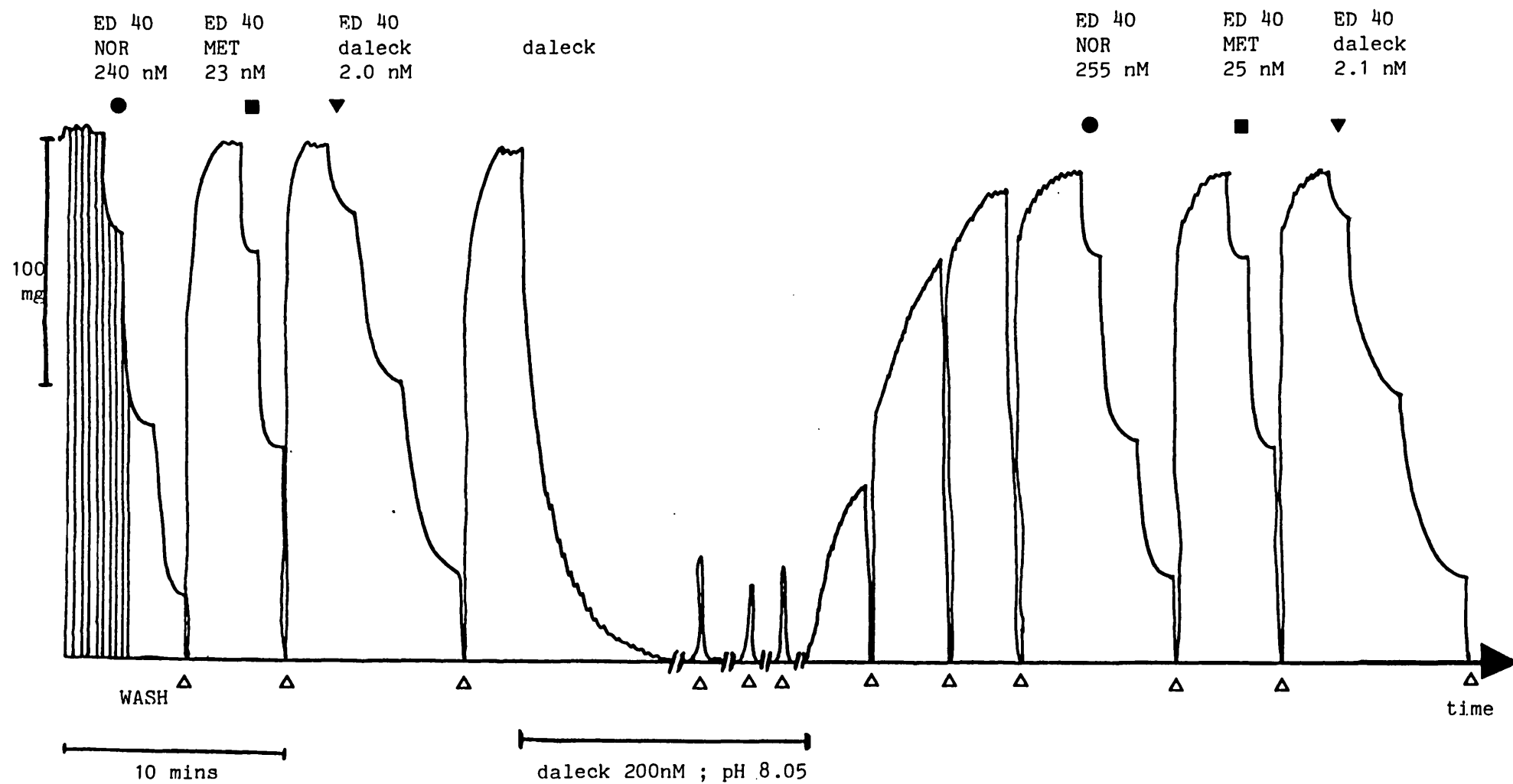


Fig. 35 Inability of Daleck to inactivate receptors in MVD

preparation displayed an increase of 5-10% in twitch height with ED_{40} values not significantly altered. Three preparations displayed an apparent irreversible depression of twitch height of 5-20%, i.e. naloxone was unable to reverse the depression. However, these preparations' twitch heights continued to fall off, although sensitivities to normorphine, met-enkephalin and daleck were not much changed. This implies that the apparent irreversible depression of twitch height was simply a decrease in twitch height during the time of the incubation and not irreversible activation of receptors by daleck.

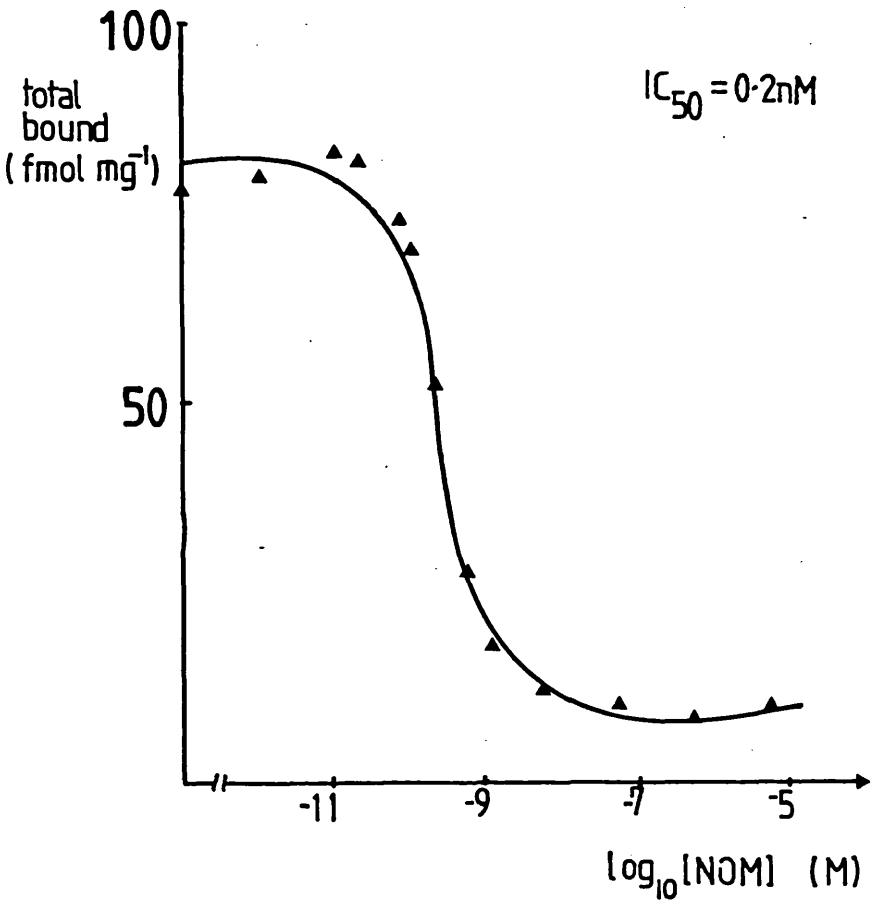
4. Membrane binding studies with NOM

Initial experiments demonstrated that NOM was able to occlude binding sites of ^3H -DAGO or ^3H -DHM and ^3H -DADL (Fig. 36a, b). The IC_{50} values obtained for NOM under these conditions were 0.2nM (^3H -DAGO) and 0.5nM (^3H -DADL) showing NOM's high affinity at these sites. A concentration of 2nM NOM was chosen for further experiments.

Since binding studies with NOM were commenced after the studies in MVD and GPI, the propensity of the compounds to persist in tissue was well-known. Ethanol and dimethylformamide were used in concentrations of 1%-5% (in buffer) in an attempt to aid wash-out of NOM from brain membranes. 5% Ethanol had no lasting effect on binding (Table 7a) but neither was it able to increase wash-out of NOM (Table 7b). Use of 1%-5% dimethylformamide gave similar results to those obtained using ethanol.

Fig. 36a

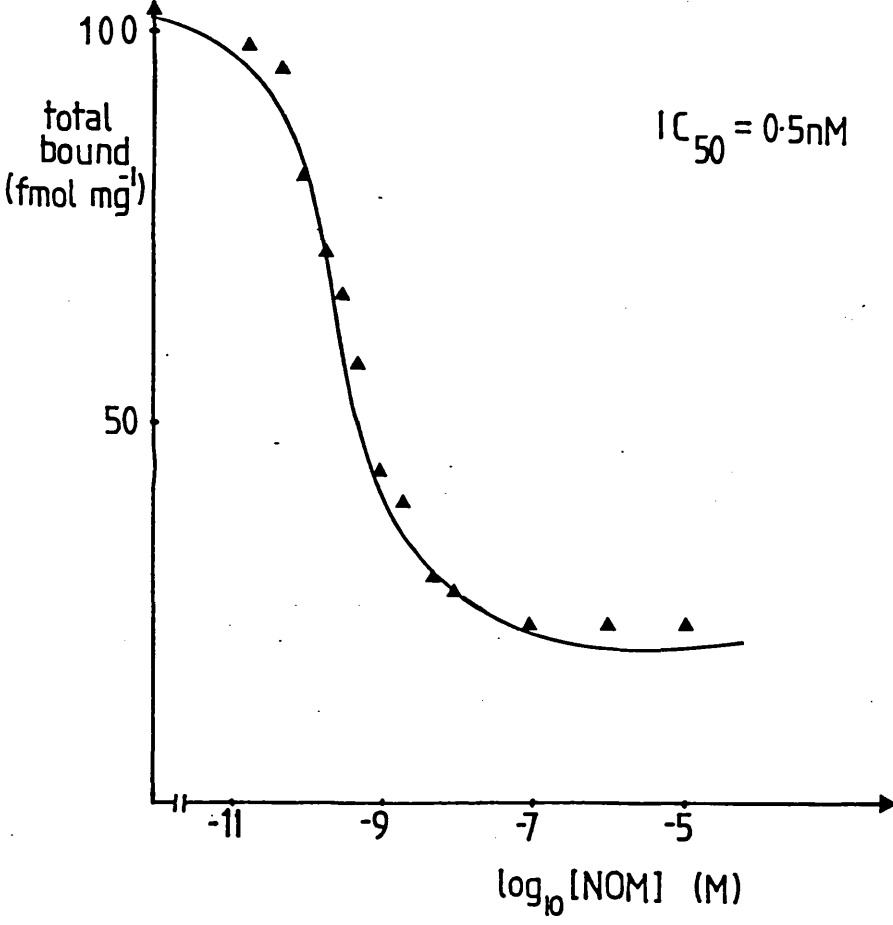
Rat brain membranes : displacement of ^3H -DAGO binding by NOM (0.66nM).



A typical result of 3 experiments

Fig. 36b

Rat brain membranes: displacement of ^3H -DADL binding by NOM
(0.73nM)



A typical result of 3 experiments

TABLE 7a Effect of 5% ethanol in washes on specific binding
(fmol mg⁻¹) of ³H-etorphine (1nM) to rat brain
membranes (single experiment)

	Number of washes				
	0	1	2	3	4
Control	222	226	220	219	170
5% ethanol	-	224	215	230	165

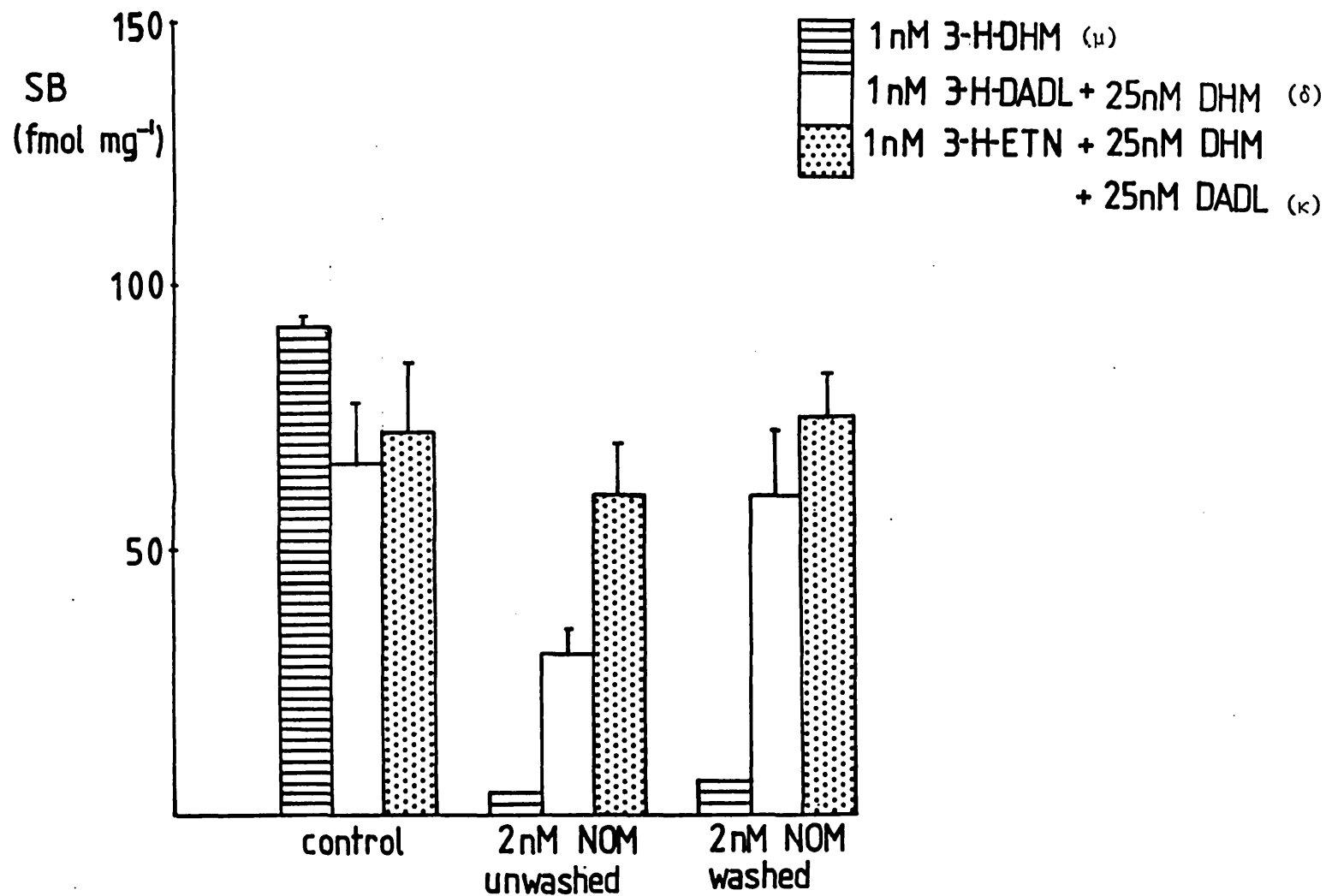
TABLE 7b Effect of 5% ethanol washes on specific binding (fmol
mg⁻¹) of ³H- DAGO (1nM) to rat brain membranes
(single experiment)

		0 washes	3 washes
<u>Control</u>	No ethanol	88	90
	5% ethanol	80	90
<u>2nM NOM</u>	No ethanol	3	2
	5% ethanol	2	6
824nM Naloxone	5% ethanol	4	90

After membranes were preincubated with 2nM NOM for 30 minutes and washed three times, they were assayed for remaining activity of μ -, δ - and κ -binding sites (Fig. 37). ^3H -DAGO or ^3H -DHEM binding was completely suppressed and not restored by washing; ^3H -DADL binding was partly suppressed and could be restored by washing, while κ -binding appeared unaffected. In an attempt to suppress δ -binding fully before washing, the concentration of NOM in the incubation was increased to 10nM. However, although this concentration of NOM did suppress δ -binding further, it was not possible to restore full δ -binding activity after three washes.

Fig. 37

Rat brain membranes : effect of 2nM NOM on specific binding of ^3H ligands



Each histogram represents the mean of 5 observations + or - s.e.m.

Fig. 37 Rat brain membranes : effect of 2nM NOM on specific binding of ^3H -ligands

III.4 DISCUSSION

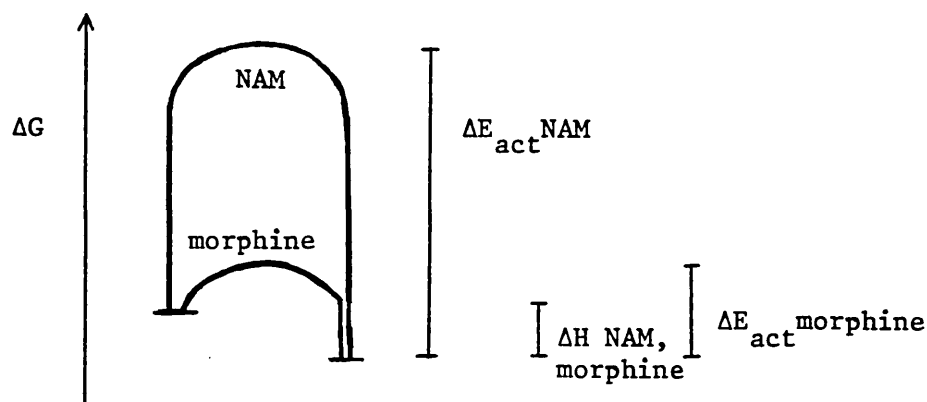
It was possible to photolyse NAM in the absence and presence of the Triton-X-100 filter and there was less photodestruction of opioid binding sites when the filter was present. The ligand synthesized before NAM lacked the o-nitro group (Rance et al, 1981; Fig. 20a) and this was the moiety which allowed photolysis of NAM using higher wavelength UV light (Knowles, 1972). These results confirmed the rationale behind NAM's synthesis, that a ligand photolysable at higher wavelength UV would lead to less photodestruction of opioid binding sites. However, that there still remained a degree of photodestruction even when radiation of wavelength greater than 295nm was used (Fig. 28) shows that opioid receptors in brain membranes are sensitive to longer wavelength UV light as well as short wavelength (254nm) UV light (Glaser & Venn, 1981). Indeed, the only report in which opioid binding has been shown not to be attenuated by radiation used light of visible wavelength to photolyse an o-nitro, p-azido derivative of leu-enkephalin (Zioudrou et al, 1983).

It was useful to have both a partial agonist (NOM) and an antagonist (NAM) in the determination of receptor selectivity of the ligands. Both showed action at opioid receptors in isolated tissues and had apparently no action on other receptor systems. Results from binding studies using brain membranes confirmed and extended these observations. In isolated tissues and membrane preparations NOM and NAM showed very slow onset of

action with no demonstrable offset, and were μ -selective. NAM was a pure antagonist at μ -receptors from which it did not show dissociation during the lifetime of isolated tissue preparations. However, its action at δ -receptors was competitive in nature (Fig. 31b), a result confirmed in binding studies using NOM, when it was shown that it was possible to wash out NOM from brain membrane δ -opioid binding sites.

The selectivity thus expressed for one subtype of opioid receptors is a desirable property for a photoaffinity probe, in this case, of μ -receptors. However, the other obvious property of the ligands, i.e. their slow kinetic behaviour, meant that NAM was not a suitable ligand for photoaffinity labelling of opioid receptors. It was not possible to distinguish blockade of opioid binding sites by photolysed (covalently-bound) NAM, non-photolysed NAM and photodestruction of opioid binding sites, when brain membranes were irradiated in the presence of NAM.

The nature of the prolonged actions of NAM and NOM at μ -receptors suggested that they bound with high affinity to these receptors, and that once bound, they remained associated with the receptors. This observation may be explained by postulating that there is a higher activation energy (E_{act}) for the binding of NAM to μ -receptors than for readily reversible ligands such as normorphine or naloxone (Kosterlitz et al, 1975).



This would explain why naloxone was unable to reverse the effects of NOM, although a high concentration of naloxone was able to delay NOM's onset; and also why NAM's antagonism of normorphine responses was non-competitive. It was not possible to reverse the effects of NOM by washing the tissue, and this observation is paralleled by the finding that a similar compound (p-azidophenyl-ethylnorlevorphanol) showed exceptionally prolonged action in GPI, although the effects of this compound were apparently reversible by naloxone (Winter and Goldstein, 1972).

Because the action of NAM is that of an irreversible antagonist, it could be used as a ligand to denude isolated tissues of μ -receptors in order to study the actions of opioids on κ and/or δ -receptors. However, because of its photosensitive azide moiety, it is not a convenient ligand to use; a more chemically stable analogue would be of greater use. Such a ligand would have advantages over chemical affinity ligands (such as daleck) which require special conditions, and those (such as β -FNA) which show initial agonist action. Also, its action at μ -receptors is irreversible because of its kinetic behaviour at these receptors, i.e. once bound it does not dissociate from

them, while its action at δ -receptors is not irreversible and 143
can be washed out, leaving an unchanged population of
 δ -receptors. However, the irreversible action of chemical
affinity ligands relies on a covalent link occurring between
ligand and receptor for irreversibility, and presumably if a
 μ -selective ligand becomes chemically linked to a κ - or
 δ -receptor it would not be possible to remove it.

Daleck was an affinity ligand whose ability to label opioid
receptors in brain membranes had previously been demonstrated
(Venn & Barnard, 1981). Examination of the relative potency of
daleck in MVD, GPI and MI, and the K_e values of naloxone and
Mr2266 against normorphine, met-enkephalin, ethylketocyclazocine
and daleck, showed that daleck was about 80 times more potent at
 μ -receptors than δ -receptors, with no demonstrable activity at
 κ -receptors. A Schild plot (Fig. 34) showing the effect of
naloxone on daleck's dose-response curve in MVD was a clear
indication that daleck acted preferentially at μ -receptors.
However, as naloxone's concentration increased, the antagonist
was able to block more μ -receptors, so daleck's action at δ -
-receptors was revealed, and is shown by a Schild plot of
gradient less than unity. Similar Schild plots obtained with
bupranolol in guinea-pig tracheal strips have been used to
postulate the existence of two β -adrenoceptor subtypes in this
tissue (Lemoine & Kaumann, 1983).

The conclusion that daleck was a μ -selective ligand in
reversible binding to opioid receptors was also drawn from
binding studies (E.L. Newman, personal communication).
However, although covalent labelling of opioid receptors by

daleck had been demonstrated in brain membranes, it was not demonstrable in isolated tissues; this was in spite of the necessary conditions being reproduced as far as possible, and the incubation time being doubled. The reason for the apparent inability of daleck to label these opioid receptors may be due to different accessibilities of opioid receptors in each system. In brain membrane suspensions, the membrane fragments are small and the conditions uniform throughout the suspension, so that the pH at receptors is that of the bulk phase. However this assumption cannot be made for opioid receptors in MVD, where receptors are presumably located in synaptic clefts within the tissue, where the pH may be different to that of the bulk phase in the organ bath. Hence, daleck would have been unable to react irreversibly with opioid receptors in MVD because its chemically reactive chloromethylketone moiety requires alkaline conditions for covalent reaction (E.L. Newman, personal communication).

The ligands synthesized in the 14- β -amino morphinone series, NOM and NAM, had large groups attached to the 14- β position. These ligands still retained high affinity for opioid receptors, showing that such groups as the o-nitro, p-azidophenyl moiety in NAM at this position have little effect on opioid affinity. However, the slow kinetic behaviour of the ligands, to which their lipophilicity probably also contributed, made them unsuitable photoaffinity probes. Therefore, use was made of the ability to extend the molecule at the 14- β position to synthesize related ligand to couple to agarose beads in the preparation of an affinity column for a soluble preparation of opioid receptors.

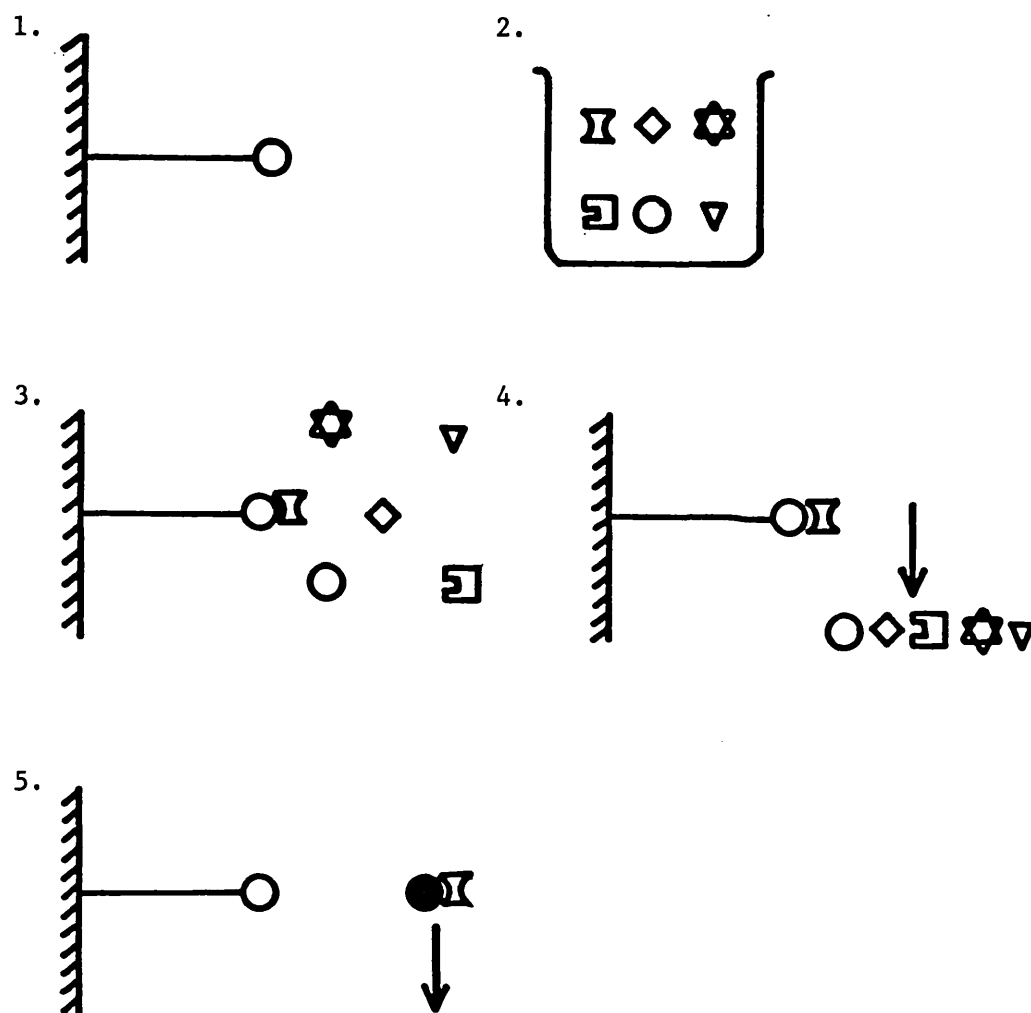
CHAPTER IV

APPROACHES TO AFFINITY CHROMATOGRAPHY OF OPIOID RECEPTORS

IV.1 INTRODUCTION

Affinity chromatography was developed as a technique in the 1950s to isolate antibodies (Campbell et al, 1951; Lerman, 1953). The principles of affinity chromatography are shown in Fig. 38; the technique relies on the specificity of the association of the protein to be purified with the ligand coupled to the column. Examples of such associations include antigen-antibody, lectin-glycoprotein, enzyme-substrate and hormone/neurotransmitter-receptor interactions. An affinity column is set up and has an immobilised ligand attached to a matrix which usually consists of beads (e.g. agarose). A crude soluble preparation of the protein to be purified is applied to the column, and the proteins which possess a binding-site for the immobilised ligand are retained on the column, while the other proteins pass through into the eluant during washing. The retained molecules are then eluted with a solution of another ligand for which they have a higher affinity, or which is in a higher concentration, than the immobilised ligand. This binding must be easily reversible so that the purified protein may then be recovered in active form.

The matrix to which ligands are coupled (immobilised) must be stable to all the conditions used during the affinity chromatographic procedures, and form a loose porous network of inert and uniform rigid beads. Chemically cross linked agarose beads are commonly used.

Fig. 38The principle of affinity chromatography

1. Ligand immobilised to gel
2. Crude solubilised preparation
3. Application of crude preparation to affinity column
4. Desired molecule retained on column, others are eluted
5. Desired molecule eluted by specific ligand (rather than change in pH, ionic strength etc.)

The proteins first purified were soluble proteins (antibodies and cytoplasmic enzymes). However, the development of techniques to solubilise membrane proteins in active form has allowed purification of hormone and neurotransmitter receptors. One of the first such receptors was the nicotinic acetylcholine receptor. In early experiments, it was not easy to elute the receptor from the affinity columns used because of the high affinity of such toxins as α -bungarotoxin from *Naja nigricollis* for the receptor (Meunier et al, 1971). However, use of further toxins such as the α -neurotoxin from the venom of *Naja naja siamensis*, which has a lower affinity for the nicotinic acetylcholine receptor, allowed elution of active receptor using such ligands as gallamine (Dolly & Barnard, 1975). Purification of other receptors has been less easy; reasons for the relative ease of AChR purification include the existence of fish such as *Torpedo* and *Electrophorus* species with electric organs containing a very high concentration of nicotinic acetylcholine receptor, and the existence of ligands of high affinity for this receptor which could be immobilised on an affinity column without loss of binding activity.

Other hormone or neurotransmitter receptors purified by affinity chromatography include the β -adrenergic receptor (Limbird & Lefkowitz, 1977), the CNS optic lobe nicotinic acetylcholine receptor (Norman et al, 1982) and the GABA-benzodiazepine receptor (Sigel et al, 1982). Usually, a solubilised preparation of all membranes would be applied to the column, although partial purification of membrane-bound β -adrenergic

receptor was claimed for small membrane fragments (less than $2\mu\text{M}$ in diameter) applied to a preparation of isoprenaline coupled to glass beads (Venter & Kaplan, 1974). Later, this receptor was solubilised in active form by 0.6% digitonin in the presence of 5mM EGTA, and concentrated on a Sepharose 6B column. Adenylate cyclase (to which β -adrenergic receptors appear to be coupled) was solubilised concurrently, and the components of β -adrenergic receptor binding and adenylate cyclase activity were shown to be separate entities by differential elution of each from the column (Limbird & Lefkowitz, 1977).

Opioid receptors have proved difficult to solubilise in active form. Several detergents have been employed in attempts to purify the receptor. The first report of a soluble opioid receptor was in fact of a soluble ^3H -etorphine-opioid receptor complex, solubilised from rat brain membranes using the detergent Brij 36T (Simon et al, 1975). It was not shown to be possible to liberate active receptor from the complex. There has been a similar report of solubilisation of ^3H -ligand-opioid receptor complex from the membranes of NG-108 cells using Brij 36T. This solubilised preparation was applied to a Sepharose 6B column, and although the radioactivity was eluted from this column, active receptor was not (Dornay & Simantov, 1982).

Other detergents used successfully for solubilisation of other neurotransmitter receptors in active form have not been so satisfactory for solubilisation of active opioid receptors; e.g. Triton-X-100 which has been used to solubilise the

GABA-benzodiazepine receptor (Chang & Barnard, 1982). Although solubilisation of active opioid receptors from rat brain membranes using Triton-X-100 has been reported (Bidlack et al, 1981), it has not proved possible to repeat this (e.g. Ruegg et al, 1982 and C. Demoliou, this laboratory, unpublished data).

Triton-X-100 in conjunction with sonication has also been used to prepare a soluble extract of rat brain membranes.

Purification of this extract was attempted using an affinity column consisting of a morphine derivative coupled through a spacer arm to a matrix of agarose beads. However, it was not possible to determine the binding activity of the solubilised preparation, so it is difficult to estimate the success of the procedure (Cho et al, 1983).

Digitonin, used to solubilise the β -adrenergic receptor (Limbird & Lefkowitz, 1977) has proved useful to solubilise active opioid receptors from toad brain (Ruegg et al, 1981) and from rat brain, in the presence of 1M sodium chloride which inhibits opioid binding to the soluble receptor (Howells et al, 1982). However, digitonin can solubilise active opioid receptors from rat brain in the absence of 1M sodium chloride, but in the presence of 50mM magnesium sulphate, 1mM EDTA and protease inhibitors, as will be illustrated in this thesis.

The novel zwitterionic detergent 3-(3-cholamidopropyl) dimethylammonio-1-propanesulphonate (CHAPS) has been used to solubilise rat brain opioid receptors (Simonds et al, 1980) and

GABA-benzodiazepine receptors (Stephenson & Olsen, 1982), in active forms. More recently, CHAPS has been used to solubilise κ - and μ -binding activity from rat brain, using a method involving precipitation and resuspension of proteins from the supernatant obtained after solubilisation with CHAPS (10mM or 13mM) and centrifugation (Chow & Zukin, 1983; Quirion *et al*, 1983).

Although the above preparations of solubilised opioid receptors have been purified using chromatography, the purification procedure has not been based on affinity chromatography. There have been two reported attempts to use affinity chromatography (mentioned above) and both these have used derivatives of morphine coupled to an inert matrix through positions of the morphine molecule which are not important for binding to receptors, i.e. the 14- and 6- positions. The first of these used 14- β -bromacetamidomorphine coupled to ω -aminohexyl sepharose. The crude Triton-X-100 extract of rat brain membranes was applied to the column and the non-specifically bound proteins eluted with 50mM Tris-HCl pH 7.5. The specifically bound proteins were eluted with 1M levorphanol or etorphine (Bidlack *et al*, 1981). In theory, this procedure constitutes true affinity chromatography.

The second attempt (Cho *et al*, 1983) used 6-succinylmorphine coupled to an Affigel-102 support. A crude soluble extract of rat brain was applied to the column and non-specifically bound proteins were eluted with Tris buffer. However, binding activity was not apparently eluted from the column when an

opioid ligand was used, but by a salt gradient, giving two protein peaks, 'A' and 'B'. 1mM naloxone was unable to elute any further receptor protein. There was no demonstrable binding activity in A or B until acidic lipids which had been eluted from the column using organic solvents were added to A, producing stereo specific binding of μ -type ligands to the protein-lipid mixture. K_d values were about two orders of magnitude greater than those for membrane-bound receptor (Cho et al, 1983).

There have been no successful attempts to repeat the result described in the first report, and the second attempt suffered from the drawback that it was not possible to measure the amount of binding activity of the soluble preparation.

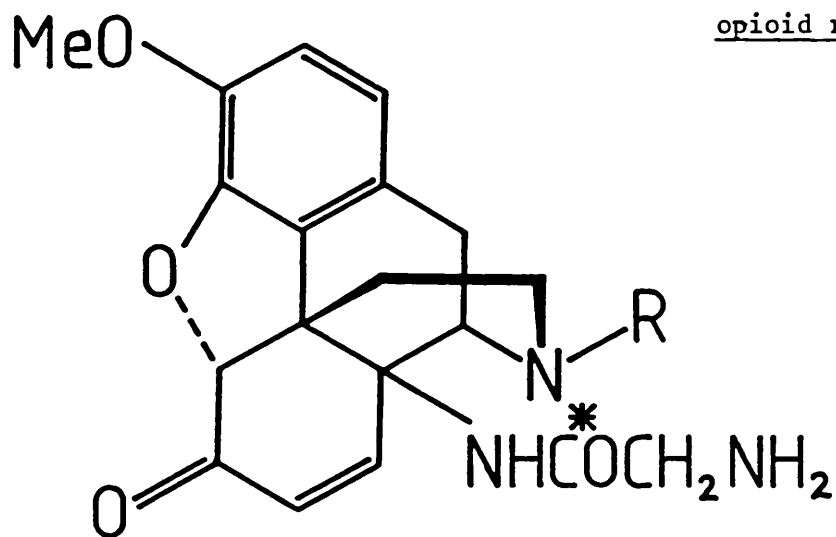
In the design of an affinity column, the choice of ligand to be immobilised is important:

1. The ligand must bind to the biologically relevant receptor.
2. The ligand must be able to be covalently linked to the matrix via a biologically irrelevant part of the molecule without significant loss in activity.
3. It should be shown that the immobilised ligand still retains its biological activity.
4. The amount of ligand released from the support should be too low to produce a biological response.

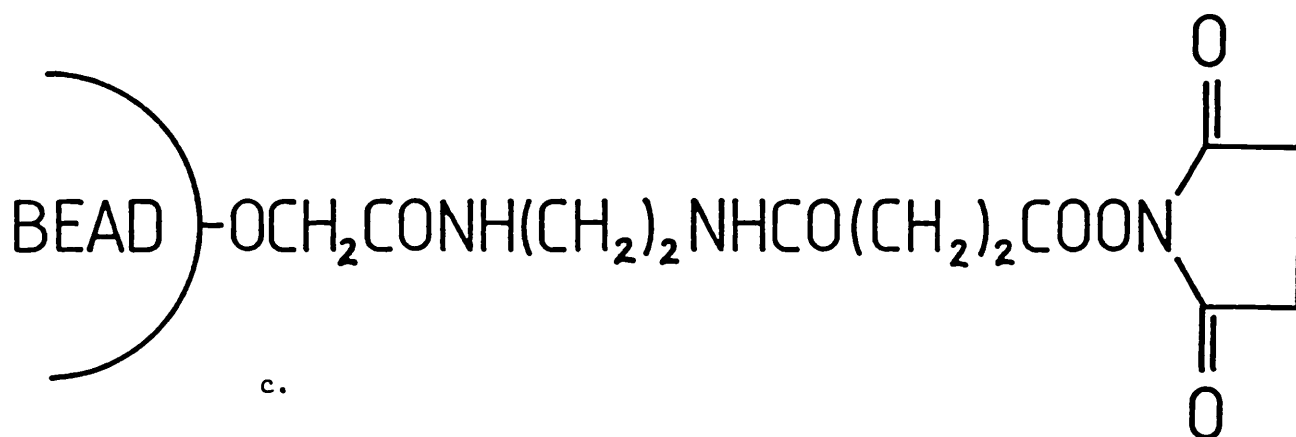
5. The affinity of the immobilised ligand for the soluble protein should be neither too low (not retained) nor too high (not eluted). (Venter, 1982).

The ligand used for the following studies was chosen on the basis of knowledge gained about the 14- β -aminomorphinone series (Ch. III). It had been shown that compounds in this series, with various large substituents at the 14- β position, bound with high affinity to opioid receptors in isolated tissues and brain membrane preparations (Rance *et al*, 1981; this work, Chapter III). Therefore, a link through this position to the matrix was not likely to change significantly the biological activity of the ligand. However, since the 14- β substituted morphinones showed such high affinity for opioid receptors, it was decided to use derivatives of 14- β -aminocodeinone so that the affinity of receptor proteins for the immobilised ligands would not be too great to preclude elution from the column. Because these ligands are lipophilic, and non-specific hydrophobic binding of proteins to affinity columns can cause difficulties in elution (Lowe & Dean, 1974; Scouten, 1981), a hydrophilic 'link' was included between the chosen ligand and the matrix (Fig. 39a, b). This consisted of a glycine molecule (Koman & Terenius, 1980) labelled with a ^{14}C -atom so that it would be possible to measure directly the incorporation of ligand into the column. The matrix chosen was Affigel-10 which consists of chemically cross-linked agarose beads with a neutral 10-carbon spacer to an N-hydroxysuccinimide ester moiety (Fig. 39c). Ligands with primary amino groups can be coupled to this support easily and

Fig. 39 Ligands synthesised for affinity chromatography of
opioid receptors.



a. b.



c.

a. R = N-cyclopropylmethyl : GANC
 (14-β-(¹⁴C-glycyl)-amido-(N-cyclopropylmethyl)-norcodeinone)

b. R = methyl : GAC
 (14-β-(¹⁴C-glycyl)-amido-norcodeinone)

c. Affigel-10

effectively for use in affinity chromatography (e.g. Kanellopoulos et al, 1979).

155

As in Ch. III, more than one ligand was synthesized, a putative agonist and antagonist:

14- β -(^{14}C -glycyl)amidocodeinone (GAC; agonist; Fig. 39b) and 14- β -(^{14}C -glycyl)amido(N-cyclopropylmethyl)norcodeinone (GANC; antagonist; Fig. 39a). The reason for this was that it had been proposed that there might be differential binding of opioid agonists and antagonists to soluble opioid receptors (Howells et al, 1982); however, recent studies have not shown differential binding of opioid ligands on the basis of their agonist or antagonist activities as will be illustrated in this thesis, and unpublished observations, C. Demoliou. Also, the biological activities of the ligands to be used were studied first in the non-immobilised form, in isolated tissues and brain membrane preparations to determine their receptor selectivity and approximate affinities for membrane-bound opioid receptors. The ligands were then coupled to the matrix for use in affinity chromatography of solubilised opioid receptors.

IV.2 METHODS

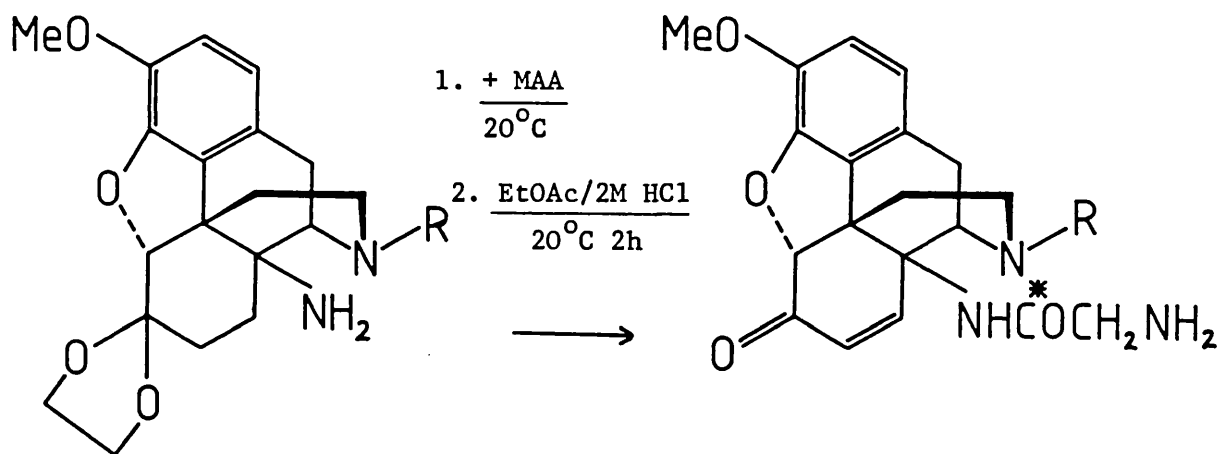
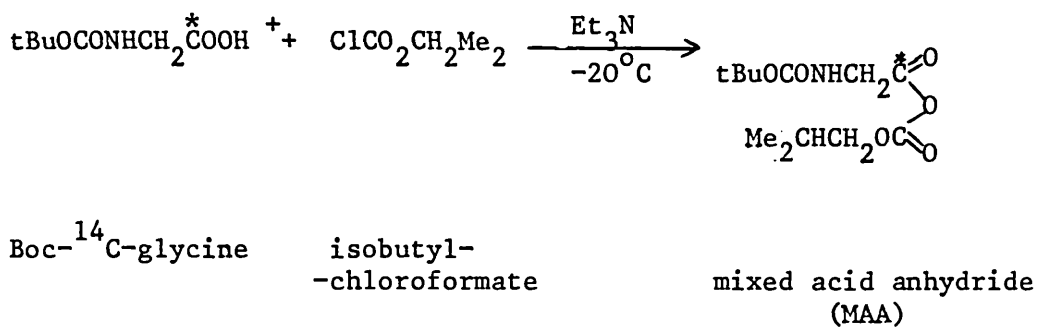
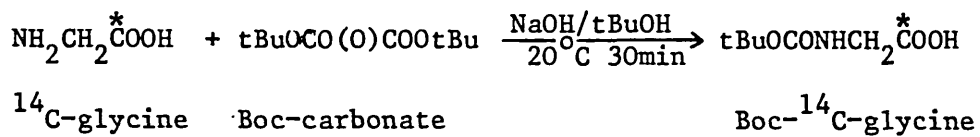
1. Synthesis of 14- β (^{14}C -glycyl)amido(N-cyclopropylmethyl)-norcodeinone (GANC) and 14- β (^{14}C -glycyl)amido-codeinone (GAC)

The scheme for the syntheses of each of the above was the same and is shown in Fig. 40. As well as preparing radiolabelled GANC and GAC, the unlabelled compounds were prepared for use in isolated tissues and membrane binding assays.

^{14}C -L-glycine was obtained from Amersham International at a specific activity of 56mCi mmol^{-1} ; it was diluted about 1400-fold with unlabelled glycine to yield a specific activity of 40 Ci mmol^{-1} .

1. Synthesis of boc- ^{14}C -glycine

t-Butyl alcohol (7ml) and water (2.8ml) were mixed in a round-bottomed flask. Sodium hydroxide (550mg) was added and the mixture stirred, before addition of ^{14}C -L-glycine (0.69mg) and unlabelled L-glycine (938mg). The resulting mixture was turbid and oily. Di-tertbutyl-dicarbonate (Boc-carbonate, 3g) was added slowly, over 75 mins, maintaining the temperature between 20° and 30°C . The mixture was stirred overnight at room temperature. The reaction was followed by t.l.c. in CMAW (chloroform:methanol:acetic acid:water, 60:18:2:3). When the reaction was judged complete, water (5ml) was added to dilute

Fig. 40 Synthesis of GAC and GANC

R = methyl : GAC

R = N-cyclopropylmethyl : GANC

the solution, and the aqueous layer was washed 3 times with petrol (60°C-80°C boiling fraction, 10ml).

The aqueous layer was acidified to pH3 with citric acid (s) and product extracted into ethyl acetate (30ml). This organic layer was washed with water (10ml) and saturated brine (10ml), then dried over sodium sulphate (s) before evaporating to dryness under reduced pressure to an oil. The oil was triturated in petrol (20°C-40°C boiling fraction) to give fine white crystals in a yield of 61% (1.35g).

2. Preparation of mixed acid anhydride

Isobutylchloroformate (0.3ml, 1.1 mole equivalent) was added to a mixture of triethylamine (0.32ml, 1.1 mole eq.) in DCM (20ml) at -20°C. Boc-¹⁴C-L-glycine (400mg) was added over 10s, and the resulting colourless solution stirred at -20°C for 30 mins. This solution was used immediately in the following reaction.

3. Coupling of Boc-¹⁴C-L-glycine to 14 β aminocodeinone-ethylene ketal

14- β -aminocodeinone ethylene ketal (762mg) was dissolved in 5ml DCM and added dropwise to the above solution. The temperature was allowed to rise to 20°C and the reaction followed by t.l.c. run in DM10. After 15 mins, the reaction was judged complete and the solution was washed twice with water, dried

over sodium sulphate (s) and evaporated to dryness under reduced pressure. The resulting product was eluted from a 10cm x 2cm alumina column (70-230 mesh) with DCM. Fractions containing the pure product were combined and evaporated to dryness to give pure product in a yield of 80% (640mg).

4. Hydrolysis of 14- β -(^{14}C -Boc-L-glycyl) amidocodeinone ethylene ketal

14- β -(^{14}C -Boc-L-glycyl) amidocodeinone ethylene ketal (600mg) was dissolved in 2M HCl/ethylacetate (6ml) and water (1.5ml) added. The mixture was stirred overnight at room temperature. T.l.c. showed disappearance of starting material; the mixture was diluted into 40ml water and neutralised, ($\text{NaHCO}_3(\text{s})$), and product extracted with DCM (4x20ml). The organic layer was dried over sodium sulphate (s) and evaporated to dryness under reduced pressure to give an oil. After trituration in ether, cream-coloured powdery crystals were obtained in a yield of 55% (250mg).

NMR, IR and MS showed that this product was 14- β -(^{14}C -glycyl)amidocodeinone, and UV-HPLC showed it was 96% pure.

The methods of synthesis of 14- β -(^{14}C -glycyl)amido(N-cyclopropylmethyl)norcodeinone (GANC) and the unlabelled analogues of GAC and GANC were the same and the products were of a similar purity (in all cases $> 95\%$).

2. Bioassay of GAC and GANC

The MVD and GPI were used as described in chapter II to estimate the potencies and receptor selectivity of GAC and GANC, with the exception that DADL was used as standard δ -agonist. The behaviour of the compounds was studied for agonist and antagonist properties, and in the presence of such selective antagonists as naltrexone (8nM, 26nM) and ICI-154,129 (4 μ M) to determine receptor selectivity. The rat vas deferens was also used to examine the antagonism of μ -selective enkephalin responses by GAC and GANC, since it had been suggested that morphine and related compounds showed antagonism of μ -receptors in this tissue (Smith & Rance, 1983). Aspects of this work were carried out by Dr. C.F.C. Smith at Reckitt and Colman.

3. Membrane binding assays with GAC and GANC

The membrane preparation described in chapter II was used, and the standard μ - and δ -agonists ^3H -DAGO, ^3H -naloxone and ^3H -DADL (all 1nM) were used to demonstrate the effect of increasing concentrations of GAC and GANC (0-10 μ M) on μ - and δ -binding activities.

4. Solubilised receptor binding assays with GANC

Solubilised opioid receptors were prepared and assayed for opioid ligand binding activity as described in chapter II. To study the ability of the compounds to inhibit binding of opioid

ligands to the solubilised preparation, ^3H -naloxone (c.10nM) or ^3H -etorphine (c.5nM) was incubated with the solubilised receptor in the presence of increasing concentrations of GANC(0-500 μM).

5. Incorporation of ^{14}C -GANC into Affigel-10

The method described by the manufacturers (Biorad) was followed. A pilot experiment to examine the most efficient of several buffer systems was carried out and 0.1M Hepes pH7.5 was chosen. The suspension of Affigel-10 (25ml) was shaken, and filtered under reduced pressure to give a moist gel cake which was washed 3 times with ice-cold isopropanol (25ml), 3 times with deionised water (25ml) and twice with 0.1M Hepes buffer pH7.5 (25ml). The moist gel cake was then transferred to a centrifuge tube and 0.1M Hepes pH7.5 (24ml) added with ^{14}C -GANC to a final concentration of 0.5mM. This mixture was incubated at 4°C for 4 hours on a rotating wheel before addition of 2.4ml 1M glycine to block unreacted groups on the gel. The gel was incubated a further hour at 4°C on the wheel before filtering under reduced pressure. The gel cake was copiously washed to remove uncoupled GANC with first the Hepes buffer, followed by deionised water and methanol. The coupled gel was stored in 10mM Tes-KOH pH 7.4 containing 0.1% digitonin at 4°C .

The incorporation of ^{14}C -GANC into the gel was first estimated by scintillation counter. Increasing aliquots of coupled gel or

^{14}C -GANC in the presence of an equivalent amount of blocked gel (to estimate quenching by the gel), were added to a xylene-Triton scintillant (18g PPO, 11 Triton-X-100, 31 xylene) and ^{14}C -radiation counted in an Intertechnique scintillation counter for 10 mins. To obtain a more precise estimate of the incorporation of ^{14}C -GANC into the gel, aliquots of coupled gel were burnt in a tissue oxidiser (Intertechnique IN 4101), and radioactive gases counted in scintillant (phenylethylamine, scintillation grade (330ml), absolute methanol (220ml), toluene (400ml), distilled water (50ml), butyl PBD (7g)). ^{14}C -hexadecane standards (Amersham) were used to estimate the efficiency of the processes.

In order to show that GANC coupled to Affigel-10 was still able to block membrane-bound opioid receptors, membranes (c.150 μg protein) were incubated with GANC-gel (100 μl) in a total volume of 1ml for increasing lengths of time; membranes were then assayed for remaining ^3H -DAGO or ^3H -naloxone binding activity. The incubation periods were for up to 4 hours at 18°C on a revolving wheel. Control experiments in which membranes were incubated with gel alone (Biogel A-5M, 100 μl) and/or GANC alone (33 μM) were also carried out.

6. Use of GANC-gel in column chromatography of solubilised opioid receptors

The initial aim was to demonstrate a decrease in binding activity of solubilised receptor after passage over the coupled

gel, compared to the same preparation of solubilised receptor before passage over the gel. Since the column set up would first be used to concentrate the solubilised receptor rather than separate it from other solubilised proteins, a short wide column was used rather than a long narrow one. The volume of coupled gel chosen for the column was 2ml, and this had a theoretical capacity far in excess of the amount of solubilised receptor available. The column was set up as shown in Fig. 41 in a 5ml syringe containing a sinter support for the gel and washed several times with the buffer to be used for diluting the solubilised receptor and with the addition of 0.1% digitonin.

Since the solubilised receptor was known to bind opioid ligands at a digitonin concentration of 0.1%, the concentrated stock of solubilised receptor containing 1% digitonin was diluted 10-fold with buffer (50mM NaCl, 50mM KCl, 10mM MgCl_2 , 1mM EGTA, 10mM Tes-KOH pH 7.4). A sample of the diluted preparation was always taken before passage over the column. The protein concentration in the diluted preparations was between 120 and $150 \mu\text{g ml}^{-1}$.

The diluted solubilised receptor preparation was passed over the column at a steady rate by a Pharmacia peristaltic pump P-1, and the eluant collected in fractions for assay of remaining binding activity. For this, ^3H -naloxone or ^3H -etorphine (5-10nM) was added to 0.5ml aliquots of fractions, in the presence or absence of excess unlabelled ligand, and to a final volume of 0.6ml. All fractions collected were assayed together. The

Peristaltic
pump
(variable rate)

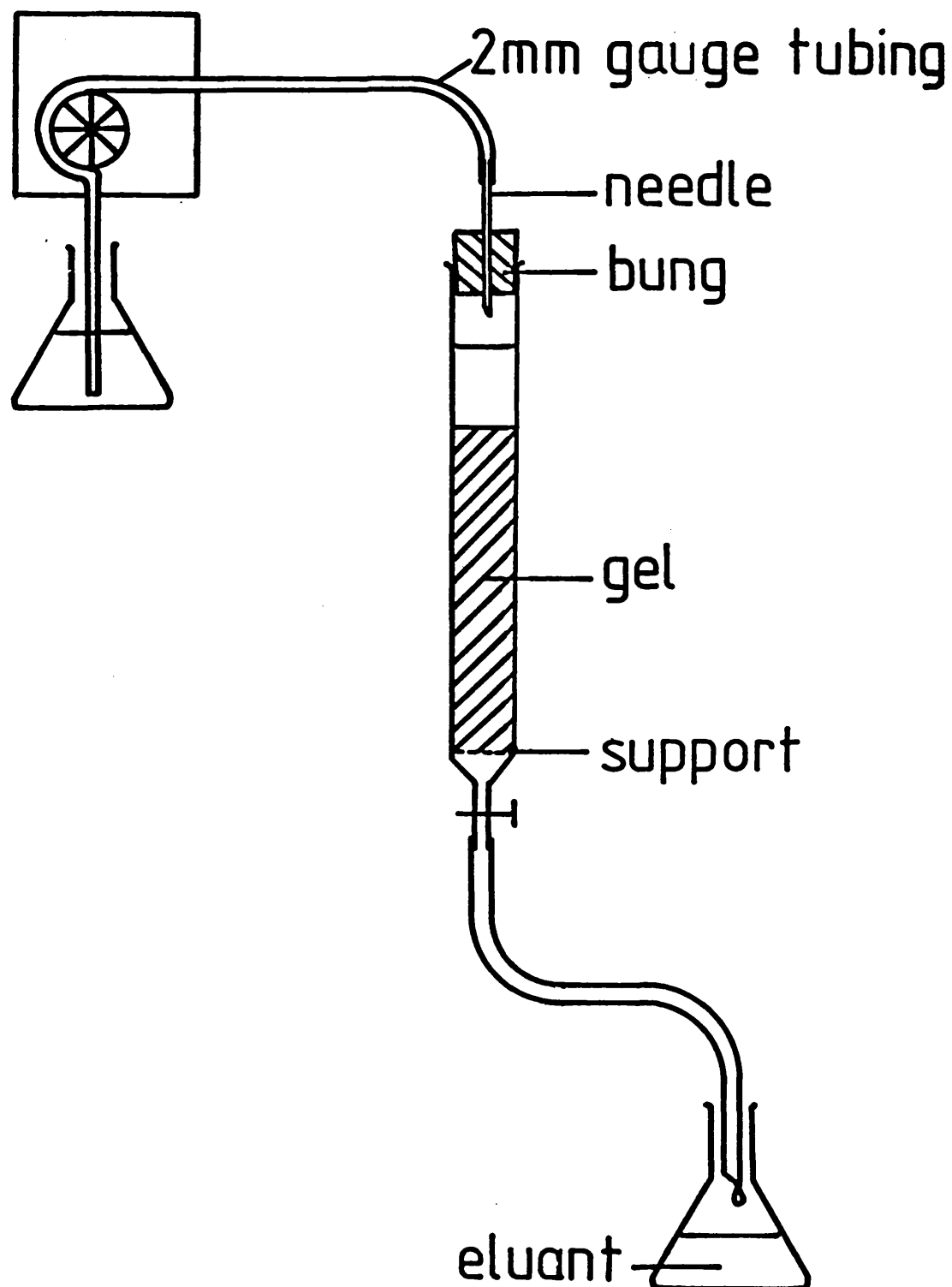


Fig. 41

Apparatus used in affinity chromatography of
solubilised opioid receptors

experiments using the column were carried out at room temperature (which was at one time around 30°C).

The first attempt to concentrate the solubilised receptor on the column used 40ml diluted preparation and a flow rate of 40ml hr⁻¹ for passage over the column. 2.2ml fractions were collected for assay; the column was washed with buffer containing 0.1% digitonin and fractions from the washing were also assayed.

Later modifications involved slower rates for passage of the solubilised receptor over the column and also passing the solubilised receptor preparation over the column twice.

In a different approach, the coupled gel was incubated with solubilised receptor at a concentration of 0.1% digitonin for 16 hours at 4°C on a revolving wheel. A control sample was incubated on the wheel in the absence of coupled gel, although the sample was not diluted to the same extent as the experimental sample.

IV.3 RESULTS

1. Actions of GAC and GANC at opioid receptors in bioassay

Both compounds were partial agonists in MVD and GPI; however, the amount of depression of twitch achieved varied between preparations, the maximum effect being between 20% and 70%. In those tissues which responded with a depression of twitch of 50% or more, GAC and GANC were about twice and 100 times respectively more potent than morphine, their ED_{40} values being about 100nM and 2nM respectively.

In MVD, the μ -selective antagonist naltrexone shifted the dose response curves of GAC and GANC to similar extents, i.e. almost as much as it shifted the normorphine dose-response curve, and considerably more than it shifted the DADL dose-response curve (Table 8). Use of the δ -selective peptide antagonist ICI-154,129 showed the reverse: GAC, GANC and normorphine dose-response curves showed no shift, while the DADL dose-response curve shifted about ten-fold (Table 8).

In RVD, both GANC and GAC, like morphine, were weak antagonists of the effects of DAGO. Their K_e values against DAGO were about 1 μ M and greater than 10 μ M respectively (morphine approx. 1 μ M). Thus there existed a discrepancy in the potencies of the compounds estimated as agonists and as antagonists.

The effects of both GAC and GANC were readily reversed by washing tissues twice, unlike those of NOM and NAM (Chapter III).

TABLE 8 Relative shifts in ED₄₀ of normorphine, DADL and GANC
to naltrexone and ICI-154,129 (MVD)

	<u>Antagonist</u>		
	Naltrexone 8nM	Naltrexone 26nM	ICI-154,129 4 μ M
<u>Agonist</u>			
Normorphine	12	40	1
DADL	1.5	3	10
GANC	10	30	1

Each value represents the mean of 3 observations in 3 separate preparations

2. Membrane binding assays with GAC and GANC

GAC and GANC were able to inhibit the binding of ^3H -DAGO to opioid receptors in brain membranes with IC_{50} values of 300nM and 40nM respectively (Fig. 42a). Because GAC showed relatively low affinity for opioid receptors, both in isolated tissues and in brain membranes, it was not studied further. GANC was also able to inhibit the binding of ^3H -naloxone to brain membrane opioid receptors with an IC_{50} value of 80nM, similar to its IC_{50} value against ^3H -DAGO. In its inhibition of ^3H -DADL binding, GANC showed two components with IC_{50} values of about 40nM and $30\text{ }\mu\text{M}$ (Fig. 42b).

3. Solubilised receptor binding assays with GANC

The behaviour of the solubilised opioid receptors (prepared as described in Chapter II) was different to that of membrane-bound opioid receptors. The assay system for solubilised receptors was less precise than that for membrane-bound receptors i.e. there was more variation in results when measuring binding of ^3H -ligands to the solubilised receptors. However, it was clear that ^3H -peptides, whether μ - or δ -selective, were not able to bind specifically to solubilised receptor preparations under any of the conditions studied. Alkaloids such as ^3H -etorphine and ^3H -naloxone, an agonist and an antagonist, did however show specific binding to solubilised opioid receptors, with affinities five- to ten-fold lower than for membrane-bound opioid receptors.

Fig. 42a

Rat brain membranes: inhibition of ^3H -DAGO
specific binding by GANC

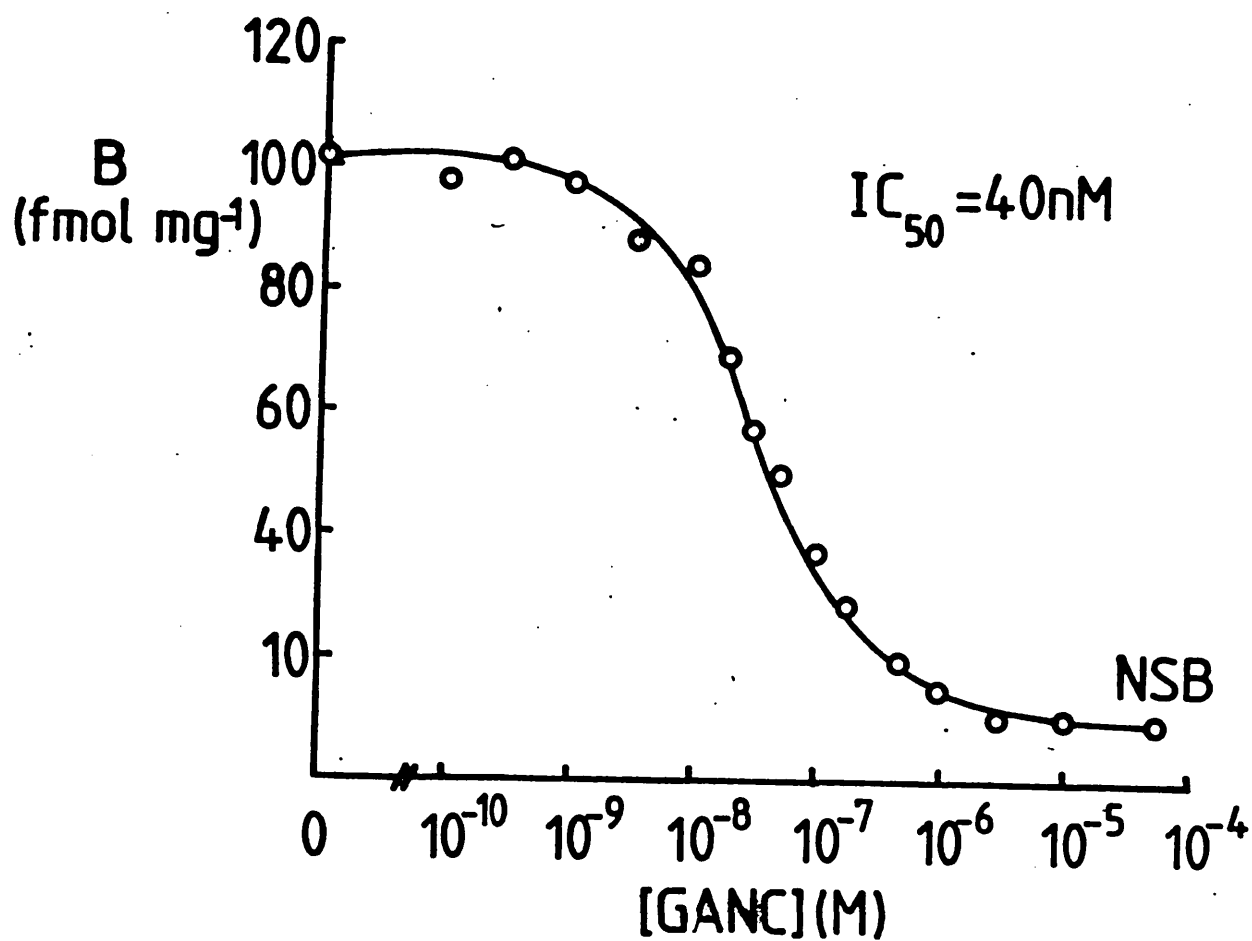


Fig. 42b
Rat brain membranes: inhibition of ^3H -DADL
specific binding by GANC

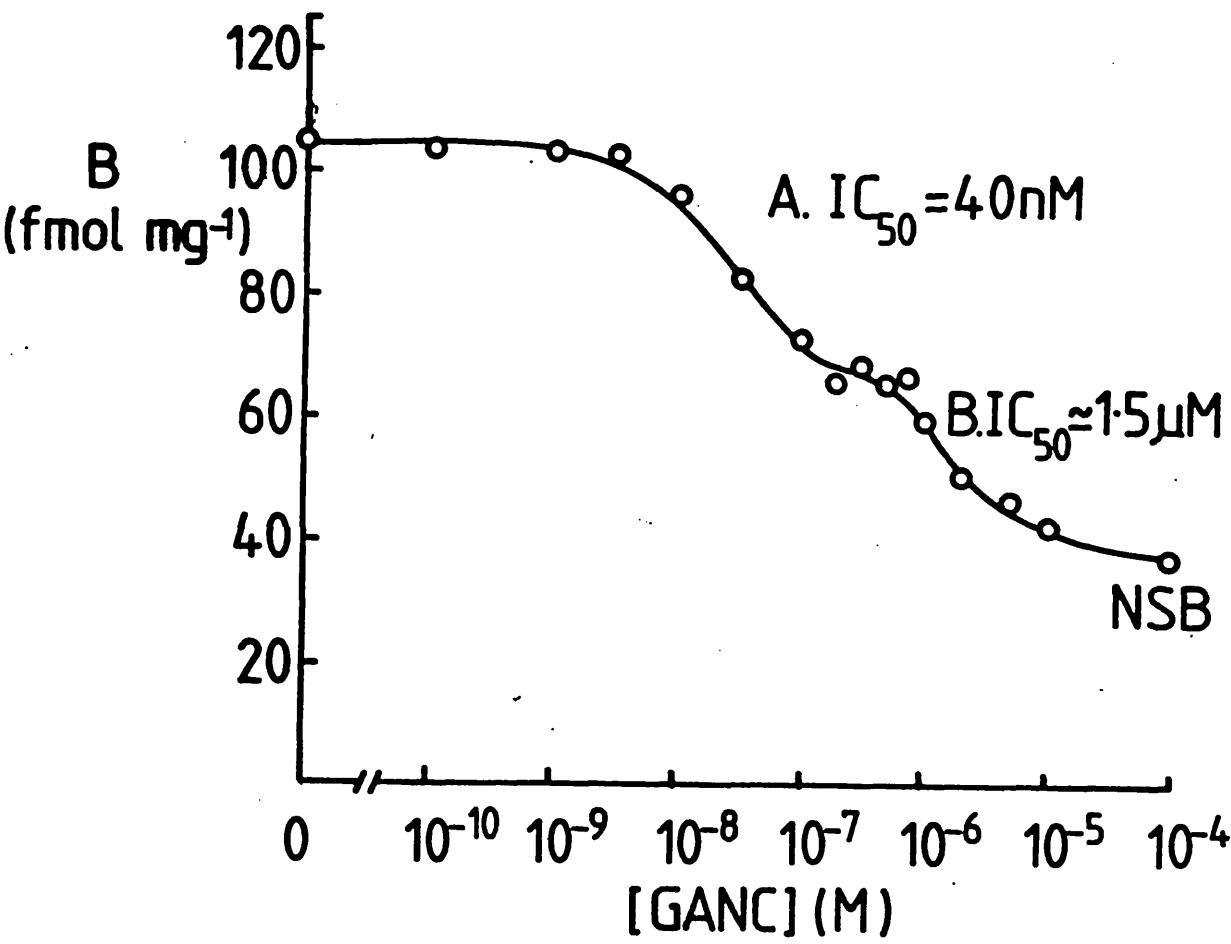
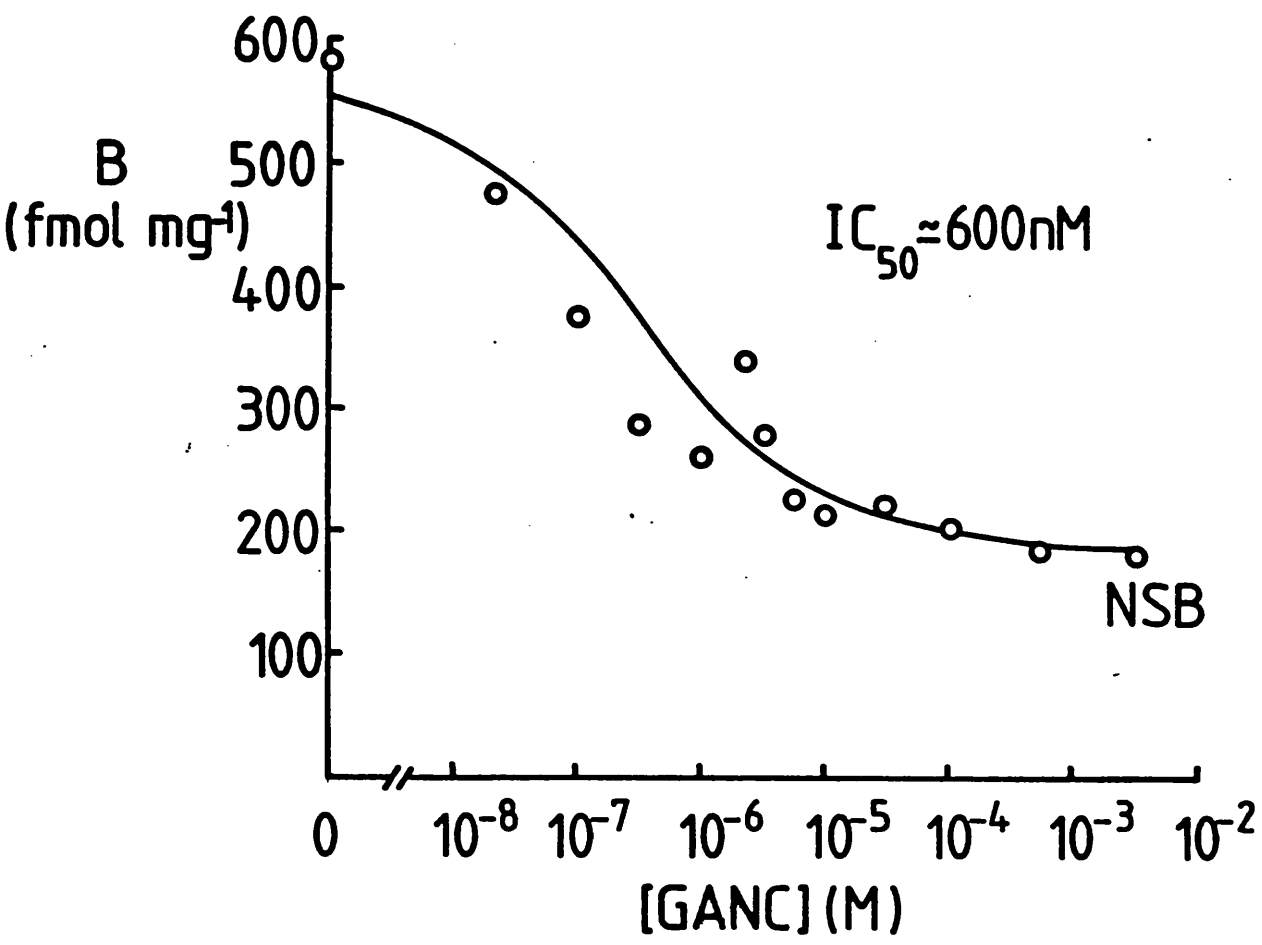


Fig. 43

Solubilised opioid receptors: inhibition of
 ^3H -naloxone binding



GANC was able to inhibit the binding of ^3H -naloxone and ^3H -etorphine to solubilised receptors, although the scatter in the results of such experiments made it more difficult to estimate an IC_{50} value for solubilised than for membrane-bound opioid receptors. However, the IC_{50} values were of the order of ten times higher for solubilised than membrane-bound receptors (Fig. 43), a result consistent with the approximately ten-fold lower affinities of ^3H -alkaloids for solubilised receptors compared to membrane-bound receptors.

4. Incorporation of ^{14}C -GANC into Affigel-10

When the degree of incorporation of ^{14}C -GANC into Affigel-10 was estimated, allowance was made for quenching by the (agarose) Affigel beads by the inclusion of an equivalent amount of blocked gel with uncoupled ^{14}C -GANC in 'control' vials. However, ^{14}C -GANC was presumably distributed evenly in the scintillant in the control vials while being held in a slurry of Affigel-10 to which it was attached in the other tubes. Therefore a strict comparison between counts from the two sets of tubes could not be made, although results indicated an incorporation of more than 75%. In order to obtain a more exact measure of incorporation, aliquots of coupled GANC and free GANC in the presence of blocked gel were combusted. This process showed that incorporation of ^{14}C -GANC into the gel was 90-95%.

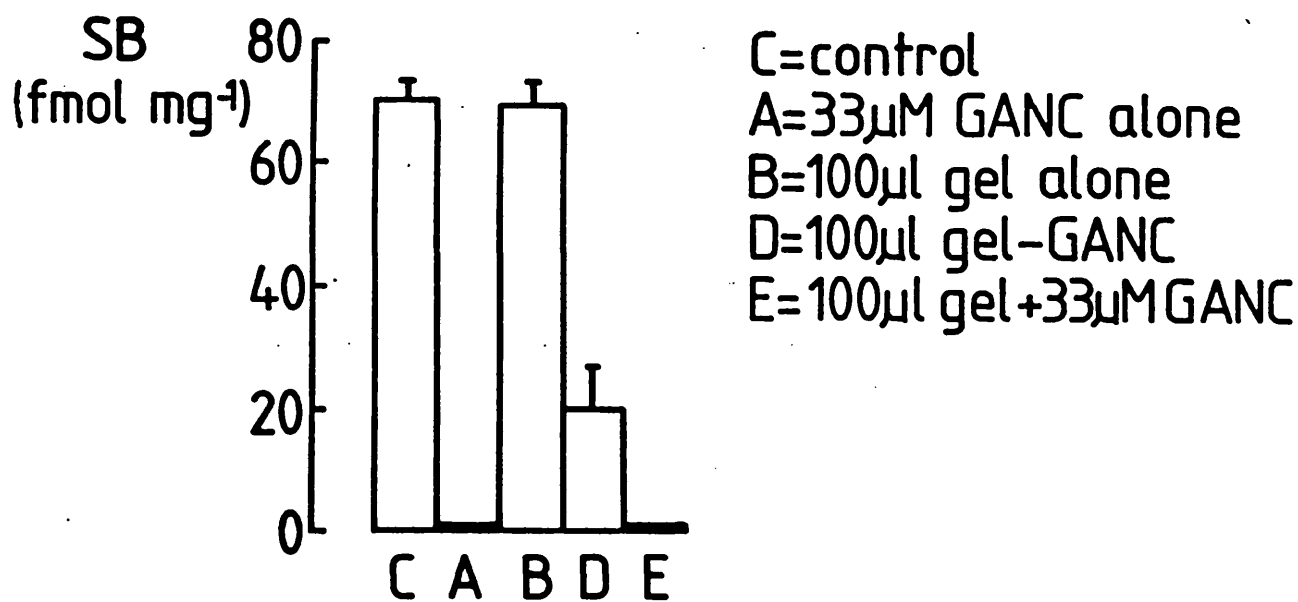
The GANC-gel was also tested for its ability to inhibit binding of ^3H -DAGO and ^3H -naloxone to membrane-bound opioid receptors (Fig. 44). Free GANC in presence and absence of gel was able to inhibit all specific binding of ^3H -DAGO or ^3H -naloxone (data not shown) to membrane-bound opioid receptors. Gel alone had no effect on either specific or non-specific binding, while GANC-gel was able to inhibit specific binding of the ^3H -ligands, although not completely. This was not unexpected since the ligand was now coupled to large agarose beads. Lengthening incubation time to 4 hours did not significantly increase the degree of inhibition.

5. Use of GANC-gel in chromatography of solubilised opioid receptors

The initial step in using GANC-gel as an affinity column for purification of opioid receptors was to demonstrate a decrease in specific binding activity of a solubilised opioid receptor preparation after passage down a column of the coupled gel. At the same time, the preparation itself should be shown not to lose such activity when not exposed to the column, i.e. the inhibition seen was not because of, for example, proteolysis or progressive denaturation. However, first attempts to demonstrate such a decrease in specific binding activity of solubilised opioid receptors after passage down a GANC-gel column were not successful, in spite of various manipulations of the conditions under which solubilised receptors were exposed to the gel. These manipulations included altering the flow rate

Fig. 44

Rat brain membranes: inhibition by GANC-gel
of ^3H -DAGO specific binding



and temperature, and passing the solubilised receptor down the column twice. An example of a typical result is shown in Fig. 45. In this experiment, room temperature was 30°C, flow rate 40ml hr⁻¹, volume of GANC-gel 1ml (capacity 400 nmoles GANC), and approximately 500 fmoles of solubilised receptor in a volume of 20ml buffer at a digitonin concentration of 0.1% were passed down the column. Aliquots (2ml) of eluant were taken and assayed for specific binding of ³H-etorphine (6nM). There was little effect on binding activity by passage of the preparation down the column.

When the coupled gel was incubated with solubilised opioid receptors on a rotating wheel at 4°C for 16 hours, there was a decrease in specific binding of ³H-ligands to the solubilised preparation. However, a 'control' preparation kept under the same conditions also showed a decrease in specific binding, and although this was less than for the sample incubated with the gel, it was not significantly different.

Experiments by Dr. Demoliou-Mason of this laboratory are continuing in an attempt to find conditions under which solubilised opioid receptors are able to bind to the GANC column. Modification of the method for preparation of solubilised opioid receptors has resulted in a solubilised preparation which is able to bind μ - and δ -selective enkephalins, in contrast to the preparation described and used in experiments described in this thesis; experiments using the new preparation are in progress.

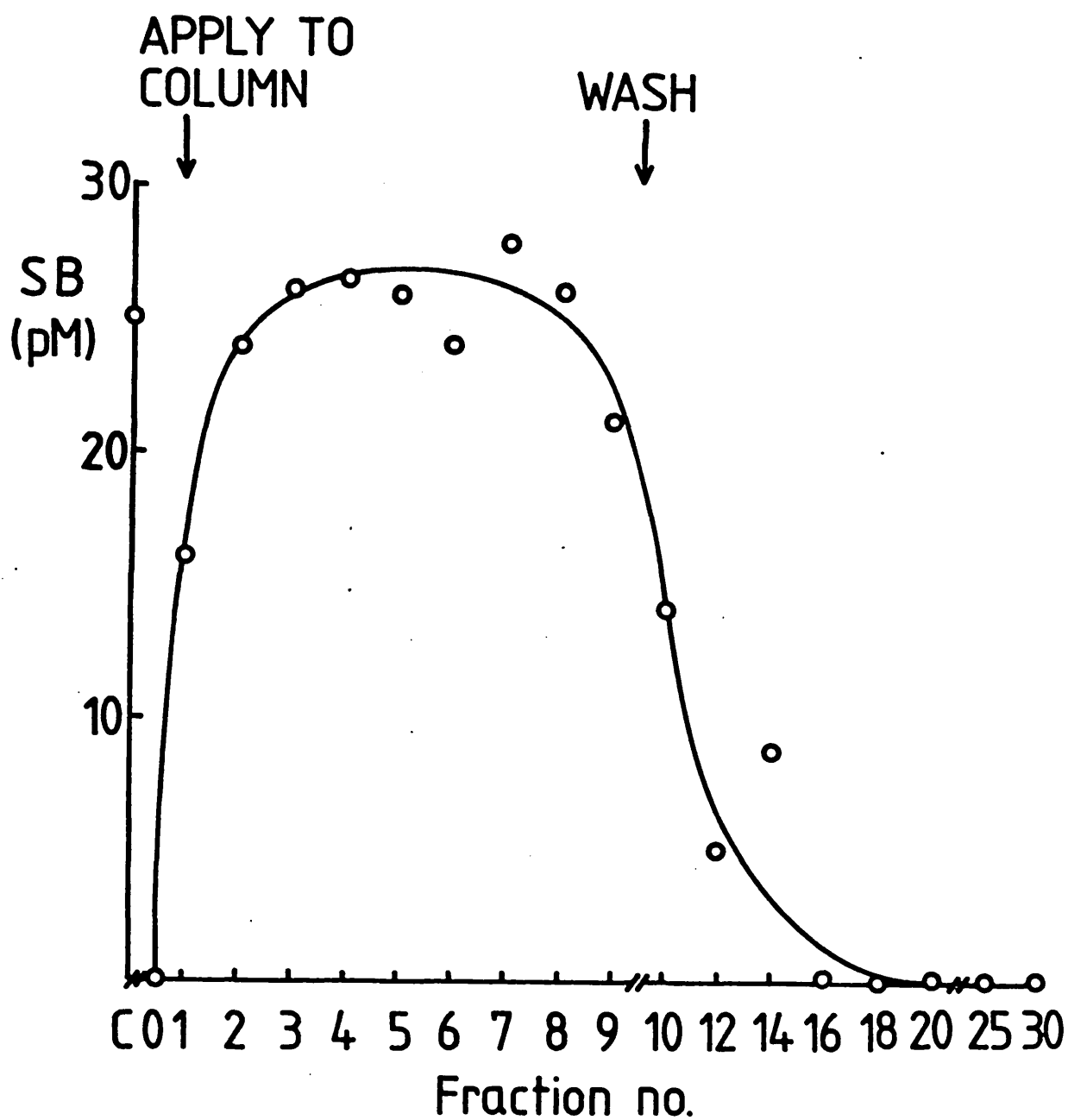


Fig. 45 Elution profile of solubilised opioid receptor
binding collected from GANC-column

IV.4 DISCUSSION

The ligand synthesized, GANC, possessed several properties which indicated that it could be used successfully in affinity chromatography of solubilised opioid receptors. GANC showed high affinity for membrane-bound opioid receptors in isolated tissues and brain membranes. In spite of having a methoxy group at position 3 (i.e. a derivative of codeine), GANC showed a higher potency than normorphine at receptors in MVD. In RVD on the other hand, its potency was unexpectedly low; one explanation might be that the glycyl side chain predisposed the molecule to metabolism in this tissue (C.F.C. Smith, personal communication). However, apart from this inconsistency, GANC showed high affinity for membrane-bound opioid receptors in isolated tissues, indicating that the introduction of the glycyl side-chain at position 14 did not decrease the affinity of the molecule for opioid receptors. This was not unexpected, since neither did the larger o-nitro, p-azido cinnamoyl side-chain at the 14-position in NAM decrease the affinity of that molecule. The receptor profile was not altered either, with GANC showing μ -selectivity; the molecule was not especially lipophilic, a useful property in a ligand for use in affinity chromatography.

GANC retained affinity for opioid receptors in brain membranes when coupled to Affigel-10. The ability of an immobilised ligand to inhibit the specific binding of ^3H -ligands to membrane-bound opioid receptors has not been reported before, and although membrane fragments containing opioid receptors

have been shown to be retained on a column, the specificity of the interaction was uncertain (H.H. Loh, personal communication). That the immobilised ligand inhibited specific binding to opioid receptors in brain membranes suggested that GANC retained biological activity when it was coupled to the gel, a prerequisite for success in affinity chromatography (Venter, 1982). It would have been difficult to design experiments to determine the change in affinity brought about by immobilisation of GANC, although it would most probably be decreased (Lowe & Dean, 1974). It was sufficient to show that the coupled gel, later to be used as a column, could inhibit the specific binding of ^3H -ligands to membrane-bound opioid receptors.

There was a report of a similar immobilised ligand (6-succinyl-morphine coupled to Sepharose 4B) removing antibodies to morphine from solution, whereas Sepharose 4B alone did not (Simon et al, 1972). Antibodies however are not membrane-bound receptors of complex constitution but are soluble glycoproteins. 6-Succinyl-morphine itself was an agonist in the GPI preparation, but once coupled to Sepharose 4B, this effect was no longer elicited, presumably because the ligand was no longer accessible to the receptors in the preparation.

GANC itself was also able to inhibit the specific binding of ^3H -ligands to solubilised opioid receptors, with IC_{50} values in the submicromolar range; this indicated about a 10-fold lower affinity for solubilised than membrane-bound receptors, and

paralleled the shift in affinity with ^3H -etorphine and ^3H -naloxone demonstrated here.

Given the inherent properties of GANC outlined above, it was paradoxical that the GANC-column did not retain solubilised opioid receptors, despite manipulations of the conditions. This suggests that the apparent lack of success was not due to the ligand chosen or to unfavourable conditions but rather to the properties of the solubilised receptor preparation itself. As alluded to above, the method used to produce solubilised receptors altered their properties, such that affinity for enkephalins was lost, and affinity for opiate alkaloids and GANC decreased about 10-fold, although GANC itself still retained affinity for solubilised receptors. Since immobilisation of ligands is also known to reduce their affinities for receptors, it is likely that the combination of using solubilised receptors of reduced capability and the immobilisation of GANC was enough to preclude the retention of these receptors on the GANC-column.

For these reasons, experiments are continuing with modified methods of solubilised opioid receptor preparation. There is now evidence that binding activity may be retained on the column (C. Demoliou-Mason, personal communication). An alternative approach might be to synthesize a new ligand, the morphine analogue of GANC since this would show a higher affinity for opioid receptors than GANC (e.g. Kosterlitz & Waterfield, 1975). However, the effect this change might have on the

affinity of the molecule for solubilised opioid receptors cannot be predicted so readily, and must await use of such a ligand. Also the synthesis would need to be slightly more sophisticated, since the 3-hydroxy group would need to be protected during coupling of glycine to the 14-position. However the experience gained in synthesis of NUM, NOM and NAM would be useful here.

There was no retention of binding activity on the column which indicated that non-specific interactions between the column and solubilised receptors were minimal, and that passage of the receptors down the column had no adverse effects on their binding activity. These are valuable assets if a column is to be used successfully in affinity chromatography.

A further advantage was the presence of the ^{14}C -atom in the GANC molecule. This allowed easy and precise estimation of ligand incorporation into the gel by combustion scintillography. The method was quicker, easier and more sensitive than previous methods used to estimate incorporation, which depend on changes in spectrophotometric absorption of gels before and after coupling to ligands (e.g. Schurz & Rudiger, 1982) and were not satisfactory for estimating the incorporation of GANC into Affigel-10.

CHAPTER V

RADIATION INACTIVATION OF
OPIOID RECEPTORS

V.1 INTRODUCTION

Radiation inactivation analysis, or target-size analysis, is a technique which involves irradiation of samples of biological material with high energy radiation, such as X-rays, γ -rays or high energy electrons. It shows that with increasing doses of radiation there is increasing loss of biological activity, whether this is measured by disappearance of binding-sites for a particular ligand (receptor) or of catalysing activity (enzyme). One of the advantages of the technique is that it is not necessary to purify a molecule to gain information about its size, i.e. the molecule may be studied in situ. This is a considerable advantage for membrane-bound proteins such as receptors, which consequently do not need to be solubilised, with the possible changes in nature this may bring about (e.g. Ch. IV). Also, the technique gives the size of the functional unit rather than the size of a particular subunit, such as a binding site.

The technique rests on the premise that high energy radiation transfers energy to the target material in discreet 'bursts'. Each burst destroys a molecule in the target material, and it follows that the larger a molecule is, the more likely it is to be hit. Since the energy transfer, which leads to ionisation, is so great (66eV; Kepner & Macey, 1968) the molecules which are hit are assumed to be completely destroyed and there do not remain partly functional molecules. Destruction of molecules does not depend on the presence of certain labile groups, such

as sulphhydryl bridges. Clearly only those molecules which escape ionisation remain viable, and the remaining activity measured after exposure to different doses of radiation is related to the molecular size and therefore molecular weight of the particular entity studied.

The above applies to the direct effects of radiation, which occur when the target material is irradiated in a solid state (frozen or freeze-dried). If liquid is present however, there are indirect effects which occur as a result of the formation of ions and radicals which can then diffuse and react with other molecules. These are then destroyed chemically by this secondary effect rather than as the result of a direct hit by high energy radiation. Radiation inactivation analysis is not applicable under such conditions.

However, under conditions where liquid is not present, it has been shown empirically that target size is reciprocally related to the dose of radiation required to reduce the activity to 37% of the initial value (D_{37}):

$$A = A_0 e^{-V D} \quad \begin{array}{l} A = \text{activity after dose } D(\text{Mrad}) \\ A_0 = \text{initial activity} \\ V = \text{target volume of entity (cm}^3\text{)} \end{array}$$

$$\text{For } A = 0.37A_0,$$

$$0.37 = e^{-V D_{37}}$$

$$\Rightarrow \ln 0.37 = -V D_{37} = -1$$

$$\Rightarrow V = \frac{1}{D_{37}}$$

$$D_{37}$$

By allowing for protein density and using Avogadro's number, the target size, V , can be converted into a molecular weight by the following empirical equation:

$$V \times (6.4 \times 10^{11}) = \text{molecular weight}$$

(Kepner & Macey, 1968)

However, it is difficult to determine D_{37} in the specimen itself with any certainty, because of the variability of quenching, although standard dosimetric techniques (such as the darkening of a perspex block according to different doses) have been used (Berry & Marshall, 1969). Clearly it is essential to know the precise D_{37} if the values obtained for target size and hence molecular weight are to have relevance.

A more recent method developed in this laboratory obviates the requirement to determine accurately the D_{37} by the inclusion of enzyme markers, whose molecular weights and behaviour upon irradiation are known, in the target material. In this way, the enzyme 'standards', S , are irradiated concurrently with the material of interest, R . This gives the inactivation rate of the enzyme marker, i_s , which can then be directly compared with that of the material of interest, i_r :

$$\log_{10} \frac{R}{R_0} \div \text{dose} = i_r$$

$$\log_{10} \frac{S}{S_0} \div \text{dose} = i_s$$

Since the relative inactivation rates are independent of dose, and

$$V = \frac{MW}{dN}$$

d = density of molecule

N = Avogadro's number

MW = molecular weight

then $V = MW$

constant

and $\frac{i_r}{i_s} = \frac{MW_r}{MW_s}$

Lo et al, 1982

This eliminates the need to determine D with accuracy, which simplifies measurements. However, the method was first checked by taking various enzymes of known molecular weight and using some as 'standards' and others as 'unknowns' to ensure the procedure gave the expected values (Lo et al, 1982).

There have been numerous reports of use of radiation inactivation analysis to estimate molecular sizes of soluble and membrane-bound enzymes (For review, see Kempner & Schlegel, 1979). These used the D_{37} method to estimate molecular weights. The technique has also been applied to membrane-bound receptors for hormones and neurotransmitters, and associated proteins such as adenylate cyclase and regulatory subunits, e.g. it has been proposed that the glucagon receptor in hepatic membranes existed uncoupled to adenylate cyclase until a ligand bound to it and then the receptor would be able to couple with a molecule of adenylate cyclase (Houslay et al, 1979).

For the nicotinic acetylcholine receptor, it has been shown that the receptors of Torpedo electric organ, chick embryonic muscle and cat denervated muscle are the same size (Lo et al, 1982). Radiation inactivation analysis has also been used to show by the method of enzyme markers that benzodiazepines (^3H -flunitrazepam) and GABA (^3H -muscimol) bind to sites of the same size (220K M.W.) in bovine brain membranes (Chang et al, 1981; Chang & Barnard, 1982). However, to illustrate the considerable variation in estimates for molecular weight made by different groups for what is presumably the same benzodiazepine binding site, other reported values have been 90K (Doble & Iversen, 1982) and 54K (Nielsen & Braestrup, 1983). Since there also appears to be a chloride ionophore of 130K (Nielsen & Braestrup, 1983), it is possible that the large 220K site reported by Chang et al consists of the GABA and benzodiazepine binding sites together with a chloride ionophore (F.A. Stephenson, personal communication).

Finally, the size of the opioid receptor has been estimated by radiation inactivation analysis. The published data have indicated a size of around 200K (McLawhon et al, 1983 a,b). However, this estimate was made by the empirical method (D_{37}), not by use of internal marker enzymes and was made in neuroblastoma X glioma cultured cells. The opioid receptor in membranes of these cells is of the σ -subtype only, and the aim of this work was to determine whether μ - and σ -receptors were of a similar molecular size.

V.2 METHODS

Since freeze-drying of bovine brain membranes had no adverse effect on the GABA and benzodiazepine binding-sites (Chang et al, 1981; Chang & Barnard, 1982), rat brain membranes were first freeze-dried for irradiation. However, since inconsistent results were obtained, membranes were then irradiated in the frozen state and this proved more satisfactory.

1. Preparation of samples for irradiation

Rat brain membranes were prepared as described in Ch. II up to the point of centrifugation in 10mM Tes-KOH pH7.4. Thence, instead of pellets being resuspended in 15-20ml Tes buffer and stored, they were resuspended in 40ml deionised water and recentrifuged. This procedure was to remove buffer and any remaining protease inhibitors from the membranes before freeze-drying or freezing prior to irradiation. These pellets were resuspended into 10ml deionised water and the standard enzymes were added and well mixed with the suspension which was then divided into 0.5ml aliquots in 8cm x 1cm Pyrex glass test-tubes.

2. Irradiation of freeze-dried samples

For freeze-dried rat brain membranes, it was found advantageous to include 1mM dithiothreitol (DTT) in the membrane suspension before it was transferred to the Pyrex test-tubes. Before

freezing the samples in liquid nitrogen, the uppermost part of each test-tube was heated in a gas-oxygen flame, and stretched, to give a narrow 'neck'. This made later sealing of tubes more convenient. At no time did the membrane suspension receive any heat. Samples were then frozen and left to freeze-dry on a Virtis Freezemobile II overnight. The following morning, the freeze-dried samples were taken from the machine and each tube was sealed under vacuum at the neck using the gas-oxygen flame. Samples were then co-transported with dry ice to and from the Department of Radiotherapeutics, Addenbrooke's Hospital, Cambridge, where they were irradiated by high-energy electrons from a Philips 15MeV linear accelerator in doses from 0.5-10 Mrad. The samples were placed in a metal block in the path of the beam of electrons, and the block was cooled by a stream of air cooled over dry ice. The temperature of the samples usually stayed below 10°C and did not rise above 25°C. However, to minimise changes in temperature, samples did not receive doses of more than 4 Mrad at one time (i.e. a 10 Mrad dose was split into 4 + 4 + 2, a 6 Mrad dose into 3 + 3 etc.). The samples were then stored at -20°C overnight before assay.

3. Irradiation of frozen samples

As with the samples for freeze drying, the membrane suspension was frozen in liquid nitrogen, and then were sealed under nitrogen in the gas-oxygen flame. The samples were co-transported with dry ice to and from Hammersmith Hospital for irradiation with high energy electrons from a Vickers linear accelerator. A diagram of the device used to keep them frozen

at constant temperature during irradiation is shown in Fig. 46. Samples were irradiated in doses from 1.5-18 Mrad, in increments of 3 Mrad. After irradiation, samples were stored at -20°C overnight before assay.

4. Resuspension of samples after irradiation

For resuspension of freeze dried membranes, the tubes were opened and c. 0.5ml buffer added by Pasteur pipette. Membranes were crudely resuspended and transferred to a small eppendorf tube, and the exercise repeated to remove all the freeze dried matter from the Pyrex tubes. Then, as for resuspension of the frozen membranes, the crudely resuspended membranes were passed through a fine gauge needle three times. The final volume was again 5ml and 100 μl samples were assayed immediately for opioid binding activity.

Frozen samples were allowed just to thaw; the tubes were then opened and the suspension removed by Pasteur pipette. This suspension was diluted to c. 1ml with buffer (50mM KCl, 50mM NaCl, 2mM MgCl_2 , 10mM Tes-KOH pH 7.4) and resuspended by passage through a fine gauge (25G) needle and 1ml syringe three times. The final volume was made up to 5ml with the same buffer. 100 μl samples were assayed immediately for opioid binding activity.

50 μl samples of membrane suspensions were also taken for assay of marker enzyme activity.

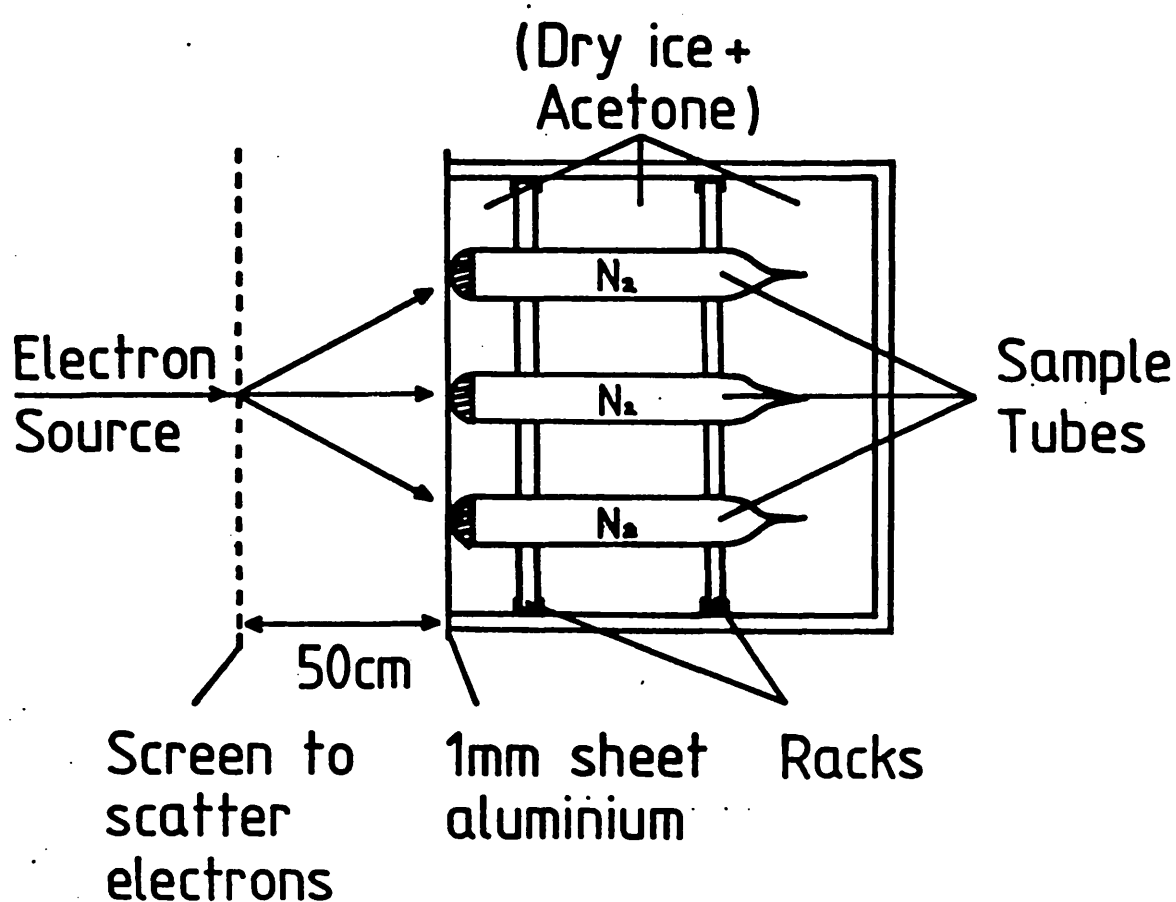


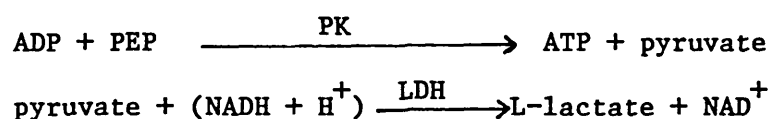
Fig. 46 Irradiation of frozen samples at -80°C

^3H -Opioid binding assays were carried out as described in Ch. II. Briefly, μ -sites were measured by $1\text{nM } ^3\text{H-DAGO}$ and δ -sites by $1\text{nM } ^3\text{H-DADL}$, in the presence of 25nM unlabelled DAGO.

5. Standard enzyme markers

Several enzymes were used as internal and external standards : β -galactosidase (β -GAL) E.C. 3.2.1.23 (E.Coli); alcohol dehydrogenase (ADH) E.C. 1.1.1.1 (horse liver); pyruvate kinase (PK) E.C. 2.7.1.40 (rabbit muscle); lactate dehydrogenase (LDH) E.C. 1.1.1.28 (rabbit muscle). All were obtained from Sigma.

Assay of PK was adapted to automated sampling and recording of activity by an LKB 8600 reaction rate analyser, using NADH as substrate and measuring its disappearance by the decrease in absorbance at 340nm . The buffer used was (at final concentrations) 123mM triethanolamine-HCl pH 7.5, 92mM KCl, 16.2mM , MgSO_4 , 1mM EDTA. NADH, phosphoenolpyruvate (PEP) and LDH were added to final concentrations of 0.18mM , 1mM and 3.7Uml^{-1} respectively. ADP (final concentration 3.1mM) was used to start the reactions:



Other assays were carried out with a Cecil spectrophotometer, and the rates of enzyme activity were measured as the gradient of the slopes indicating disappearance of substrate or appearance of product (by changes in absorption).

LDH : the buffer was 50mM Tris-HCl pH 7.4 with 5mM sodium pyruvate and 0.15 mM NADH. The change in absorption at 340nm was measured following addition of enzyme (in the membrane suspension).

β -GAL : the buffer was 50mM sodium phosphate pH 7.5, with 10mM 2-mercaptoethanol and 1mM $MgCl_2$. The substrate used was o-nitrophenylgalactopyranoside-6-phosphate (final concentration 1mM) and the absorbance change at 410nm was followed after addition of enzyme (in the membrane suspension)(Craven et al, 1965).

ADH : the buffer was 100mM Tris-HCl pH 8.8 with 200mM ethanol. The substrate was NAD (final concentration 1mM) and the absorbance change at 340nm was followed after addition of enzyme. (Vallee & Hoch, 1955).

The following quantities of enzymes were used, per tube : β -GAL 10U, ADH 3U, PK 5U and LDH 5U. The enzymes were not all present in every irradiation series.

A protein assay (Ch. II) was carried out on every tube to ensure that the activities of samples were normalised in this respect.

V.3 RESULTS

1. Effect of freeze-drying and freezing in water alone on opioid receptor binding

It was shown that the processes of freeze-drying, transport to and from the linear accelerator at Cambridge and resuspension of the freeze-dried samples in the absence of irradiation had no detrimental effect on the specific binding of ^3H -etorphine to opioid receptors in rat brain membranes (Fig. 47). Similarly, there was no adverse effect of freezing in deionised water alone in the absence of buffer on the specific binding of ^3H -ligands to rat brain membranes in the absence of irradiation (data not shown). It was necessary to establish that these procedures had no effect on opioid receptor binding because irradiation has to be carried out in the absence of liquid water, because of the large indirect effect of secondary radiation that would arise from its radiolytic products (see section V.1).

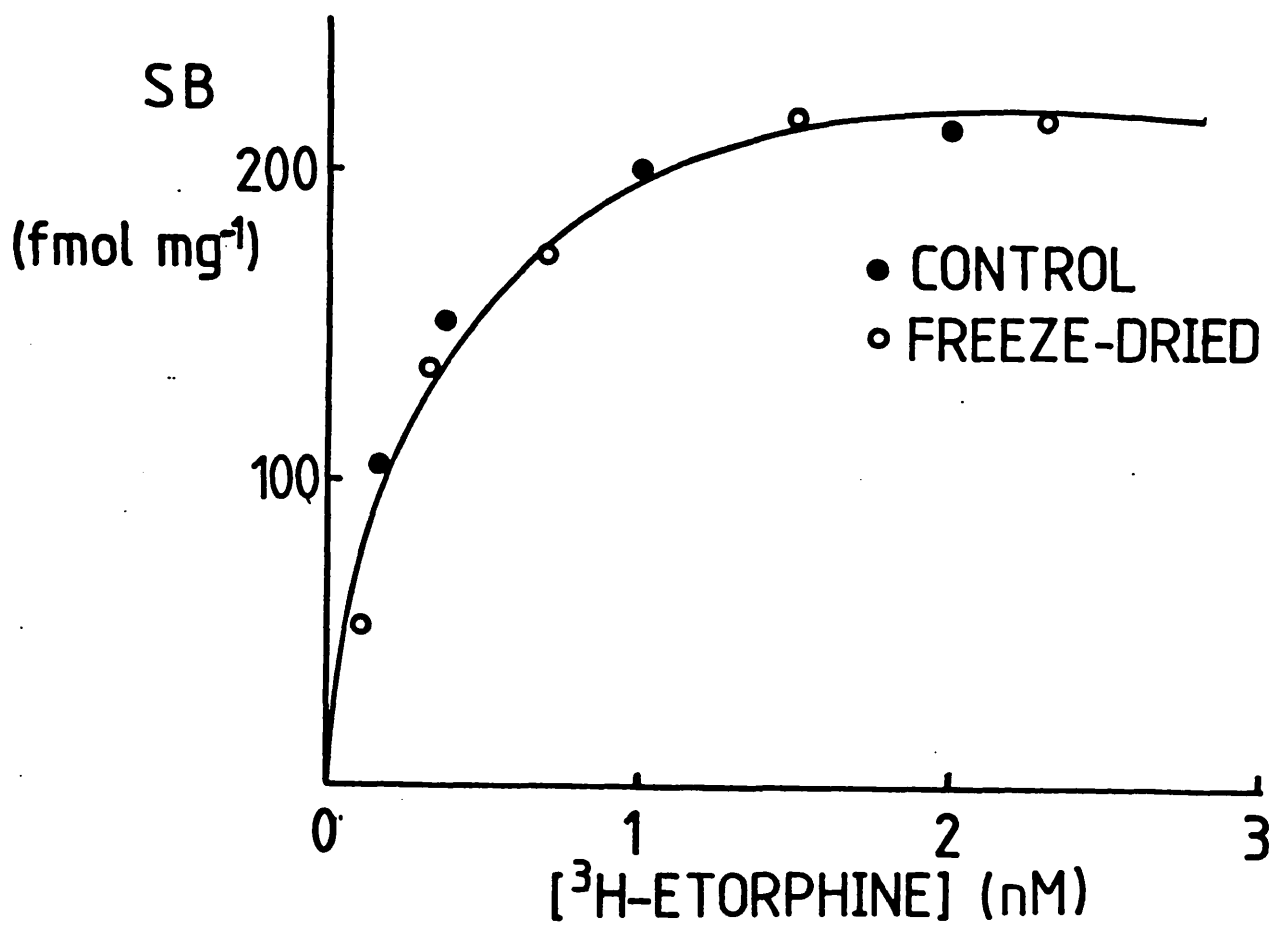
Since during irradiation the temperature of freeze-dried samples rose to 25°C (4 Mrad dose), samples were heated for the same length of time to 25°C in the absence of irradiation; there was no demonstrable effect on opioid receptor binding.

2. The effect of irradiation on freeze-dried samples and standard enzymes

The standard enzymes used in irradiation of membranes in the freeze-dried state were ADH and β -GAL. It had already been

Fig. 47

Rat brain membranes: effect of freeze-
-drying on specific binding of ^3H -etorphine



established that freeze-drying and redissolving did not alter the activity of β -GAL, although it did decrease the activity of ADH by about 10%. After irradiation, the relative inactivation rates of these two enzymes were similar, although the actual inactivation rates varied from one occasion to another due to differences in beam strength or inaccuracy of dose calibration (Table 9). These results were in agreement with those previously reported from this laboratory (Lo et al, 1982).

Initial experiments showed fast inactivation of opioid receptor binding, with almost complete inactivation. This behaviour was also seen for opioid receptor binding in bovine brain membranes after a dose of only 1 Mrad and would indicate a very large molecular size of several million. However it was found amongst various manipulations tried that the addition of 1mM DTT to the membrane suspension before freeze-drying abolished this effect (Fig. 48). Presumably DTT prevented aggregation of opioid receptors which apparently occurred in its absence during the process of freeze-drying. Indeed, it has been shown that sulphhydryl reagents inhibit aggregation of opioid receptors in neuroblastoma cells (Hazum et al 1979 a, b). Specific binding of ^3H -ligands to opioid receptors in rat brain membranes was not affected by the presence of any remaining DTT in the assay system.

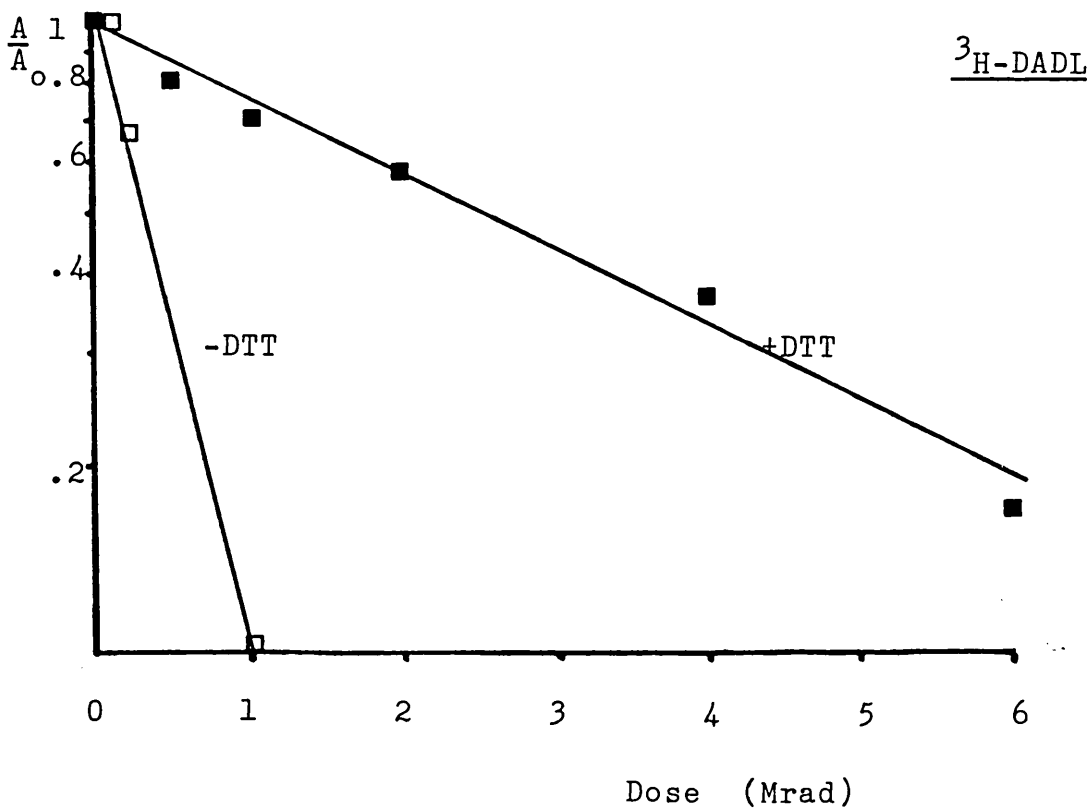
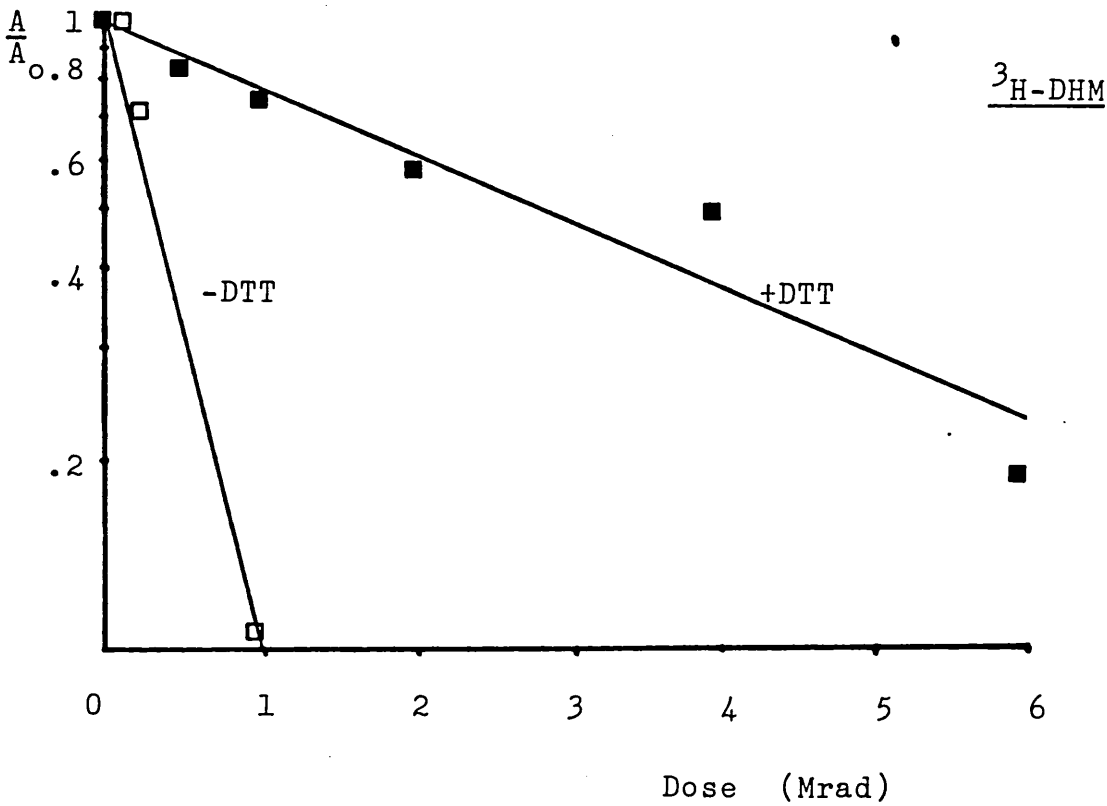
The effect of DTT on the inactivation rates of ADH and particularly β -GAL was less consistent; the rate decreased, but to a variable extent. Since β -GAL consists of 4 subunits, it

TABLE 9 Inactivation rates (i) of β -GAL, ADH, μ -and σ -binding
sites in freeze dried rat brain membranes

$i_{\beta\text{-GAL}}$	i_{ADH}	$\frac{i_{\beta\text{-GAL}}}{i_{\text{ADH}}}$	i_{μ}	i_{σ} (all cmMrad ⁻¹)
1.31	0.43	3.0	0.93	0.99
1.39	0.45	3.0	1.0	1.27
1.39	0.45	3.0	1.23	1.13
-	-	-	3.8	4.2
1.63	0.62	2.6	0.97	0.97
0.78	0.28	2.8	1.48	1.70
-	0.51	-	1.34	1.29
1.12	0.28	4.0	3.4	4.6

All data obtained from samples containing DTT, and with β -GAL and ADH as external standards in the absence of DTT.

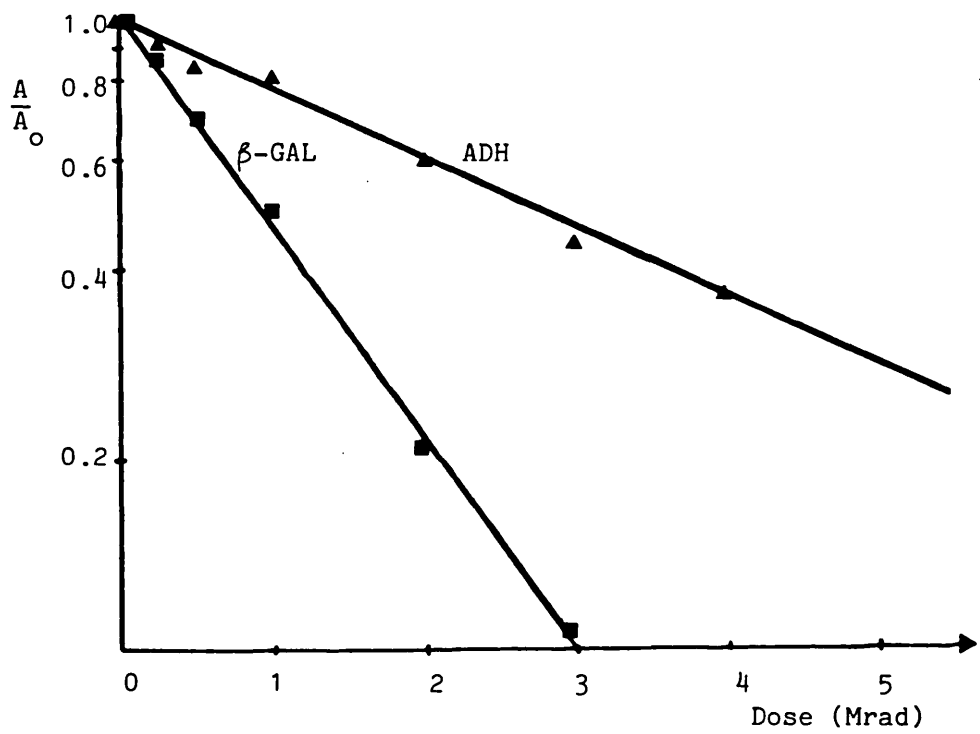
Fig. 48 Rat brain membranes : effect of 1mM DTT on the inactivation rates of μ - and δ -binding sites in the freeze-dried state



is possible that on the occasions when DTT was included, different mixtures of monomer/dimer/tetramer were produced. For this reason, further experiments were executed using external standards, i.e. using 2 sets of tubes containing rat brain membranes, the first containing DTT and no standard enzymes and the second containing standard enzymes but no DTT. The tubes were paired and irradiated together so that both received the same dose. Opioid receptor binding was then measured in membranes from the tubes containing DTT and enzyme activity from the tubes without DTT. The presence of rat brain membranes in the second set of tubes was considered necessary to ensure similar quenching characteristics for each set of tubes. This method constituted an extension of the internal standard method first described by Lo et al (1982).

A typical result is shown in Fig. 49. The relative inactivation rates remained reproducible in the absence of DTT. However, although early experiments using DTT give reproducible results for inactivation of opioid receptor binding later experiments showed considerable variation in inactivation rates (although there was little difference between the μ - and δ -binding sites). Further, there was no correlation between the occasions when inactivation rates of the standard enzymes were relatively high and those when the inactivation of opioid receptor binding were relatively high (Table 9).

Fig. 49 Rat brain membranes : inactivation rates of ADH and β -GAL
in the freeze-dried state in the absence of DTT



3. The effect of irradiation on frozen samples

In view of the inconsistent values obtained from freeze-dried membranes, it was decided to use frozen membranes. It was not possible to irradiate samples in the frozen state at Addenbrookes, Cambridge, so the linear accelerator at Hammersmith Hospital was used (Fig. 46). Two series of experiments were performed, using internal standards in the absence of DTT, and another series in the presence of DTT, again with internal standards.

In the first series, ADH and β -GAL were used, as in the freeze-dried samples. The results are shown in Fig. 50. Clearly, ADH inactivation took place at a rate not dissimilar to the rate seen in freeze-dried samples, but β -GAL inactivation was 3-4 fold slower than in freeze-dried samples. The reasons for this were unclear, but the result has been confirmed independently in this laboratory (A. Lai, unpublished observations). The result of ^3H -DAGO and ^3H -DADL inactivation are shown in Fig. 51.

Assuming the molecular weight of ADH is 84K, this means that approximate values for the μ - and σ -binding sites can be calculated on the following basis:

$$\frac{^1_{\text{DAGO}}}{^1_{\text{ADH}}} \times \text{mol. wt. ADH} = \text{mol. wt. DAGO binding site}$$

Therefore $\frac{0.28}{0.23} \times 84\text{K} = 102\text{K} (\mu\text{-site})$

and $\frac{0.29}{0.23} \times 84\text{K} = 105\text{K} (\sigma\text{-site})$

Fig. 50 Inactivation rates of ADH and β -GAL in the absence
of DTT (frozen rat brain membranes, series 1)

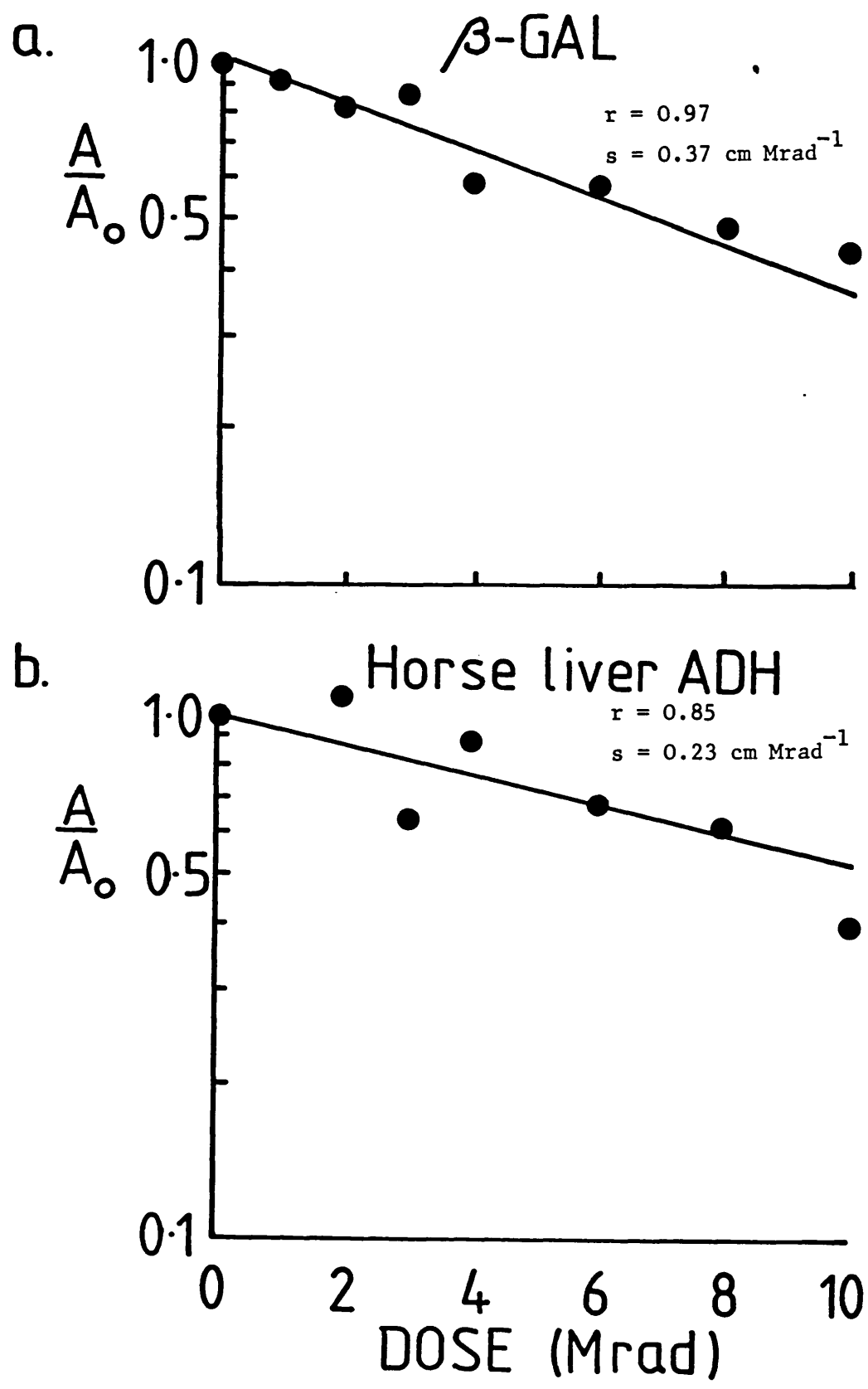
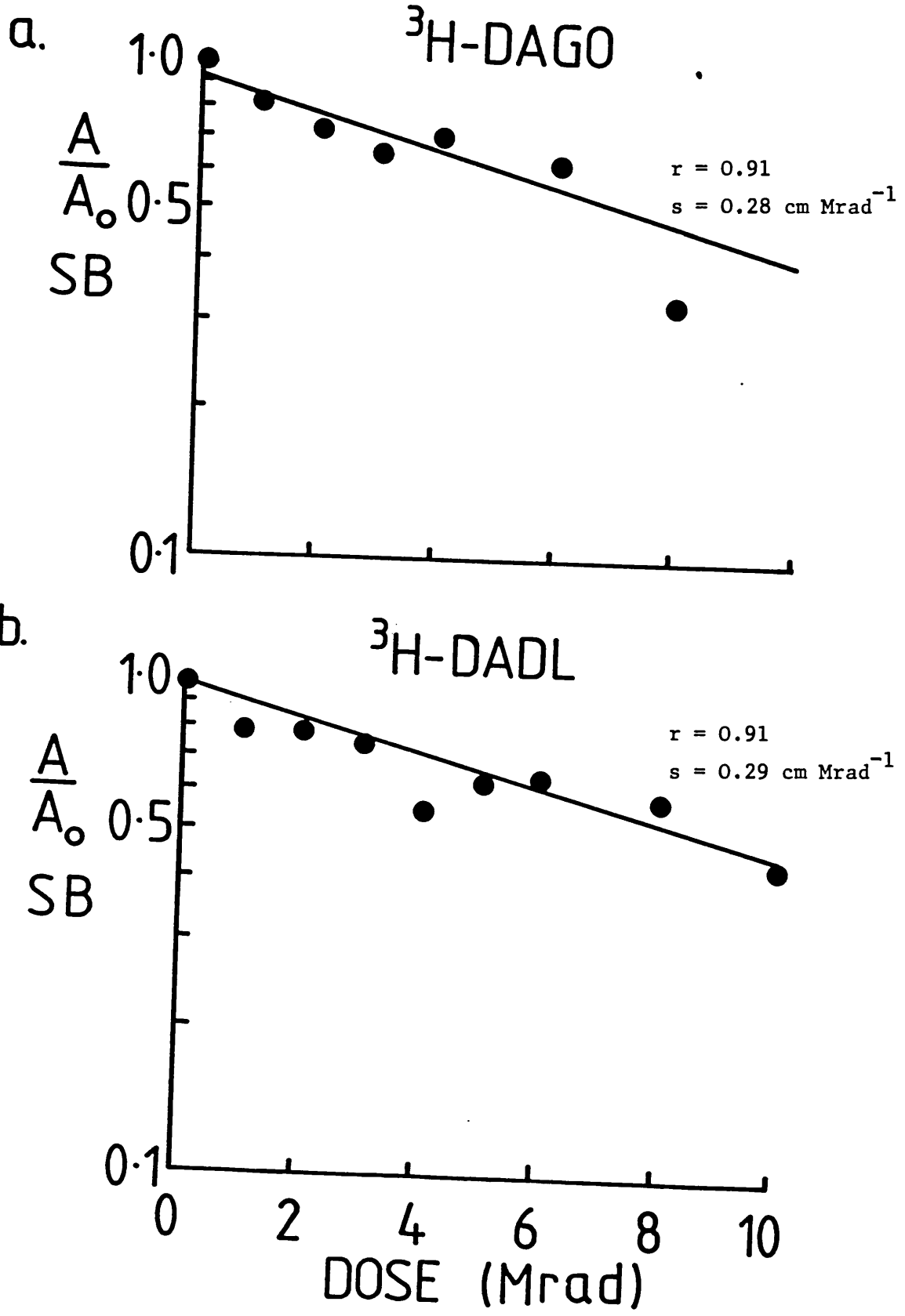


Fig. 51 Inactivation rates of μ - and δ - binding sites in the
absence of DTT (frozen rat brain membranes series 1)



Whereas β -GAL appeared to inactivate as a monomer rather than as a tetramer:

$$\frac{0.37 \times 84K}{0.23} = 136K$$

In the second series, PK and LDH were used as standards, and their inactivation is shown in Figs. 52 + 53. Assuming the mol. wt. of PK is 230K, then:

$$\frac{0.32 \times 230K}{0.63} = 117K (\mu\text{-site})$$

and

$$\frac{0.31 \times 230K}{0.63} = 113K (\sigma\text{-site})$$

whereas LDH, whose mol. wt. measured by other methods is 140K, inactivated as a larger size ($>500K$).

In the third series, the presence of DTT gave the result that PK and the σ -site inactivated at the same rate (PK : 0.38cm Mrad^{-1} , $r=0.99$; σ -site : 0.38cm Mrad^{-1} , $r=0.97$). The rate of σ -site inactivation is slightly higher than that above, but that of PK is about half. Since it has been shown that DTT causes PK to inactivate at a slower rate than in its absence, to give a molecular weight half that expected (A. Lai, unpublished observations), the estimate for the size of the σ -site in this series (115K) is consistent with the values previously obtained.

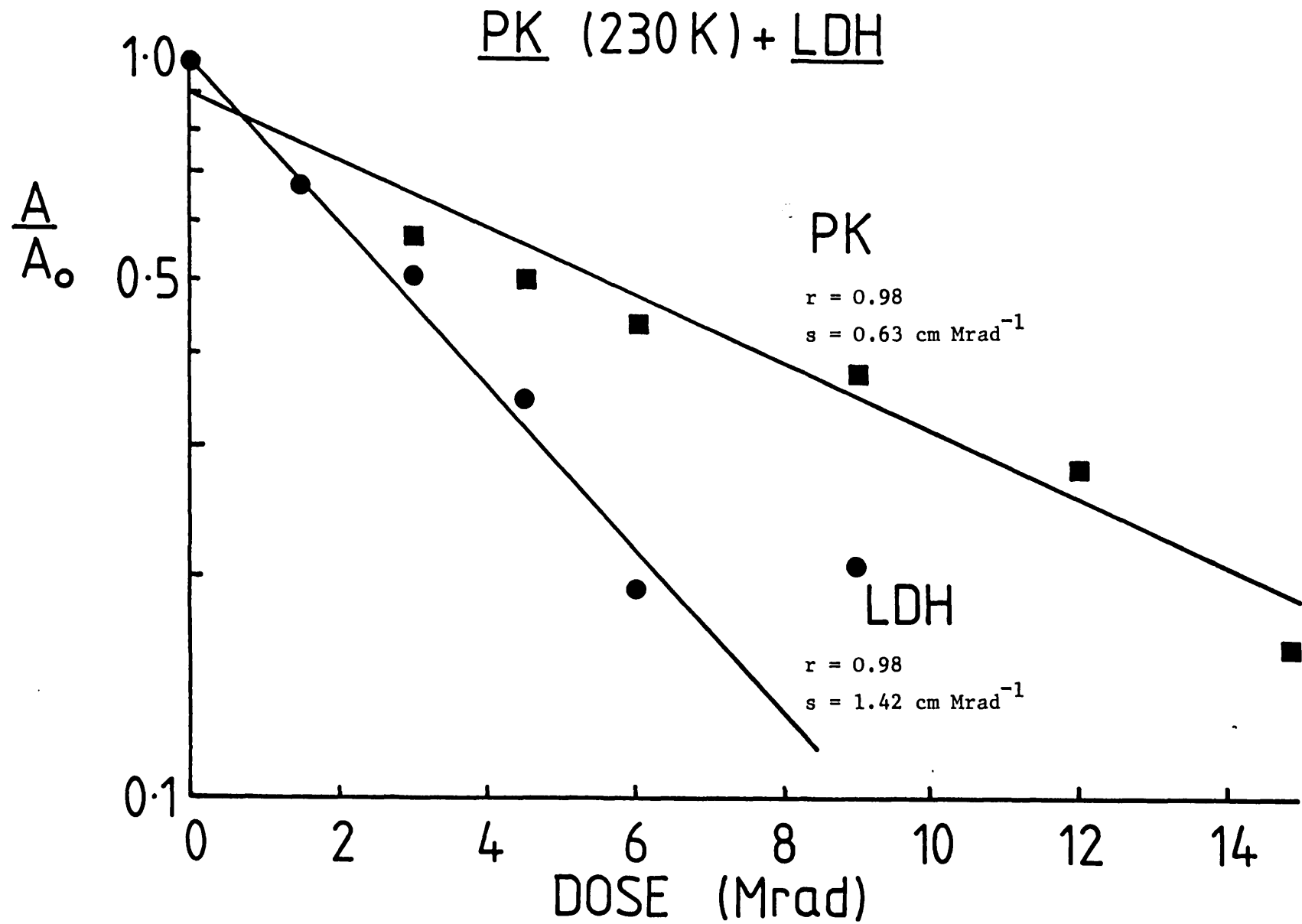
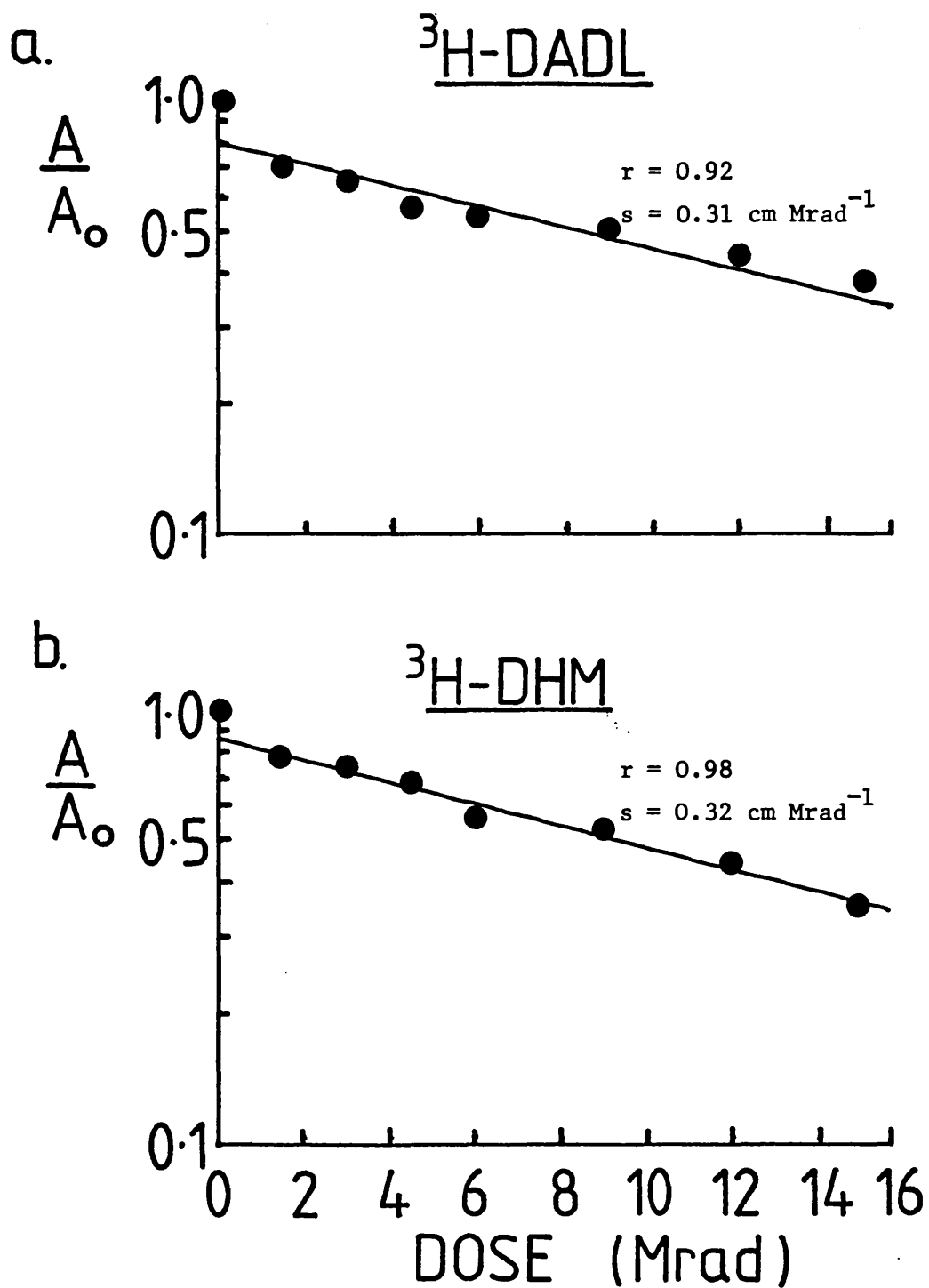


Fig. 52 : Inactivation rates of PK and LDH activities in the absence of DTT (rat brain membranes, frozen; series 2)

Fig. 53 Inactivation rates of μ - and δ -binding sites in frozen rat brain membranes in the absence of DTT (series 2)



The sizes of the μ - and δ -binding sites in rat brain membranes in the above experiments have been shown to be a similar size, both in freeze-dried and in frozen membranes. While the variation among results from freeze-dried samples precluded estimation of that size, the results from frozen membranes did allow such a determination to be made. Using two different enzyme standards on separate occasions (PK and ADH), the size of the μ - and δ -sites was estimated to be 110,000 $\pm$ 7,000.

V.4 DISCUSSION

The technique of target size analysis by radiation inactivation has been used to estimate the sizes of many proteins (Kempner & Schlegel, 1979), both soluble and membrane-bound, with the particular advantage that the minimum molecular size of a structure carrying the biological activity measured may be estimated in situ. This advantage is particularly useful for obtaining the sizes of proteins otherwise intractable to standard purification procedures, including receptors for a variety of hormones and neurotransmitters. The technique was applied here in order to determine unambiguously whether the binding sites of μ - and δ -opioid receptors reside on molecules of different sizes.

The technique was in the past applied on the basis that there existed an empirical relationship between the D_{37} and the size of the molecule studied which led to varying estimates of the sizes of proteins e.g. the glucagon receptor-adenylate cyclase unit was estimated as 1,300K by one group (Schlegel et al, 1979) and as 328K by another (Martin et al, 1979). Since it is difficult to determine D_{37} (see V.1), the method used was that developed in this laboratory which obviates the requirement to determine the precise D_{37} by including enzyme markers as standards in the same tube as the sample itself (Lo et al, 1982; Chang & Barnard, 1982). When frozen samples of rat brain membranes were used, the sizes of the μ - and δ -opioid binding sites were both consistently around 110K (Fig. 51) by simple

proportionality to the dose-dependence of the internal standards whose molecular sizes were known. However, the freeze-dried samples did not give consistent results, a finding in agreement with Ott et al (1983), although it was evident that the μ -binding site behaved very similarly to the δ -binding site in each series (Table 9).

The value of 110K for the target size of opioid receptors is in conflict with the present report of 200K by McLawhon et al (1983a). However, there are possible reasons for the discrepancy. McLawhon et al used a freeze-dried preparation of membranes from NG108-15 neuroblastoma X glioma hybrid cells instead of the frozen rat brain membranes used here. It may be that the δ -receptor in these cell lines is able to undergo irradiation after freeze-drying without showing the rapid inactivation seen here in the absence of DTT (Fig. 48) and by Ott et al (1983). Further, the size of 200K was calculated by the early empirical method of estimating D_{37} rather than the internal standard calibration method.

One of the reasons for using the latter method is seen in Table 9 where, apparently, there is a two-fold variation in inactivation rates for β -GAL and ADH after irradiation by one machine at Addenbrookes Hospital on different occasions. This implies that, since the molecular weights of the enzymes must remain constant, the doses of radiation varied from one occasion to another. This finding is of particular relevance here because this equipment was that used by McLawhon et al in their analysis.

The discrepancy is also unlikely to be due to the difference in temperature during irradiation of the frozen (-70°C) and freeze-dried samples (generally $0-10^{\circ}\text{C}$), since one of the abovementioned authors had previously shown that there was no change in sensitivity when samples of acetylcholinesterase were irradiated in the frozen state at low temperature ($\geq 190^{\circ}\text{C}$) compared to the freeze-dried state ($0-10^{\circ}\text{C}$) (Levinson & Ellory, 1974). Indeed, there is no thermodynamic reason why samples should show a decreased sensitivity at lower temperatures, so it ought not be necessary to apply an empirical 'temperature correction factor', as has been suggested elsewhere (Kempner & Schlegel, 1979).

The results presented in this thesis have been confirmed and extended by recent studies in this laboratory which examined the effect of magnesium ions on the target sizes of μ - and δ -binding sites in frozen rat brain membranes (Lai et al, 1984). The conclusion that the μ - and δ -sites both have a size of around 110K under a variety of conditions and compared to several marker enzymes is in accord with the result of around 90K obtained by the empirical method for μ - and δ -sites in frozen rat brain membranes (Ott et al 1983).

Since the results from frozen rat brain membranes conflict with those obtained using the freeze-dried NG108-15 cell membranes, it seems probable that the δ -receptor of the cell line has a different size to the δ -receptor in rat brain, and may possess

properties such that it can be irradiated in the freeze-dried state without rapid loss of activity. If so, the validity of extrapolating results from the opioid receptor of such cell lines to the opioid receptors in mammalian brain is called into question.

Meanwhile, it has been demonstrated here that the μ - and δ -opioid receptors have a similar size, although whether these receptors both exist on one molecule of size 110K or are each of size 110K is a question of considerable interest.

CHAPTER VI

GENERAL DISCUSSION

VI GENERAL DISCUSSION

There has accumulated evidence for the existence of subtypes of opioid receptor since its identification over ten years ago (Simon et al, 1973; Terenius, 1973; Pert & Snyder, 1973). The μ - and δ -subtypes were the first to be demonstrated (Lord et al, 1976; 1977), and of the remaining putative subtypes (κ , ϵ , σ), only the κ -subtype is now widely accepted as such. In spite of the recent advent of relatively specific ligands for the various subtypes, such as DAGO (μ -agonist; Handa et al, 1981), ICI-154,129 (δ -antagonist; Shaw et al, 1982) and tifluadom (κ -agonist; Romer et al, 1982), it has remained difficult to characterise the subtypes; reasons for this include low potency and scant availability of selective ligands. Also, the ligands have not so far been readily available in radiolabelled form (with the exception of DAGO), and therefore can only be used indirectly for binding studies.

In spite of this, various models have been proposed to account for the differences between opioid receptor subtypes, although some of these have a narrow applicability. One such is the hypothesis that there is only one opioid receptor, i.e. that which mediates analgesia and is inactivated by naloxonazine, (the μ_1 site of Pasternak et al), and that other receptors are merely 'recognition sites' (Laduron, 1983). However there is much pharmacological evidence against this e.g. the demonstration that there must exist at least two opioid receptors in MVD (Lord et al, 1977; Ward et al, 1982c; Miller & Shaw, 1983) and in the chronic spinal dog (Gilbert & Martin, 1976; Martin, 1976).

Related to this is the multiple environment model which also proposes the existence of a sole opioid receptor whose characteristics, including receptor selectivity and receptor-effector coupling, are determined by surrounding molecules such as proteins, lipids, ions and nucleotides (Morley, 1983b). The existence of a spectrum of opioid receptors might be expected according to this model, whereas there appear to be discrete subtypes whose behaviour is reproducibly different from each other in a variety of preparations both pharmacological and biochemical.

This does not preclude that a receptor's environment is able to alter its function; moreover Lee and Smith (1980) proposed that the environment formed part of the opioid receptor itself i.e. the

μ -binding site has a protein moiety while the δ -binding site consisted of juxtaposed acidic lipids (Fig. 14). It appears though that such lipids would not provide a specific enough site to bind opioid ligands with the high affinities observed (Glasel et al, 1980). Further, only slight changes to an enkephalin molecule can alter receptor selectivity from δ to μ with little concomitant increase in hydrophobicity, which may be expected to be of importance in binding to lipids. Nevertheless, the extreme sensitivity of opioid receptors to detergents suggests a close association with membrane lipids.

It has been suggested by others than the μ - and δ -receptors show allosteric interaction (Vaught et al, 1981; Rothman & Westfall, 1982a, b). There is evidence that leu-enkephalin increases the

affinity of morphine and related ligands for its receptor in vitro (Vaught et al, 1981) and in vivo (Vaught & Takemori, 1979). Conversely, leu-enkephalin was also shown to inhibit the binding of ligands to the morphine receptor (Rothman & Westfall, 1982a). However, the specificities of the ^3H -ligands used were relatively poor: ^3H -etorphine and ^3H -leu-enkephalin were each assumed to label single separate sets of sites (Rothman & Westfall, 1982b) an assumption against which evidence is strong e.g. Paterson et al, 1983. It has been further proposed that μ - and δ -receptors coexist in an opioid receptor complex (Rothman & Westfall, 1983; Fig. 12), although autoradiographic evidence suggests that μ - and δ -subtypes have a different distribution (Goodman et al, 1980).

In this thesis, the initial approach to characterising opioid receptors was the synthesis of a ligand for use as a photoaffinity label (NAM). While NAM itself was unsuitable for this purpose, as discussed in Ch. III.4, its prolonged receptor interaction (e.g. Fig. 31) was a property useful for denuding preparations of μ -receptors. A prolonged duration of action was also observed for buprenorphine (Fig. 5e) which has both agonist and antagonist properties (Cowan et al, 1977). NAM and buprenorphine differ from other lipophilic opioids such as methadone, whose action in isolated tissues is readily reversed by the addition of naloxone (Kosterlitz et al, 1975); this suggested that methadone's relatively long duration of action was due to its retention in lipid depots within the tissue, while NAM and buprenorphine display a prolonged interaction with opioid receptors themselves.

Unlike NAM however, buprenorphine's prolonged action was not selective for μ -receptors (Villiger & Taylor, 1981; Sadée et al, 1982); NAM acts reversibly at δ -receptors and shows no interaction with κ -receptors at the concentrations studied (Figs. 32 & 37). This suggests that its prolonged action at μ -receptors was unlikely to be due to a covalent interaction; this is important because of its reactive azide moiety. As shown in Ch. III.4, NAM's slow rates of onset and offset at μ -receptors may be due to a high activation energy for binding together with a high affinity, without this applying for NAM's interactions at δ - and κ -receptors. Such differential kinetic behaviour has been described for ^3H -etorphine dissociation from fast dissociating-low affinity sites and slow dissociating-high affinity sites in sheep caudate nucleus (Tolkovsky, 1982) and rat brain membranes (EMP, unpublished observations).

NAM's behaviour was also apparently similar to that of β -FNA and naloxonazine in isolated tissues, although the mechanism is different, as discussed in Ch. III.4. However, there were differences in that β -FNA was unable to suppress all μ -binding in rat brain membranes (Rothman et al, 1983); in isolated tissues, β -FNA and related compounds have been proposed to differentiate between the μ -receptors in GPI and in MVD (Ward, 1983; Sayre et al, 1983). Similarly, it has been proposed that because naloxonazine only inactivates 70% of ^3H -dihydromorphine specific binding in rat brain membranes, and does not inactivate the μ -receptors in GPI, that there are μ_1 and μ_2 receptors (Gintzler and Pasternak, 1983). Daleck showed similar behaviour:

the majority of ^3H -DAGO binding sites in rat brain membranes is inactivated by daleck (E.L. Newman, personal communication) yet daleck is incapable of inactivating μ - (or any other) receptors in MVD (Ch. III.3). This suggests that the opioid receptors in GPI and MVD are inaccessible to naloxonazine and daleck respectively. There are thus no unequivocal data which show that there exist subtypes of μ -receptors.

Although NAM acted pseudoirreversibly at μ -receptors, this interaction appeared to have no effect on the interaction of δ -ligands with δ -receptors (Figs. 32 & 37). This would suggest that there is no interaction or coupling between μ - and δ -receptors in the preparations studied. Consistent with this is the observation that, while μ - and δ -receptors both exist in GPI (e.g. Egan & North, 1981), only the former are coupled to the contractile mechanism, and the role of the δ -receptors is unknown. Conversely, the MI has functional δ - and κ -receptors but no (functional) μ -receptors. All this suggests that μ - and δ -receptors are able to exist and operate independently of each other. Again, autoradiographic evidence is consistent with this.

In contrast to the pharmacological approaches discussed above, GANC was synthesized in an attempt to examine the biochemical characteristics of opioid receptors, by affinity chromatography. Solubilisation of a receptor in active form is the first stage in purification, leading to characterisation of the receptor molecule(s). However, treatment of brain membranes with various detergents has not so far resulted in solubilised opioid receptors

which retain all the properties of the membrane-bound receptors. Indeed, there is loss in activity, with alterations in the receptor profile e.g. the solubilised receptor no longer binds certain ligands, as demonstrated in this thesis and elsewhere (Quirion et al, 1983; Chow & Zukin, 1983). The one report of purification of a preparation of solubilised active opioid receptors (Bidlack et al, 1981) has not been repeated or further investigated. Further, it has been estimated that the activity of this purified preparation was less than 1% of that expected, which suggests that the purity of product was uncertain (Barnard & Demoliou-Mason, 1983).

There are several reasons why GANC coupled to gel should function well as an affinity column for solubilised opioid receptors which retain more of the properties shown by membrane-bound opioid receptors (as discussed in Ch. IV.4). The intractability of opioid receptors to solubilisation is currently being overcome (Demoliou-Mason & Barnard, 1984) and results using the GANC affinity column are anticipated.

SDS-PAGE of opioid receptors chemically labelled with various radioligands has provided several estimates of the size of opioid binding sites e.g. 35K (Zukin & Kream, 1979); 43K, 35K and 23K (Bidlack et al, 1981); 58K, 43K and 17.5K (Klee et al, 1982). However, only the 58K band was shown to be absent in an excess of competing unlabelled opioid; if this is indeed the opioid binding site it appears to be, then 58K would represent a minimum size for the receptor.

Other estimates for the size of solubilised opioid receptors by such methods as gel filtration have indicated much larger sizes than this. Opioid receptor-³H-ligand complexes have been estimated as 370K (Simon et al, 1975b), 380K (Zukin & Kream, 1979), 680K (Dornay & Simantov, 1982) and 690K (Hammonds et al, 1982). There has been only one estimate of the size of the receptor alone (from NG-108 cell line), which indicated a size between 120K and 500K (Simonds et al, 1980).

To date, then, biochemical techniques have not provided much information about the molecular characteristics of opioid receptors, and in particular have not been able to distinguish between the putative subtypes. However, it seems that opioid receptors are glycoprotein in nature (Gioannini et al, 1982), and that κ -receptors undergo solubilisation more easily than μ - and δ -receptors. The novel approach of radiation inactivation (target size) analysis was therefore used to estimate the sizes of opioid receptor subtypes in rat brain membranes. The initial studies, presented herein, showed that μ - and δ -subtypes were of the same size (c.110K). This datum alone, however, is insufficient to determine whether there are two binding sites on one molecule or two separate sites of similar size, although as discussed above, pharmacological evidence would appear to support the latter. Biochemically, the behaviour of solubilised receptors is consistent with this too (i.e. while solubilised μ -receptors partially retain ability to bind ligands, solubilised δ -receptors completely lose this property). It has also been shown that alcohol inhibits binding to (membrane-bound) δ -receptors more than to μ -receptors, again implying the two are separate.

As a continuation of the studies presented here, the size of the k-receptor has also been estimated by the same process. Results were less unequivocal than for the μ - and δ -receptors because of the lack of radiolabelled specific k-ligands. However, it was clear that the k-binding site is smaller in size than the μ - and δ -sites, being of the order of 30K (Lai et al, 1984).

There is also biochemical evidence that the k-receptor is less like the μ - and δ -receptors than they are to each other. Firstly, k-receptors have different physico-chemical properties, being more resistant to proteolytic attack by trypsin, phospholipase A₂, N-ethylmaleimide, and to heat denaturation (Wood & Pilapil, 1983). Biochemically, this may explain why k-receptors are easier to solubilise.

The pharmacological biochemical and molecular studies presented support and extend current evidence for the existence of multiple opioid receptors. The hypothesis that there are multiple types of the opioid receptor has been reiterated by several authors since 1976 (Lord et al, 1976, 1977; Chang & Cuatrecasas, 1979, 1981; Simon, 1981; Zukin & Zukin, 1981; Wood, 1982), although other models were also proposed, as discussed above. The three subtypes generally accepted are μ , δ and k; σ receptors are likely to be phencyclidine receptors (Henderson, 1982) and ϵ receptors to be μ -receptors coupled to an effector system in such a way that only compounds with high intrinsic activity can act as agonists (Smith & Rance, 1983). Data presented here also indicate that the δ -subtype of cultured cell lines exhibits

different properties to its counterpart in mammalian brain, and may be of a different molecular size (V.4).

It is clear that μ - and δ -receptors are closely related, their sizes and biochemical properties being so similar, while the κ -receptor shows differences in these respects. Nonetheless, all three subtypes share similar binding properties, as witnessed by the dearth of specific ligands for the δ - and κ -subtypes. There exist many opioid peptides as putative endogenous ligands for the receptor subtypes (Fig. 8; Table 1). It is notable however, that the μ -receptor, which appears to mediate the best-known effects of opioid drugs, such as euphoria and analgesia, and for which there exist the most selective ligands, is the opioid receptor subtype for which there has not yet emerged a specific endogenous ligand. It is tempting indeed, then, to suggest that such a paradox typifies the mystery that has surrounded opium for centuries, and presents a challenge to research today.

CHAPTER VII

REFERENCES

- AKIL, H., MAYER, D.J. & LIEBESKIND, J.C.
C.R. Acad. Sci, Ser. D. 274 3603-3605 (1972)
- AKIL, H., MAYER, D.J. & LIEBESKIND, J.C.
Science 191 961-962 (1976)
- ALBERTSON, N.F. Adv. Biochem. Psychopharmacol. 8 63-79 (1974)
- ARUNLAKSHANA, O. & SCHILD, H.O.
Brit. J. Pharmacol. 14 48-58 (1959)
- ATWEH, S.F. & KUCHAR, M.J. Brit.Med.Bull. 39 47-53 (1983)
- BAJUSZ, S., RONAI, A.Z. SZEKELY, J.I., GRAF, L., DUNAI-KOVACS, S. & BERZETEI, I. F.E.B.S. Lett 76 91-92 (1977)
- BARNARD, E.A. & DEMOLIOU-MASON, C.
Brit. Med. Bull. 39 37-45 (1983)
- BARRETT, R.W. & VAUGHT, J.L. Eur. J. Pharmacol. 80 427-430 (1982)
- BAUME, S. de la, YI, C.C., SCHWARTZ, J.C. CHAILLET, P., MARCAIS-COLLADO, H. & CONSTENTIN, J.
Neuroscience 8 143-151 (1983)
- BAXTER, M.G., GOFF, D., MILLER, A.A., SAUNDERS, I.A.
Br. J. Pharmacol. 59 455-456P (1977)
- BECKETT, A.H. & CASY, A.F.
J. Pharm. Pharmacol. 6 986-1001 (1954)
- BEHAR, M., MAGORA, F., OLSHWANG, D., DAVIDSON, J.T.
Lancet 1 527-528 (1979)
- BERRIDGE, M.J. T.I.P.S. 1 419-424 (1980)
- BERRIDGE, V. & EDWARDS, G. in Opium and the people.
Allen Lane (1982)
- BERRY, R.J. & MARSHALL, C.H.
Phys. Med. Biol. 14 585-596 (1969)

- BIDLACK, J.M., ABOOD, L.G., OSEI-GYIMAH, P. & ARCHER, S.
Proc.Nat.Acad.Sci. 78 636-639 (1981)
- BLANE, G.F., BOURRA, A.L.A., FITZGERALD, A.E. & LISTER, R.E.
Br. J. Pharmacol. 30 11-22 (1967)
- BLUMBERG, H., DAYTON, H.B., GEORGE, M. & RAPAPORT, D.N.
Fed. Proc. 20 311 (1961)
- BLUMBERG, H., DAYTON, H.B. & WOLF, P.S.
Proc. Soc. Exp. Biol. Med. 123 755-758 (1966)
- BLUME, A.J., LICHTSHTEIN, D. & BOONE, G.
Proc.Nat.Acad.Sci. 76 5626-5630 (1979)
- BRADY, K.T., BALSTER, R.L. & MAY, E.L.
Science 215 178-181 (1982)
- BRENNAN, M.J.W. CANTRILL, R.C. & WYLIE, B.A.
Life Sci. 27 1097-1101 (1980)
- BURGER, A. Introduction. In *Analgetics*, G. de STEVENS (Ed.)
 Academic Press New York (1965)
- CAMPBELL, D.H., LUESCHER, E. & LERMAN, L.S.
Proc.Nat.Acad.Sci. 37 575-578 (1951)
- CARUSO, T.P., TAKEMORI, A.E., LARSON, D.L. & PORTOGHESE, P.S.
Science 204 316-318 (1979)
- CHAILLET, P., COULAUD, A., FOURNIE-ZALUSKI, G., GACEL, G.,
 COSTENTIN, J. & ROQUES, B.P.
Life Sci. 33 (S1) 685-688 (1983)
- CHANG, K-J & CUATRECASAS, P. *J.Biol.Chem.* 254 2610-2618 (1979)
- CHANG, K-J. & CUATRECASAS, P.
Fed. Proc. 40 2729-2735 (1981)
- CHANG, K-J., MILLER R.J. & CUATRECASAS, P.
Mol. Pharmacol. 14 961-970 (1978)
- CHANG, K-J., HAZUM, E. & CUATRECASAS, P.
Proc.Nat.Acad.Sci. 77 4469-4473 (1980)

- CHANG, K-J., HAZUM, E., KILLIAN, A.K. & CUATRECASAS, P.
Mol. Pharmacol. 20 1-8 (1981a)
- CHANG, K-J., HAZUM, E. & CUATRECASAS, P.
Proc.Nat.Acad.Sci. 78 4141-4145 (1981b)
- CHANG, K-J., ECKEL, R.W. & BLANCHAR, S.G.
Nature 296 446-448 (1982)
- CHANG, L-R. & BARNARD, E.A.
J. Neurochem. 39 1507-1518 (1982)
- CHANG, L-R., BARNARD, E.A., LO, M.M.S. & DOLLY, J.O.
FEBS Lett. 126 309-312 (1981)
- CHANGEUX, J.P., MEUNIER, J.C. & HUCHET, M.
Mol. Pharmacol. 5, 538-553 (1971)
- CHAPMAN, D.B. & WAY, E.L.
Ann. Rev. Pharmacol. Toxicol. 20 (1980) 553-579
- CHAVKIN, C. & GOLDSTEIN, A.
Proc.Nat.Acad.Sci. 78 6543-6547 (1981a)
- CHAVKIN, C. & GOLDSTEIN, A. Nature 291 591-593 (1981b)
- CHENEY, B.V., LAHTI, R.A. & BARSUHN, C.
Eur. J. Pharmacol. 77 259-263 (1982)
- CHILDERS, S.R., CREESE, I., SNOWMAN, A.M. & SNYDER, S.H.
Eur. J. Pharmacol. 55 11-18 (1979)
- CHO, T.M., YAMATO, C., CHO, J.S. & LOH, H.H.
Life Sci. 28 2651-2657 (1981)
- CHO, T.M., GE, B.L., YAMATO, C., SMITH, A.P. & LOH, H.H.
Proc.Nat.Acad.Sci. 80 5176-5180 (1983)
- CHOW, T. & ZUKIN, R.S. Mol. Pharmacol. 24 203-212 (1983)

- COATES, M. & TSCHUDI, G. J. Am. Chem. Soc. 74 1109 (1952)
- COLLIER, H.O.J. & ROY, A.C. Nature 248 24-27 (1974)
- COMB, M., SEEBURG, P.H., ADELMAN, J., EIDEN, L. & HERBERT, E.
Nature 295 663-666 (1982)
- COOPER, D.M.F., LONDOS, C., GILL, D.L. & RODBELL, M.
J. Neurochem. 38 1164-1167 (1982)
- CORBETT, A.D., PATERSON, S.J., McKNIGHT, A.T., MAGNAN, J. &
KOSTERLITZ, H.W. Nature 299 79-81 (1982)
- CORBETT, A.D., KOSTERLITZ, H.W., McKNIGHT, A.T., & PATERSON, S.J.
Br. J. Pharmacol. Supp. 78 170P (1983a)
- CORBETT, A.D., PATERSON, S.J., McKNIGHT, A.T. & KOSTERLITZ, H.W.
Presented at INRC (1983b)
- COTTON, R., GILES, M.G., MILLER, I., SHAW, J. & TIMMS, D.
Eur. J. Pharmacol. 97 331-332 (1984)
- COWAN, A., LEWIS, J.W. & MACFARLANE, I.R.
Brit. J. Pharmacol. 60 537-545 (1977)
- COX, B.M. & CHAVKIN, C. Mol. Pharmacol. 23 36-43 (1983)
- CRAVEN, G.R., STEERS, E.Jr. & ANFINSEN, C.B.
J. Biol. Chem. 240 2468-2477 (1965)
- CRAVISO, G.L. & MUSACCHIO, J.M.
Mol. Pharmacol. 23 619-628 (1983)
- CUELLO, A.C. Brit.Med.Bull. 39 11-17 (1983)
- DALE, H.H. J. Pharmacol. exp. Ther. 6 147-190 (1914)
- D'AMOUR, F.E. & SMITH, D.L.
J. Pharmacol. exp. Ther. 72 74-79 (1941)
- DAVID, M., MOISAND, C., MEUNIER, J-C., MORGAT, J-L., GACEL, G
& ROQUES, B. Eur. J. Pharmacol. 78 385-387 (1982)

- DEMOLIOU, C.D. Ph.D. thesis. McMaster University 1980
- DEMOLIOU-MASON, C. & BARNARD, E.A. Submitted for publication, 1984
- DICKENS, J.M. Ph.D.. Thesis. University of Hull 1978)
- DOBLE, A. & IVERSEN, L.L. *Nature* 295 522-523 (1982)
- DOLLY, J.O., & BARNARD, E.A. *F.E.B.S. Lett* 57 267-271 (1975)
- DORNAY, M. & SIMANTOV, R. *J. Neurochem.* 38 1524-1531 (1982)
- DRESER, H. *Arch. ges. Physiol.* 76 306 (1899)
- DUGGAN, A.W. *Brit.Med.Bull.* 39 65-71 (1983)
- DUGGAN, A.W., JOHNSON, S.M. & MORTON, C.R.
Brain Res. 229 379-389 (1981)
- DUM, J., MEYER, G., HOLLT, V. & HERZ, A.
Eur. J. Pharmacol. 46 165-170 (1977)
- EDDY, N.B. & LEIMBACH, D.
J. Pharmacol. exp. Ther. 107 385-391 (1953)
- EGAN, T.M. & NORTH, R.A. *Science* 214 923-924 (1981)
- EHRLICH, P. *Lancet* 2 445 (1913)
- FADEN, A., JACOBS, C., HOLADAY, J.W. *Science* 211 493 (1981)
- FANTOZZI, R., MULLIKIN-KILPATRICK, D. & BLUME, A.J.
Mol. Pharmacol. 20 8-15 (1981)
- FELDBERG, W. & SMYTH, D.G.
Br. J. Pharmacol. 60 445-453 (1977)
- FIELDS, H.L., EMSON, P.C., LEIGH, B.K., GILBERT, R.T.F. &
IVERSEN, L.L. *Nature* 284 351-353 (1980)
- FLEET, G.W.J., KNOWLES, J.R. & PORTER, R.R.
Nature 224 511-515 (1969)
- FOOTE, R.W. & MAURER, R. *Eur. J. Pharmacol.* 85 99-103 (1982)

- FOURNIE-ZALUSKI, M-C., GACEL, G., MAIGRET, B., PREMILAT, S. & ROQUES, B.P. *Mol. Pharmacol.* 20 484-491 (1981)
- GACEL, G., FOURNIE-ZALUSKI, M-C., FELLION, E. & ROQUES, B.P. *J. Med. Chem.* 24 1119-1124 (1981)
- GADDUM, J.H. *Gaddum's Pharmacology*, 8th edition, revised by A.S.V. Burgen and J.F. Mitchell, 1978
- GARBAY-JAUREGUIBERRY, C., ROBICHON, A. & ROQUES, B.P. *Life Sci.* 33 (S1) 247-250 (1983)
- GARFIELD, E. *in* 'Current Comments' on Naloxone, on the 1982 John Scott Award to Jack Fishman & Harold Blumberg for synthesis and investigation of naloxone. *Curr. Cont.* 26 5-12 (1983)
- GENTLEMAN, S., PARENTI, M., NEFF, N. & PERT, C.B. *Cell. & Molec. Neurobiol.* 3 17-26 (1983)
- GILBERT, P.E. & MARTIN, W.R. *J. Pharmacol. exp. Ther.* 198 66-82 (1976)
- GILLAN, M.G.C., KOSTERLITZ, H.W. & PATERSON, S.J. *Br. J. Pharmacol.* 70 481-490 (1980)
- GILLAN, M.G.C., KOSTERLITZ, H.W. & MAGNAN, J. *Br. J. Pharmacol.* 72 13-15 (1981)
- GILLAN, M.G.C., JIN, W-Q., KOSTERLITZ, H.W. & PATERSON, S.J. *Br. J. Pharmacol.* 79 275P (1983)
- GIOANNINI, T., FOUCAUD, B., HILLER, J.M., HATTEN, J.M. & SIMON, E.J. *Biochem. Biophys. Res. Comm.* 105 1128-1134 (1982)
- GINTZLER, A.R. & PASTERNAK, G.W. *Life Sci.* S1 167-170 (1983)
- GLASEL, J.A. & VENN, R.F. *Life Sci.* 29 221-8 (1981)
- GLASEL, J.A., VENN, R.F. & BARNARD, E.A. *Biochem. Biophys. Res. Comm.* 95 263-268 (1980)

- GOLDSTEIN, A., ARONOW, L. & KALMAN, S.M. in Principles of drug action. 2nd edn. Wiley 33-36 (1976)
- GOLDSTEIN, A., LOWNEY, L.I. & PAL, B.K.
Proc.Nat.Acad.Sci. 68 1742-1747 (1971)
- GOODMAN, R.R., SNYDER, S.H., KUCHAR, M.J. & YOUNG, W.S., III
Proc.Nat.Acad.Sci. 77 6239-6243 (1980)
- GORMLEY, J.J., MORLEY, J.S., PRIESTLEY, T., SHAW, J.S.
- TURNBULL, M.J. & WHEELER, H. Life Sci. 31 1263-1266 (1982)
- GREENBERG, R. & O'KEEFE, E.H. Life Sci. 31 1185-1188 (1982)
- GROSSMAN, A. & REES, L.H. Brit.Med.Bull. 39 83-89 (1983)
- GULLAND, J. & ROBINSON, R.
Memoirs & Proc. Manchester Lit. & Phil. Soc. 69 79 (1925)
- GWYNN, G.J. & COSTA, E. Proc.Nat.Acad.Sci. 79 690-695 (1982)
- GYANG, E.A. & KOSTERLITZ, H.W.
Br. J. Pharmacol. 27 514-527 (1966)
- HAHN, E., FISHMAN, J., SHIWAKU, Y., FOLDES, F.F., NAGASHIMA, H. &
DUNCALF, D. Res. Comm. Chem. Path. Pharmac. 18 1-9 (1977)
- HAMBROOK, J.M., MORGAN, B.A., RANCE, M.J., & SMITH, C.F.C.
Nature 262 782-783 (1976)
- HAMMONDS, R.G., NICOLAS, P. & LI, C.H.
Proc. Nat. Acad. Sci. 79 6494-6496 (1982)
- HANDA, B.K., LANE, A.C., LORD, J.A.H., MORGAN, B.A., RANCE, M.J.,
& SMITH, C.F.C. Eur. J. Pharmacol. 70 531-540 (1981)
- HANSTEIN, W.G. Meth. Enzymol. 48 653-673 (1979)
- HART, E.R. J. Pharmacol. exp. Ther. 72 19 (1941)
- HARTHOORN, A.M. & BLIGH, J. Res. Vet. Sci. 6 290-299 (1965)
- HAYES, A.G., HILL, R.G., SKINGLE, M. & TYERS
Br. J. Pharmacol. 78 47P (1983)

- HAZUM, E., CHANG, K-J., & CUATRECASAS, P.
Science 206, 1077 (1979a)
- HAZUM, E., CHANG, E-J. & CUATRECASAS, P.
Nature 282 626-628 (1979b)
- HAZUM, E., CHANG, K-J., SCHECHTER, Y., WILKINSON, S. &
 CUATRECASAS, P. *Biochem. Biophys. Res. Commun* 88 841-846 (1979c)
- HAZUM, E., CHANG, K.J. & CUATRECASAS, P.
Proc.Nat.Acad.Sci. 77 3038-3041 (1980)
- HENDERSHOT, L.C. & FORSAITH, J.
J. Pharmacol. exp. Ther. 125 237-240 (1959)
- HENDERSON, G. *T.I.P.S.* 3 248-250 (1982)
- HENDERSON, G. *Brit.Med.Bull.* 39 59-65 (1983)
- HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W.
Br. J. Pharm. 46 764-766 (1972)
- HENDERSON, G., ROBINSON, D.S. & SIM, J.A.
Br. J. Pharmacol. 75 29P (1982)
- HERZ, A. Neuroendocrine mechanisms in Opioids : past, present
 and future. (in press, 1984)
- HERZ, A. & TESCHEMACHER, H.J. *Adv. Drug Res.* 6 79-119 (1971)
- HILLER, J.M. & SIMON, E.J.
J. Pharmacol. Exp. Ther. 214 516-519 (1980)
- HILLER, J.M., ANGEL, L.M. & SIMON, E.J.
Science 214 468-469 (1981)
- HOKFELT, T., JOHANSSON, O., LJUNGDAHL, A., LUNDBERG, J.M. &
 SCHULTZBERG, M. *Nature* 284, 515-521 (1980)
- HOLADAY, J.W. & D'AMATO, R.J. *Life Sci.* 33 (SI) 703-706 (1983)
- HOLLT, V. *T.I.N.S.* 6 24-26 (1983)
- HOLLT, V., TULUNAY, F.C., WOO, S.K., LOH, H.H. & HERZ, A.
Eur. J. Pharmacol. 85 355-356 (1982)

- HOLTZMAN, S.G. Fed. Proc. 41 2328-2831 (1982)
- HOUDE, R.W. & WALLENSTEIN, S.L. Fed. Proc. 15 440-441 (1956)
- HOUSLAY, M.D., ELLORY, J.C., SMITH, G.A., HESKETH, T.R., STEIN, J.M., WARREN, G.B. & METCALFE, J.C.
- Biochim. Biophys. Acta 467 208-219 (1977)
- HOWELLS, R., GIOANNINI, T.M., HILLER, J.M. & SIMON, E.J.
- J. Pharmacol. exp. Ther. 222 629-634 (1982)
- HUGHES, J. Brain Res. 88 295-308 (1975)
- HUGHES, J. Brit.Med.Bull. 39 25-31 (1983)
- HUGHES, J. KOSTERLITZ, H.W. & LESLIE, F.M.
- Br. J. Pharmacol. 53 371 (1975)
- HUGHES, J., SMITH, T.W., KOSTERLITZ, H.W., FOTHERGILL, L.A.,
- MORGAN, B.A. & MORRIS, H.R. Nature 258 577-579 (1975)
- HUIDOBRO, F., HUIDOBRO-TORO, J.P. & MIRANDA, H.
- Br. J. Pharmacol. 70 519-524 (1980)
- HUTCHINSON, M., KOSTERLITZ, H.W., LESLIE, F.M., WATERFIELD, A.A.
- & TERENIUS, L. Br. J. Pharmacol. 55 541 (1975)
- ISHII, K., YAMAMOTO, S., MURAKI, T. & KATO, M.
- Jap. J. Pharmacol. 31 905-911 (1981)
- ITZHAK, Y., KALIR, A. & SARNE, Y.
- Eur. J. Pharmacol. 73 229-33 (1981)
- IZQUIERDOI, R.D., DIA, A. & ELISABETSKY, L.
- Behav. Brain. Res. 1 451-469 (1980)
- JACOBS, S., HAZUM, E., SCHECHTER, Y. & CUATRECASAS, P.
- Proc.Nat.Acad.Sci. 76 4918-4921 (1979)
- JACQUET, Y.F. Science 210 95-96 (1980)
- JAFFE, J.H. & MARTIN, W.R.Ch. 15 in 'The pharmacological basis of therapeutics',
- eds. L.S. Goodman & A. Gilman, 5th edition, Macmillan N.Y. (1975)

- JASINSKI, D.F., MARTIN, W.R. & HAERTZEN, C.A.
J. Pharmacol. exp. Ther. 157 420-426 (1967)
- JESSEL, T.M. & IVERSEN, L.L. *Nature* 268 549-551 (1977)
- JOHNSON, N. & PASTERNAK, G.W. *Life Sci.* 33 985-992 (1983)
- JONES, C.R., GIBSON, W.A. & GARSKY, V. *Nature* 262 779-781
- KAKIDANI, H., FURUTANI, Y., TAKAHASHI, H., NODA, M., MORIMOTO, Y., HIRDSE, T., ASAI, M., ANAYAMA, S., NAKANISHI, S. & NUMA, S.
Nature 298 245-249 (1982)
- KANELLOPOULOS, J., ROSSI, G. & METZGER, H.
J. Biol. Chem. 254 7691-7697 (1979)
- KEMPNER, E.S. & SCHLEGEL, W. *Anal. Biochem.* 92 2-10 (1979)
- KEPNER, G.R. & MACEY, R.I. *Biochim. Biophys. Acta* 163 188-203 (1968)
- KERR, F.W.L. *Pain* 1 325-356 (1975)
- KILLIAN, A.K., SCHUSTER, C.R., WAINER, B.H. & MERZ, H.
Life Sci. 28 1239-1243 (1981)
- KLEE, W.A. & NIRENBERG, M.
Proc.Nat.Acad.Sci. 71 3474-3477 (1974)
- KLEE, W.A. & STREATY, R.A. *Nature* 248 61-63 (1974)
- KLEE, W.A., SIMONDS, W.F., SWEAT, F.W., BURKE, T.R., JACOBSON, A.E. & RICE, K.C. *F.E.B.S. Lett.* 150 125-129 (1982)
- KNOWLES, J.R. *Acc. Chem. Res.* 5 155-160 (1972)
- KOMAN, A. & TERENIUS, L. *F.E.B.S. Lett.* 118 293 (1980)
- KOOB, G.F. & BLOOM, F.E. *Brit.Med.Bull.* 39 89-95 (1983)
- KOSTERLITZ, H.W. & PATERSON, S.J.
Proc. Roy. Soc. Lond. B. 210 113-122 (1980)
- KOSTERLITZ, H.W. & PATERSON, S.J.
Br. J. Pharmacol. 73 299P (1981)

- KOSTERLITZ, H.W. & WATT, A.J.
Br. J. Pharmacol. 32 266-276 (1968)
- KOSTERLITZ, H.W. & WATERFIELD, A.A.
Ann. Rev. Pharmacol. Toxicol. 15 29-47 (1975)
- KOSTERLITZ, H.W., LESLIE, A.M. & WATERFIELD, A.A.
Eur. J. Pharmacol. 32 10-18 (1975)
- KOSTERLITZ, H.W., PATERSON, S.J. & ROBSON, L.E.
Br. J. Pharmacol. 73 939-949 (1981)
- LADURON, P.M. T.I.P.S. 3 351-352 (1982)
- LADURON, P.M. T.I.P.S. 4 333-335 (1983)
- LAI, F.A., NEWMAN, E.L., PEERS, E.M. & BARNARD, E.A.
Submitted for publication (1984)
- LAMOTTE, C., PERT, C.B. & SNYDER, S.H.
Brain Res. 113 407-412 (1976)
- LANGLEY, J.N. J. Physiol. 33 374-413 (1905)
- LASAGNA, L. & BEECHER, H.K.
J. Pharmacol. exp. Ther. 112 356-363 (1954)
- LAW, P-Y, HOM, D.S. & LOH, H.H. Reported at INRC (1983a)
- LAW, P.Y., HOM, D.S. & LOH, H.H.
Mol. Pharmacol. 23 26-35 (1983b)
- LEE, N.M. & SMITH, A.P. Life Sci. 26 1459-1464 (1980)
- LEE, N.M., LEYBIN, L., CHANG, J-K & LOH, H.H.
Eur. J. Pharmacol. 68 181-185 (1980)
- LEE, R.T., WILLIAMS, R.E. & FOX, C.F.
J. Biol. Chem. 254 11787-11790 (1979)
- LEMAIRE, S., MAGNAN, J. & REGOLI, D.
Br. J. Pharmacol. 64 327-329 (1978)

- LEMOINE, H. & KAUMANN, A.J.
Naunyn-Schmiedeberg's Arch. Pharm. 322 111-120 (1983)
- LERMAN, L.S. Proc.Nat.Acad.Sci. 39 232-236 (1953)
- LESLIE, F.M., CHAVKIN, C. & COX, B.M.
J. Pharmacol. Exp. Ther. 214 395-402 (1980)
- LEVINSON, S.R. & ELLORY, J.C. Biochem. J. 137 123-126 (1974)
- LEWIS, J.W., BENTLEY, K.B. & COWAN, A.
Ann. Rev. Pharmacol. 11 241-270 (1971)
- LEWIS, J.W. & RANCE, M.J.
Pharmaceut. J. 221 395-397 (1978)
- LIAO, C.S., DAY, A.R. & FREER, R.J.
Life Sci. 29 2617-2622 (1981)
- LILLY, L., FRASER, C.M., JUNG, C.Y., SEEMAN, P. & VENTER, J.C.
Mol. Pharmacol. 24 10-14 (1983)
- LIMBIRD, L.E. & LEFKOWITZ, R.J.
J. Biol. Chem. 252 799-802 (1977)
- LIN, H-K, HOLLAND, M-J.C. & SIMON, E.J.
J. Pharmacol. exp. Ther. 216 149-155 (1981)
- LING, G.S.F. & PASTERNAK, G.W.
Neurosci. Lett. 32 193-196 (1982)
- LING, G.S.E., SPIEGEL, K., NISHIMURA, S.L. & PASTERNAK, G.W.
Eur. J. Pharmacol. 86 487-488 (1983)
- LO, M.M.S., BARNARD, E.A. & DOLLY, J.O.
Biochemistry 21 2210-2217 (1982)
- LOH, H.H. & LAW, P.Y.
Ann. Rev. Pharmacol. Toxicol. 20 201-220 (1980)
- LOH, H.H., LEE, N.M. & SMITH, A.P.
T.I.P.S. 4 371-372 (1983)

- LOH, H.H., BRASE, D.A., SAMGATH-KHANNA, S., MAR, J.B., WAY, E.L. & LI, C.H. *Nature* 264 567-568 (1976)
- LORD, J.A.H., WATERFIELD, A.A., HUGHES, J. & KOSTERLITZ, H.W. Multiple opiate receptors in *Opiates & endogenous opioid peptides* ed. Kosterlitz 275-280. Elsevier/NH Biomed. Press (1976)
- LORD, J.A.H., WATERFIELD, A.A., HUGHES, J. & KOSTERLITZ, H.W. *Nature* 267 495-499 (1977)
- LOWE, C.R. & DEAN, P.D.G. *Affinity chromatography*. Wiley (1974)
- MACKAY, A.V.P. *T.I.N.S.* 4 (5) IX (1981)
- McKNIGHT, A.T., CORBETT, A.D. & KOSTERLITZ, H.W. *Eur. J. Pharmacol.* 86 393-402 (1983)
- MCLAWHON, R.W., CERMAK, D., ELLORY, J.C. & DAWSON, G. *J. Neurochem.* 41 1286-1296 (1983b)
- MCLAWHON, R.W., ELLORY, J.C. & DAWSON, G. *J. Biol. Chem.* 258 2102-2105 (1983a)
- McLENNAN, H. in *Synaptic transmission*. Saunders, Philadelphia (1963)
- MAAS, C.L. *Eur. J. Pharmacol.* 77 71-74 (1982)
- MARKWELL, M.A.K., HAAS, S.M., BIEBER, L.L. & TOLBERT, N.E. *Anal. Biochem.* 87 206-210 (1978)
- MARTIN, W.R. *Opioid agonists and antagonists in* *Opioids : past, present and future*. In press (1984)
- MARTIN, W.R. *Pharmacol. Rev.* 19 463-521 (1967)
- MARTIN, W.R. *Brit. J. Clin. Pharmacol.* 7 2735-2795 (1979)
- MARTIN, W.R., EADES, C.G., THOMPSON, J.A. HUPPLER, R.E. & GILBERT, P.E. *J. Pharmacol. exp. Ther.* 197, 517-532 (1976)
- MARTIN, W.R., FRASER, H.F., GORODETZKY, C.W. & ROSENBERG, G.W. *J. Pharmacol. exp. Ther.* 150 426-436 (1965)

- MARTIN, W.R., JASINSKI, D.R. & MANSKY, P.A.
Arch. gen. Psychiat. 28 784-791 (1973)
- MEO, T., GRAMSCH, C., INAN, R., HOLLT, V., WEBER, E., HERZ &
 RIETHMULLER, G. *Proc.Nat.Acad.Sci.* 4084-4088 (1983)
- MEUNIER, J-C., JUCHET, M., BOQUET, P. & CHANGEUX, J-P.
C.R. Acad. Sci. (Paris) 272 117-120 (1971)
- MICHELL, R.H., KIRK, C.J., JONES, L.M., DOWNES, C.P. & CREBA,
 J.A. *Phil. Trans. Roy. Soc. Lond. B.* 296 123-137 (1981)
- MILLER, L. & SHAW, J.S.
Eur. J. Pharmacol. 90 257-261 (1983)
- MILLER, R.J., CHANG, K-J., LEIGHTON, J. & CUATRECASAS, P.
Life Sci. 22 379-388 (1978)
- MORLEY, J.S. *Ann. Rev. Pharmacol. & Toxicol.* 20 81-110 (1980)
- MORLEY, J.S. *Brit. Med. Bull.* 39 17-25 (1983a)
- MORLEY, J.S., *T.I.P.S.* 4 370-371 (1983b)
- MURRAY, W.J. & MILLER, J.W.
J. Pharmacol. exp. Ther. 128 380-383 (1960)
- MURRIN, L.C., COYLE, J.T. & KUJAR, M.J.
Life Sci. 27 1175-1183 (1980)
- NAGAI, U., KUDO, Y., SATO, K., TAKI, N. & SHIBATA, S.
Biomed. Res. 2 229-232 (1981)
- NAKANISHI, S., INOUE, A., KITA, T., NAKAMURA, M., CHANG, A.C.Y.,
 COHEN, S.N. & NUMA, S. *Nature* 278 423-427 (1979)
- NEIL, A. & TEREINIUS, L. *Eur. J. Pharmacol.* 69 33-39 (1981)
- NIELSEN, M. & BRAESTRUP, C.
Eur. J. Pharmacol. 96 321-322 (1983)

- NORMAN, R.I., MEHRABAN, F., BARNARD, E.A. & DOLLY, J.O.
Proc.Nat.Acad.Sci. 79 1321-1325 (1982)
- NORTH, R.A. & EGAN, T.M. *Brit.Med.Bull.* 39 71-77 (1983)
- NORTH, R.A. & WILLIAMS, J.T. *T.I.N.S.* 6 337-339 (1983a)
- NORTH, R.A. & WILLIAMS, J.T.
Br. J. Pharmacol. 79 423P (1983b)
- OKA, T., NEGISHI, K., SUDA, M., MATSUMIYA, T., INAZU, T. &
 UEKI, M. *Eur. J. Pharmacol.* 73 235-236 (1981)
- OKA, T., NEGISHI, K., SUDA, M., SAWA, A., FUJINO, M. &
 WAKIMASU, M. *Eur. J. Pharmacol.* 77 137-141 (1982)
- OTT, S., COSTA, T. HIETEL, B., SCHLEGEL, W. & WUSTER, M.
Naunyn-Schmiedeberg's Arch. Pharmacol. 324 160-162 (1983)
- PASTERNAK, G.W. & SNYDER, S.H. *Nature* 253 563-565 (1975)
- PASTERNAK, G.W., SNOWMAN, A.M. & SNYDER, S.H.
Mol. Pharmacol. 11 735-757 (1975)
- PATERSON, S.J., ROBSON, L.E. & KOSTERLITZ, H.W.
Brit.Med.Bull. 39 31-37 (1983)
- PATON, W.D.M. *Br. J. Pharmacol.* 12 119-127 (1957)
- PEERS, E.M., RANCE, M.J., HAYNES, A.S., SMITH, C.F.C. & BARNARD,
 E.A. *Life Sci.* 33 (51) 439-443 (1983)
- PERT, C.B. & SNYDER, S.H. *Mol. Pharmacol.* 10 868-879 (1974)
- PERT, C.B. & SNYDER, S.H. *Science* 179 1011-1014 (1973)
- PERT, C.B. & TAYLOR, D. *in* Endogenous and exogenous opiate
 agonists and antagonists, ed. E.L. Way, 87-90 (1980)
- PERT, C.B., BOWIE, D.L., PERT, A., MORELL, J.L. & GROSS, E.
Nature 269 73-74 (1977)

- PERT, C.B., KUJAR, M.J. & SNYDER, S.H.
 Proc.Nat.Acad.Sci. 73 3729-3733 (1976)
- PFEIFFER, A. & HERZ, A.
 Biochem. Biophys. Res. Commun. 101 38-43 (1981)
- PFEIFFER, A., FEUERSTEIN, G., KOPIN, I.J. & FADEN, A.I.
 J. Pharmacol. exp. Ther. 225 735-741 (1983)
- PFEIFFER, A., & HERZ, A.
 Mol. Pharmacol. 21 266-271 (1982)
- PIERCEY, M.F., LAHTI, R.A. & SCHROEDER, L.A.
 Life Sci. 31 1197-1200 (1982a)
- PIERCEY, M.F., VARNER, K. & SCHROEDER, L.A.
 Eur. J. Pharmacol. 80 283-284 (1982b)
- POHL, J. Exp. Path. Ther. 17 370-382 (1915)
- PORRECA, F. & BURKS, T.F.
 J. Pharmacol. exp. Ther. 225 668-693 (1983)
- PORTOGHESE, P.S., LARSON, D.L., JIANG, J.B., TAKEMORI, A.E. &
 CARUSO, T.P. J. Med. Chem. 21 598-599 (1978)
- QUINCEY, Thomas de in Confessions of an English Opium Eater pt
 ii, The pleasures of opium (1856) and pt iii The pains of opium
 (1856)
- QUIRION, R., HAMMER, R., HERKENHAM, M. & PERT, C.B.
 Proc.Nat.Acad.Sci. 78 5881-5885 (1981)
- QUIRION, R., BOWEN, W.D., HERKENHAM, M. & PERT, C.B.
 Cell. & Mol. Neurobiol. 2 333-346 (1983)
- RACKHAM, A., WOOD & HUDGIN, R.L. Life Sci. 30 1337-1342 (1982)
- RAMACHANDRAN, J., MURAMOTO, K., KENEZ-KERI, M., KERI, G. &
 BUCKLEY, D.I. Proc.Nat.Acad.Sci. 77 3967-3970 (1980)

- RANCE, M.J. Br. J. Clin. Pharmacol. 7 281S-286S (1979)
- RANCE, M.J., KOBYLECKI, R.J., LANE, A.C., HOLDGATE, M.J. & BARNARD, E.A. in Adv. Endog. & Exog. opioids. Eds. H. Takagi & E.J. Simon, 80-81 (1981)
- REYNOLDS, D.V. Science 164 444-445 (1969)
- RICE, K.C., JACOBSON, A.E., BURKE, T.R., BAJWA, B.S., STREATY, R. & KLEE, W.A. Science 22- 314-316 (1983)
- ROBSON, L.E. & KOSTERLITZ, H.W. Proc. Roy. Soc. Lond. B. 205 425-432 (1979)
- RODBELL, M. Nature 284 17-22 (1980)
- ROMER, D., BUSCHER, H.H., HILL, R.C., PLESS, J., BAUER, W., CARDINAUX, F., CLOSSE, A., HAUSER, D. & HUGUENIN, R. Nature 268 547-549 (1977)
- ROMER, D., BUSCHER, H.H., HILL, R.C., MAURER, R., PETCHER, T.J., ZEUGNER, H., BENSON, W., FINNER, E., MILKOWSKI, W. & THIES, P.W. Nature 298 759-760 (1982)
- ROTH, B.L., LASKOWSKI, M.B. & COSCIA, C.J. J. Biol. Chem. 256 10117-10123 (1981)
- ROTHMAN, R.B. & WESTFALL, T.C. Eur. J. Pharmacol. 72 365-368 (1981)
- ROTHMAN, R.B. & WESTFALL, T.C. Mol. Pharmacol. 21 538-547 (1982a)
- ROTHMAN, R.B. & WESTFALL, T.C. Mol. Pharmacol. 21 548-557 (1982b)
- ROTHMAN, R.B. & WESTFALL, T.C. Neurochem. Res. 8 913-931 (1983)
- ROTHMAN, R.B., BOWEN, W.D., SCHUMACHER, U.K. & PERT, C.B. Eur. J. Pharmacol. 95 147-148 (1983)

- RUEGG, U.T., CUENOUD, S., HILLER, J.M., GIOANNINI, T., HOWELLS, R.D. & SIMON, E.J. *Proc.Nat.Acad.Sci.* 78 4635-4638 (1981)
- RUEGG, U.T., CUENOUD, S. FULPIUS, B.W. & SIMON, E.J. *Bioch. Biophys. Acta* 685 241-249 (1982)
- SADEE, W., ROSENBAUM, J.S. & HERZ, A. *J. Pharmacol. exp. Ther.* 223 157-162 (1982)
- SANGER, D.J. & MCCARTHY, P.S. *Pharmacol. Biochem. Behav.* 16 1013-1017 (1982)
- SAYRE, L.M., PORTOCHESE, P.S. & TAKEMORI, A.E. *Eur. J. Pharmacol.* 90 159-160 (1983)
- SCHAUMANN, W. *Br. J. Pharmacol.* 12 115-118 (1957)
- SCHLEGEL, W., KEMPNER, E.S. & RODBELL, M. *J. Biol. Chem.* 254 5168-5176 (1979)
- SCHULZ, R. & GOLDSTEIN, A. *Life Sci.* 16 1843-1848 (1975)
- SCHULZ, R. & WUSTER, M. *Eur. J. Pharmacol.* 76 61-66 (1981)
- SCHULZ, R., WUSTER, M., KRENSS, H. & HERZ, A. *Mol. Pharmacol.* 18 395-401 (1980a)
- SCHULZ, R., WUSTER, M., KRENZZ, H. & HERZ, A. *Nature* 285 242-243 (1980b)
- SCHULZ, R., WUSTER, M. & HERZ, A. *J. Pharmacol. exp. Ther.* 216 604-606 (1981a)
- SCHULZ, R., WUSTER, M., RUBINI, P. & HERZ, A. *J. Pharmacol. exp. Ther.* 219 547-550 (1981b)
- SCHURZ, R.H. & RUDIGER, R.L. *Anal. Biochem.* 123 174-181 (1982)
- SCOUTEN, W.H. Vol. 59 in *Chemical Analysis Monographs Wiley* (1981)
- SEIZINGER, B.R., PITTIUS, C.W., MEHRAEIN, P., PASI, A. & HERZ, A. *Life Sci.* 33 (SI) 41-44 (1983)

- SHANNON, H.E. Eur. J. Pharmacol. 84 225-228 (1982)
- SHAW, J.S., MILLER, M.J., TURNBULL, M.J., GORMLEY, J.J. & MORLEY, J.S. Life Sci. 31 1259-1262 (1982)
- SHAW, J.S., TURNBULL, M.J., DUTTA, A.S., GORMLEY, J.J. HAYWARD, C.F. & STACEY, G.J.
- in Van Ree, J.M. & Terenius, L. (eds.) Characteristics and functions of opioids 185-195 Elsevier/North Holland (1978)
- SHEARMAN, G.T. & HERZ, A. Pharmacol. Biochem. Behav. 16 249-252 (1982a)
- SHEARMAN, G.T. & HERZ, A. J. Pharmacol. Exp. Ther. 221 735-739 (1982)
- SHEARMAN, G.T. & HERZ, A. Eur. J. Pharmacol. 82 167-172 (1982)
- SIGEL, E., STEPHENSON, F.A., MAMALAKI, C. & BARNARD, E.A. J. Biol. Chem. 258 6965-6971 (1983)
- SIMON, E.J. Proc.Nat.Acad.Sci. 69 1835-1839 (1972)
- SIMON, E.J. T.I.P.S. 3 155-158 (1981)
- SIMON, E.J., DOLE, W.P. & HILLER, J.M. Proc.Nat.Acad.Sci. 69 1835-1839 (1972)
- SIMON, E.J., HILLER, J.M. & EDELMAN, I. Proc.Nat.Acad.Sci. 70 1947-1949 (1973)
- SIMON, E.J., HILLER, J.M., GROTH, J. & EDELMAN, I. J. Pharmacol. Exp. Tuer. 192 531-537 (1975a)
- SIMON, E.J., HILLER, J.M. & EDELMAN, I. Science 190 389-390 (1975b)
- SIMONDS, W.F., KOSKI, G., STREATY, R.A., HJELMELAND, L.M. & KLEE, W.A. Proc.Nat.Acad.Sci. 77 4623-4627 (1980)
- SINGH, A., THORNTON, E.R. & WESTHEIMER, F.H. J. Biol. Chem. 237 PC 3006-3008 (1962)

- SLATER, P. & PATEL, S. Eur. J. Pharmacol. 92 159-160 (1983)
- SMITH, C.F.C. & RANCE, M.J. Presented at INRC 1983
- SMITH, J.R. & SIMON, E.J.
- Proc.Nat.Acad.Sci. 77 281-284 (1980)
- SMOLARSKY, M. & KOSHLAND, D.E.
- J. Biol. Chem. 255 7244-7249 (1980)
- SMYTH, D.G. in Endogenous and exogenous opiate agonists and antagonists. ed. E. Leong Way. Elsevier/North Holland 1-4 (1979)
- SMYTH, D.G. Brit.Med.Bull. 39 25-31 (1983)
- SNYDER, S.H. Nature 279 13-14 (1979)
- SPIEHLER, V., FAIRHURST, A.S. & RANDALL, L.O.
- Mol. Pharmacol. 14 587-595 (1978)
- STEPHENSON, F.A. & OLSEN, R. J. Neurochem. 39 1579-1586 (1982)
- STEPHENSON, R.P. Br. J. Pharmacol. 11 379-393 (1956)
- STONE, T.W. Br. J. Pharmacol. 79 305-312 (1983)
- SU, T-P. Eur. J. Pharmacol. 75 (81-82) (1981)
- SU, T-P., CLEMENTS, T.H. & GORODETZKY, C.W.
- Life Sci. 28 2519-2528 (1981)
- SWILLENS, S. & DUMONT, J.E. FEBS Lett 134 29-31 (1981)
- TAKAGI, H., SHIOMI, H., UEDA, H. & AMANO, H.
- Eur. J. Pharmacol. 55 109-111 (1979)
- TAKEMORI, A.E., LARSON, D.L. & PORTOGHESE, P.S.
- Eur. J. Pharmacol. 70 445-451 (1981a)
- TERENIUS, L. Acta Pharmacol. Toxicol. 32 317-320 (1973)
- TERENIUS, L. & WAHLSTROM, A.
- Acta Pharmacol. Toxicol. 35 (Supp. I) 87 (1974)

- TESCHEMACHER, H., OPHEIN, K.E. COX, B.M. & GOLDSTEIN, A.
Life Sci. 16 1771-1782 (1975)
- TOLKOVSKY, A.M. *Mol. Pharmacol.* 22 648-656 (1982)
- TRABER, J., FISHER, K., KATZIN, S. & HAMPRECHT, B.
Nature 253 120-122 (1975)
- TYERS, M.B. *Life Sci.* 31 1233-1236 (1982)
- VALLEE, B.L. & HOCH, F.L. *Proc.Nat.Acad.Sci.* 41 327-338 (1955)
- VAUGHT, J.L. *Eur. J. Pharmacol.* 76 453-356 (1981)
- VAUGHT, J.L. & TAKEMORI, A.E.
J. Pharmacol. Exp. Ther. 208 86-89 (1979)
- VAUGHT, J.L., KITANO, T. & TAKEMORI, A.E.
Mol. Pharmacol. 19 236-241 (1981)
- VAUGHT, J.L., ROTHMAN, R.B. & WESTFALL, T.C.
Life Sci. 30 1443-1455 (1982)
- VAUPEL, D.B. *Eur. J. Pharmacol.* 92 269-274 (1983)
- VENN, R. & BARNARD, E.A. *J. Biol. Chem.* 256 1529-1532 (1981)
- VENTER, J.C. *Pharmacol. Rev.* 34 153-188 (1982)
- VENTER, J.C. *J. Biol. Chem.* 258 4842-4848 (1983)
- VENTER, J.C. & KAPLAN, N.O. *Meth. Enzymol.* 38 187-191 (1974)
- VIGNON, J., VINCENT, J-P., BIDARD, J-N, KAMENKA, J-M.,
GENEST, P., MONIER, S. & LAZDUNSKI, M.
Eur. J. Pharmacol. 81 531-542 (1982)
- VILLARREAL, J.E. & SEEVERS, M.H.
Bull. Probl. Drug Depend. 34 (add. 7) 1040-1053 (1972)
- VILLIGER, J.W. & TAYLOR, K.M. *Life Sci.* 29 2699-2708 (1981)
- VIVEROS, O.H., DILIBERTO, E.J., HAZUM, E. & CHANG, K-J.
Mol. Pharmacol. 16 1101-1108 (1979)
- WAMSLEY, J.K. *Pharmacol. Rev.* 35 69-83 (1983)

- WARD, S.J. Presented at I.N.R.C. 1983
- WARD, S.J., PORTOGHESE, P.S. & TAKEMORI, A.E.
J. Pharmacol. Exp. Ther. 220 494-498 (1982a)
- WARD, S.J., PORTOGHESE, P.S. & TAKEMORI, A.E.
Eur. J. Pharmacol. 80 377-383 (1982b)
- WARD, S.J., PORTOGHESE, P.S. & TAKEMORI, A.E.
Eur. J. Pharmacol. 85 163-170 (1982c)
- WATKINS, L.R. & MAYER, D.J. Science 216 1185-1192 (1982)
- WEBER, E., EVANS, C.J. & BARCHAS, J.D.
Nature 299 77-79 (1982)
- WEBER, E., EVANS, C.J., BARCHAS, J.D., BOHLEN, P. & ESCH, F.
Presented at INRC (1983)
- WEI, E. J. Pharmacol. Exp. Ther. 216 12-18 (1981)
- WERZ, M.A. & MACDONALD, R.L. Presented at INRC (1983)
- WESSELS, P.L., FEENEY, J., GREGORY, H. & GORMLEY, J.J.
J. Chem. Soc. Perkin II 1691-1698 (1973)
- WINTER, B.A. & GOLDSTEIN, A.
Mol. Pharmacol. 6 601-611 (1972)
- WOOD, P.L. Neuropharmacol. 21 487-497 (1982)
- WOOD, P.L. & PILAPIL, C. Eur. J. Pharmacol. ** 281-282 (1983)
- WOOD, P.L. & RICHARD, J.W.
Neuropharmacol. 21 1305-1310 (1982)
- WOOD, P.L., RACKHAM, A. & RICHARD, J.
Life Sci. 28 2119-2125 (1981)
- WOOD, P.L., SANSCHAGRIN, D., RICHARD, J.W. & THAKUR, M.
J. Pharmacol. Exp. Ther. 226 545-550 (1983)
- WU, K.M., MARTIN, W.R., KAMERLING, S.G. & WETTSTEIN, J.G.
Life Sci. 33 1831-1838 (1983)

- YOSHIMURA, K., HUIDOBRO-TORO, J.P. & WAY, E.L.
Eur. J. Pharmacol. 84 17-24 (1982)
- YOSHIMURA, M. & NORTH, R.A.
Nature 305 529-530 (1983)
- YOUNG, E.A., WALKER, J.M., HOUGHTEN, R. & AKIL, H.
Eur. J. Pharmacol. 91 327-329 (1983)
- ZHANG, A-Z. & PASTERNAK, G.W.
Eur. J. Pharmacol. 67 323-324 (1980)
- ZHANG, A-Z. & PASTERNAK, G.W. Life Sci. 29 843-851 (1981)
- ZIOUDROU, C., VAROUCOA, D., LOUKAS, S., STREATY, R.A. & KLEE, W.
Life Sci. 31 1671-1674 (1982)
- ZIOUDROU, C., VAROUCOA, D., LOUKAS, S., NICOLAOU, N. STREATY,
R.A. & KLEE, W.A. J. Biol. Chem. 258 10934-10937 (1983)
- ZUKIN, R.S. & KREAM, R.M. Proc.Nat.Acad.Sci. 76 1593-1597 (1979)
- ZUKIN, R.S. & ZUKIN, S.R. Mol. Pharmacol. 20 246-254 (1981a)
- ZUKIN, R.S. & ZUKIN, S.R. Life Sci. 29 2681-2691 (1981b)